



Ibuprofen-mediated potential inhibition of biofilm development and quorum sensing in *Pseudomonas aeruginosa*

Lu Dai^{a,g}, Tian-qi Wu^b, Yi-song Xiong^c, Hong-bing Ni^d, Ye Ding^a, Wen-chen Zhang^e, Shao-peng Chu^a, Shao-qing Ju^a, Juan Yu^{a,f,*}

^a Department of Laboratory Medicine, Affiliated Hospital of Nantong University, 20 Xi Si Road, Nantong, 226001, PR China

^b Glenelg Country School, 12793 Folly Quarter Rd, Ellicott City, MD, 21042, USA

^c Department of Laboratory Medicine, Chengdu Military General Hospital, 270 Tian Hui Road, Chengdu, 610000, PR China

^d Department of Business and External Cooperation, Affiliated Hospital of Nantong University, 20 Xi Si Road, Nantong, 226001, PR China

^e Department of Clinical Laboratory, Wuxi Children's Hospital, No. 299 at Qingyang Road, Wuxi, 214023, PR China

^f Institute of Public Health, Nantong University, 9 Se Yuan Road, Nantong, 226001, PR China

^g Department of Laboratory Medicine, Kunshan affiliated Hospital of Nanjing University of Chinese Medicine, 189 Chao Yang Road, Kunshan, 215300, PR China

ARTICLE INFO

Keywords:

Pseudomonas aeruginosa
Ibuprofen
Quorum sensing
Anti-QS activity
N-acyl homoserine lactones

ABSTRACT

Aims: *Pseudomonas aeruginosa* is one of the leading causes of opportunistic and hospital-acquired infections worldwide, which is frequently linked with clinical treatment difficulties. Ibuprofen, a widely used non-steroidal anti-inflammatory drug, has been previously reported to exert antimicrobial activity with the specific mechanism. We hypothesized that inhibition of *P. aeruginosa* with ibuprofen is involved in the quorum sensing (QS) systems.

Main methods: CFU was utilized to assess the growth condition of *P. aeruginosa*. Crystal violet staining and acridine orange staining was used to evaluate the biofilm formation and adherence activity. The detection of QS virulence factors such as pyocyanin, elastase, protease, and rhamnolipids were applied to investigate the anti-QS activity of ibuprofen against *P. aeruginosa*. The production of 3-oxo-C₁₂-HSL and C₄-HSL was confirmed by liquid chromatography/mass spectrometry analysis. qRT-PCR was used to identify the QS-related gene expression. Furthermore, we explored the binding effects between ibuprofen and QS-associated proteins with molecular docking.

Key findings: Ibuprofen inhibits *P. aeruginosa* biofilm formation and adherence activity. And the inhibitory effects of ibuprofen on C₄-HSL levels were concentration-dependent ($p < 0.05$), while it has no effect on 3-oxo-C₁₂-HSL. Moreover, ibuprofen attenuates the production of virulence factors in *P. aeruginosa* ($p < 0.05$). In addition, the genes of QS system were decreased after the ibuprofen treatment ($p < 0.05$). Of note, ibuprofen was binding with LuxR, LasR, LasI, and RhlR at high binding scores.

Significance: The antibiofilm and anti-QS activity of ibuprofen suggest that it can be a candidate drug for the treatment of clinical infections with *P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa, the most common Gram-negative non-fermentative bacteria, is one of the leading causes of opportunistic and hospital-acquired infections worldwide [1]. The infection can cause serious complications in patients with burns, cystic fibrosis, or in immunocompromised patients with respiratory infections, sepsis, osteomyelitis, endocarditis, and urinary tract infections (UTIs) [2]. The infection with *P. aeruginosa* has been linked with difficulties in clinical treatment because of significant resistance of the bacterium to

antibiotics [3], which is further aggravated by the worldwide abuse of antibiotics. Specifically, every year approximately 700,000 people die from antibiotic resistance worldwide, which can likely increase to 10 million by 2050 if no action is taken to reduce drug resistance or to develop new antibiotics. In 2017, the World Health Organization (WHO) published a list of drug-resistant bacteria threatening the lives of people which critically requires new antibiotics. *P. aeruginosa* was listed second on the WHO list [4]. Therefore, there is an imminent need to develop alternative strategies to treat *P. aeruginosa* infections.

P. aeruginosa possesses complex mechanisms, wherein quorum

* Corresponding author. Department of Laboratory Medicine, Affiliated Hospital of Nantong University, 20 Xi Si Road, Nantong, 226001, PR China.
E-mail address: yujuanjs@163.com (J. Yu).

<https://doi.org/10.1016/j.lfs.2019.116947>

Received 21 July 2019; Received in revised form 28 September 2019; Accepted 8 October 2019

Available online 09 October 2019

0024-3205/ © 2019 Elsevier Inc. All rights reserved.

sensing (QS) plays a significant role in virulence and pathogenicity. The system responsible for cell-to-cell communication in bacteria is mediated by QS, which relies on small signal molecules, N-acyl homoserine lactones (AHLs), that bind transcriptional activators to regulate gene expression. Studies report that QS controls expression of more than 300 genes involved in virulence factors synthesis, motility, phenotypic changes, biofilm formation, antibiotic resistance, and metabolic pathways that regulate stress response [5]. *P. aeruginosa* has four QS systems: las, rhl, pqs, and iqs. Specifically, the LasI synthases N-3-oxododecanoyl-homoserine lactone (3-oxo-C₁₂-HSL), which is recognized by the transcriptional regulator LasR. The las system requires sufficient levels of 3-oxo-C₁₂-HSL to activate virulence genes [6,7]. The rhl system produces N-butanoyl-L-homoserine lactone (C₄-HSL) to modulate virulence gene expression [8]. The pqs system regulates the production of 2-heptyl-3-hydroxy-4(1H)-quinolone or simple quinolone and has been reported to control biofilm formation and the production of virulence factors [5,9]. Finally, the iqs system produces 2-(2-hydroxyphenyl)thiazole-4-carbaldehyde but its exact role requires additional research.

Studies have shown that QS may increase the expression of virulence factors, such as pyocyanin, elastase, proteases, and rhamnolipids, to adapt to the environmental pressures [10]. Pyocyanin, a part of blue redox-active pigment called phenazine, has been reported to modulate bis-(3',5')-cyclic dimeric guanosine (c-di-GMP) messenger and extracellular polymeric substance (EPS) [11]. Moreover, pyocyanin regulates secretion of the extracellular DNA in bacteria (eDNA) to mediate biofilm development [11]. Elastase is one of the extracellular virulence factor which also called LasB. It is a zinc metalloprotease acting as a vital virulence factor to control the infection with *P. aeruginosa* [12]. Proteases are involved in the pathogenesis of acute lung injury caused by *P. aeruginosa* infection and are linked to the invasion and destruction of host tissue [13,14]. Rhamnolipids are biosurfactants that contribute to motility and biofilm development under stress conditions [15]. Studies have shown that the level of virulence factors in QS-defective isolates is lower compared to wild-type isolates [16]. Moreover, there is a relationship between the reduction in antibiofilm activity and increase in rhlR gene expression [17]. Potential therapeutic strategies, attracting a lot of research attention, including targeting and inhibition of the QS system to treat *P. aeruginosa* infections [18,19].

Ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), is one of the most popular non-prescription drugs given its high efficacy and safety. Early studies by Lee et al. discovered that ibuprofen inhibited pulmonary vasoconstriction and bronchiolar constriction in pigs infected with *P. aeruginosa* [20]. Moreover, in an acute *P. aeruginosa* pulmonary infection in mice, ibuprofen decreased the recruitment of granulocytes to airways and suppressed lung inflammation [21]. Additionally, ibuprofen effectively inhibited the production of inflammatory factor-leukotriene B₄ (LTB₄) in turn reducing lung inflammation in a rat model of chronic pulmonary infection [22]. Importantly, in a randomized controlled trial, two-thirds of female patients with uncomplicated UTIs who took ibuprofen recovered without additional antibiotic treatment [23]. Until more studies are done to carefully identify patients needing antibiotics, ibuprofen cannot be recommended as a stand-alone therapy for UTI patients. However, it is an attractive treatment alternative to antibiotics that does not cause drug resistance.

Other studies have reported that ibuprofen inhibits the growth of *P. aeruginosa* in a dose-dependent manner [24]. Moreover, ibuprofen can slow the progression of lung deterioration in patients with cystic fibrosis [25]. Although multiple studies have suggested that ibuprofen exerts antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria, fungi, and viruses [26–29], the underlying mechanism of antimicrobial activity of ibuprofen against *P. aeruginosa* remains unclear. In this study, we hypothesized that the inhibition of *P. aeruginosa* induced by ibuprofen is related to the QS systems.

2. Materials and methods

2.1. Bacterial strain, growth conditions and chemicals

P. aeruginosa PAO1 (ATCC15692) strain was cultured in Luria-Bertani (LB) medium (Sangon Biotech, China). Ibuprofen was purchased from Sigma-Aldrich (Cat# 14883, St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) (Biosharp). Bacteria were streaked from –80 °C skim milk stocks onto blood agar plates and incubated at 37 °C. Single colonies were incubated in LB medium, shaking at 120 rpm overnight at 37 °C.

2.2. Growth curve assessment

The cultures were diluted to 10⁶ colony-forming units (CFU)/mL as the initial concentration. The growth curve of PAO1 was determined in the presence or absence of different ibuprofen concentrations (0, 50, 75, and 100 µg/mL). The final DMSO concentration in all of the samples was 0.2% (v/v). All of the cultures at all of the time points (12 h, 18 h, and 24 h) were inoculated in the plates after dilution with sterile phosphate-buffered saline (PBS). The cell numbers of all of the groups were analyzed by the CFU plate count [24].

2.3. Biofilm formation assay

PAO1 was cultured in 96-well plates in the presence or absence of different ibuprofen concentrations (0, 50, 75, and 100 µg/mL) for 24 h at 37 °C. The cultures were removed and the plates were washed twice with PBS. The resulting biofilm was stained with 0.5% crystal violet for 15 min and solubilized in 95% alcohol. The absorbance was measured at 570 nm [30].

2.4. Microscopic assessment of adherence

PAO1 was incubated in 24-well plates in the presence or absence of ibuprofen with coverslips for 24 h at 37 °C. The coverslips were washed with PBS and stained with 4 µL of 0.01% acridine orange (AO, Cat#A6014, Sigma) for 10 min in the dark. The samples were immediately observed under the fluorescent microscope (Olympus BX41, Japan) at 40 × lens magnification [30].

2.5. Evaluation of virulence factor production

We used four different assays to assess the levels and activity of (1) pyocyanin, (2) elastase, (3) protease, and (4) rhamnolipids. Specific details for each assay are outlined below.

- 1) Pyocyanin assay: PAO1 was cultured in the presence or absence of ibuprofen at 37 °C. The supernatants were collected and extracted with 3 mL of chloroform, and subsequently mixed with 1 mL of 0.2 N HCl. The absorbance was measured at 520 nm.
- 2) Elastase activity: The elastase activity was determined by Elastin-Congo red (ECR, Sigma) test according to previously published methods [31,32]. Briefly, 600 µL of supernatant was mixed with 200 µL of ECR buffer (20 mg/mL in 0.1 M Tris-HCl at pH 8 with 1 mM CaCl₂) and the mixture was incubated under shaking conditions (200 rpm) for 15 h at 37 °C. Next, 100 µL of 0.12 M EDTA was used to stop the reaction. The undissolved ECR was removed by centrifugation at 5000 × g at 10 min and the absorbance was measured at 495 nm.
- 3) Protease activity: The activity of protease was determined with 2% azocasein (Sigma) [32]. Equal volumes of azocasein dissolved in PBS and supernatant were mixed and the cultures were incubated for 4 h at 4 °C. Next, 500 µL of 10% trichloroacetic acid was added to stop the reaction and the cultures were spun at 10,000 × g for 10 min. Finally, 500 µL of NaOH was mixed with the suspension and

the absorbance was measured at 440 nm.

- 4) Rhamnolipid assay: Rhamnolipids were extracted from the supernatant by ethyl acetate using equal volumes [32]. The samples were vortexed for 10 min and spun at $10,000 \times g$ for 5 min at 4 °C. The upper layer was collected and the extraction process with ethyl acetate was repeated three times. The collected liquid was purified under N₂ gas evaporation system (Agela, USA). Finally, 900 µL of orcinol reagent (0.19% orcinol in 53% H₂SO₄) was added to the precipitate and the samples were incubated for 30 min at 80 °C and the absorbance was measured at 420 nm.

2.6. High-performance liquid chromatography with triple quadrupole mass spectrometry analysis of 3-oxo-C₁₂-HSL and C₄-HSL

The extraction method of AHL is similar to that for rhamnolipids [32]. Based on previous reports, we used 200 µL of acetonitrile (ACN) with 0.1% formic acid to reconstitute AHLs [30,32]. Samples were injected through the C₁₈ reversed-phase column (3.5 µm, 2.1 mm × 150 mm) (Waters, Milford, MA USA). The samples were analyzed using Shimadzu Nexera X2 HPLC system (Shimadzu Corporation, Japan) conjugated to AB Sciex 55000 triple quadrupole mass spectrometer (AB Sciex, Redwood City, CA USA). The flow rate of system was set to 0.3 mL/min with 0.1% (v/v) formic acid in water (as mobile phase A) and 0.1% (v/v) formic acid in ACN (as mobile phase B). The mobile phase gradient was increased to 60% B for the first 2 min after the column was equilibrated. Then the gradient was ramped to 100% B for up to 5 min and maintained for 7 min. Afterwards, the gradient was dropped to 50% B and the process was stopped at 12 min. The volume of the system was 5 µL and the column temperature was maintained at 37 °C. The conditions were as follows: capillary voltage, 4000V; temperature, 350 °C; GS1, 40 psi; GS2: 40 psi; collision energy, 15 eV. Analysis was conducted with the following required parameters: *m/z* 298.2/197 for 3-oxo-C₁₂-HSL and *m/z* 172.0/102.1 for C₄-HSL. The Analyst software (v. 1.2.1, AB Sciex) was used to create the calibration curve based on peak areas and analyze the data.

2.7. Gene expression analysis by quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the cultures using TRIzol (TRIzol reagent, Invitrogen, Carlsbad, CA USA) and cDNA synthesis was conducted using a kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The concentration and purity of RNA was determined using the UV spectrophotometer (Implen, Munchen, Germany). qRT-PCR was performed using the LightCycler® 480 SYBR Green I Master (Roche, Germany) with a final reaction volume of 20 µL. The expression of target genes was normalized to the expression of 16S used as a housekeeping gene. Primer sequences are listed in Table 1.

Table 1

List of primers used in qRT-PCR.

Target name	Type	Primer sequence	References
<i>lasI</i>	Fw	5'-CGCACATCTGGGAACCTCA-3'	[46]
	Rev	5'-CGGCACGGATCATCATCT-3'	
<i>lasR</i>	Fw	5'-CTGTGGATGCTCAAGGACTAC-3'	
	Rev	5'-AACTGGTCTTCCCGATGG-3'	
<i>rhII</i>	Fw	5'-GTAGCGGGTTTGGGATG-3'	
	Rev	5'-CGGCATCAGGTCTTCATCG-3'	
<i>rhIR</i>	Fw	5'-GCCAGCGTCTTGTTCCGG-3'	
	Rev	5'-CGGTCTGCCTGAGCCATC-3'	
<i>pqsA</i>	Fw	5'-GACCGGTCTATTGATTC-3'	
	Rev	5'-GCTGAACCAGGAAAGAAC-3'	
<i>pqsR</i>	Fw	5'-CTGATCTGCCGTAATTGG-3'	
	Rev	5'-ATCGACGAGGAACTGAAGA-3'	
16S	Fw	5'-GAGGAAGGTGGGATGACGT-3'	[47]
	Rev	5'-AGGCCCGGAAACGTATTAC-3'	

2.8. Binding analysis of ibuprofen and QS proteins

The binding interaction between the ibuprofen and proteins associated with *P. aeruginosa* QS was performed by AutoDock [33]. Crystal protein structures (LasR, LasI, LuxR, pqsA, and pqsR) were downloaded from PDB (<http://www.pdb.org>). Since there was no available structure for RhlR, we constructed it based on the homology model using Modeller 9.17 [34]. Structure of ibuprofen was drawn in ChemBioDraw (v.14) software. The structures of ibuprofen and proteins were analyzed using AutoDockTools (v. 1.5.6) and docking was carried out by AutoDock Vina.

2.9. Functional complementation assays with 3-oxo-C₁₂-HSL and C₄-HSL

Exogenous 3-oxo-C₁₂-HSL and C₄-HSL at 2 µM concentration were added to the PAO1 medium with ibuprofen in order to carry out functional complementation assays [35]. The virulence factors were determined according to methods described above.

2.10. Statistical analysis

All of the experiments were conducted in triplicate and data are expressed as mean ± SD. The data were analyzed by one-way analysis of variance (ANOVA) using SPSS 20.0 software and the threshold for significance was set at a *p*-value < 0.05.

3. Results

3.1. Ibuprofen inhibits *P. aeruginosa* biofilm formation

The bacterial burden showed a concentration-dependent reduction with increasing concentrations of ibuprofen (0, 50, 75, and 100 µg/mL) at all examined time points (12 h, 18 h, and 24 h). Although all of the cultures treated with 100 µg/mL ibuprofen had the lowest CFU counts at all of the examined time points, it has no effectively inhibitory effect on bacterial growth until 2log₁₀ CFU decline achieved (Fig. 1A). We hypothesized that ibuprofen may exert specific antibiofilm action against *P. aeruginosa*. Antibiofilm activity of ibuprofen was evaluated at several concentrations of ibuprofen by crystal violet staining. The results revealed that ibuprofen partly attenuated biofilm formation in *P. aeruginosa*, with the maximum reduction in biofilm formation (55%) found at 100 µg/mL (Fig. 1B). Next, the fluorescent microscopy was used to confirm the antibiofilm activity of ibuprofen. Specifically, *P. aeruginosa* was treated with ibuprofen and then AO was used for staining. Captured images indicated significant decrease in the attachment of biofilm cells at 100 µg/mL ibuprofen and the changes reflected a concentration-dependent pattern (Fig. 1C). These data clearly suggest that although ibuprofen had no significant inhibitory effect on the growth of *P. aeruginosa*, it could inhibit the biofilm formation.

3.2. Ibuprofen reduces QS signal synthesis in *P. aeruginosa*

To evaluate the effect of ibuprofen on QS signaling molecules, we confirmed the structures of 3-oxo-C₁₂-HSL and C₄-HSL (Fig. 2A and B). The HPLC/MS analysis demonstrated that levels of 3-oxo-C₁₂-HSL were lower (17.08 ± 0.74 ng/mL) only at 100 µg/mL ibuprofen at 12 h compared to the control group and there was no evidence of concentration-dependent changes at other examined time points (Fig. 2C). In contrast, we observed a significant (*p* < 0.05) time-dependent reduction in C₄-HSL levels (Fig. 2D). PAO1 cultures treated with ibuprofen had significantly decreased levels of C₄-HSL (up to 132.76 ng/mL) at all time points (12 h, 18 h, and 24 h). Overall, the inhibitory effects of ibuprofen on C₄-HSL levels were concentration-dependent, with the unexceptional changes at 18 h and 24 h with the 75 µg/mL concentration (Fig. 2C).

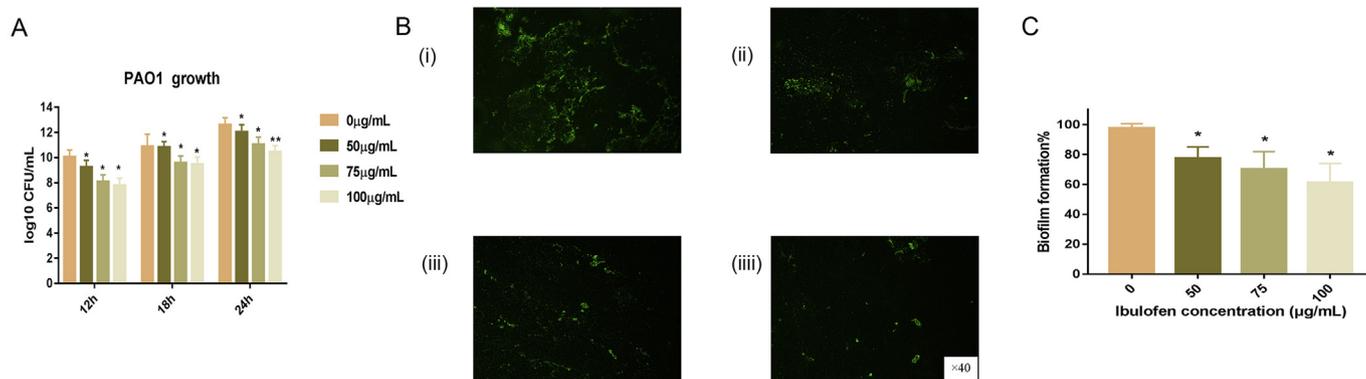


Fig. 1. Concentration-dependent effects of ibuprofen on inhibiting the growth and biofilm formation in *P. aeruginosa*. (A) Growth conditions. (B) Adherence ability. a. 0 µg/mL ibuprofen; b. 50 µg/mL ibuprofen; c. 75 µg/mL ibuprofen; d. 100 µg/mL ibuprofen (original magnification, × 40). (C) Biofilm formation. *P. aeruginosa* PAO1 treated with different concentrations of ibuprofen (0, 50, 75, and 100 µg/mL). Data are represented as mean of four independent experiments and presented as mean ± SD. * indicates $P < 0.05$, ** indicates $P < 0.01$.

3.3. Ibuprofen attenuates the production of virulence factors in *P. aeruginosa*

Since our analysis identified that ibuprofen affected bacterial signaling, we next investigated the production of virulence factors regulated in *P. aeruginosa* PAO1 strain by QS. Upon treatment, 100 µg/mL ibuprofen significantly ($p < 0.05$) reduced the release of pyocyanin (up to 24.5%) (Fig. 3A). And the significant reduction in rhamnolipid production by ibuprofen was also seen at 100 µg/mL ibuprofen (34.5%) (Fig. 3B). There were no significant effects of ibuprofen on rhamnolipid levels after 50 µg/mL ibuprofen treatment (Fig. 3B). Similarly, the production of protease was effectively inhibited by ibuprofen at 75 and 100 µg/mL ibuprofen (Fig. 3C). In contrast, only moderate, but significant ($p < 0.05$), reduction (16.9%) in the total elastase production was observed at 100 µg/mL ibuprofen (Fig. 3D). Overall, ibuprofen exerted the inhibitory effect on the virulence factor production.

3.4. The expression of QS genes decreases after ibuprofen treatment

To study whether ibuprofen affected changes in *P. aeruginosa* on a molecular level, we analyzed gene expression levels associated with QS system by qRT-PCR (Fig. 4). We noted significant ($p < 0.05$) reduction in the expression of *lasI*, *lasR*, *rhII*, *rhIR*, *pqsA*, and *pqsR* at 18 h. Among all of the examined concentrations, 75 µg/mL exerted the most pronounced effects in suppressing QS system gene expression. The relative expression of *lasI* and *lasR* was decreased 1.5- and 3.5-fold, respectively (Fig. 4A and B), while the expression of *pqsA* and *pqsR* was reduced by approximately 1.5–2.0-fold (Fig. 4C and D). Most interesting, the levels of *rhII* were reduced 4.7-fold and the levels of *rhIR* was suppressed 8.3-fold (Fig. 4E and F). These data suggest that ibuprofen mainly acted on the Rhl and Las system.

Given the inhibition of QS signaling molecules, we examined whether exogenous addition of 3-oxo- C_{12} -HSL and C_4 -HSL could functionally complement the activity of ibuprofen. Specifically, we observed a significant increase ($p < 0.05$) in biofilm formation (Fig. 5A), pyocyanin production (Fig. 5B), elastase activity (Fig. 5C), protease activity

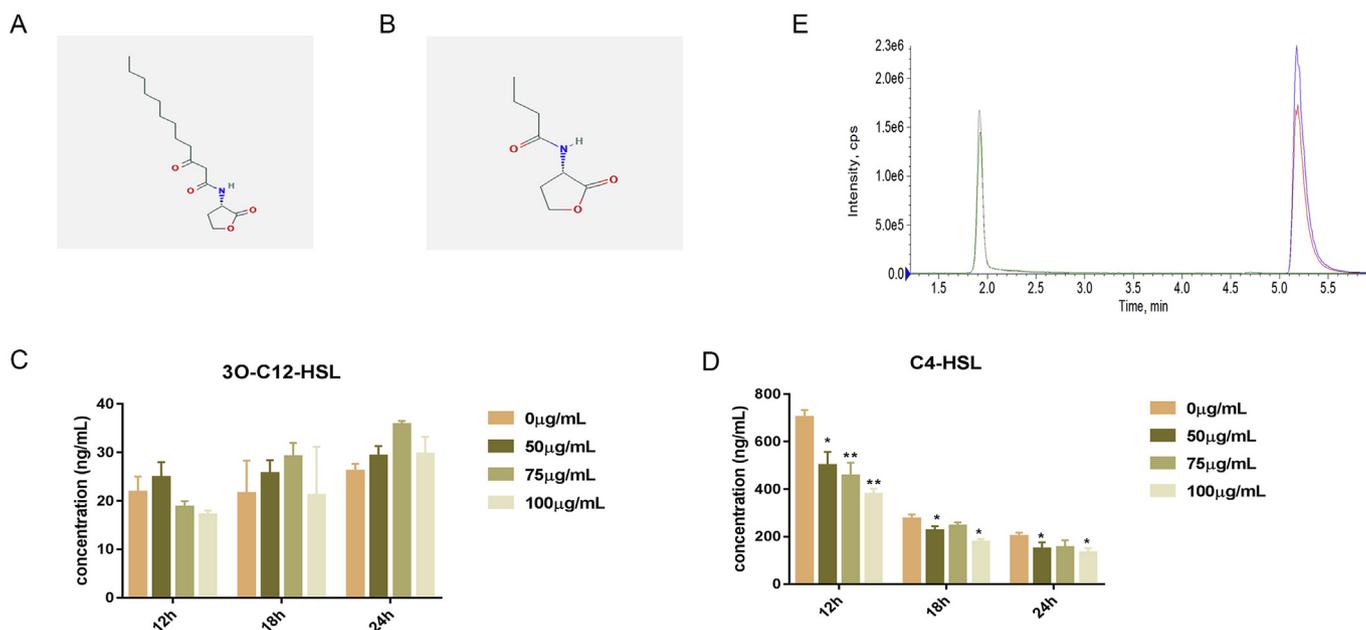


Fig. 2. Effect of ibuprofen on AHL concentrations as determined by LC/MS. (A) Structure of 3-oxo- C_{12} -HSL. (B) Structure of C_4 -HSL. (C) 3-oxo- C_{12} -HSL production. (D) C_4 -HSL production. (E) LC/MS peaks of 3-oxo- C_{12} -HSL and C_4 -HSL. AHL concentrations of *P. aeruginosa* PAO1 treated with different concentrations of ibuprofen (0, 50, 75, and 100 µg/mL) evaluated at three time points (12 h, 18 h, and 24 h). Data are represented as mean of three independent experiments and presented as mean ± SD. * indicates $P < 0.05$, ** indicates $P < 0.01$.

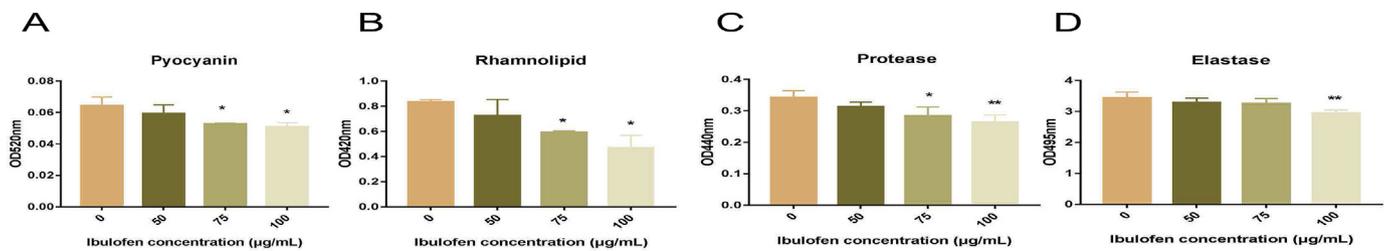


Fig. 3. Extracellular virulence factor levels in cell-free culture supernatants treated with ibuprofen. (A) Pyocyanin. (B) Rhamnolipids. (C) Protease. (D) Elastase. *P. aeruginosa* PAO1 treated with different concentrations of ibuprofen (0, 50, 75, and 100 µg/mL) and extracellular virulence factors in the supernatants. Data are represented as mean of three independent experiments and presented as mean ± SD. * indicates $P < 0.05$, ** indicates $P < 0.01$.

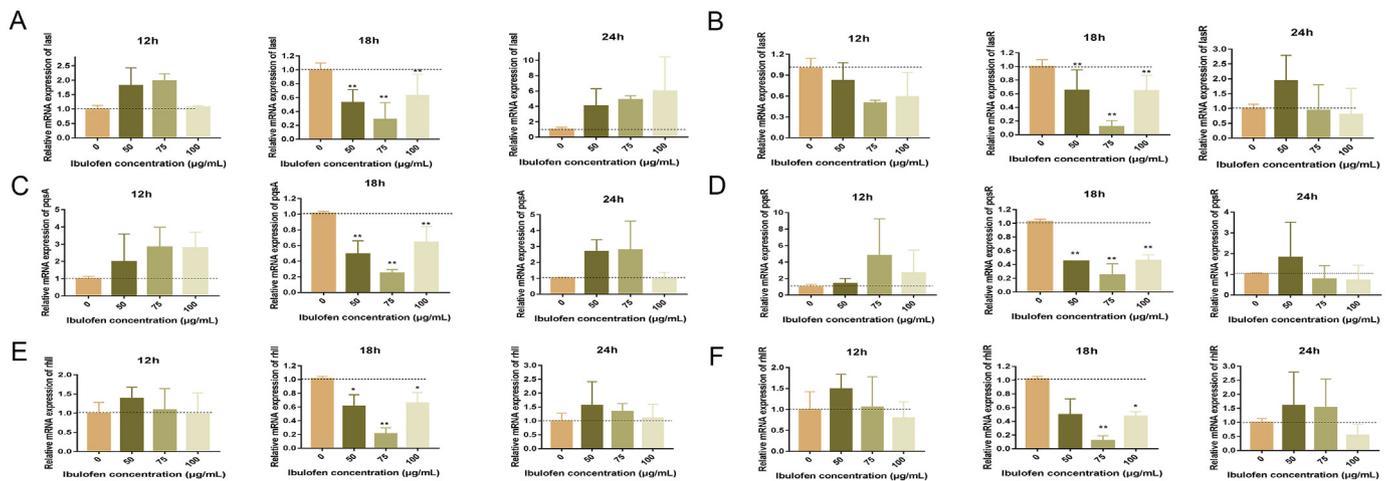


Fig. 4. The changes in gene expression related to the QS system in *P. aeruginosa* treated with ibuprofen. (A) *lasI*. (B) *lasR*. (C) *pqsA*. (D) *pqsR*. (E) *rhlI*. (F) *rhlR*. *P. aeruginosa* PAO1 was treated with different concentrations of ibuprofen (0, 50, 75, 100 µg/mL) and the gene expression changes were evaluated at three time points (12 h, 18 h, and 24 h). Data are represented as mean of three independent experiments and presented as mean ± SD. * indicates $P < 0.05$, ** indicates $P < 0.01$.

(Fig. 5D), and rhamnolipid production (Fig. 5E) upon addition of exogenous C₄-HSL. Importantly, we did not observe any statistically significant changes in the level of virulence factors upon treatment with exogenous 3-oxo-C₁₂-HSL (Fig. 5).

3.5. Assessment of molecular docking of ibuprofen with QS-related proteins in *P. aeruginosa*

To further analyze the role of ibuprofen, molecular docking study was performed to better understand the effect of ibuprofen on attenuating QS-related proteins (*LasI*, *RhlR*, *LuxR*, *LasR*, *PqsA*, and *PqsR*). Crystal structures of the QS-related proteins were found online: *LasR* (PDB ID: 2UV0), *LasI* (PDB ID: IRO5), *LuxR* (PDB ID: 3JPU), *PqsA* (PDB ID: 5OE3), and *PqsR* (PDB ID: 4JVD). Although the crystal structure of *RhlR* was not available, we obtained it by homology modeling. The

native receptors and binding affinity of ibuprofen with QS-related proteins is summarized in Table 2. The results of AutoDock analysis suggested that ibuprofen only formed a hydrogen bond with Ile107 of *LasI* (Fig. 6A). The binding energy between ibuprofen and *LasI* was -7.4 kcal/mol (Table 2). We constructed a virtual homology model of *RhlR* since the crystal structure was not available. Here, ibuprofen formed two hydrogen bonds with Asp80 of *RhlR* at a distance of 1.5 and 3.3 Å (Fig. 6B). The docking score between ibuprofen and *RhlR* was -7.2 kcal/mol (Table 2). Moreover, ibuprofen formed two hydrogen bonds with Thr75 and Asp73 in *LuxR* at a distance of 3.2 and 1.9 Å, respectively (Fig. 6C). The result showed that binding energy between ibuprofen and *LuxR* was -7.9 kcal/mol (Table 2). The docking studies showed that ibuprofen binds into the active site of *LasR* with Asp73, Thr75, Thr115, and Ser129 residues by hydrogen bond interactions (Fig. 6D). The binding energy between ibuprofen and *LasR* was

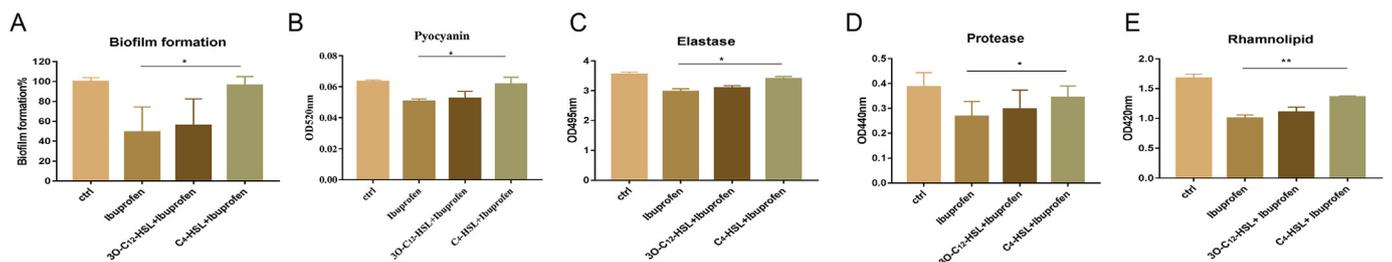


Fig. 5. Functional complementation of ibuprofen-treated *P. aeruginosa* with exogenous 3-oxo-C₁₂-HSL and C₄-HSL. (A) Biofilm formation. (B) Pyocyanin. (C) Elastase. (D) Protease. (E) Rhamnolipids. The determination of virulence factor production and biofilm formation in *P. aeruginosa* PAO1 treated with ibuprofen (100 µg/mL) in the presence of 3-oxo-C₁₂-HSL and C₄-HSL. Data are represented as mean of three independent experiments and presented as mean ± SD. * indicates $P < 0.05$, ** indicates $P < 0.01$.

Table 2

Binding affinity of QS regulator proteins from *P. aeruginosa* with ibuprofen. AutoDock provides a binding score revealing the binding affinity measured in kcal/mol. Negative scores indicate high binding affinity.

Receptor protein	PDB ID	Native ligand	Probable inhibitor	Hydrogen bonds	Binding energy(kcal/mol)
LasI	1RO5	perhenate	Ibuprofen	Ile107	-7.4
RhlR	4Y15	–	Ibuprofen	Asp80	-7.2
LuxR	3JPU	TY4	Ibuprofen	Thr75, Asp73	-7.9
LasR	2UV0	OHN	Ibuprofen	Asp73, Thr75, Thr115, Ser129	-7.7
PqsA	5OE3	3UK	Ibuprofen	Asp299, Ser280, Gly279, Gly300	-6.8
PqsR	4JVD	NNQ	Ibuprofen	Ser196, Gln194	-6.7

–7.7 kcal/mol (Table 2). PqsA was shown to interact with four different ligands, and 5'-O-[(S)-[(2-aminobenzoyl)oxy](hydroxy)phosphoryl]adenosine (3UK) with high binding scores (–6.8 kcal/mol) (Table 2). Ibuprofen formed hydrogen bonds with Asp299, Ser280, Gly279, and Gly300 residues at a distance of 1.9, 3.2, 2.5, and 3.4 Å, respectively (Fig. 6E). Furthermore, ibuprofen was found to interact with the Ser196 and Gln194 of PqsR (Fig. 6F) and showed binding energy of –6.8 kcal/mol (Table 2). After docking of ibuprofen into the original ligand binding pockets of these proteins, we found that ibuprofen was binding with LuxR (–7.9 kcal/mol), LasR (–7.7 kcal/mol), LasI (–7.4 kcal/mol), and RhlR (–7.2 kcal/mol) at high binding scores (Table 2). Thus, the molecular docking data further demonstrate that ibuprofen may inhibit the LuxR, Las, and Rhl systems in *P. aeruginosa*.

4. Discussion

Inhibitors of QS which act to directly prevent biofilm formation and limit virulence factor release are being considered as a novel strategy for treating *P. aeruginosa* infections [36–38]. Although many studies examined the action of ibuprofen in bacterial infections, the antibacterial mechanism of action of ibuprofen requires further exploration. The first mention of the antifungal and antimicrobial activity of ibuprofen was reported by Hersh and colleagues in 1991. Many reports have demonstrated that ibuprofen exerts antibacterial effects in high concentrations that exceed levels seen in normal human blood [26,39,40]. Previous report indicated that ibuprofen was able to prevent AHL inhibition of the responses to nucleotides in cystic fibrosis airway epithelium [41]. Moreover, ibuprofen has been reported as an alternative drug for the treatment of local infections (e.g., UTIs) and showed a promising therapeutic potential when combined with clinical antibiotics [23,26,42–44].

In this study, we confirmed that ibuprofen specifically inhibits the QS system to exert the activity. According to the clinical trial data, the efficacy and safety of high-dose ibuprofen (50–100 µg/mL plasma concentration) was shown in cystic fibrosis patients [24]. Based on these data, we designed a concentration gradient of ibuprofen that was tested. We discovered that ibuprofen can affect the growth of *P. aeruginosa* but it may not have a complete bactericidal effect (Fig. 1A). The OA staining assay demonstrated that the attachment activity of *P. aeruginosa* was inhibited by ibuprofen in a concentration-dependent manner (Fig. 1C). Biofilm is defined as the community of bacteria attached to both biological and abiotic surfaces, and the presence of biofilm further exacerbates the crisis of antibiotic resistance worldwide [45]. Crystal violet staining confirmed that ibuprofen exerted a significant effect on the reduction of biofilm formation by *P. aeruginosa* (Fig. 1B), with the 100 µg/mL concentration having the greatest effect on biofilm inhibition.

We next determined that the synthesis of C₄-HSL was inhibited by ibuprofen in a concentration- and time-dependent manner (Fig. 2C). However, ibuprofen did not reduce 3-oxo-C₁₂-HSL secretion, suggesting that ibuprofen exerts anti-QS effects through the reduction of C₄-HSL levels instead of the direct cell death effects (Figs. 1 and 2).

Many studies have been conducted to identify that QS molecules act

as regulators mediating the production of virulence factors [38,46]. Studies in different animal models demonstrate that QS plays a critical role in the pathogenicity of *P. aeruginosa* infections [10,17,47,48]. In this study, we found that the virulence factors, including pyocyanin, elastase, protease, and rhamnolipids, were reduced upon treatment with 75 and 100 µg/mL ibuprofen (Fig. 3). We concluded that treatment with ibuprofen targets QS molecules and virulence factors in *P. aeruginosa* and can therefore be a promising clinical therapy.

Las and Rhl are involved in the LuxR-type QS system of *P. aeruginosa*. Gene expression analysis revealed significant decreases in genes encoding QS proteins (lasI, lasR, rhlI, rhlR, pasA, and pqsR) following ibuprofen treatment within 18 h (Fig. 4). Despite these findings, the inhibition by ibuprofen did not show concentration-dependent changes and future studies are needed to address this finding in detail. Work by Somaia. et al. suggested that aspirin, another NSAID, reduces lasR gene expression by competing with 3-oxo-C₁₂-HSL [49]. In our findings, ibuprofen significantly repressed lasR and rhlR gene expression by 88.3% and 88%, respectively. The inhibitory activity of ibuprofen against *P. aeruginosa* is dependent on lasR and rhlR. The reduction in pqsA and pqsR gene expression levels by ibuprofen was almost 75%. In our study, the decreased level of virulence factors was consistent with the reduction in QS systems related to the effects on Las and Rhl gene expression (Figs. 3 and 4).

To further investigate the anti-QS activity of ibuprofen, we examined the molecular interaction of QS-associated LasR, LasI, LuxR, RhlR, PqsA, and PqsR proteins with ibuprofen. Our docking studies of ibuprofen with proteins suggested that ibuprofen specifically binds to ligand binding pockets in four of the QS proteins LuxR, LasR, LasI, and RhlR with a relatively high binding affinity (Fig. 6 and Table 2). Amino acids presenting in the binding sites of these four proteins play crucial roles in the dimerization and activation of the receptors. Based on our results, we propose that the binding of ibuprofen to the amino acids might be directly responsible for the inactivation of the QS proteins. We believe that this occurs either by preventing protein dimerization or there are direct effects on the DNA resulting in suppression of gene expression downstream in the QS system. Overall, the data suggest that the proteins related to QS may be a promising target for anti-QS and antibiofilm action of ibuprofen.

Moreover, we also examined whether exogenous addition of 3-oxo-C₁₂-HSL and C₄-HSL can functionally complement the activity of ibuprofen. We observed that C₄-HSL was capable of reversing the inhibitory action of ibuprofen on biofilm formation and virulence factors, whereas 3-oxo-C₁₂-HSL had no effect (Fig. 5). These data recapitulated the LC/MS results. Although 3-oxo-C₁₂-HSL-LasR can directly activate transcription of target genes and stimulate the production of C₄-HSL and RhlR, the action of ibuprofen was not inhibited by 3-oxo-C₁₂-HSL. These results warrant additional investigation to further explore the specific anti-QS role of ibuprofen.

In summary, we demonstrated the molecular mechanism of anti-QS activity of ibuprofen against *P. aeruginosa* [50]. In our study, ibuprofen inhibited the *P. aeruginosa* by suppressing the Las and Rhl QS system. Considering the gene expression results and effect on virulence factors, treatment with ibuprofen may be a promising therapy when dealing

with *P. aeruginosa* infections. Additionally, because other studies suggest that ibuprofen is effective in treating UTIs and cystic fibrosis, we propose ibuprofen as a candidate drug to be used in the management of clinical infections with *P. aeruginosa*.

Funding

This work was supported by the Science and Technology Project of Nantong city [JC2018067 and MS12017010-2].

Authors' contributions

All authors listed made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. In details: Juan Yu and Shao-qing Ju conceived the project. Lu Dai, Yi-song Xiong and Hong-bing Ni designed and carried out the experiments. Tian-qi Wu, Ye Ding, Wen-chen Zhang and Shao-peng Chu analyzed the data. Lu Dai and Tian-qi Wu wrote the paper and all authors reviewed the manuscript. All authors were also involved in drafting the manuscript and have given final approval of the version to be published.

Declaration of competing interest

The authors declare they have no financial conflicts of interest.

Acknowledgements

We thank the staff of the Department of Laboratory Medicine and Research Center of Clinical Medicine in Affiliated Hospital of Nantong University for technical assistance. This manuscript has been released as a Pre-Print at Biorxiv.

References

- Z. Pang, et al., Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies, *Biotechnol. Adv.* 37 (1) (2019) 177–192, <https://doi.org/10.1016/j.biotechadv.2018.11.013>.
- M.F. Moradali, et al., *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence, *Front Cell Infect Microbiol* 7 (2017) 39, <https://doi.org/10.3389/fcimb.2017.00039>.
- S. Chevalier, et al., Structure, function and regulation of *Pseudomonas aeruginosa* porins, *FEMS Microbiol. Rev.* 41 (5) (2017) 698–722, <https://doi.org/10.1093/femsre/fux020>.
- C. Willyard, The drug-resistant bacteria that pose the greatest health threats, *Nature* 543 (7643) (2017) 15, <https://doi.org/10.1038/nature.2017.21550>.
- M.W. Azam, A.U. Khan, Updates on the pathogenicity status of *Pseudomonas aeruginosa*, *Drug Discov. Today* 24 (1) (2019) 350–359, <https://doi.org/10.1016/j.drudis.2018.07.003>.
- D.G. Davies, et al., The involvement of cell-to-cell signals in the development of a bacterial biofilm, *Science* 280 (5361) (1998) 295–298.
- T.H. Jakobsen, et al., Targeting quorum sensing in *Pseudomonas aeruginosa* biofilms: current and emerging inhibitors, *Future Microbiol.* 8 (7) (2013) 901–921, <https://doi.org/10.2217/fmb.13.57>.
- P.N. Jimenez, et al., The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*, *Microbiol. Mol. Biol. Rev.* 76 (1) (2012) 46–65, <https://doi.org/10.1128/mmb.05007-11>.
- J. Lee, L. Zhang, The hierarchy quorum sensing network in *Pseudomonas aeruginosa*, *Protein Cell* 6 (1) (2015) 26–41, <https://doi.org/10.1007/s13238-014-0100-x>.
- O. Karatuna, A. Yagci, Analysis of quorum sensing-dependent virulence factor production and its relationship with antimicrobial susceptibility in *Pseudomonas aeruginosa* respiratory isolates, *Clin. Microbiol. Infect.* 16 (12) (2010) 1770–1775, <https://doi.org/10.1111/j.1469-0691.2010.03177.x>.
- L.A. Meirelles, D.K. Newman, Both toxic and beneficial effects of pyocyanin contribute to the lifecycle of *Pseudomonas aeruginosa*, *Mol. Microbiol.* 110 (6) (2018) 995–1010, <https://doi.org/10.1111/mmi.14132>.
- D. Szamosvari, et al., Synthetic quinolone signal analogues inhibiting the virulence factor elastase of *Pseudomonas aeruginosa*, *Chem Commun (Camb)*. 52 (92) (2016) 13440–13443, <https://doi.org/10.1039/c6cc06295d>.
- E. Kipnis, et al., Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis, *Med Mal Infect* 36 (2) (2006) 78–91, <https://doi.org/10.1016/j.medmal.2005.10.007>.
- S. Lindsay, et al., The detrimental impact of extracellular bacterial proteases on wound healing, *Int. Wound J.* 14 (6) (2017) 1237–1247, <https://doi.org/10.1111/iwj.12790>.
- R.S. Reis, et al., Gene regulation of rhamnolipid production in *Pseudomonas aeruginosa*—a review, *Bioresour. Technol.* 102 (11) (2011) 6377–6384, <https://doi.org/10.1016/j.biortech.2011.03.074>.
- R. Garcia-Contreras, Is quorum sensing interference a viable alternative to treat *Pseudomonas aeruginosa* infections? *Front. Microbiol.* 7 (2016) 1454, <https://doi.org/10.3389/fmicb.2016.01454>.
- M. Gholamrezazadeh, et al., Effect of nano-silver, nano-copper, deconex and benzalkonium chloride on biofilm formation and expression of transcription regulatory quorum sensing gene (rhIR) in drug-resistance *Pseudomonas aeruginosa* burn isolates, *Burns* 44 (3) (2018) 700–708, <https://doi.org/10.1016/j.burns.2017.10.021>.
- T. B, et al., Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections, *Microbiology (Reading, Engl.)*. 151 (null) (2005) 3873–3880, <https://doi.org/10.1099/mic.0.27955-0>.
- D. S, New perspectives in the management of *Pseudomonas aeruginosa* infections, *Future Microbiol.* 9 (7) (2014) 917–928, <https://doi.org/10.2217/fmb.14.42>.
- C.C. Lee, et al., Effects of ibuprofen on a pig *Pseudomonas* ARDS model, *J. Surg. Res.* 40 (5) (1986) 438–444, [https://doi.org/10.1016/0022-4804\(86\)90212-x](https://doi.org/10.1016/0022-4804(86)90212-x).
- D.O. Sordelli, et al., Ibuprofen modifies the inflammatory response of the murine lung to *Pseudomonas aeruginosa*, *Eur. J. Respir. Dis.* 67 (2) (1985) 118–127.
- M.W. Konstan, et al., Ibuprofen attenuates the inflammatory response to *Pseudomonas aeruginosa* in a rat model of chronic pulmonary infection. Implications for anti-inflammatory therapy in cystic fibrosis, *Am. Rev. Respir. Dis.* 141 (1) (1990) 186–192, <https://doi.org/10.1164/ajrccm/141.1.186>.
- I. Gagyor, et al., Ibuprofen versus fosfomycin for uncomplicated urinary tract infection in women: randomised controlled trial, *BMJ* 351 (2015), <https://doi.org/10.1136/bmj.h6544>.
- P.N. Shah, et al., Antimicrobial activity of ibuprofen against cystic fibrosis-associated gram-negative pathogens, *Antimicrob. Agents Chemother.* 62 (3) (2018) 1–22, <https://doi.org/10.1128/aac.01574-17>.
- L.C. Lands, S. Stanojevic, Oral non-steroidal anti-inflammatory drug therapy for lung disease in cystic fibrosis, *Cochrane Database Syst. Rev.* 4 (2016) Cd001505, <https://doi.org/10.1002/14651858.CD001505.pub4>.
- J. Obad, et al., Antimicrobial activity of ibuprofen: new perspectives on an "Old" non-antibiotic drug, *Eur. J. Pharm. Sci.* 71 (2015) 93–98, <https://doi.org/10.1016/j.ejps.2015.02.011>.
- A.O. Ogundeji, et al., Repurposing of aspirin and ibuprofen as candidate anti-cryptococcus drugs, *Antimicrob. Agents Chemother.* 60 (8) (2016) 4799–4808, <https://doi.org/10.1128/aac.02810-15>.
- T. Pan, et al., Non-steroidal anti-inflammatory drugs (NSAIDs) potentially inhibit the replication of zika viruses by inducing the degradation of AXL, *J. Virol.* (2018), <https://doi.org/10.1128/jvi.01018-18>.
- A.E. Laudy, et al., The influence of efflux pump inhibitors on the activity of non-antibiotic NSAIDs against gram-negative rods, *PLoS One* 11 (1) (2016) e0147131, <https://doi.org/10.1371/journal.pone.0147131>.
- M. Chatterjee, et al., Mechanistic understanding of Phenylacetic acid mediated inhibition of quorum sensing and biofilm development in *Pseudomonas aeruginosa*, *Appl. Microbiol. Biotechnol.* 101 (22) (2017) 8223–8236, <https://doi.org/10.1007/s00253-017-8546-4>.
- M.C. Das, et al., Attenuation of *Pseudomonas aeruginosa* biofilm formation by Vitexin: a combinatorial study with azithromycin and gentamicin, *Sci. Rep.* 6 (2016) 23347, <https://doi.org/10.1038/srep23347>.
- A.A. Jack, et al., Alginate oligosaccharide-induced modification of the lasI-lasR and rhlI-rhlR quorum-sensing systems in *Pseudomonas aeruginosa*, *Antimicrob. Agents Chemother.* 62 (5) (2018) 1–14, <https://doi.org/10.1128/aac.02318-17>.
- O. Trott, A.J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, *J. Comput. Chem.* 31 (2) (2010) 455–461, <https://doi.org/10.1002/jcc.21334>.
- A. Fiser, A. Sali, Modeller: generation and refinement of homology-based protein structure models, *Methods Enzymol.* 374 (2003) 461–491, [https://doi.org/10.1016/s0076-6879\(03\)74020-8](https://doi.org/10.1016/s0076-6879(03)74020-8).
- F. Babic, et al., Tobramycin at subinhibitory concentration inhibits the Rhl/R quorum sensing system in a *Pseudomonas aeruginosa* environmental isolate, *BMC Infect. Dis.* 10 (2010) 148, <https://doi.org/10.1186/1471-2334-10-148>.
- J.C. Janssens, et al., Small molecules for interference with cell-cell-communication systems in Gram-negative bacteria, *Curr. Med. Chem.* 15 (21) (2008) 2144–2156, <https://doi.org/10.2174/092986708785747580>.
- A.M. Hardman, et al., Quorum sensing and the cell-cell communication dependent regulation of gene expression in pathogenic and non-pathogenic bacteria, *Antonie Leeuwenhoek* 74 (4) (1998) 199–210, <https://doi.org/10.1023/a:1001178702503>.
- R. Le Berre, et al., [Quorum sensing: a new clinical target for *Pseudomonas aeruginosa*?], *Med Mal Infect* 36 (7) (2006) 349–357, <https://doi.org/10.1016/j.medmal.2006.01.008>.
- K.T. Elvers, S.J. Wright, Antibacterial activity of the anti-inflammatory compound ibuprofen, *Lett. Appl. Microbiol.* 20 (2) (1995) 82–84, <https://doi.org/10.1111/j.1472-765x.1995.tb01291.x>.
- C. Pina-Vaz, et al., Antifungal activity of ibuprofen alone and in combination with fluconazole against *Candida* species, *J. Med. Microbiol.* 49 (9) (2000) 831–840, <https://doi.org/10.1099/0022-1317-49-9-831>.
- A. Saleh, et al., *Pseudomonas aeruginosa* quorum-sensing signal molecule N-(3-oxododecanoyl)-L-homoserine lactone inhibits expression of P2Y receptors in cystic fibrosis tracheal gland cells, *Infect. Immun.* 67 (10) (1999) 5076–5082.
- P. Little, Antibiotics or NSAIDs for uncomplicated urinary tract infection? *BMJ* 359 (2017) j5037, <https://doi.org/10.1136/bmj.j5037>.
- I. Vik, et al., Ibuprofen versus pivmecillinam for uncomplicated urinary tract infection in women—A double-blind, randomized non-inferiority trial, *PLoS Med.* 15 (5) (2018) e1002569, <https://doi.org/10.1371/journal.pmed.1002569>.

- [44] C. Pina-Vaz, et al., Potent synergic effect between ibuprofen and azoles on *Candida* resulting from blockade of efflux pumps as determined by FUN-1 staining and flow cytometry, *J. Antimicrob. Chemother.* 56 (4) (2005) 678–685, <https://doi.org/10.1093/jac/dki264>.
- [45] H. Mathur, D. Field, Fighting biofilms with lantibiotics and other groups of bacteriocins, *NPJ Biofilms Microbiomes* 4 (2018) 9, <https://doi.org/10.1038/s41522-018-0053-6>.
- [46] A.B. Alayande, et al., Correlation between quorum sensing signal molecules and *Pseudomonas aeruginosa*'s biofilm development and virulency, *Curr. Microbiol.* 75 (7) (2018) 787–793, <https://doi.org/10.1007/s00284-018-1449-5>.
- [47] P. Cosson, et al., *Pseudomonas aeruginosa* virulence analyzed in a *Dictyostelium discoideum* host system, *J. Bacteriol.* 184 (11) (2002) 3027–3033.
- [48] L.G. Rahme, et al., Common virulence factors for bacterial pathogenicity in plants and animals, *Science* 268 (5219) (1995) 1899–1902, <https://doi.org/10.1126/science.7604262>.
- [49] S.A. El-Mowafy, et al., Aspirin is an efficient inhibitor of quorum sensing, virulence and toxins in *Pseudomonas aeruginosa*, *Microb. Pathog.* 74 (2014) 25–32, <https://doi.org/10.1016/j.micpath.2014.07.008>.
- [50] L. Dai, et al., Ibuprofen-mediated Potential Inhibition of Biofilm Development and Quorum Sensing in *Pseudomonas aeruginosa*, (2019) Biorxiv.