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1 **Novel quinoline-based derivatives as the PqsR inhibitor against *Pseudomonas***

2 ***aeruginosa* PAO1**

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24 **Abstract**:

25 **Aims:** The emerging of drug resistant *Pseudomonas aeruginosa* is a critical challenge and 26 renders an urgent action to discover innovative antimicrobial interventions. One of these 27 interventions is to disrupt the pseudomonas quinolone signal (pqs) quorum sensing (QS) system, 28 which governs multiple virulence traits and biofilm formation. This study aimed to investigate the 29 QS inhibitory activity of a series of new PqsR inhibitors bearing a quinoline scaffold against *Ps.* 30 *aeruginosa*.

31 **Methods and Results:** The results showed that compound **1** suppressed the expression of QS- 32 related genes and showed the best inhibitory activity to the pqs system of wild-type *Ps. aeruginosa* 33 PAO1 with an IC_{50} of 20.22 μmol l^{-1}. The virulence factors including pyocyanin, total protease, 34 elastase, and rhamnolipid were significantly suppressed in a concentration-dependent manner with 35 the compound. In addition, **1** in combination with tetracycline inhibited synergistically the bacterial 36 growth and suppressed the biofilm formation of PAO1. The molecular docking studies also 37 suggested that **1** could potentially interact with the ligand-binding domain of the Lys-R type 38 transcriptional regulator PqsR as a competitive antagonist.

39 **Conclusions:** The quinoline-based derivatives were found to interrupt the quorum sensing 40 system via the pqs pathway and thus the production of virulence factors was inhibited and the 41 antimicrobial susceptibility of *Ps. aeruginosa* was enhanced.

42 **Significance and Impact of Study:** The study showed that the quinoline-based derivatives 43 could be used as an anti-virulence agent for treating *Ps. aeruginosa* infections.

44

45 **Keywords**: Quinoline derivatives, *Pseudomonas aeruginosa*, PqsR inhibitor, Quorum 46 sensing, Virulence factors, Drug resistance.

47 **1. Introduction**

48 Antibiotic drug resistance has been a critical public health problem worldwide. The multidrug-
49 resistant (MDR) bacteria such as MDR *Pseudomonas aeruginosa* are commonly associated with
50 health care and cause a substantial health burden on society and health systems (Jernigan *et al.*
51 2020). High and increasing prevalence of MDR *Ps. aeruginosa* was observed in many tertiary
52 hospitals in Mainland China. Declining susceptibility to most antibiotics in *Ps. aeruginosa* isolates
53 was also found (Peng *et al.* 2014; Fan *et al.* 2016). A 16-year retrospective report undertaken to
54 examine antimicrobial resistance of *Ps. aeruginosa* in Southern China stated that ampicillin,
55 ampicillin-sulbactam, ceftriaxone, and trimethoprim-sulfamethoxazole almost lost their action on
56 *Ps. aeruginosa*, with resistance rates reaching 90% (Xie *et al.* 2017). In addition, a 2019 joint report
57 by the United Nations, World Health Organization and World Organization for Animal Health
58 stated that drug-resistant diseases could cause 10 million deaths each year by 2050 and force up to
59 24 million people into extreme poverty by 2030 if no action was taken (Mohammed and
60 Ghebreyesus 2019).

61 *Ps. aeruginosa* is the Gram-negative opportunistic bacteria, which are a major source of
62 nosocomial infections, especially in individuals with cystic fibrosis and those who are
63 immunocompromised (Azam and Khan 2019). *Ps. aeruginosa* antimicrobial resistance can be
64 adaptive, intrinsic, or acquired. Overexpression of efflux pump, production of modified enzymes
65 and the reduction of outer membrane protein have important roles in antibiotic resistance (Azam
66 and Khan 2019). In addition, *Ps. aeruginosa* acquires the external genes responsible for resistance
67 through horizontal gene transfer and chromosomal gene mutations. The dissemination of β-lactams
68 resistance in *Ps. aeruginosa* has been largely attributed to the horizontal transfer of β-lactamase
69 genes by plasmids (Xu *et al.* 2021). Biofilm production also gives an additional advantage to

70 antimicrobial resistance by lowering the accessibility of antimicrobial agents to the cells (Sharma
71 *et al.* 2019). Moreover, *Ps. aeruginosa* release virulence factors, which disrupt the normal
72 physiological function of host cells and may cause death (Gellatly and Hancock 2013). The
73 virulence agents secreted by *Ps. aeruginosa* including elastase, pyocyanin, rhamnolipid, hydrogen
74 cyanide and exotoxin A assist in invasion and damage of host tissues (Blackwood *et al.* 1983;
75 Wretlind and Pavlovskis 1983; Lau *et al.* 2004; Caiazza *et al.* 2005; Soheili *et al.* 2019). As an
76 alternative to direct killing bacteria, the use of effective anti-infective agents can also suppress the
77 expression of bacterial virulence factors, as well as the biofilm formation, and thereby achieving
78 the aim of combating bacteria-mediated diseases (Rasko and Sperandio 2010). Since anti-virulence
79 approaches may stimulate a milder evolutionary pressure towards the development of resistance
80 than conventional antibiotics, the selective pressure on bacteria to evolve antibiotic resistance may
81 be reduced (Mühlen and Dersch 2016; Fleitas *et al.* 2019).

82 Quorum sensing (QS) is an intercellular signal transduction mechanism that relies on bacterial
83 density to govern group behavior among bacteria by using signal molecules called autoinducers.
84 The QS system in *Ps. aeruginosa* is made up of two *N*-acyl homoserine lactone (AHL)-dependent
85 systems (las and rhl) and a pseudomonas quinolone signal (pqs) system that uses alkylquinolone-
86 derived autoinducers (Smith 2003; De Kievit 2009; Lee and Zhang 2014). These QS systems work
87 in a hierarchical fashion to control the production of virulence factors and formation of biofilms.
88 Signal molecular synthetases (LasI and RhlI) and transcriptional regulator proteins (LasR/RhlR)
89 make up the las and rhl systems. LasI catalyzes the formation of *N*-(3-oxododecanoyl) homoserine
90 lactone (3-oxo-C12-HSL), which binds to the receptor protein LasR and activates the expression
91 of genes involved in the production of virulence factors including alkaline protease, LasA protease,
92 elastase and that of the rhl system (Gambello *et al.* 1993; Latifi *et al.* 1996; Pesci *et al.* 1997). The

93 *N*-butyryl-homoserine-lactone (C4-HSL) generated by RhlI synthase binds to RhlR, then the
94 activating genes involve in pyocyanin, elastase, rhamnolipid and hydrogen cyanide production
95 (Lee and Zhang 2014; Mukherjee *et al.* 2017). Alkyl quinolones, such as 2-heptyl-4-quinolone
96 (HHQ) and 2-heptyl-3-hydroxy-4-quinolone (PQS), are used in pqs system to activate the Lys-R
97 type transcriptional regulator PqsR, which is also known as MvfR (Pesci *et al.* 1999; Cao *et al.*
98 2001). The receptor PqsR in *Ps. aeruginosa* upon forming complex with PQS or HHQ is able to
99 regulate positively several genes including the biosynthesis of *pqsABCDE* operon and the release
100 of various virulence factors such as elastases, lectins, pyocyanin, and hydrogen cyanide (Soheili *et*
101 *al.* 2019). It was reported that *Ps. aeruginosa* could influence the growth of other pathogens, such
102 as *Staphylococcus aureus* via QS system (Xu *et al.* 2019). Another quinolone QS signaling
103 molecule induced by PqsL, 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO), inhibits electron
104 transfer, causing *S. aureus* respiration to shift from aerobic to anaerobic (Radlinski *et al.* 2017).
105 Pyocyanine has also been shown to inhibit *S. aureus* aerobic respiration and growth. Moreover, *Ps.*
106 *aeruginosa* exoproducts such as HQNO, LasA protein, and rhamnolipids were discovered to alter
107 *S. aureus* susceptibility to bactericidal antibiotics (Radlinski *et al.* 2017; Xu *et al.* 2019).
108 These QS systems work together to integrate and govern the activity of each other and regulate
109 the diverse virulence factors at different levels (**Figure 1A**). The las system placed at the top of the
110 regulatory network is considered able to influence positively the expression of the *rhlI/R*, *pqsR*,
111 and *pqsABCDE* operons (Pesci *et al.* 1997; Wade *et al.* 2005). Between the las and rhl system, the
112 pqs system serves as a regulatory connection. The synthesis of PQS signal molecules is regulated
113 by two systems (las and rhl) (McKnight *et al.* 2000; Wade *et al.* 2005), on the other hand, pqs
114 system also regulates the expression of genes related to las and rhl system (Pesci *et al.* 1999; Diggle
115 *et al.* 2003). PqsR has also been found to bind and influence directly the expression of additional

116 35 loci across the *Ps. aeruginosa* genome including the key regulators such as *lasR* and *rhlR*, and
117 the genes involved in protein translation, secretion and oxidative stress response (Maura *et al.* 2016;
118 García-Reyes *et al.* 2020). Moreover, the importance of PqsR on controlling the virulence factors
119 is further highlighted by the dramatic drop in the production of virulence factors in a mutant
120 (knockout) PqsR strain (Diggle *et al.* 2003). Therefore, PqsR is a potential drug target because it
121 plays a critical and central role in *Ps. aeruginosa* QS network. Rational design of structural
122 analogues of PQS and HHQ may be able to block the pqs pathway and could be a promising
123 strategy for reducing pathogenicity and fighting antibiotic-resistant *Ps. aeruginosa* infections. In
124 recent years, some small molecules derived from benzimidazole, quinolinone, quinazolinone,
125 pyridine, and pyrrole have been demonstrated as the potential PqsR inhibitors (Figure 2), which
126 reduced pathogenicity of *Ps. aeruginosa* by selectively inhibiting the pqs pathway (Lu *et al.* 2014;
127 Starkey *et al.* 2014; Aleksic *et al.* 2019; Grossman *et al.* 2020; Sabir *et al.* 2021; Liu *et al.* 2022).
128 Compound **I** (**M64**) demonstrated effective anti-pyocyanin and anti-biofilm properties (IC_{50} = 300
129 nmol l^{-1} and 1 μmol l^{-1}, respectively), which are the best anti-virulence activity reported in literature.
130 In addition, **M64** was the first PqsR inhibitor to exhibit *in vivo* action in a mouse lung infection
131 model (Starkey *et al.* 2014). In preclinical stage, it also appears to be one of the most promising
132 PqsR inhibitors. However, there is no lead compound reported for clinical trials. In the present
133 study, we designed a series of novel quinoline-based derivatives as the potential inhibitor of PqsR.
134 The compounds showed good inhibition potency in the production of virulence factors against the
135 wild type *Ps. aeruginosa* PAO1.
136

137 ## Materials and methods

138 ### Reagents and compound characterization

139 All chemicals and reagents used in this work were purchased commercially in AR grade and
140 were utilized without further purification. Aglient produced high-resolution mass spectra (HRMS).
141 Bruker Avance III 300 MHz Superconducting Fourier Nuclear Magnetic Resonance Spectrometer
142 was used to record ^{1}H and ^{13}C NMR in deuterated solvent (DMSO-d_6) at 24 °C using TMS as a
143 reference. All the compounds synthesized were characterized with ^{1}H NMR, ^{13}C NMR, and high
144 resolution MS before examined in bioassays (**Figure S1-S6**).

145 ### MIC assay

146 The minimum inhibitory concentration (MIC) tests were determined using 96-well microtiter
147 plates with *Ps. aeruginosa* PAO1. An overnight culture of PAO1 (grown in LB medium at 37 ℃,
148 200 rpm) was diluted in MHB (Mueller-Hinton broth) medium to a final concentration of 5×10^{5}
149 CFU ml^{-1} in a 96-well microtiter plates. A series of concentrations of the compounds were then
150 added. After incubation at 37 ℃ for 24 hours, the minimum inhibitory concentration is defined as
151 the lowest concentration of test compounds that prevents visible growth of bacterium.

152 ### Growth curve assay

153 To construct growth curves, an overnight culture of PAO1 (grown in LB medium at 37 ℃,
154 200 rpm) was diluted in LB medium to OD_{600} of 0.05. The growth curve was recorded every 15
155 minutes with Spectramax M3 Microplate Reader (Molecular Devices, USA) for 16 hours.

156 ### MTT assay

157 Normal cells HK2 cells and 16HBE cells were used in the MTT experiments. Culture the cells
158 into a 96-well microtiter plate with a cell density of approximately 5000 cells per well at 37 ℃
159 and 0.5% CO_2. The culture medium was F12 (HK2) and DMEM (16HBE). After culturing for 24

160 hours, the 96-well microtiter plate was washed 3 times with PBS, and then the cells were treated
161 with a series of concentrations of compounds solution for 48 hours. The medium was sucked with
162 a pipettor while 200 μl of 0.5 mg ml^{-1} MTT solution was added to each well, and then incubated in
163 the dark for 4 hours. Next, MTT solution in the well was sucked with a pipettor, and 100 μl of
164 DMSO was added to dissolve the blue-purple crystal formazan in the cells. These treated 96-well
165 plates were measured for absorbance with an Enzyme label and the absorption wavelength was 570
166 nm. Finally, the cell survival rate and the IC_{50} of compounds on the cells were calculated from the
167 obtained absorbance values.

168 **GFP reporter strain assay**

169 The assay for QS inhibition activity was performed using PAO1-*pqsA*-*gfp*, PAO1-*lasB*-*gfp*,
170 and PAO1-*rhlA*-*gfp* bioreporter strains (Liu *et al.* 2020). An overnight culture of reporter strain
171 (grown in LB medium at 37 ℃, 200 rpm) was diluted in MHB medium to the OD_{600} of 0.05 in a
172 bottom transparent black 96-well microtiter plate (Greiner Bio-one). A series of concentrations of
173 the compounds were then added. The microtiter plate was incubated at 37 ℃ in Spectramax M3
174 Microplate Reader (Molecular Devices, USA) to measure the OD_{600} and GFP fluorescence
175 (excitation at 485 nm, emission at 535 nm) with 15 minutes intervals for at least 16 hours. The
176 fluorescence values shown in the graph were normalized with respect to OD_{600}. The inhibition
177 assays for all compounds tested and controls were performed in triplicate.

178 **qRT-PCR assay**

179 To investigate the expression of QS-related genes (*lasI*, *lasR*, *rhlI*, *rhlR* and *pqsA*), efflux
180 pump genes (*mexA* and *mexE)* and exopolysaccharide genes (*pelA* and *pslA*), primers were
181 designed (**Table S1**) and *16S rRNA* was used as the reference gene. An overnight culture of PAO1
182 (grown in LB medium at 37 ℃, 200 rpm) was diluted in LB medium to the OD_{600} of 0.05. PAO1

183 cells treated with **1** and 0.8% DMSO were harvested for total RNA isolation after 24 hours
184 incubation. Total RNA was isolated using the RNA extraction reagent (Servicebio). The quality of
185 purified RNA was analyzed by quantified by Nanodrop 1000 Spectrophotometer (Thermo). Then,
186 the HiScriptII one-step qRT-PCR SYBR Green kit was used for reverse transcription of RNA and
187 quantitative analysis of transcribed cDNA. The RT-PCR reactions were performed on a Real-Time
188 PCR System (Bio-Rad) and the cycling parameters were set according to the manufacturer's
189 recommendations. The relative transcript abundance was calculated according to the $2^{-\triangle\triangle Ct}$ method.

190 **Virulence factors assay**

191 An overnight culture of PAO1 (grown in LB medium at 37 ℃, 200 rpm) was diluted in LB
192 medium to the OD_{600} of 0.05. A series of concentrations of the compounds were then added. The
193 sterile supernatant was obtained by centrifugation after culture at 37 ℃ for 24 hours.

194 Total proteolytic activity was measured as described previously (El-Mowafy *et al.* 2014).
195 Briefly, 0.25 ml PAO1 sterile supernatant was mixed with 0.5 ml 1.25% skim milk. The absorbance
196 at 600 nm was measured after incubated at 37 ℃ for 15 minutes.

197 Pyocyanin was determined according to the procedures reported by Liu *et al* (Liu *et al.* 2022).
198 Briefly, 5 ml supernatant were mixed with 3 ml chloroform, and then the chloroform was isolated
199 and acidified with 1 ml 0.2 mmol l^{-1} HCl. The mixture was centrifuged, and the absorbance at 520
200 nm was measured with the pink water phase in the supernatant.

201 Elastase activity was determined using Microbial Elastase LasB ELISA Kit (MEIMIAN). The
202 experimental steps are operated according to the manufacturer's recommendations.

203 Rhamnolipids were assessed using the orcinol method (Zhou *et al.* 2018). Briefly, an organic
204 phase containing rhamnolipid was obtained by extracting 500 μl sterile supernatant with ethyl
205 acetate. After ethyl acetate was volatilized at room temperature, the residue was dissolved in 100

206 μl deionized water. Then, 900 μl orcinol solution (0.19% orcinol in 53% [v/v] H_2SO_4) was mixed
207 with the sample. The orange liquid was obtained after the sample was heated at 80 ℃ for 30
208 minutes, and the absorbance at 421 nm was measured. To avoid the influence of the absorbance of
209 the compound, solvent control containing the compounds were set up in the experiment.

210 **Motility assay**

211 **Swimming motility assay**

212 Swimming motility was assayed according to the procedures reported by Liu *et al* (Liu *et al.*
213 2020). LB medium with 0.3% agar (soft agar) was poured into a petri dish. Different concentrations
214 of compounds **1-6** were then added before the medium solidify. The medium was solidified at room
215 temperature for 2 hours prior to inoculation. An overnight culture of PAO1 (grown in LB medium
216 at 37 ℃, 200 rpm) was used as inoculum. Sterile toothpicks were used to inoculate PAO1 into the
217 center of the medium without touching the bottom of petri dish. The swimming zone area was then
218 observed after incubated at 37 ℃ for 12 hours.

219 **Swarming motility assay**

220 Swarming motility was assayed according to the procedures reported by Liu *et al* (Liu *et al.*
221 2020). LB medium with 0.5% agar (soft agar) was poured into a petri dish. Different concentrations
222 of compounds **1-6** were then added before the medium solidify. The medium was solidified at room
223 temperature for 2 hours prior to inoculation. Each petri dish was inoculated with 3 μl of a liquid
224 culture that was grown as described above. The swarming zone area was then observed after
225 incubated at 37 ℃ for 24 hours.

226 **Preformed biofilm inhibition assay**

227 Measurement of compound **1** and tetracycline in biofilm inhibition was by crystal violet assay
228 (Zhou *et al.* 2018). An overnight culture of PAO1 (grown in LB medium at 37 ℃, 200 rpm) was

229 diluted in LB medium to the OD_{600} of 0.05 in a 96-well microtiter plates. Once biofilm formed, the
230 suspension cultures were removed and wells were washed three times with sterile PBS solution to
231 remove the planktonic cells. LB medium contain compound **1** and tetracycline was then added into
232 96-well microtiter plates. The suspension cultures were handled as described above after incubated
233 at 37 ℃ for 8 hours, then MeOH as a fixative. After 30 minutes, the MeOH was removed, and the
234 microtiter plates were dried at room temperature. The attached biofilms were stained with crystal
235 violet (0.1%) for 15 minutes. The excess crystal violet solution in the well was washed with distilled
236 water and bound crystal violet was solubilized in 100 μl of 33% acetate solution. Biofilms were
237 quantified by reading the absorbance at 600 nm.

238 **3D imaging of biofilm**

239 For visual observation of the preformed biofilms, samples were captured using confocal laser
240 scanning microscopy (ZEISS LSM 800 with Airyscan). Briefly, the preformed biofilms were
241 established in confocal dishes and treated with compound **1**, tetracycline, or their combination as
242 in the preformed biofilm inhibition assay. The supernatant was removed and the dish surface was
243 rinsed briefly with PBS three times. Then, 100 μl of PBS containing 0.01% acridine orange was
244 added into the dish and incubated for 15 minutes in the dark (Zhou *et al.* 2018). 3D images were
245 obtained using a 10X objective lens and Z-stack program.

246 **Molecular docking analysis**

247 The molecular docking procedures were performed using Discovery Studio version 2016 (DS
248 2016). The *X*-ray crystal structure of MvfR (PqsR) ligand binding domain in complex with **M64**
249 (PDB ID: 6B8A) (Kitao *et al.* 2018) was downloaded from RCSB PDB database. Water molecules
250 and co-crystal ligands were removed, and the protein was prepared using DS automated procedures.
251 The structure of compound **1** was converted into 3D and minimized using DS small molecule tool.

252 The docking studies were implemented using DS-CDocker protocol. The docking results were
253 analyzed and visually inspected in DS 2016.
254

255 **Results**

256 **Antibacterial activity evaluation and growth curve analysis**

257 The screening criteria for potential inhibitors of PqsR mainly emphasize on the reduction of
258 bacterial virulence rather than direct killing of bacteria (Rasko and Sperandio 2010; Abd El-Aleam
259 *et al.* 2021). Antibacterial activity of the new inhibitors of PqsR was evaluated in terms of the
260 minimum inhibitory concentrations (MICs) using broth microdilution protocols specified in the
261 Clinical and Laboratory Standards Institute (CLSI) recommendations (Ferraro 2000) against the
262 *Ps. aeruginosa* standard strain PAO1 (**Table 1**). The PqsR inhibitors tested were found no inhibitory
263 effect on the growth of PAO1 even at high concentration (260 μmol l^{-1}). Ciprofloxacin and
264 tetracycline were also conducted as the control. It was found that PAO1 was susceptible to
265 ciprofloxacin (MIC = 4 μmol l^{-1}) but not tetracycline (MIC > 260 μmol l^{-1}), indicating that PAO1
266 was resistant to tetracycline. In addition, the growth kinetics of PAO1 (**Figure S7**) showed no
267 significant difference for the compound-treated groups (concentration ranged from 16.25 μmol l^{-1}
268 to 260 μmol l^{-1}) compared to the control group.
269

270 **Evaluation of cytotoxicity of the compound against human cells**

271 To examine whether the new compounds exhibited cytotoxicity against human cells, HK2
272 cells and 16HBE cells were investigated with MTT assays (**Figure S8**). From the IC_{50} values
273 obtained in the assays (**Table 2**), it was found that some compounds, **1**, **4** and **6**, which have an
274 oxygen atom at the amine substituent group generally showed significantly lower cytotoxicity

275 effect ($IC_{50} > 40$ µmol l^{-1}) than the other compounds **2**, **3** and **5** ($IC_{50} < 20$ µmol l^{-1}). Therefore, the
276 analogues of **1**, **4** and **6** may be more favorable for using as the PqsR inhibitor against PAO1.
277
278 **Evaluation of QS inhibition effect of the compound against PAO1**
279 According to the previous studies (Lee and Zhang 2014; García-Reyes *et al.* 2020), PqsR was
280 found able to trigger the expression of the *pqsABCDE* and *phnAB* operons and was implicated in
281 the induction of *rhlR* and *lasR*. Therefore, the function of las, rhl and pqs QS systems may be
282 interrupted when the function of PqsR is inhibited with inhibitors. To understand whether our new
283 inhibitors could influence the pqs quorum sensing system by targeting PqsR, four reporter strains
284 including PAO1-*pqsA*-*gfp*, PAO1-*lasB*-*gfp*, PAO1-*rhlA*-*gfp* and PAO1-*gfp* were selected for
285 investigations. The green fluorescent protein (*gfp*) gene was integrated downstream from the
286 promoters of *pqsA*, *lasB*, and *rhlA*. The production of green fluorescent protein (GFP) indicated
287 that these promoters were active in the strains (Liu *et al.* 2020). The *Ps. aeruginosa* PAO1-*pqsA*-
288 *gfp* reporter strain was firstly used to evaluate the compounds for the inhibitory activity (**Figure**
289 **S9A**). The inhibitory activity of compound **1** was shown in **Figure 3A**. The expression level of
290 *pqsA* in the control experiment was found peaked at about 6 hours. Moreover, compared with the
291 control, **1** was markedly suppressed the *pqsA* expression in a concentration-dependent manner.
292 Among the compounds tested, **1** with a morpholine substituent group at the 2-position of 1-
293 methylquinolinium showed the best inhibitory activity against *pqsA* ($IC_{50} = 20.22$ µmol l^{-1}).
294 Moreover, compound **2** bearing a pyrrolidine substituent ($IC_{50} = 30.13$ µmol l^{-1}) was found slightly
295 less active than **1**. For compounds **3**-**6** bearing the non-cyclic amine substituent group, the
296 inhibitory activity observed was just moderate ($IC_{50} > 50$ µmol l^{-1}). The results suggested that the

297 molecular scaffold at the 2-position of 1-methylquinolinium showed remarkable influence in the
298 suppression of *pqsA* expression (**Table 3**).

299 To study the inhibitory effects of the compounds against the las and rhl systems, PAO1 *lasB*-
300 *gfp* and *rhlA*-*gfp* reporter strains were selected for investigation (**Figure S9B-C**). From **Figure 3B-**
301 **C**, the expression of *lasB* and *rhlA* was significantly suppressed by compound **1** in a concentration-
302 dependent manner. The inhibitory effects of **1** and **2** against the las and rhl systems were generally
303 better than that of **3**-**6** (**Table 3**). In addition, to further confirm that compound **1** may target the QS
304 genes of the strains rather than the GFP protein, a PAO1-*gfp* strain was utilized as a control to
305 examine the effect of **1** on the fluorescence of GFP protein. From **Figure 3D**, it was found that **1**
306 showed no significant influence on the GFP fluorescence.

307 **The effect of the compound on the expressions of QS-related genes**

308 qRT-PCR assays were performed to investigate the effects of compound **1** on the expressions
309 of five QS-related genes of *Ps. aeruginosa* PAO1, which include *lasI*, *lasR*, *rhlI*, *rhlR* and *pqsA*.
310 The results (**Figure 4**) showed that the expression levels of these genes were all downregulated
311 markedly. Among these, the expression of *pqsA* decreased most obviously. In addition to the QS-
312 related genes, the efflux pump genes, *mexA* and *mexE*, were found downregulated significantly.
313 Interestingly, the exopolysaccharide genes, *pelA* and *pslA*, were found upregulated with the
314 compound.

315 **The effect of the compound on bacterial virulence factors**

316 The effect of compound **1** on the bacterial virulence factors (pyocyanin, total protease, elastase
317 and rhamnolipid) regulated by QS system was investigated. Pyocyanin is a blue redox-active
318 secondary metabolite synthesized from chorismate by phz operons and the synthesis is positively
319 regulated by the LysR-like transcriptional activator PqsR, with additional regulatory signals from

320 the las and rhl systems (Lau *et al.* 2004). It was reported that pyocyanin had a role in the persistence
321 of *Ps. aeruginosa* in the lungs of cystic fibrosis (CF) patients and induced severe cellular damage
322 (Lau *et al.* 2004). Elastase, encoded by *lasB*, also has tissue-damaging activity and can degrade
323 various plasma proteins (McKnight *et al.* 2000). The expression of *lasB* is regulated by las, rhl and
324 pqs systems (Wretlind and Pavlovskis 1983; García-Reyes *et al.* 2020). Rhamnolipid acts as a
325 surfactant and plays an important role in the swarming motility and biofilm formation in *Ps.*
326 *aeruginosa*. The expression of rhamnolipid synthesis gene *rhlA* was directly activated by *N*-butyryl
327 homoserine lactone-activated RhlR (Caiazza *et al.* 2005). The effect of compound **1** on these
328 bacterial virulence factors was shown in **Figure 5**. As expected, the compound significantly
329 decreases the level of pyocyanin in a concentration-dependent manner (**Figure 5A**), with an IC_{50}
330 value of 17.38 ± 0.41 μmol l^{-1}. However, the inhibitory effect of **1** on the activity of total protease,
331 elastase and rhamnolipidis was found obviously weaker than pyocyanin (**Figure 5B-D**). The
332 inhibitory assays with compounds **2-6** on these virulence factors also gave similar observation
333 (**Figure S10-S14**).

334 **Effects of the compound on swimming and swarming motility**

335 Swimming motility is associated with flagella, which are the motor organ and assist *Ps.*
336 *aeruginosa* in obtaining nutrients, escaping toxic environments, and finding adequate adhesion
337 sites (Conrad *et al.* 2011). Swimming motility is dependent on flagella rotation and is the movement
338 of bacteria in a liquid or semi-solid media with a low agar concentration (< 0.3% w/v). Swarming
339 motility is a sort of colony behavior in which bacteria coordinate and move rapidly in a semi-solid
340 environment (0.5 ~ 0.7% w/v) (Rashid and Kornberg 2000). It is connected to the synthesis of
341 bacterial flagella, type IV flagella, and rhamnolipids (Caiazza *et al.* 2005; Conrad *et al.* 2011).
342 These motility modes are regarded as virulence properties of *Ps. aeruginosa* because of its

343 important role in bacterial adhesion and biofilm formation (Wang *et al.* 2014). We found that, as
344 shown in **Figure 6**, compound **1** suppressed both swimming and swarming motilities of PAO1 in a
345 concentration-dependent manner. Compounds **2-6** also showed a similar inhibitory effect for PAO1
346 swimming and swarming motilities (**Figure S15-S16**). These experimental results were also found
347 consistent with the previously established anti-virulence activity of compound **1**.

348 **Synergistic effects of the compound with tetracycline against *Pseudomonas aeruginosa***

349 From the MIC results shown in **Table 1**, compounds **1-6** and tetracycline were found no
350 significant killing effect against PAO1. It was reported that the tetracycline-resistance of PAO1 was
351 related to the efflux pump (Morita *et al.* 2001). We found that compound **1** exhibited markedly
352 effects on the downregulation of efflux pump genes. It may be able to enhance the susceptibility of
353 PAO1 in the tetracycline treatment combined with **1**. As expected, the combination of **1** with
354 tetracycline exhibited a remarkably synergistic effect (**Figure 7A**). In addition, the growth curve
355 of the indicated that the combined treatment inhibited the growth rate of bacteria (**Figure 7B**). In
356 the control experiments, neither **1** nor tetracycline alone was able to inhibit PAO1 growth within
357 24 hours. We also found that the combined treatment with 32.5 μmol l^{-1} **1** and 65 μmol l^{-1}
358 tetracycline significantly suppressed PAO1 proliferation. Compounds **2-6** with tetracycline also
359 showed similar synergistic inhibitory effect against *Ps. aeruginosa* (**Figure S17**).

360 **Effects of the compound on *Pseudomonas aeruginosa* biofilm formation**

361 The pre-established PAO1 biofilms (24 hours) grown in LB medium were treated with
362 compound **1** and tetracycline, respectively. The biofilm contents were then evaluated using crystal
363 violet tests. The results indicated that **1** alone did not suppress the production of biofilm (**Figure**
364 **8A**). However, when the pre-established biofilm was treated with **1** and tetracycline together for 8
365 hours, the level of biofilm production in the well plate was reduced by 40% approximately (**Figure**

366 **8A**). In addition to quantitative analysis, the treated biofilms were also visualized using a confocal
367 laser scanning microscopy as shown in **Figure 8B**. The biofilm of PAO1 become denser under the
368 treatment of compound **1** alone, while it was significantly reduced under the combined treatment
369 with **1** and tetracycline.

370 **Molecular docking study**

371 To have a better understanding in the possible binding mode of compound **1** with the PqsR
372 (MvfR) receptor, we conducted *in silico* molecular docking study using Discovery Studio version
373 2016 (DS 2016). The energy minimized ligand, compound **1**, was docked into the active site of a
374 model created from the crystal structure of the PqsR protein (PDB: 6B8A) in complex with the
375 previously reported competitive inhibitor **M64** (Kitao *et al.* 2018). According to the best docking
376 model (**Figure 9A**), compound **1** was predicted to bind to the PqsR receptor in the hydrophobic
377 pocket same as **M64**. The predicted interactions between **1** and PqsR residues were depicted in a
378 2D ligand interaction diagram (**Figure 9B**). The phenyl moiety of **1** binds to Leu208, Val211, and
379 Ile236 of the PqsR protein primarily via π-alkyl hydrophobic interactions. Between the morpholine
380 substituent group of **1** and Thr265, two carbon hydrogen bonds were established. In addition, a
381 number of amino acids such as Ala168, Leu207, and Gln194 in the binding pocket may also form
382 Van der Waals forces with **1**.

383 **Discussion**

384 Quinoline had been used as a potent scaffold for the development of PqsR inhibitors because
385 of its structural similarity to the endogenous signal molecules PQS and HHQ (Aleksić *et al.* 2017;
386 Aleksic *et al.* 2019). In the present study, a series of quinoline-based derivatives was designed via
387 the integration of different amine substituents (linear or cyclic) at the 2-position of 1-
388 methlyquinolinium core scaffold (**Figure 1B**) as the new inhibitors of PqsR. Recently, hordenine

389 had been demonstrated as an effective QS inhibitor (Zhou *et al.* 2018). To enhance the anti-QS
390 activity of these inhibitors, in the present study, a 4-dimethylaminostyrene group with similar
391 structure to hordenine was introduced at the 4-position of quinoline. The newly designed quinoline-
392 based derivatives could be the potential inhibitor of PqsR. In addition, these compounds were
393 readily obtained with good yields via a multi-step synthesis under simple and mild reaction
394 conditions as shown in **Scheme S1**.

395 *Ps. aeruginosa* has been found with high levels of antibiotic resistance and threaten greatly
396 the global public health. The emerging of drug resistant bacteria is a critical challenge and renders
397 an urgent action to discover innovative antimicrobial interventions (Jernigan *et al.* 2020). The
398 potential use of anti-virulence therapies to combat bacterial infections has attracted great interests
399 in recent decades, as the approach may potentially diminish the pathogenicity of target germs with
400 little selection pressure and thus possibly limiting the establishment of resistance (Abd El-Aleam
401 *et al.* 2021). One of these strategies is to disrupt the bacterial pqs quorum sensing system, which
402 governs multiple virulence traits and biofilm formation through the interaction with PqsR, a Lys-R
403 type transcriptional regulator, with its cognate signal molecules HHQ and PQS (Soheili *et al.* 2019).

404 In the present study, a series of new PqsR inhibitors bearing a quinoline scaffold similar to the
405 core structure of PQS was designed and synthesized. The biology activity of these inhibitors against
406 *Ps. aeruginosa* PAO1 was also evaluated. In MICs assay and growth curve analysis, we found that
407 these compounds were not able to interrupt the primary bacterial metabolism and exhibited very
408 mild effect on the cell growth or reproduction of PAO1. Moreover, to understand whether our new
409 inhibitors could influence the pqs quorum sensing system by targeting PqsR, GFP reporter strains
410 were selected for investigations. The results showed that all compounds could inhibit the
411 expression of *pqsA*, *lasB* and *rhlA* in a concentration-dependent manner. Among the compounds

412 tested, **1** with a morpholine substituent group at the 2-position of 1-methylquinolinium showed the
413 best inhibitory activity against *pqsA* (IC_{50} = 20.22 μmol l^{-1}). Furthermore, the results generally
414 suggested that the compound exhibited relatively stronger inhibition effects against *pqsA* than *lasB*
415 and *rhlA*, which may suggest that the compounds could probably interrupt the QS system via the
416 pqs pathway as proposed in **Figure 1B**.

417 qRT-PCR assays were also performed to investigate the effects of **1** on the expressions of five
418 QS-related genes of *Ps. aeruginosa* PAO1, which include *lasI*, *lasR*, *rhlI*, *rhlR* and *pqsA*. The results
419 (**Figure 4**) showed that the expression levels of these genes were all downregulated markedly. It is
420 noteworthy that the inhibitory effect of **1** on *pqsA* was found much stronger than other genes
421 examined. The results may support the hypothesis that **1** targeting PqsR could selectively inhibit
422 the pqs pathway and consequently repress the QS system of *Ps. aeruginosa* PAO1.

423 In *Ps. aeruginosa*, QS has been demonstrated to play a crucial role in virulence factors
424 production (Smith 2003). Compound **1** was found significantly decrease the level of pyocyanin in
425 a concentration-dependent manner, with an IC_{50} value of 17.38 ± 0.41 μmol l^{-1}. However, the
426 inhibitory effect of **1** on the activity of total protease, elastase and rhamnolipidis was found
427 obviously weaker than pyocyanin. Taken together, these results demonstrated that the compounds
428 could inhibit the production of virulence factors through the inhibition of pqs quorum sensing
429 system. The motility modes are regarded as virulence properties of *Ps. aeruginosa* because of its
430 important role in bacterial adhesion and biofilm formation (Wang *et al.* 2014). We also found that
431 **1** suppressed both swimming and swarming motilities of PAO1 in a concentration-dependent
432 manner. These experimental results were also found consistent with the previously established anti-
433 virulence activity of **1**.

434

435 In addition to virulence factor production, we found that PAO1 treated with compounds
436 showed increased susceptibility. When PAO1 was treated in combination with the compound and
437 tetracycline, the bacterial growth was inhibited and the mature biofilms were broken down
438 markedly. It was reported that the tetracycline-resistance of PAO1 was related to the efflux pump
439 (Morita *et al.* 2001). Efflux pumps are membrane proteins that involve in the export of noxious
440 substances from the bacterial cell into the external environment (Alav *et al.* 2018). The
441 downregulation of efflux pump genes may provide a possible explanation that the bacteriostatic
442 activity of tetracycline increases when combined with **1**. The inhibition of efflux pump activity
443 may increase the intracellular concentration of tetracycline in individual cell or a group of cells.
444 Consequently, bacterial proliferation was inhibited (Cernicchi *et al.* 2021).

445 Nevertheless, the biofilm treated with **1** showed a slight increase in the production. In
446 literature, several PqsR inhibitors have been reported to show a positive impact on PAO1 biofilm
447 development (Soukarieh *et al.* 2020; Soukarieh *et al.* 2021). From the qRT-PCR experiments
448 (**Figure 4**), we found that the exopolysaccharide production genes of *pelA* and *pslA* were
449 upregulated after administered with **1**. In non-mucoid *Ps. aeruginosa* strains, such as PAO1 and
450 PA14, the main forms of exopolysaccharides in biofilms are Pel and Psl polysaccharides (Ryder *et*
451 *al.* 2007). We speculated that these PqsR inhibitors, due to their similar structures to PQS, activated
452 the expression of exopolysaccharide genes after binding to PqsR, thereby increasing biofilm
453 production.

454 The molecular docking study suggested that the compound could potentially binds to the
455 ligand-binding domain (LBD) of MvfR, which is the PQS responsive quorum-sensing transcription
456 factor of *Ps. aeruginosa*, and acts as a competitive antagonist. Besides the central ligand-binding
457 site, PqsR (MvfR) has an adjacent hydrophobic pocket lined by residues Leu183, Ile186, Leu189,

458 and Tyr258 that are coupled to the core ligand-binding site by a narrow channel. Hydrophobic
459 interactions between the phenoxy group of **M64** and the aforementioned amino acid residues allow
460 it to occupy this extra hydrophobic pocket in PqsR (MvfR) LBD. Due to the short side group of
461 compound **1**, it is unable to occupy this site and this may probably result in the PqsR antagonistic
462 activity of **1** not as potent as **M64** (Kitao *et al.* 2018; Sabir *et al.* 2021). This discovery may serve
463 as a theoretical foundation for future structural optimization of lead compounds to boost PqsR
464 inhibitor efficacy.

465 In conclusion, a series of new quinoline-based derivatives was synthesized and demonstrated
466 as the effective PqsR inhibitor against *Ps. aeruginosa* PAO1. The inhibitory activity observed was
467 at micro-molar level. The compounds were found to inhibit the pqs pathways by targeting the
468 receptor protein PqsR but without affecting normal bacterial growth of PAO1. The bacterial
469 production of a number of virulence factors including pyocyanin, total protease, elastase, and
470 rhamnolipid was significantly suppressed in a concentration-dependent manner. In addition, the
471 compounds showed remarkable influence in the bacterial motility, biofilm formation and efflux
472 pump expression in PAO1. Furthermore, when PAO1 was treated in combination with the
473 compound and tetracycline, the bacterial growth was inhibited and the mature biofilms were broken
474 down markedly. It was found that compound **1** bearing a heterocyclic amine substituent at the 2-
475 position of 1-methylquinolinium scaffold exhibited high potency in inhibition of the pqs pathway
476 and the IC_{50} down to 20.22 $\mu mol\ l^{-1}$ was observed. The molecular docking study suggests that the
477 compound could potentially interact with the ligand-binding domain of MvfR and acts as a
478 competitive antagonist targeting the PqsR receptor in the pqs system of PAO1.
479

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Conflict of interest statement

The authors declare no conflicts of interest.

Author contribution statement

All authors were contributed to the conception and design of the study. Project administration, supervision, conceptualization and writing-reviewing and editing were performed by N.S., J.X.Z., W.L.W., Y.J.L. Material preparation and data collection were performed by X.H.H., D.X.Z., Y.H.Z. Experiments were designed and performed by X.H.H., M.T.S., Y.H.Z., Y.F.L. The manuscript was wrote by X.H.H. All authors reviewed the manuscript and approved the finalized manuscript.

Data availability statement

Research data are not shared.

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671 **Table 1**. MICs of compounds **1-6** against *Ps. aeruginosa* standard strain PAO1 (μmol l^{-1}).

Compound	**1**	**2**	**3**	**4**	**5**	**6**	CIP	TCY
MICs	>260	>260	>260	>260	>260	>260	4	>260

672 CIP: ciprofloxacin; TCY: tetracycline.

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675 **Table 2**. The cytotoxicity of compounds **1-6** against HK2 cells and 16HBE cells.

	IC_{50} (μmol l^{-1})					
Compound	**1**	**2**	**3**	**4**	**5**	**6**
HK2	42.56 ± 2.54	10.5 ± 0.55	16.49 ± 1.03	48.93 ± 3.12	17.71 ± 1.85	53.56 ± 3.11
16HBE	46.01 ± 1.84	12.61 ± 0.38	14.03 ± 0.45	40.84 ± 2.18	18.83 ± 1.69	40.06 ± 1.36

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678 **Table 3**. The QS inhibitory activity of the compounds using quorum sensing reporter assay.

Compound	IC_{50} (μmol l^{-1})		
	pqsA-gfp PAO1	*lasB-gfp* PAO1	*rhlA-gfp* PAO1
1	20.22 ± 2.97	57.37 ± 5.25	38.41 ± 2.12
2	30.13 ± 2.34	40.41 ± 4.63	35.11 ± 3.75
3	65.80 ± 3.85	79.22 ± 3.53	41.53 ± 3.53
4	58.61 ± 4.53	156.73 ± 14.54	55.88 ± 7.75
5	51.12 ± 6.67	75.29 ± 5.67	43.05 ± 4.45
6	73.85 ± 7.43	144.41 ± 18.75	78.13 ± 5.67

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