Synthesized 3-(2-isocyano-6-methylbenzyl)-1H-indole Enhanced Susceptibility of Serratia marcescens to Kanamycin by Occluding Quorum Sensing

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Research Article

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Synthesized 3-(2-isocyano-6-methylbenzyl)-1H-indole Enhanced Susceptibility of *Serratia marcescens* to Kanamycin by Occluding Quorum Sensing

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Abstract

Quorum sensing (QS) inhibition is recognized as a novel drug target for infections caused by drug-resistant pathogens and is an attractive strategy for the development of antipathogenic agents. Herein, we designed and synthesized three parts of 3-(2-isocyanobenzyl)-1H-indole derivatives and evaluated their activity as novel quorum sensing inhibitors (QSIs). We found that 3-(2-isocyanobenzyl)-1H-indole derivatives showed promising QS inhibitory activity in the minimum inhibitory concentrations (MICs), biofilm and prodigiosin inhibition assays in Serratia marcescens. In particular, 3-(2-isocyano-6-methylbenzyl)-1H-indole (IMBI, 32) was the best candidate by biofilm and prodigiosin inhibition assays screening strategies. Further studies demonstrated, exposure IMBI (1.56 μg/mL, 1/8MICs) to S. marcescens NJ01 significantly inhibited the formation of biofilms by 42%. The IMBI treatment (1.56 μg/mL) on S. marcescens NJ01 notably enhanced the susceptibility of the formed biofilms, destroying the architecture of biofilms up to 40% as evidenced by scanning electron microscopy (SEM) and confocal laser scanning microscope (CLSM). For interference of virulence factors in S. marcescens NJ01, IMBI (3.12 μg/mL, 1/4MICs) inhibited the activity of protease, and extracellular polysaccharides (EPS) by 17% and 51% respectively, which were higher than that of the positive control vanillic acid (VAN). Furthermore, IMBI downregulated the expressions of QS- and biofilm-related genes bsmA, pigP, flhC, rssB, rsmA, and fimC by 0.13-fold to 1.46-fold. To confirm these findings, molecular docking was performed, which indicated that the binding of IMBI to SmaR, RhlI, RhlR, LasR, and CviR could antagonize the expression of QS-linked traits. In addition, molecular dynamic simulations (MD) and energy calculations indicated that the binding of receptors with IMBI was extremely stable. Most importantly, the biofilms of S. marcescens NJ01 were markedly reduced by 50% under IMBI (0.39 μg/mL, 1/32MICs) combining with 0.15 μg/mL kalamycin (KAN). In conclusion, this study highlights the potency of IMBI in inhibiting the virulence factors against S. marcescens. IMBI could be developed as an effective QS inhibitor.
and anti-biofilm agent to restore or improve drug sensitivity for drug-resistant pathogens.

Key points

- Designed and synthesized 3-(2-isocyanobenzyl)-1H-indole derivatives, IMBI is one potent QSI against *S. marcescens*.
- IMBI inhibited the formation biofilm, virulence factors and expressions of *pigA* and *pigC* of *S. marcescens*.
- Synergistic effects of IMBI combined with KAN against *S. marcescens* NJ01 on erosion of biofilms
- IMBI is one potent QSI and anti-biofilm agent for drug-resistant pathogens.

Keywords 3-(2-isocyano-6-methylbenzyl)-1H-indole, *Serratia marcescens*, quorum sensing, biofilm, virulence factors, kanamycin
Introduction

The emergence of antimicrobial resistance (AMR) due to current strategies to battle infectious diseases is recognized as a major threat and considered a slow-moving pandemic that is worsening every day. The rise in AMR infection is inevitable due to the abuse of antibiotics and prolonged hospital stay during the ongoing COVID-19 pandemic, leading to the patients more vulnerable to attack by opportunistic pathogens (Miethke et al. 2021). This fact provokes an urgent need to discover for a new drug for instant use in case of an outbreak. Drug repurposing approach is a process to recognize new pharmacological indications for existing drugs or prodrugs. It will strengthen our preparedness and fulfill our demand of fully exhausting the arsenal of antimicrobial drugs in use (Christopher JL Murray 2022). The majority of pathogens causing nosocomial infections regulate their virulence factor production via QS (Castillo-Juárez et al. 2015). One of the well-studied opportunistic pathogens is *S. marcescens*. It causes a range of diseases such as pneumonia, nosocomial bacteremia, or wound and urinary tract infections (Jones 2010).

Some *Serratia* spp. utilize QS to measure their population density by sensing external signals, such as C4-C8 homoserine lactones in order to regulate motility, biofilm formation, secondary metabolite production, and the production of virulence factors (Brackman et al. 2011). *S. marcescens* can secrete multiple extracellular virulence factors, and most importantly can form biofilms (Devi et al. 2018). And biofilms are protective barriers adopted by bacteria against the host immune system and antibacterial agents. They are exopolysaccharide protein polymers secreted by bacteria that attach themselves to membranes on abiotic or biotic surfaces. Moreover, as one of the vital virulent factors regulated by QS in *S. marcescens*, prodigiosin plays a critical role in host invasion and pathogenicity (Liu and Nizet 2009). So, interference
with QS systems in pathogenic bacteria has been discussed as a promising strategy to control *S. marcescens* infection.

To date, a number of natural products, such as alkaloids, phenols, and cinnamic acids, and their synthetic analogous have been reported as QS inhibitors (QSIs) and antibiofilm agents (Zhou et al. 2018; Xu et al. 2022), for example, norharmane (Luo et al. 2021) and hordenine (Zhou et al. 2019) can QSmediate virulence factors and biofilm formation against *S. marcescens* NJ01. VAN isolated from kiwifruit can significant decrease the pathogenicity of *S. marcescens* through QS inhibiting (Sethupathy et al. 2017). 4-dimethylaminocinnamic acid was identified as potential QSI and anti-biofilm agent in *C. violaceum* (Cheng et al. 2020), 3-ethoxycinnamic acid was investigated as QSI against *Agrobacterium tumefaciens* (Pan et al. 2022), and (E)-3-phenylprop-2-en-1-aminewas obtained as QSI against *S. marcescens* (Liu et al. 2021) (Fig. 1). Therefore, the development of a variety of novel QSI is of great medical interest to control AMR infection, in particularly *S. marcescens* infection, and revised the abuse of anti-bacterial drugs and prolonged hospital stay and so on.

The indol-isonitrile derivatives often as a reaction intermediate or reactive material in organic chemistry(Hohlman et al. 2021), such as synthesized biologically intriguing pyroglutamic acids(Isaacson et al. 2007). But few studies focus on their own biological activity. As an important isonitrile-containing indole alkaloid skeleton derivatives, hapalindoles are a large family of 80+ polycyclic indole-isonitrile alkaloids produced by branching cyanobacteria of the order Stigonematales, and most of them exhibit antibacterial, and cytotoxic activities(Walton and Berry 2016). For instance, hapalindole H is a potent inhibitor of the NF-kB pathway in prostate cancer cells(Munoz Acuna et al. 2018)(Kim et al. 2012) and is active against several important fungal and bacterial pathogens(Mo et al. 2009). Consider that cyanobacteria natural products (cNP) and NP-inspired derivatives constitute to new pharmaceutical
lead compounds, (Newman and Cragg 2016) cyanobacteria hold tremendous potential as sources of new bioactive molecules (or core scaffolds) useful to medicine. Based on possible structure-activity relationships (SARs) and analyzed active fragment, we focus on exploring the hapalindole H’ sub-structure indol-isonitrile and focus on their own biological activity.

Based on the above analysis, a similar structure compound, 3-indoleacetonitrile (3-ICN) (Fig. 1), is a plant growth hormone auxin, produced by cruciferous vegetables, such as broccoli, cabbage, and cauliflower (Vos et al. 2008). Recent literatures have reported that 3-ICN decrease biofilm formation, motility, and inhibit prodigiosin by interfering with QS in S. marcescens (Sethupathy et al. 2020). It was also found to inhibit biofilm formation and motility against Acinetobacter baumannii (Kashyap et al. 2022). In addition, some papers have reported that indoles had antibiofilm and anti-virulence properties against Serratia marcescens. But with QS inhibition values, mostly their potency remains relatively lower, and the studies were unthoroughly and unsystematically. Selective, high-activity novel candidates are thus needed to enable further investigation of the QS inhibition in S. marcescens and in-use anti-bacterial drugs for the management of pneumonia, urinary tract infections and so on. As one important indol-isonitrile skeleton compounds, 3-(2-isocyanobenzyl)-1H-indole (IBI) was first synthesized in our previous work (Wang et al. 2022). Herein, we systematically designed, synthesized and assessed indol-isonitrile candidates (A-C three parts) for identified a new class of QSIs of S. marcescens. After our preliminary QS inhibiting screening work, found IBI could inhibit the QS of C. violaceum CV026, S. marcescens NJ01 and S. marcescens 4547. Building on these findings, we herein expanded the SARs of indol-isonitrile derivatives by employing a ligand-based design and synthesis strategy. Functional evaluation of the novel derivatives in a biofilm inhibition rates and prodigiosin inhibition rates assay revealed that incorporation in part B-C, especially the indol-isonitrile scaffold, leaded to highly potent QS activity.
with increased efficacy compared to IBI. Moreover, to improve QS inhibitory activity against *S. marcescens* and based on QS inhibitory screening strategies including anti-biofilm formation activities and inhibition prodigiosin effects, we performed structural optimization of IBI to synthesis 35 indol-isonitrile derivatives (1–35).

To be specific, the QS inhibitory activity of the designed and synthesized derivatives was preliminarily evaluated by determining the expression levels of *S. marcescens* NJ01 vital virulence factors prodigiosin and biofilm formation inhibition assay was also performed. For designing indol-isonitrile derivatives towards specific virulence factor (prodigiosin) inhibition of *S. marcescen* were also discussed. As one of the vital virulent factors regulated by QS, prodigiosin plays a critical role in host invasion and pathogenicity. Prodigiosin is a prominent red pigment produced by *S. marcescens* and is essential for its invasion, survival, and pathogenicity. From gene control respective of virulence factor in QS system, prodigiosin could be more representative and the synthesis of prodigiosin is regulated by QS system. So, we tested the prodigiosin inhibition rates of indol-isonitrile derivatives against *S. marcescens*. Besides, IBI was used as lead compound to design and synthesize 35 derivatives and their QS inhibitory and anti-biofilm formation activities were evaluated (Scheme 1 and Fig. 2).

Among them, derivative 32 exhibited potent QS inhibitory activity against *S. marcescens* (inhibited biofilm by 60% and inhibited prodigiosin by 68% at 1/2MICs). It is noted that the inhibitory activity of derivative 32 was significantly improved compared to that of IBI (inhibited biofilm by 46% and inhibited prodigiosin by 67% at 1/2MICs). Also, it is more potent than the classic QS inhibitor VAN (inhibited biofilm by 42% and inhibited prodigiosin by 58% at 1/2MICs). Based on the results of preliminary bioassays, derivative 32 was selected to further evaluate their QS inhibitory activity and targeting effect against *S. marcescens* by testing bacterial growth inhibition, biofilm formation, biofilm erasion, virulence factors such as...
swarming motility, and QS and the biofilm related genes. Further, the inhibitory activity of derivative 32 against the *C. violaceum* CV026 reporter strain was also investigated. More importantly, the toxicity of derivative 32 was evaluated on the mortality of *Tenebrio molitor* larvae. Besides, molecular docking and molecular dynamic simulations and energy calculations was also performed to obtain a closer insight into the anti-virulence and anti-biofilm forming potential of the derivative 32. Further mechanism of action study showed that derivative 32 inhibited *S. marcescens* through inhibiting the virulence factors and biofilm in *S. marcescens* in QS system. Moreover, derivative 32 downregulated the expressions of *S. marcescens* genes *bsmA, pigP, flhC, rssB, rsmA*, and *fimC* by 0.13-fold to 1.46-fold in QS system. Further, the synergistic effects of derivative 32 when combined with antimicrobials KAN against *S. marcescens* NJ01 were determined. And showed IMBI was also further investigated to confirm its partially restoring or improving anti-bacterial drug KAN sensitivity. The QSIs can competitively quench QS signaling systems, providing a new way for combating microbial infection. Since derivative 32 can partially restore or improve drug sensitivity, it could be helpful in clinical administration and may merit to be developed for clinical drugs against infection with anti-bacterial drugs related diseases in the future.
Materials and methods

Bacterial Strains

The bacterial strains used included the biomarker strains *C. violaceum* CV026, the plant pathogen *S. marcescens* NJ01 and the human pathogen *S. marcescens* 4547 in this study. Specifically, *C. violaceum* CV026 was received from the Guangdong Provincial Center for Microbial Strains (Guangzhou, China). The *S. marcescens* NJ01 was kindly provided by W. Wang (Nanjing Agricultural University, Nanjing, China). The *S. marcescens* 4547 strain was a kind gift from H. Huang (Haikou Municipal People's Hospital, China). All the strains were cultured at 28 °C, with Luria Bertani (LB) broth (pH 7.0) medium unless otherwise specified.

QS inhibitory screening of the 3-(2-isocyanobenzyl)-1H-indole derivatives

Total 35 3-(2-isocyanobenzyl)-1H-indole derivatives were investigated for their QS inhibitory activities. All the chemical data were performed. We initially evaluated the QS inhibitory activities of the 3-(2-isocyanobenzyl)-1H-indole derivatives against *S. marcescens* according to published methods (Kim et al. 2015). *C. violaceum* CV026 was used as a biomarker strain for the screening of QSI (Sybiya Vasantha Packiavathy et al. 2012). The pathogen strains including the biomarker strains *C. violaceum* CV026, the plant pathogen *S. marcescens* NJ01, and the human pathogen *S. marcescens* 4547 were all used as strains for the screening of QSIs.

Determination of MICs and growth curves

After confirming the QS inhibitory activities of 3-(2-isocyanobenzyl)-1H-indole derivatives, MICs of derivatives against *S. marcescens* were determined using the 2-
fold serially diluted method (Lee et al. 2013). In brief, 200 μL of compound was added to 96-well plates and cultured overnight at 28 °C. Absorbance was determined at OD$_{620}$.

**Formed biofilm erosion assay**

For biofilm erosion assay (Ramanathan et al. 2018), briefly, biofilm was cultivated in LB broth in 24-well plates at 28 °C overnight. Then, the biofilm was washed with PBS, and then added with fresh LB broth and IMBI. After culture overnight again, the biofilm was washed with PBS, then fixed with methanol, dyed with crystal violet, dissolved with ethanol, and eventually quantified by OD$_{570}$. For microscope analysis, as mentioned above, *S. marcescens* biofilms were cultivated in LB broth in 24-well plates with a round slide. Then, after fixation and dehydration, some biofilms were detected by SEM. The other biofilms were treated with AO/EB (1:1, v/v) and detected with CLSM.

**Molecular docking**

The three-dimensional structures of SmaR (Khayyat et al. 2021), RhII (PDB ID: 4NG2), RhlR(PDB ID: 7KGW), LasR (PDB ID: 3RKR), and CviR (PDB ID: 3QP5), were retrieved from the Protein Data Bank (PDB). These target proteins were individually docked with IMBI and their native ligands. After docking, the energy of the ligands docked to the proteins was minimized for MD analysis. The chemical structures of all ligands were constructed by using ChemDraw 15.1. Molecular docking was performed using the AutoDock program (Nam et al. 2020). Nacyl-L-homoserine lactones (AHLs), which are secreted by Gram-negative bacteria, serve as the main QS signaling molecules (Lee et al. 2013). C4 to C8-HSL synthesis is involved in the pathogenesis of *S. marcescens*, which mediates biofilm formation,
motility, and the production of extracellular components (Bakkiyaraj et al. 2012). The two QS systems are known to be hierarchically organized, with the las system controlling both rhlR and rhlI transcription (Ramanathan et al. 2018). LasR, a crucial component of the circuit, requires 3-oxo-C12-HSL for proper folding to control the entire QS system (Bakkiyaraj et al. 2012). The CviR protein, which is implicated in QS and biofilms, was molecularly docked with IMBI in light of the fact that C. violaceum CV026 was employed as a biomarker strain for the screening of QSI in the study (Chen et al. 2011). The IMBI and the co-crystallized natural ligand C4-HSL were docked into the active site of SmaR protein in the docking experiment (Khayyat et al. 2021).

Molecular dynamic studies on binding stability and interaction energy

On an Ubuntu (18.06) Linux operating system with the NVIDIA CUDA-supported GPU hybrid parallel computation platform, the Groningen Machine for Chemical Simulation (GROMACS, 2020.03) was used to perform molecular dynamic analyses. In the analysis, the Amber99SB force field was used. Each target protein was positioned in the center of a dodecahedron unit cell, with a spacing of 3.0 nm between the proteins and the edge. The cells were filled with H2O, then neutralized by replacing H2O with Na+ and Cl− with finally concentrations of 0.1 M. After a brief energy minimization and temperature and pressure equilibration, the analysis was carried out using the Amber99SB force field, which is optimized for ab-initio calculation of protein three-dimensional structure, and the TIP3P explicit water model. Each analysis has 50,000,000 time steps, generating 8 Gb data approximately. The root mean square deviation (RMSD) of the position of the heavy atoms in a ligand was analyzed to evaluate the binding stability of the ligand by equation (1):

\[
RMSD_{t_1,t_2} = \left[ \frac{1}{M} \sum_{i=1}^{N} m_i ||r_i(t_1) - r_i(t_2)||^2 \right]^{\frac{1}{2}}
\]  

where \( r \) is the position vector, \( t \) time, and \( M \) the mass of all atoms.
The interaction energy between a pair of atoms \((i,j)\) is the sum of Lennards-Jones and Coulombic potential between atom pairs by equation (2) and (3):

\[
V_{LJ(ij)} = \frac{C^{(12)}_{ij}}{r_{ij}^{12}} - \frac{C^{(6)}_{ij}}{r_{ij}^{6}}
\]

\[V_{C(ij)} = f \frac{q_i q_j}{\varepsilon r_{ij}}\]

where \(q\) is the elementary charge which equals to \(1.602176565 \times 10^{-19}\) C, and \(r\) the vector length, and \(f\) the electric conversion factor.

Mortality experiment of T. molitor larvae.

An overnight culture of \(S. marcescens\), and centrifuged. Through a 0.22 μm filter, filtered supernatant (2 μL) was injected into larvae with syringe needles. The larvae were kept in petri dishes at 25 °C for 3 days. The mortality of larvae was observed every day.

Biofilm erasure of IMBI combined with KAN.

The prepared culture medium was placed in a 24-well plate and cultured at 28°C for 24 h. The overnight culture was then removed, and the biofilm was washed three times with PBS. Both IMBI (0.39 μg/mL) with 0.15 μg/mL KAN were added to fresh LB broth and mixed well. The mixed medium was taken (1 mL) and added to a 24-well plate with culture at 28°C for 24 h. After incubation, the biofilm was processed and quantified according to the Biofilm Inhibition section.

Synthesis of 3-(2-isocyanobenzyl)-1H-indole derivatives
3-(2-isocyanobenzyl)-1H-indole derivatives used in this study were designed and synthesized as compounds targeting QS for anti-biofilm drug development (more data in support information).

320 **Statistical analysis**

322 All the results were reported as mean ± standard deviation and the statistical analyses were done using the GraphPad Prism. Dunnett’s one-way analysis of variance (one-way ANOVA test) was used to compare the differences among tests and controls.
Results

Chemistry

According to the possible structure-activity relationship (SAR), three parts (part A, part B, and part C) were divided (design strategy and leading compound in Scheme S3). The isocyano group in part A was essential to the activity. Comparing the indole ring, benzene ring and alkane in part B, indole ring was better than others. In addition, the electron withdrawing or electron-donating group on the indole ring, and changing the positions or numbers of these substituents were all taken into account. Moreover, indole N-H was protection by t-Butyloxy carbonyl group or alkyl substitution, and so on. In part C, phenyl and alkyl isocyano groups were introduced, then the electron withdrawing or electron-donating group on the benzene ring were introduced, and also changed the positions or numbers of these substituents that were all taken into account. So dominant skeleton derivatives were designed and screened. All synthesized 3-(2-isocyanobenzyl)-1H-indole derivatives used for testing QSI activities were listed in Scheme 1. Detail chemical identification of these 3-(2-isocyanobenzyl)-1H-indole derivatives and their QS inhibiting activities were presented in the supporting information.

QS inhibitory screening of the 3-(2-isocyanobenzyl)-1H-indole derivatives

In this study, we initially evaluated the effects of 3-(2-isocyanobenzyl)-1H-indole derivatives on QS inhibitory activities of C. violaceum CV026, S. marcescens NJ01, and S. marcescens 4547. Our results indicated that derivatives 11, 17, 25 and 32 exhibited well QS inhibitory activities against C. violaceum and S. marcescens (Fig. S1-S3). Next, we tested the MICs of all synthesized 3-(2-isocyanobenzyl)-1H-indole derivatives (Table S1) and tested the biofilm inhibition of these derivatives on S.
marcescens NJ01 and used VAN as a positive control. The results showed a concentration-dependent relation between the derivatives and biofilm inhibition in Fig. 2. Finally, IMBI showed the comprehensive highest inhibitory effect on S. marcescens NJ01 by 60% at 1/2MICs, 48% at 1/4MICs. Based on this finding, IMBI was used as the target compound for further research. Details of the QS inhibitory screening of the 3-(2-isocyanobenzyl)-1H-indole derivatives experiment results was shown in Table S2.

Prodigiosin inhibition effects of 3-(2-isocyanobenzyl)-1H-indole derivatives against S. marcescens

Prodigiosin is one vital virulence factors for QS system and play an important role in S. marcescens [7]. We tested prodigiosin inhibition by treatment with 3-(2-isocyanobenzyl)-1H-indole derivatives. As shown in Fig. 3, prodigiosin was significantly decreased after treatment with 3-(2-isocyanobenzyl)-1H-indole derivatives at 1/2MICs. Prodigiosin inhibition of derivatives 4, 6, 7, 13, 17, 18, 21, 24, 31, and 32 were better than the positive control VAN (58%), especially derivatives 4, 7, and 32 (67%, 66%, 68%, respectively). Details of the QS inhibitory for prodigiosin inhibition effects of 3-(2-isocyanobenzyl)-1H-indole derivatives against S. marcescens experiment results were shown in Table S3.

Biofilm inhibition effects of 3-(2-isocyanobenzyl)-1H-indole derivatives against S. marcescens and SAR analysis

Next, we investigated the inhibitory effects of synthesized 35 3-(2-isocyanobenzyl)-1H-indole derivatives on S. marcescens biofilm formation at 1/4 MICs (Fig. 2), and the potential SAR analysis were carried out.

(1)Based on the contributions of investigators (Sethupathy et al. 2020), we
hypothesize that the isocyano is essential for biofilm inhibition activity. Derivatives 5 (34%), with amino group substitution, absence of isocyano structure, the analog showed lower inhibition effects comparing to the lead derivative 4 (38%). This finding confirmed our hypothesis, that is, the isocyano group in part A was essential for the biofilm activity. In addition, tryptamine-derived isocyanides, derivative 1 (9%), 2 (6%), 3 (13%), with alkyl substituent on isocyano groups, showed lower inhibition effects, comparing to phenyl substituent of derivative 4 (38%), so phenyl substituent on the isocyano maybe appropriate. Focus on 3-(2-isocyanobenzyl)-1H-indole skeleton maybe desirable in the next study. By above analysis indicated that more modifying work in part B or C should be done next.

(2) Next, from the results of derivatives 6 (27%), 7 (35%), and derivative 4 (38%), bearing the different substituents: derivatives substituted by the indole ring derivative 4 (38%), benzene ring derivative 7 (35%) and alkane derivative 6 (27%) in part B, indicated indole ring was advisable. Based on this, continued to explore in part B for indole ring. In part B, bearing the different substituents in indole ring: derivatives substituted by the electron withdrawing or electron-donating group on the indole ring, and changed the positions or numbers of these substituents. Such as methyl, methoxy, halogen, phenyl, t-Butyloxy carbonyl group, and so on. Specifically, Derivative 8 (40%) substituent with 6-methoxy in indole ring, was comparable to unsubstituted derivatives of indole ring derivative 4. Derivative 18 (52%) substituent with 3- methyl in indole ring was higher biofilm inhibition to unsubstituted derivatives of indole ring derivative 4. Comparing to the derivatives bearing the different substituents in indole ring in part B, such as derivative 10 (33%), 11 (19%) and so on, all of the results, derivatives 18 and 8 show that the inhibition activity of biofilm for 3-methoxy or 6-methyl in indole ring structure for 3-(2-isocyanobenzyl)-1H-indole derivatives were higher than that of the lead compound 4, and the others all lower, which preliminary confirmed that 3-(2-isocyanobenzyl)-1H-indole maybe a dominant skeleton. In addition, indole ring N-H maybe important for biofilm activity, so we altered indole
N-H was protective by t-Butyloxy carbonyl group derivative 20 (27%) and derivative 21 (39%), and alkane substitution, such as derivative 22 (28%) with N-methyl substituents. So indicated indole ring N-H free was important for biofilm activity which confirmed that 3-(2-isocyanobenzyl)-1H-indole is a dominant skeleton.

Finally, to obtain more highly active derivatives, we explored the influence of substituents on the activity of benzene ring in part C. Comparing with the results of derivatives 23-33, different substitution on the benzene ring. Through derivatives 23 (31%), 26 (31%), 28 (20%), 32 (48%), were all methyl group substituted, found 16-Me may be a dominant position. By the way, derivative 29 (42%), the 15-Br-substitution on the benzene ring is higher than that substituents with methyl, fluorine or chlorine, which was the best highly active derivative of 15-substitution on the benzene ring. Noteworthily, derivative 32 (48%), the 16-Me-substitution on the benzene ring is higher than that of the position in the 13-, 14-, and 15- positions, which was also the best highly active derivative of 16-substitution on the benzene ring. Furthermore, in part C, from the results of derivatives 28 (38%), 32 (48%), and 29 (42%), bearing the different substituents on benzene ring suggested that the order of inhibition is 16-Me>15-Br>15-F=lead compound. Other derivatives, such as derivative 24 (30%), bearing the different substituents on benzene ring, have lower inhibition effects. So, 16-methyl-substitution of benzene maybe a dominant structure.

Overall, SAR analysis indicated that the biofilms inhibition effects of 3-(2-isocyanobenzyl)-1H-indole derivatives are related to part A, B, and C. Specifically, for part A, the isocyano group was essential to the activity, and phenyl substituent of on the isocyano was appropriate. For part B, indole ring is more suitable for biofilm inhibitory activities of 3-(2-isocyanobenzyl)-1H-indole derivatives, and 7-Me-substitution and 4-OMe-substitution of indole rings have stronger activity. For part C, 15-Br substitution, 15-F substitution, and 16-methyl-substitution of benzene was stronger activity. Finally, derivative 18 (52%), with 3- methyl in indole ring structure
for in part B, derivative 32 (48%), the 16-Me-substitution on the benzene ring in part C, were the best for biofilm inhibition effects of 3-(2-isocyanobenzyl)-1H-indole derivatives against \( S.\ marcescens \). Combined with the prodigiosin inhibition effects of 3-(2-isocyanobenzyl)-1H-indole derivatives against \( S.\ marcescens \), derivative 32 was the best candidate in the study. Therefore, more in-depth research on derivative 32 on biofilm inhibition was conducted in the next.

**Growth profile**

The MIC of IMBI was determined to be 12 μg/mL. The growth curves were drawn to identify the influence on the growth of \( S.\ marcescens\) NJ01. As shown in Fig. S4, IMBI had no inhibition effect on growth of \( S.\ marcescens\) NJ01 at the concentrations ranging from 0.39 to 1.56 μg/mL and 1.56 to 3.12 μg/mL.

**IMBI suppressed biofilm formation**

The inhibitory effects of IMBI on biofilm formation was analyzed by crystal violet assay. The IMBI at 0.39, 0.78, and 1.56 μg/mL significant inhibit biofilms by 10%, 17%, and 42%, respectively (Fig. 4A). IMBI treatment showed a remarkably decreased compared to the DMSO control. Furthermore, IMBI showed a suppressed biofilm formation of \( S.\ marcescens\) 4547, reduced biofilms by 8%, 13%, and 23%, respectively (Fig. 4B). The inhibitory potential of IMBI against biofilm was evaluated by SEM (Fig. 4C) and CLSM (Fig. 4D). SEM images of the blank DMSO group biofilm was a dense net-structured system connected by fibrous structures. Then, IMBI treatment at 1.56 μg/mL, biofilm was remarkably decreased. CLSM images showed that biofilm biomass was also remarkably decreased, and scattered appearance was presented after treatment with 1.56 μg/mL IMBI.
Erasion of formed biofilms

The erasing effect of IMBI on formed biofilms was determined through crystal violet assay. The IMBI at 0.39, 0.78, and 1.56 μg/mL significant reduced formed biofilms by 17%, 27%, and 40%, respectively (Fig. 5A). Furthermore, IMBI showed a suppressed biofilm formation of *S. marcescens* 4547, reduced biofilms by 23%, 27%, and 35%, respectively (Fig. 5B). Biofilms were remarkably reduced after IMBI treatment. The scattered appearance and disrupted the integrity of the formed biofilms after treatment with IMBI at 1.56 μg/mL, was detected through SEM (Fig. 5C) and CLSM (Fig. 5D) images.

IMBI suppressed virulence factors

At firstly, IMBI was determined to suppress *S. marcescens* NJ01 virulence factors at 0.39, 0.78, and 1.56 μg/mL. Unfortunately, the results of effect for virulence factors were less than ideal (detail see the Fig. S5), so we explored virulence factors which were changed at 1.56, 2.34, 3.12 μg/mL. And IMBI had no inhibition effect on growth of *S. marcescens* NJ01 at the concentrations ranging from 1.56 to 3.12 μg/mL (Fig. S4-C). So IMBI was determined to suppress *S. marcescens* NJ01 virulence factors (Fig. 6). More detail suppress *S. marcescens* NJ01 virulence factors were presented in the supporting information. (1) Effects of IMBI on protease production, Protease, a key virulence factor controlled by QS system, can affect host immune responses (Sethupathy et al. 2017). Exposure to IMBI at 3.12 μg/mL resulted in a 17% inhibition in protease activity compared to the negative control. This is comparable to the positive control VAN (6%) (Fig. 6A). (2) Effects of IMBI on EPS production. EPS is a vital ingredient in biofilms and play important roles in maintaining cohesion, obtaining nutrition, and blocking entry of antimicrobial agents into cells (Padmavathi et al. 2014). The results showed a remarkable decreased production of EPS with IMBI...
treatment. At 3.12 μg/mL, EPS decreased by 51%. Then at 3.12 μg/mL with VAN, only 10% reduction in EPS (Fig. 6B). (3) Effects of IMBI on prodigiosin. Prodigiosin is a prominent red pigment produced by *S. marcescens* (Liu and Nizet 2009). The synthesis of prodigiosin is regulated by QS system. At 3.12 μg/mL IMBI suppressed the prodigiosin activity by 26%, which was more potent than that of VAN (12%) (Fig. 6C). (4) Effects of IMBI on hemolysin production. Hemolysin is another well-studied virulence factor secreted by *S. marcescens* NJ01. At 3.12 μg/mL, IMBI inhibited hemolysin by 26% compared to 17% by VAN (Fig. 6D). (5) Effects of IMBI on lipase production. Lipolytic enzymes are involved in degrading the phospholipid bilayer and mediating cell signaling pathways of the host (Sethupathy et al. 2017). In this study, lipase was induced by 23% and 8% at 3.12 μg/mL IMBI and VAN treatments, respectively (Fig. 6E). (6) Swarming motility and swimming motility. We also investigated IMBI on swarming motility, and found a similar result compared with the prodigiosin in *S. marcescens* (Fig. S5-E).

**Expression of QS and biofilm related genes**

The qRT-PCR was carried out to investigate the effects of IMBI on QS-mediated genes *bsmA, pigP, flhC, rssB, rsmA*, and *fimC* in *S. marcescens* NJ01 (Fig. 7). The genes *flhC, rsmA, fimC* and *rssB*, that were relevance with motility, fimbria production and adherence were down-regulated by 0.49-, 0.13, 0.59 and 1.01-fold, respectively. The genes *bsmA* responsible for biofilm formation were inhibited by 1.46-fold, and the gene *pigP* involved in the biosynthesis of prodigiosin and hemolysin were down-regulated by 1.19-fold. The *rplU* gene was the internal control (Srinivasan et al. 2017) The primers are shown in Table S4.

**Molecular docking**
To obtain a closer insight into the anti-virulence and anti-biofilm forming potential of IMBI, molecular docking was performed. In consideration of the *C. violaceum* CV026 was used as biomarker strain for the screening of QSI in the study, molecular docking of IMBI was performed for *C. violaceum* at first. CviR is an important receptor of *C. violaceum* CV026 (Chen et al. 2011), and ligand-binding domain (LBD) and DNA-binding domain (DBD) are two important domains of CviR in QS system (Ahmed et al. 2013). Specifically, the benzene ring of isocyano group of IMBI (part C) interacts with Try88 (3.81 Å) formed π-π interaction, with Try88 (3.60 Å) formed H-π interaction (Fig. S6 E). The methylene of IMBI with Try80 (4.69 Å) formed H-π interaction (linked by 6-ring structure formed by amino residues), with Asp97 (2.93 Å) formed H-bond of CviR (Fig. S6 E). The indole benzene ring of IMBI (part B) interacts with Phe126 (4.47 Å) formed H-π interaction of CviR (Fig. S6 E). And the isocyano group of IMBI (part A) formed H-bond with the amino acid residue Asp97 (2.82 Å) of CviR (Fig. S6 E).

SmaR is a transcriptional activator of *S. marcescens* QS systems. The isocyano group of IMBI formed H-bond with the amino acid residue Asn49 (3.14 Å) of SmaR (Fig. S6 A). The 6-ring structure formed by amino residues with methylene group of IMBI interacts with Try57 (3.96 Å) of SmaR formed H-π interaction (Fig. S6 A). RhII is also a transcription regulator in *S. marcescens* QS systems. The isocyano group of IMBI formed H-bond with the amino acid residue Try47 (3.29 Å) of RhII. The indole 5-ring interacts with Gly38 (4.61 Å) and Try56 (3.70 Å) of RhII formed H-π interaction, respectively (Fig. S6 C). The benzene ring of isocyano group interacts with Try56 (4.17 Å) of RhII formed H-π interaction (Fig. S6 C). The indole N-H of IMBI formed H-bond with the amino acid residue Try47 (2.71 Å) of RhII (Fig. S6 C).

RhII is a transcription regulator in *S. marcescens* QS systems where binding of butanoyl-homoserine lactone with the regulator activates the certain virulent gene.
expression. The indole N-H of IMBI formed H-bond with the amino acid residue Asp178 (2.61 Å) of RhlR (Fig. S6 B) and the indole 5-ring interacts with His221 (4.58 Å) of RhlR formed H-π interaction (Fig. S6 B). LasR is a transcriptional activator of numerous genes of S. marcescens QS systems. The benzene ring of isocyano group of IMBI formed H-π bonds with the amino acid residue Arg16 (4.47 Å), Ala37 (4.33 Å), Ala91 (4.37 Å) of LasR (Fig. S6 D). The C13 of IMBI formed H-bond with the amino acid residue Gly13 (3.24 Å) of LasR (Fig. S6 D). The indole benzene ring interacts with Gly92 (4.05 Å, 4.29 Å) to formed H-π interaction (Fig. S6 D). And the indole N-H of IMBI formed H-bond with the amino acid residue Gly13 (2.87 Å) of LasR (Fig. S6 D). Then, the isocyano group of IMBI formed H-bond with the amino acid residue Cys62 (3.05 Å), Asn90 (3.15 Å), Arg16 (3.42 Å) of LasR (Fig. S6 D). Finally, methylene group of IMBI interacts with Asn90 (3.11 Å) formed H-bond interaction of SmaR (Fig. S6 D).

Overall, blocking AHL binding sites by IMBI is considered to be an effective strategy against transcription factors SmaR, RhlI, RhlR, LasR and CviR. The results showed that the combination of IMBI with SmaR, RhlI, RhlR, LasR and CviR might antagonise the expression of QS linked traits. And the interaction between IMBI and pilus protein may also be a possible mechanism to inhibit the testing of bacterial biofilm from above analysis.

**Molecular Dynamic Simulations and Energy Calculations**

To determine the binding stability, the complexes of IMBI with different target proteins of QS and biofilms were further studied using MD simulations. Five proteins (SmaR, RhlI, RhlR, LasR and CviR) of different targets were selected as initial conformations for MD simulation studies. The RMSD and interaction potential energy (E_{iap}), which is the sum of the Lennards-Jones potential and the Columbic energy,
were calculated as functions of time. The RMSD of the position of the heavy elements of the ligands bound to the proteins was calculated to assess the validity of the binding of the ligands to target proteins (Fig. S7). The RMSD of most ligands binding to proteins was less than 0.25 nm. For instance, the RMSD of SmaR-IMBI complex was found to be 0.09 ± 0.029 (Fig. S7-A). Following a brief initial equilibrium, the RMSD held steady in all conditions, demonstrating a stable binding of the ligands to the target proteins. The $E_{iap}$ between proteins and ligands ranged from -80 to -180 kcal/mol (Fig. 8). For instance, the $E_{iap}$ of SmaR-IMBI complex was found to be -147.4±8.766 kcal/mol (Fig. 8-A). In all situations, the $E_{iap}$ also attained equilibrium and became stable after a few picoseconds.

Cytotoxicity

Encouraged by the results of in vitro experiments, we investigated the effect of IMBI on the mortality of Tenebrio molitor larvae. The larvae injected with S. marcescens NJ01 started to die at the beginning of incubation and almost 90% of them died in the 3rd incubation period. The survival rate made a highly improvement of IMBI -treated larvae, with probably 50% larvae survived. In addition, when the larvae were treated with IMBI at 0.39 - 1.56 μg/mL, their mortality was increased depending on the IMBI concentration, emphasizing the key role of $shlA$ may be in the production of the virulence factor. More details of the T. molitor experiment results can be found in the Fig. S8-S9. On account of in vitro and in vivo results, we guess that the pure $shlA$ antagonist IMBI inhibits prodigiosin and hemolysin production by down-regulation of virulence factor production gene expression (e.g., $pigA$, $pigC$ and $shlA$ genes), as described in a working model (Srinivasan et al. 2017).

Effects of IMBI and anti-bacterial drugs acceleration on biofilms
To assess whether IMBI could increase biofilm sensitivity to anti-bacterial drugs, the *S. marcescens* NJ01 biofilms were grown in the presence of IMBI (0.39 μg/mL), or DMSO (negative control) and then treated with KAN (0.15 μg/mL) in 24 h for biofilm inhibition assay. Interestingly, IMBI enhanced the impact of KAN on biofilm inhibition. As quantified by OD$_{570}$, the biofilms were markedly reduced by 50% under IMBI (0.39 μg/mL) with 0.15 μg/mL KAN (Fig. 9A). At the same time, the biofilms were slightly reduced by 15%, 5% under only IMBI (0.39 μg/mL), and KAN (0.15 μg/mL) respectively (Fig. 9A). Next, to assess whether IMBI could increase erasion biofilm to anti-bacterial drugs, the *S. marcescens* NJ01 biofilms were grown in the presence of IMBI (0.39 μg/mL), or DMSO (negative control) for 24 h, and then treated with KAN (0.15 μg/mL) for another 24 h. Interestingly, IMBI enhanced the impact of KAN on biofilm biomass. As quantified by OD$_{570}$, the biofilms were markedly reduced by 44% under IMBI (0.39 μg/mL) with 0.15 μg/mL KAN (Fig. 9B). At the same time, the biofilms were slightly reduced by 16%, 6% under only IMBI (0.39 μg/mL), and KAN (0.15 μg/mL) respectively (Fig. 9B). Under the inhibition of IMBI, the biofilms were thinner and more fragile, allowing KAN to more easily penetrate and kill the bacteria. So bacterial sensitivity to KAN was partially recovered and the decrease in the biofilms was a direct reflection of a decrease in biomass (Fig. 9). Thus, when IMBI were combined with KAN, the mature biofilms were effectively destroyed. So, a bacterial biofilms inhibition and erosion assay using a combination of IMBI and KAN demonstrated that the drug combination (which we name I-K here) could significantly enhance the inhibitory effect of KAN on the growth of *S. marcescens* NJ01 strains in a concentration-dependent manner.
Discussion

Due to the massive use and abuse of anti-bacterial drugs, AMR has become a major threat to public health and economic development (Larsson and Flach 2022). Traditional antibacterial drugs usually interfere with the efflux pump system, DNA replication, protein synthesis, or bacterial cell wall biosynthesis (Zhao et al. 2020), (Bai et al. 2023). Therefore, new targets are needed to treat these bacterial infections.

The QS system is a bacterial communication mechanism that can regulate the formation of bacterial resistance (Liu et al. 2022), biofilm formation (Li et al. 2022), and virulence factors (Defoirdt 2018). In addition, biofilms formed by many pathogens also lead to strong bacterial resistance (Rajput et al. 2018). The QS can reduce anti-bacterial drugs resistance by regulating biofilm formation (Zhao et al. 2020). Therefore, QS inhibition can be developed a new antibacterial strategy and recognized as a novel drug target for infections.

In addition, these virulence factors are not necessary for the growth and survival of bacteria. Therefore, centralized treatment will not inhibit the growth of bacteria and generate selective pressure. It can effectively reduce the development of bacterial resistance (Sully et al. 2014). The progress of QS research could lead to the development of new antibacterial compounds with new modes of action, possibly providing a new strategy against antimicrobial resistance. The bacterial SmaR protein plays a key role in the QS process of S. marcescens and is an effective target (Khayyat et al. 2021). In the present study, the SmaR protein was used as a target. The new structural inhibitors inhibiting QS that were obtained through QS activities plate screening of reporter strain C. violaceum CV026.

Based on our continuing research on QSIs, we identified 3-(2-isocyanobenzyl)-1H-indole derivatives, as a new class of potential QSIs of S. marcescens. Besides, S.
marcescens can secrete multiple extracellular virulence factors, and most importantly form biofilms (Brackman et al. 2011). And biofilms are protective barriers adopted by bacteria against the host immune system and antibacterial agents. They are exopolysaccharide protein polymers secreted by bacteria that attach themselves to membranes on abiotic or biotic surfaces. So, we used biofilm inhibition rates to screen of indol-isonitrile derivatives against S. marcescens initially. To improve QS inhibitory activity against S. marcescens and based on QS inhibitory screening strategies including anti-biofilm formation activities. Biofilm inhibition rates was performed at 1/2MICs and 1/4MICs to investigate the effect on biofilm inhibition (Fig. 2). At 1/2MICs most of the 3-(2-isocyanobenzyl)-1H-indole derivatives inhibited biofilm formation against S. marcescens NJ01. However, at 1/4MICs the inhibition rates declined, even so, IMBI exhibited excellent effect on S. marcescens NJ01 at 1/4MICs (48%). Moreover, designing indol-isonitrile derivatives to inhibit prodigiosin, one vital virulent factor of S. marcescens, was also discussed. Prodigiosin is a prominent red pigment produced by S. marcescens and is essential for its invasion, survival, and pathogenicity (Liu and Nizet 2009). As one vital virulent factor regulated by QS, prodigiosin plays a critical role in pathogenicity. It is a representative virulence factor against S. marcescens and the synthesis of itself is also regulated by QS. So, we used prodigiosin inhibition rates to evaluate the QSI and anti-biofilm of indol-isonitrile derivatives against S. marcescens. According to similar work reported (Chen et al. 2023). Our results indicated that IMBI could inhibit prodigiosin production dramatically, that is to say, it may be a potent QS inhibition activity.

In addition, the SAR analysis revealed that derivatives with 3-(2-isocyanobenzyl)-1H-indole as backbone is in general more potential than those with benzene ring or alkane core in part B. Moreover, in part C, phenyl substituent is more appropriate than alkyl in isocyano group. So, 3-(2-isocyanobenzyl)-1H-indole may be a dominant structure.
in biofilm inhibiting against *S. marcescens* specifically. This structure information emphasizes that the 1H-indole and 3-2-isocyanobenzyl should be two vital chemical factors. Comparing with all 35 3-(2-isocyanobenzyl)-1H-indole derivatives, IMBI shows an excellent QSI and anti-biofilm agent against *S. marcescens*. Considering its significant role in inhibiting prodigiosin production at sub-MICs, IMBI showed the most promising potency in QS inhibition against *S. marcescens* (Fig. 3). And combined with the biofilm inhibition effects of 3-(2-isocyanobenzyl)-1H-indole derivatives against *S. marcescens*. IMBI was the best candidate in the study.

Furthermore, the potential QSI activities of IMBI were further evidenced by suppressed QS related virulence factors of *S. marcescens*, such as prodigiosin, lipase, protease and swarming motility (Fig. 6). In this study, the potential QSI activities of IMBI were evidenced by downregulation of QS-related genes such as related genes *bsmA*, *pigP*, *flhC*, *rssB*, *rsmA*, and *fimC* in *S. marcescens* NJ01 (Fig. 7), two important virulence factors, hemolysin and prodigiosin (Liu and Nizet 2009), which all play a critical role in host invasion and pathogenicity in *S. marcescens* QS system were suppressed dramatically by IMBI and accordingly with the downregulation of the related genes *pigP*. And the IMBI also suppressed lipase production and extracellular protease by *S. marcescens* NJ01, which play important roles in cytolytic activities and host infection respectively (Sybiya Vasantha Pakiavathy et al. 2012). Swarming plays a key role in pathogen colonization, including initial attachment and biofilm formation (Lee et al. 2013). Swarming motility mediated by flagella and dominated by *flhC*, and benefit for forming biofilm. Moreover, the gene *fimC* regulated fimbriae subunits, remarkable for adhesin attachment and colonization by *S. marcescens* (Lee et al. 2011). The gene *bsmA* dominate biofilm formation in *S. marcescens* (Labbate et al. 2004). Here, exposure to IMBI, and down-regulation of relevant genes were coincident for the inhibition in the biofilm formation, attachment capacity, and swarming motility (Fig. 7). Biofilms can be formed on both biotic or abiotic surfaces
and are highly structured by bacterial populations that are attached to a surface or embedded in a scaffold of self-produced extracellular matrix (Solano et al. 2014). As an important component of biofilms (Jayathilake et al. 2017), EPS is significantly suppressed after exposure to IMBI up to 51% at 3.12 μg/mL (Fig. 6E). In addition, molecular docking of IMBI with the target protein (Fig. S6) showed a closer insight between anti-virulence, anti-biofilm and IMBI in QS system.

Using QSI is an important approach to attenuate S. marcescens pathogenicity and reduce the probability of development of resistant S. marcescens strains. Bacteria change phenotypically in response to their environment. Free-swimming cells transition to biofilm communities that promote cellular cooperativity and resistance to stressors and anti-bacterial drugs. Biofilms are the first barrier to antimicrobial drugs entering cells and are one of the main causes of pathogenetic microbial drug resistance (Dodson et al. 2022). After a S. marcescens biofilm forms, drug resistance is unavoidable (Liu et al. 2020). In the study, IMBI inhibit biofilm formation (Fig. 4) and erase the formed biofilms (Fig. 5) against S. marcescens NJ01 by 42% and 41% at 1.56 μg/mL respectively (Fig. 4 and 5). With other strains such as S. marcescens 4547, IMBI can also demonstrated adorable inhibiting activities. The effect of IMBI on biofilm erasion was further confirmed by SEM (Fig. 5C) and CLSM (Fig. 5D) images. These assays showed significant biofilm inhibitory and erasion effects of IMBI at each concentration level. IMBI maybe a good choice for resolving drug resistance used the new antibacterial strategy by QS. Besides, one main promising to QSI development will be to demonstrate that their combination therapy with anti-bacterial drugs has a clear clinical benefit (Soukarieh et al. 2018). The ability of nosocomial pathogens to form biofilms is of significant clinical interest, since biofilm formation influences the efficacy of antimicrobial therapy and the outcome of an infection. Moreover, biofilm formation may contribute to the establishment and long-term survival of bacterial pathogens in the hospital environment. Traditional detection
methods of combination therapy based on MICs and an MIC checkerboard assay of anti-bacterial drugs can be used to evaluate the coadministration of anti-virulence drugs and anti-bacterial drugs. To be enjoyed, our results showed the outstanding effect of IMBI in erosion of formed biofilms against *S. marcescens* NJ01 by 50% at 0.39 μg/mL, only KAN (0.15 μg/) by 5% (Fig. 9).

The combination of a QS inhibitor and a conventional anti-bacterial drug is known as a promising approach for eradicating preformed biofilms and decreasing the magnitude of the infection (Xu et al. 2022). Here, our results showed I-K could reduce the use of anti-bacterial drugs and, consequently, attenuate the risk of anti-bacterial drugs resistance.
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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at
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Graphical abstract

35 3-(2-isocyano benzyl) IH-indole analog candidates

Inhibitory on biofilm formation (60%)
Synergistic effects on biofilms inhibition (50%)

Concentration(µg/mL)
Fig. 1 Representatives of natural products and synthetic bioactive candidates for QSIs.

3-methoxycinnamic acid  4-dimethylaminocinnamic acid  (E)-3-phenylprop-2-en-1-amine  IMBI
Scheme 1. Synthesized 3-(2-isocyanobenzyl)-1H-indole derivatives for QS inhibiting evaluation against S. marcescens.

Conditions: I. LiAlH$_4$, THF, 0 ℃ - r.t. overnight. II. TFA, DCE, 50 ℃, overnight. III. HCOOH, Ac$_2$O, THF, r.t. IV. POCl$_3$, TEA, DCM, 0 ℃ - r.t., gave the products e.
Fig. 2 Effects of 3-(2-isocyanobenzyl)-1H-indole analogs candidates on biofilm inhibition on *S. marcescens* NJ01 at 1/2 MICs and 1/4 MICs. Each experiment was performed at least three times in triplicate and, each time, at least 400 cells were counted for each treatment. Compounds were dissolved in DMSO, and the amount of DMSO used as the solvent for the compounds, and the VAN was used as a control. Data represent the means and the standard deviations of three independent experiments. Statistical differences were determined by one-way ANOVA test. * p < 0.05, ** p < 0.01, and *** p < 0.001 vs. the VAN control. ns: no difference.
Fig. 3 Effects of 3-(2-isocyanobenzyl)-1H-indole derivatives on prodigiosin inhibition rates of *S. marcescens* NJ01 at 1/2MICs. Each experiment was performed at least three times in triplicate and, each time, at least 400 cells were counted for each treatment. Compounds were dissolved in DMSO, and the amount of DMSO used as the solvent for the compounds was used as a control. Data represent the means the standard deviations of three independent experiments. VAN served as the positive control. Statistical differences were determined by one-way ANOVA test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the DMSO control.
Fig. 4 Inhibitory effects of IMBI on biofilm formation of *S. marcescens*. (A) Inhibitory effect of IMBI on *S. marcescens* NJ01 biofilm formation. DMSO was used as negative control. (B) Inhibitory effect of IMBI on *S. marcescens* 4547 biofilm formation. DMSO was used as negative control. Statistical differences were determined by one-way ANOVA test. * \( p < 0.05 \), ** \( p < 0.01 \), and *** \( p < 0.001 \) vs. the DMSO control. (C) SEM images of *S. marcescens* NJ01 treated with (a) DMSO, (b) 0.39 μg/mL, (c) 0.78 μg/mL, and (d) 1.56 μg/mL of IMBI. (D) CLSM images of *S. marcescens* NJ01 treated with (a) DMSO, (b) 0.39 μg/mL, (c) 0.78 μg/mL, and (d) 1.56 μg/mL of IMBI.
Fig. 5 Erosion effect of IMBI on the formed biofilms of *S. marcescens*. (A) Erosion effect of IMBI on *S. marcescens* NJ01 formed biofilm. DMSO was used as a negative control. (B) Erosion effect of IMBI on *S. marcescens* 4547 formed biofilm. DMSO was used as negative control. Statistical difference was determined by one-way ANOVA test. *p* < 0.05, **p** < 0.01, and ***p*** < 0.001 vs. the DMSO control. (C) SEM images of *S. marcescens* NJ01 treated with (a) DMSO, (b) 0.39 μg/mL, (c) 0.78 μg/mL, and (d) 1.56 μg/mL of IMBI. (D) CLSM images of *S. marcescens* NJ01 treated with (a) DMSO, (b) 0.39 μg/mL, (c) 0.78 μg/mL, and (d) 1.56 μg/mL of IMBI.
Fig. 6 Inhibitory effects of IMBI on virulence factor production of *S. marcescens* NJ01. (A) Protease; (B) Extracellular polysaccharides (EPS); (C) Prodigiosin; (D) Hemolysin; (E) Lipase; VAN as
positive control, and DMSO as negative control. Statistical difference was determined by one-way ANOVA test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. DMSO control.

Fig. 7 Relative fold difference in the expression of QS and the biofilm related genes of S. marcescens NJ01 after being treated with IMBI. The results are expressed as means ± SD (n = 3). Statistical difference was determined by one-way ANOVA test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the rplU.
Fig. 8 E$_{iap}$ profiles. Molecular dynamic simulations (MD) studies for target proteins bound with IMBI. Time-dependent plots for E$_{iap}$ of atomic positions, IMBI for the H-bonds, H-π interaction, and π-π interaction with target proteins. (A) SmaR; (B) CviR; (C) RhlI; (D) RhlR; and (E) LasR.
Fig. 9 Enhanced effects of IMBI in combination with kanamycin (KAN) on inhibition(A) and erosion(B) of biofilms. Quantification of biofilms with IMBI using a crystal violet staining. Erasure effect of IMBI combined with KAN on *S. marcescens* NJ01 formed biofilm. DMSO was used as a negative control. Statistical difference was determined by one-way ANOVA test. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. DMSO control.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementarydataNC0330.pdf