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A New Quorum-Sensing Inhibitor Attenuates Virulence and Decreases Antibiotic Resistance in *Pseudomonas aeruginosa*

Yu-Xiang Yang[1†], Zhen-Hua Xu[1†], Yu-Qian Zhang[1], Jing Tian[2], Li-Xing Weng[1*], and Lian-Hui Wang[2*]

[1]*Department of Microbiology and Microbial Engineering, School of Life Sciences, Fudan University, Shanghai 200433, P. R. China*
[2]*Institute of Advanced Materials, Nanjing University of Posts and Telecommunications, Nanjing, Jiangsu 210046, P. R. China*

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Quorum sensing (QS) has been a novel target for the treatment of infectious diseases. Here structural analogs of *Pseudomonas aeruginosa* autoinducer *N*-acyl homoserine lactone (AHL) were investigated for QS inhibitor (QSI) activity and a novel QSI was discovered, *N*-decanoyl-L-homoserine benzyl ester (C2). Virulence assays showed that C2 downregulated total protease and elastase activities, as well as the production of rhamnolipid, that are controlled by QS in *P. aeruginosa* wild-type strain PAO1 without affecting growth. C2 was also shown to inhibit swarming motility of PAO1. Using a microdilution checkerboard method, we identified synergistic interactions between C2 and several antibiotics, tobramycin, gentamycin, cefepime, and meropenem. Data from real-time RT-PCR suggested that C2 inhibited the expression of *lasR* (29.67%), *lasI* (21.57%), *rhlR* (28.20%), and *rhlI* (29.03%).

Keywords: *Pseudomonas aeruginosa*, quorum sensing, inhibitor, virulence factor, swarming, antimicrobial susceptibility

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen that causes acute infections in immunocompromised hosts, such as those with burns, acquired immune deficiency syndrome (AIDS) or neutropenia while undergoing chemotherapy. *P. aeruginosa* has also been recognized as the main cause of complications in cystic fibrosis (CF) (Bjarnsholt and Givskov, 2007). *P. aeruginosa* infections are difficult to eradicate due to various virulence factors, such as extracellular proteases and toxins, high levels of antibiotic resistance, and the formation of biofilms (Driscoll *et al.*, 2007). These virulence factors are related to quorum sensing (QS), a cell-cell communication mechanism that has been discovered in many kinds of bacteria and dimorphic fungi (Tarkka *et al.*, 2009; Antunes *et al.*, 2010).

At least two LuxR-type receptors control the QS regulatory network and *N*-acyl-L-homoserine lactones (AHLs) are used as signal molecules (Geske *et al.*, 2008). *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL, OdDHL) is produced by the AHL synthase, LasI, in the *las* system (Pearson *et al.*, 1994). Once OdDHL reaches a critical threshold concentration, it binds to transcriptional regulatory protein, LasR. Dimers of OdDHL-LasR then bind to target promoters and upregulate the expression of downstream genes (Passador *et al.*, 1993; Pearson *et al.*, 1994, 1997; Kiratisin *et al.*, 2002). The *rhl* system is similar to *las* but consists of *N*-butanoyl-L-homoserine lactone (C4-HSL, BHL), the cognate receptor RhlR, and AHL synthase, RhlI (Ochsner *et al.*, 1994; Ochsner and Reiser, 1995; Pearson *et al.*, 1995, 1997). The *las* and *rhl* systems control a complicated regulatory network involving several hundred genes (Schuster *et al.*, 2003; Wagner *et al.*, 2003).

Due to its essential role in *P. aeruginosa* infections, QS has been investigated as a potential therapeutic target. Natural QS inhibitors (QSIs), including halogenated furanones (Manefield *et al.*, 2002), garlic extracts (Rasmussen *et al.*, 2005a), patulin, and penicillin (Rasmussen *et al.*, 2005b), have all been shown repress virulence of *P. aeruginosa*. Compared to the unbiased screening efforts from natural products, the design and study of non-native AHL signals is much more well-targeted (Mattmann and Blackwell, 2010). As the first LuxR-type QS receptor discovered in *P. aeruginosa*, LasR has received the most attention for the development of new QSIs. Some of these compounds were synthesized without altering the AHL lactone group but with a modified acyl tail, for example, changing the length or degree of saturation of the alkyl chain (Passador *et al.*, 1996). Other approaches that varied the lactone group while maintaining the side chain have also had some success as QSIs (Smith *et al.*, 2003a, 2003b; Ishida *et al.*, 2007). However, further studies are needed to clarify the interaction between the LuxR-type receptors and their ligands.

Several non-antibiotic compounds have been used as 'helpers' for the treatment of multidrug-resistant (MDR) pathogenic bacteria that use intracellular efflux pumps as defense mechanisms (Kristiansen *et al.*, 2007; Mazumdar *et al.*, 2009). This suggests that combinations of non-antibiotics and antibiotics can generate synergistic effects and broaden antimicrobial effectiveness (Ejim *et al.*, 2011). The current study screened a small library of compounds based on the chemical structure of AHLs and identified a novel QSI, *N*-Decanoyl-L-

†These authors contributed equally to this work.
*For correspondence. (L. Weng) E-mail: wenglixing@fudan.edu.cn; Tel.: +86-21-65642817; Fax: +86-21-55664575 / (L. Wang) E-mail: iamlhwang@njupt.edu.cn; Tel.: +86-25-85866333; Fax: +86-25-85866333

homoserine benzyl ester (C2). In this report, we also demonstrated the antimicrobial susceptibility of *P. aeruginosa* PAO1 in the presence of C2.

Materials and Methods

Bacterial strains and culture conditions

P. aeruginosa strains wild-type PAO1 (Holloway *et al.*, 1979) and a *lasI/rhlI* double-mutant PAO-MW1 (Whiteley *et al.*, 1999) were kindly provided by Dr. Mark Willcox (University of New South Wales, Sydney, Australia) and Professor Lianhui Zhang (Institute of Molecular Cell and Biology, Singapore), respectively. All strains were kept frozen at -75°C in trypticase soy broth (TSB; Thermo Fisher Biochemicals, Beijing, China) containing 30% glycerol. Strains from these frozen stocks were inoculated onto Luria-Bertani (LB) agar plates for PAO1 or LB agar plates containing 50 μg/ml tetracycline (Sigma, USA) for PAO-MW1. For culturing mutants in broth, a single colony of each strain was inoculated into 5 ml LB broth containing 50 μg/ml tetracycline.

Chemical synthesis

Synthesis of *N*-decanoyl-L-homoserine benzyl ester (C2) was performed by an esterification reaction between *N*-decanoyl-L-homoserine and benzyl bromide, as previously described (Wu *et al.*, 2005; Shim *et al.*, 2008; Auvinet *et al.*, 2009).

Growth assays

Cells were grown in LB medium in the presence or absence of C2 (20–200 μM) dissolved in methanol (final concentration of the solvent was 1% [v/v]). Bacterial culture turbidity was measured at 600 nm at intervals of 5 h up to 25 h (Rudrappa and Bais, 2008). Each experiment was repeated twice with three replicates.

Virulence factor assays

Azocasein assays were used to assess total protease activity, as previously described (Brint and Ohman, 1995). A bacterial culture was grown overnight in PTSB (5% polypeptone [Nihon Seiyaku, Japan], 0.1% TSB), washed, then resuspended to an optical density at 600 nm (OD_{600}) of 0.05. This culture was then aliquoted into 50 ml centrifuge tubes in the absence or in the presence of C2 (20–100 μM). In control experiments, the addition of 1 μM OdDHL and 2 μM BHL (both dissolved in methanol) to cultures at 0 h were required for consistent induction of PAO-MW1 virulence factors. Following 6 h of growth, the supernatant was collected and filter-purified using a 0.22 μm nylon filter. A 100 μl filtered culture supernatant was then added to the reaction buffer (400 μl 50 mM K_2HPO_4 [pH 7.0] containing 0.8% azocasein [Sigma]). The reaction mixture was incubated at 30°C for 3 h. After adding 400 μl 1.5 M HCl and incubation for 30 min on ice, the reaction mixture was centrifuged and 0.5 ml 1 M NaOH was added. The absorbance at 440 nm was measured.

Elastase activity was assessed by the elastin-Congo red assay, as previously described (Rudrappa and Bais, 2008). Briefly, 100 μl filtered supernatant from 6 h PTSB cultures was added to tubes containing 10 mg of elastin-Congo red (Sigma) and 900 μl Na_2HPO_4 (pH 7.0). Tubes were incubated for 4 h at 37°C with agitation and the precipitate was removed by centrifugation and the absorbance at 495 nm was measured.

For the pyocyanin assay, 1 ml supernatant from a 24 h culture in PPB (2% proteose peptone [Oxoid, UK], 1% K_2SO_4, 0.3% $MgCl_2{\cdot}6H_2O$) was extracted using 500 μl chloroform. The chloroform layer was re-extracted with 150 μl 0.2 M HCl. Pyocyanin concentration in the top layer was measured at OD_{520} (Zaborina *et al.*, 2007).

Rhamnolipid was quantified by orcinol assays, as previously described (Koch *et al.*, 1991). Briefly, 300 μl supernatant from PPB (containing 2% glycerol) culture was extracted twice using 600 μl diethyl ether. The ether layer was transferred to a new tube and allowed to evaporate. Residues were dissolved in 100 μl H_2O, 100 μl 1.6% orcinol (Sigma), and 800 μl 60% H_2SO_4. After heating for 30 min at 80°C, tubes were cooled at room temperature for 15 min and the absorbance at 421 nm was measured. Rhamnose concentrations were obtained using the standard curve equation: y=0.0033x-0.0077, and concentrations of rhamnolipids were calculated by multiplying rhamnose values by a coefficient of 2.5, as previously described (Pearson *et al.*, 1997).

Swarming assays

Swarming motility was observed on plates containing 0.5% (w/v) agar noble (Becton Dickinson, USA), 8 g/L nutrient broth (Fluka), and 5 g/L glucose (Rashid and Kornberg, 2000). Swarm plates were dried at room temperature for 30 min before use. Bacterial cells from overnight LB agar plates were spot inoculated onto the swarm plates supplemented with C2 (50–200 μM) and incubated for 24 h at 37°C.

Determination of minimum inhibitory concentration (MIC)

The MICs of piperacillin, ceftazidime, cefepime, imipenem, meropenem, tobramycin, and gentamycin on *P. aeruginosa* PAO1 and *P. aeruginosa* ATCC 27853 (used as an internal control) were based on CLSI M100-S20 guidelines (CLSI, 2010). The inoculum of approximately 5×10^5 CFU/ml (final concentration) was prepared from an overnight culture. Each antibiotic concentration was diluted in cation-adjusted Mueller-Hinton broth (CAMHB). An aliquot of 200 μl from each suspension, containing 100 μl of the serial dilutions of each antibiotic and 100 μl adjusted bacteria culture, was placed in 96-well flat-bottomed MIC plates. After incubating at 37°C for 24 h, the OD was measured using a spectrophotometer and MICs were determined from these values. Positive

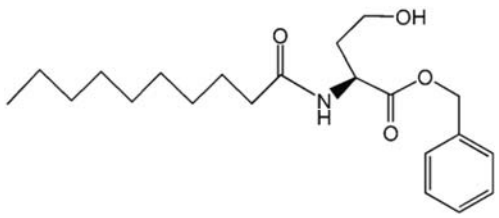

Fig. 1. **Chemical structure of *N*-decanoyl-L-homoserine benzyl ester (C2).**

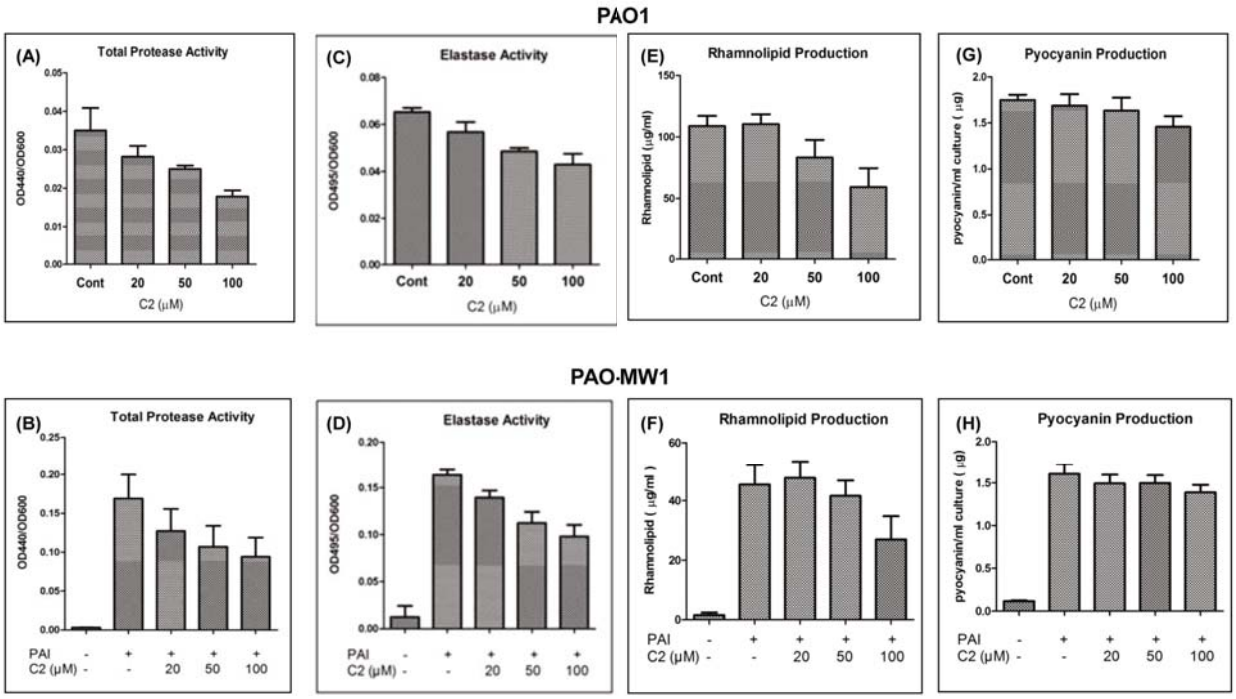

Fig. 2. Effect of C2 on extracellular virulence factors of *P. aeruginosa* PAO1 and PAO-MW1. Levels of total protease activity (A, B), elastase activity (C, D), rhamnolipid (E, F), and pyocyanin (G, H) in absence or presence of C2. PAI means culture medium containing 1 μM OdDHL and 2 μM BHL.

(bacterial suspension) and negative (broth) controls were also included. Experiments were performed three times with three replicate wells for each experiment.

Microdilution checkerboard and heat map

The antibacterial activity of antibiotics in combination with C2 was assessed by the microdilution checkerboard method (Rand *et al.*, 1993). Using a two-fold serial dilution method, the antibiotics were prepared at suitable concentrations and placed in 96-well plates. The final C2 concentrations after the addition of 100 μl of inoculum ranged from 2–128 μg/ml. Plates were incubated at 37°C for 24 h and read using a spectrophotometer. Heat maps were created using Excel (Microsoft, USA) to illustrate inhibition percentages of bacterial growth compared to the controls (Ejim *et al.*, 2011).

Fractional inhibitory concentration (FICs) index determination

The fractional inhibitory concentrations (FICs) and FIC index (FICI) were determined using the checkerboard method described in the preceding paragraph. The following formula was used to calculate FIC: FIC=[X]/MICx, where [X] was the lowest inhibitory concentration of drug in the presence of the co-drug (C2 with each antibiotic). The FICI is the sum of two FICs, when it was ≤0.5, >0.5–≤4, and >4, indicating synergy, no interaction, and antagonism, respectively (Odds, 2003).

Real-time RT-PCR

Primers used to amplify *lasR*, *lasI*, *rhlR*, and *rhlI* genes, as well as the reference gene, *rpsL*, are shown in Table 2. An overnight culture of PAO1 was washed, then used to inoculate fresh LB medium supplemented with 100 μM of C2 at an initial OD_{600} of 0.05. Cells were collected at 10 h (OD_{600}, ~2.0) incubation at 37°C with agitation when the majority of QS-controlled genes were maximally regulated (Hentzer *et al.*, 2003). Control and experimental groups

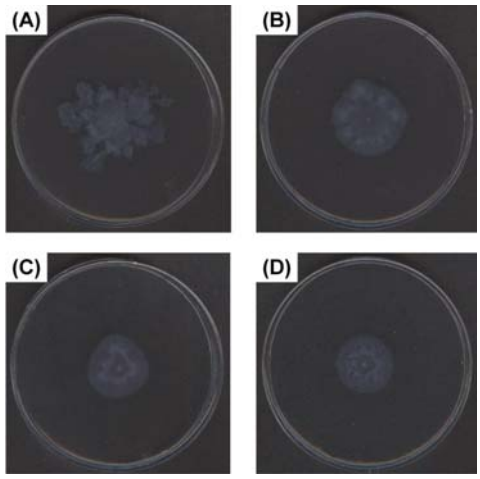

Fig. 3. Effect of C2 on the swarming motility of *P. aeruginosa* PAO1. Swarming agar (0.5%) plates supplemented with C2 at 0, (A), 50 μM (B), 100 μM (C), and 200 μM (D) respectively, were stab inoculated with a sterile needle to the bottom of the medium and incubated for 24 h at 37°C.

were each repeated in three replicates. Total RNA was extracted using the EASYspin Bacteria RNA Fast Extracting kit (Beijing Yuanpinghao Biotechnology, China). Total RNA was treated with DNase (TiangenBiotech, China), and reverse transcribed using the TevertAid First Strand cDNA Synthesis kit (Fermentas, USA) according to the manufacturer's instructions. Real-time PCR was performed using the SYBR Premix Ex Taq kit (TaKaRa Bio, Japan) with a Stratagene Mx3000p System. The reaction procedure involved incubation at 95°C for 5 min, 40 cycles of (95°C for

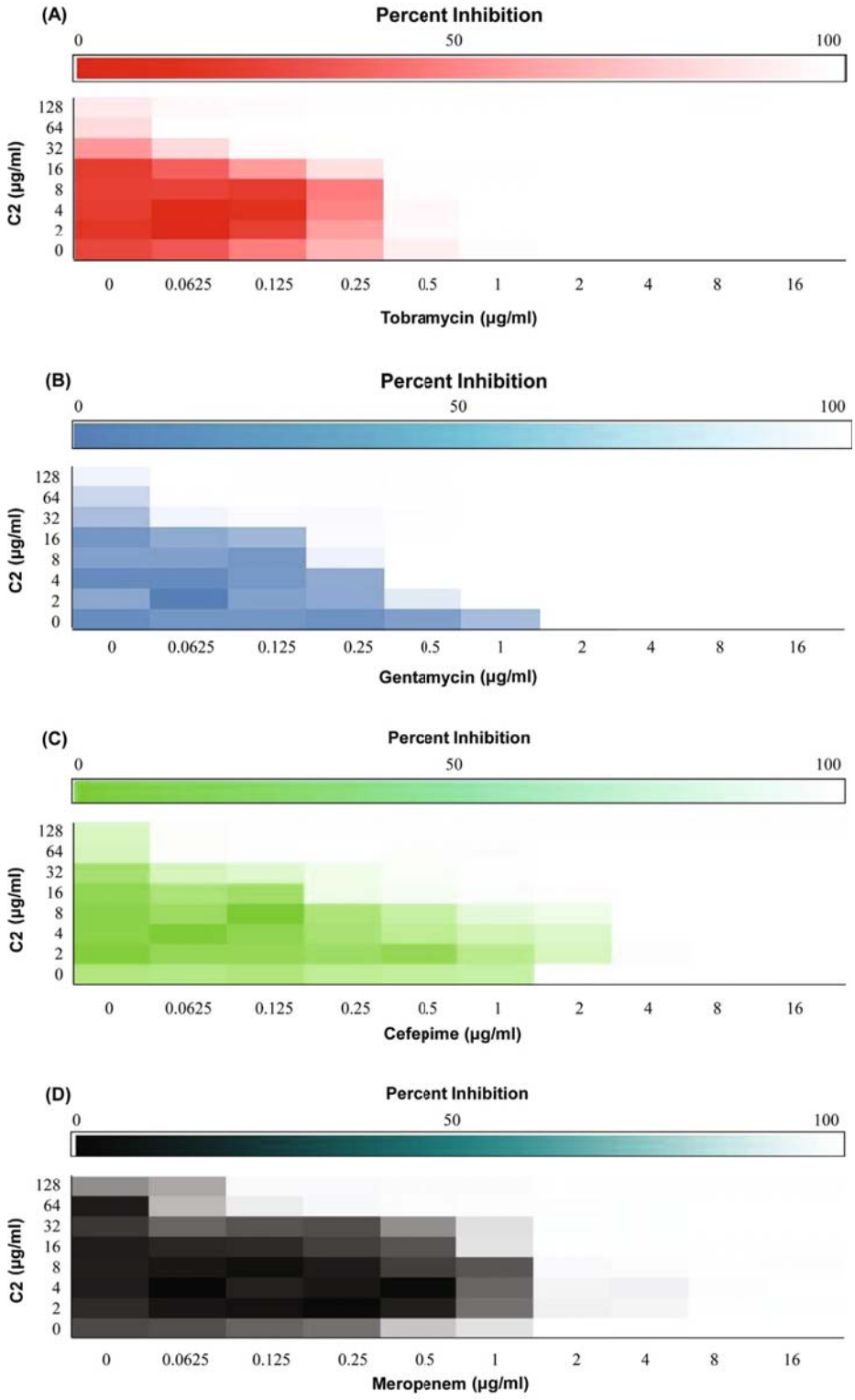

Fig. 4. **Heat map showing synergistic growth inhibition of *P. aeruginosa* PAO1 by C2 in combination with tobramycin (A), gentamycin (B), cefepime (C), and meropenem (D) antibiotics.**

Table 1. Checkerboard assay of C2 and antibiotics against *P. aeruginosa* PAO1

	P. aeruginosa PAO1				
	MIC (μg/ml) of each antibotic				
Antibotics	Alone	Combination	FIC	FICI	Outcome
C2	>128	64	<0.5	<0.5	Synergy
Meropenem	2	0.25	0.125		
C2	>128	32	<0.25	<0.3125	Synergy
Gentamycin	2	0.125	0.0625		
C2	>128	32	<0.25	<0.5	Synergy
Cefepime	2	0.5	0.25		
C2	>128	32	<0.25	<0.375	Synergy
Tobramycin	1	0.125	0.125		

FIC, fractional inhibitory concentration (FIC=MIC combination/MIC alone); FICI=FIC of C2+FIC of antibiotics (Tobramycin, Gentamycin, Cefepime, Meropenem, Imipenem, Piperacillin or Ceftazidime).

15 sec, 58°C for 20 sec, and 72°C for 20 sec). The ribosomal gene, *rpsL*, was chosen as an internal control to normalize the real-time RT-PCR data and to calculate the relative fold changes in gene expression. Experiments were repeated independently three times with different RNA samples each time.

Results

Effect of C2 on *P. aeruginosa* growth

To test the efficacy of C2 (Fig. 1) for the inhibition of *P. aeruginosa* virulence controlled by QS, we first investigated its effect on the growth of wild-type strain PAO1. At <200 μM, C2 did not affect growth of the planktonic cultures. The *lasI/rhlI* double-mutant PAO-MW1 showed similar growth rates to the wild-type strain under C2 treatment (data not shown).

Inhibition of virulence factor production

To study the ability of C2 to down-regulate *P. aeruginosa* virulence factors, the levels of these extracellular virulence factors controlled by QS were measured. Virulence factors examined included total protease and elastase, which are directly controlled by the *las* system, and pyocyanin and rhamnolipid, which are mainly regulated by the *rhl* system. *P. aeruginosa* wild-type strain PAO1 and its QS-deficient strain, PAO-MW1, were both used in the following experiments. The activity of total protease and elastase were shown to be inhibited by C2 with a significant dose-dependent manner in PAO1 (Figs. 2A and 2C). A similar inhibitory effect was observed for PAO-MW1 (Figs. 2B and 2D). Rhamnolipids were reduced to 50% of control levels in the presence of 100 μM C2 in both PAO1 (Fig. 2E) and PAO-MW1 (Fig. 2F); however, inhibition of pyocyanin was not significant (Figs. 2G and 2H).

Table 2. PCR primers for real-time RT-PCR

Gene	Primer direction	Sequence (5′-3′)	Amplicon size (bp)
lasR	Forward	ACGCTCAAGTGGAAAATTGG	111
	Reverse	TCGTAGTCCTGGCTGTCCTT	
lasI	Forward	GGCTGGGACGTTAGTGTCAT	104
	Reverse	AAAACCTGGGCTTCAGGAGT	
rhlR	Forward	CATCCGATGCTGATGTCCAACC	101
	Reverse	ATGATGGCGATTTCCCCGGAAC	
rhlI	Forward	AAGGACGTCTTCGCCTACCT	130
	Reverse	GCAGGCTGGACCAGAATATC	
rpsL	Forward	GCAACTATCAACCAGCTGGTG	231
	Reverse	GCTGTGCTCTTGCAGGTTGTG	

Inhibition of swarming

P. aeruginosa is able to grow in a 'swarming' manner to propagate on semisolid surfaces, which is beneficial for nutrient acquisition, toxic environment avoidance, and colonization (Rashid and Kornberg, 2000). This type of motility requires rhamnolipids that act as a biosurfactant and are dependent on QS (Kohler *et al*., 2000). Thus, we tested the effect of C2 on swarming motility of *P. aeruginosa* to provide further evidence of its ability to interfere with QS. After incubation for 24 h, PAO1 not treated with C2 propagated on the plate with an irregular branching appearance at the periphery of the colony (diameter=4.6–5 cm), forming dendritic structures (Fig. 3A). In the presence of 50 μM C2, PAO1 formed a regular colony (diameter=3.2–3.5 cm), without scalloping or finger-like extrusions (Fig. 3B). At 100 μM C2, the swarming colony had a smoother edge (Fig. 3C) and smaller size (diameter=2.6–2.9 cm) than observed for treatment with 50 μM C2. When the concentration of C2 was 200 μM, the colony was noticeably small (diameter=2.2–2.4 cm) and circular (Fig. 3D).

Synergistic interaction with antibiotics

P. aeruginosa exhibits a high level of antibiotic resistance that can be attributed to multiple intrinsic and acquired mechanisms, such as outer membrane impermeability, efflux pumps, and β-lactamase production (Hancock, 1998). Thus, drug combinations, including two or more antibiotics or an antibiotic with a non-antibiotic, are an important strategy for clinical treatment (Doering *et al*., 2000; Kristiansen *et al*., 2007; Ejim *et al*., 2011). The present study assessed drug synergy using the checkerboard method where the MICs of all tested antibiotics alone against PAO1 were significantly higher than MICs in combination with C2. Tobramycin

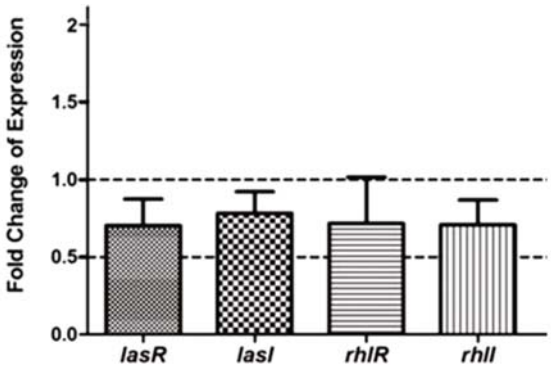

Fig. 5. Comparison of *lasR*, *lasI*, *rhlR*, and *rhlI* gene expression levels relative to expression of the internal control *rpsL* gene. Expression levels were determined by real-time RT-PCR.

(FICI<0.3125), gentamycin (<0.3125), cefepime (<0.5), and meropenem (<0.5) showed synergistic interactions with C2 (Fig. 4; Table 1). On the other hand, piperacillin, ceftazidime, and imipenem showed no change in effectiveness with the addition of C2 (data not shown).

Expression analysis with real-time RT-PCR

To investigate whether the effect of C2 on the virulence of *P. aeruginosa* was the result of inhibition of quorum sensing, real-time RT-PCR was used to monitor expression of *lasR*, *lasI*, *rhlR*, and *rhlI* reporter genes. At a concentration of 100 μM, C2 inhibited the expression of *lasR* (29.67%), *lasI* (21.57%), *rhlR* (28.20%), and *rhlI* (29.03%) (Fig. 5). Although the down-regulation of transcription of these genes was not dramatic, C2 did significantly inhibit *P. aeruginosa* virulence.

Discussion

As QS inhibition is widely accepted as a promising tool for the treatment of *P. aeruginosa* infections, natural products and non-native analogues capable of blocking AHL/LuxR-type receptor binding have been targeted and applied more frequently (Mattmann and Blackwell, 2010). In this study, we demonstrated that C2 repressed the activity of virulence factors, including total protease and elastase, as well as the production of rhamnolipids by *P. aeruginosa* without affecting growth. Swarming is a flagella-driven movement by which bacteria can spread as a biofilm over a surface (Daniels *et al.*, 2004). It requires not only flagella and type IV pili, but also rhamnolipids that are controlled by *las* and *rhl* signaling in *P. aeruginosa* (Kohler *et al.*, 2000). Here, we found that C2 significantly inhibited the swarming ability of PAO1 and suggests that C2 interferes with the QS system to reduce rhamnolipids and inhibit swarming.

Quantitative analysis of gene expression showed that C2 (100 μM) inhibited the expression of *lasR*, *lasI*, *rhlR*, and *rhlI* to varying degrees. This indicated that C2 affected virulence of *P. aeruginosa* by repression of the *las* and *rhl* system activity.

The use of traditional antibiotics has led to failed treatments because of the development of antibiotic-resistant bacteria, including *P. aeruginosa*. Thus, more powerful antibiotics or non-antibiotics that provide a synergistic effect with current antibiotics are needed (Mazumdar *et al.*, 2009). These non-antibiotic compounds are used in combination with antibiotics, and alter membrane permeability by controlling the modulation of the efflux pumps of bacteria or by enhancing the killing activity of macrophages. The current study proved the susceptibility of *P. aeruginosa* to antibiotics using a QSI. Using the checkerboard method and the time-kill assay, we found that *P. aeruginosa* strains PAO1 were inhibited by the synergistic interaction of C2 and several antibiotics. The current study suggests that these antibiotics may be used in combination with C2 to treat *P. aeruginosa* infections. However, further studies are required, such as testing in *in vivo* animal models and clinical studies, in order to confirm the synergistic activity of C2 in a clinical setting.

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