Differential effects of alkyl gallates on quorum sensing in *Pseudomonas aeruginosa*

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Virulence factors and biofilms constitute attractive targets for the prevention of infections caused by multidrug-resistant bacteria. Among alkyl gallates, propyl gallate (PG) and octyl gallate (OG) are used as food preservatives. Here we found that alkyl gallates differentially affect virulence, biofilm formation, and quorum sensing (QS) in *Pseudomonas aeruginosa*. Ethyl gallate (EG), PG, and butyl gallate (BG) inhibited biofilm formation and virulence factors including elastase, pyocyanin, and rhamnolipid, in *P. aeruginosa* without affecting cell viability by antagonizing the QS receptors LasR and RhlR. PG exhibited the most potent activity. Interestingly, hexyl gallate (HG) inhibited the production of rhamnolipid and pyocyanin but did not affect elastase production or biofilm formation. Notably, OG inhibited the production of rhamnolipid and pyocyanin but stimulated elastase production and biofilm formation. Analysis of QS signaling molecule production and QS gene expression suggested that HG inhibited RhlR, while OG activated LasR but inhibited PqsR. This mechanism was confirmed using QS mutants. Additionally, PG prevented the virulence of *P. aeruginosa* in *Caenorhabditis elegans* and a mouse model. This is the first report of the differential effects of alkyl gallates on QS systems and PG has great potential as an inhibitor of the virulence and biofilm formation of *P. aeruginosa*.

Quorum sensing (QS) is a bacterial communication system that uses small diffusible signal molecules known as autoinducers. Bacterial population behaviors regulated by QS include bioluminescence, sporulation, biofilm formation, virulence factor production, and antibiotic resistance1–3. Many pathogenic bacteria utilize QS for the production of virulence factors and biofilms. Thus, the inhibition of QS is considered a new promising target for the control of bacterial infections. Traditional strategies for the prevention and treatment of bacterial infections are based on the use of antibacterial compounds that kill bacteria or inhibit the growth of bacteria. These strategies have resulted in substantial stress on target bacteria, causing the rapid growth of resistant populations4. Virulence factors and biofilm are not essential for bacterial survival. Thus, interference with bacterial virulence factors and biofilms is a new therapeutic strategy because this method produces less selection pressure for the development of resistance than traditional strategies5.

*Pseudomonas aeruginosa* is a Gram-negative bacteria that is highly resistant to existing antibiotics and cause many opportunistic and nosocomial infections. In particular, chronic lung infections with *P. aeruginosa* are the major causes of mortality in cystic fibrosis (CF) patients6. *P. aeruginosa* has been shown to form biofilms in the CF lung, which increase bacterial resistance to antibiotics7, and also produce several virulence factors including elastase, rhamnolipid, and pyocyanin. Thus, the inhibition of virulence factor production and biofilm formation may be highly attractive for the prevention and treatment of *P. aeruginosa* infections8.

QS in *P. aeruginosa* is tightly regulated by three main QS systems organized in a hierarchical manner. LasR-LasI and RhlR-RhlI use acyl homoserine lactones (AHLs) as signaling molecules, while PqsR-PqsABCD uses 2-alkyl-4-quinolones. LasI and RhlI, the AHL synthases, synthesize N-(3-oxododecanoyl)-l-homoserine lactone (OdDHL) and N-butanoyl homoserine lactone (BHL), respectively, whereas PqsABCD, the quinolone synthase, produces 2-heptyl-3-hydroxy-4(1 H) quinolone (PQS). When activated by OdDHL, the LasR-OdDHL complex activates the transcription of *lasI* and *lasR*, which leads to autoinduction. The LasR-OdDHL complex also initiates the expression of the rhl and pqs QS systems and directs the gene expression of biofilms and virulence factors.

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virulence factors such as elastase. The RhlR-BHL complex, in turn, activates the expression of rhlI and activates many QS-dependent virulence factors, such as rhamnolipid and pyocyanin. The PqsR-PQS complex also activates the gene cascades associated with the PQS system and virulence factors such as pyocyanin and rhamnolipids. Recently, IQS (2-(2-hydroxyphenyl) thiazole-4-carbaldehyde), was discovered as a fourth QS signal molecule. The IQS system is tightly controlled by las under normal conditions, but take over the functions of the central las system under phosphate depletion stress conditions.

Gallic acid (GA) and alkyl gallates are found in several natural and industrial products. For example, GA and propyl gallate (PG) are abundant in green tea and octyl gallate (OG) has been found in the medicinal plant *Terminalia bellerica*. Notably, PG and OG have been approved for use as antioxidant additives in the food and pharmaceutical industries for over 60 years because these compounds exhibit significantly low toxicity both *in vitro* and *in vivo*. PG and OG are used at concentrations of up to 0.015% (0.7 mM) and 0.4% (1.4 mM), respectively, in foods. To date, in addition to their antioxidant activity, alkyl gallates have been reported to exhibit various other biological activities and the chain lengths of alkyl gallates differentially affects the pharmacological activity of these compounds depending on the specific pharmacological activity. Alkyl gallates were reported to have antimicrobial activity against Gram-positive bacteria and some fungi, but almost no antibacterial activity against Gram-negative bacteria. Very recently, phenolic compounds, including methyl gallate (MG), have been reported to exhibit antibiotic activity against *Streptococcus mutans* and anti-QS activity against *P. aeruginosa*, but the antivirulence activity of alkyl gallates, especially PG and OG, has not been studied to date.

While screening for an inhibitor of *P. aeruginosa* virulent factors from a microbial metabolite library, we identified gallate-like compounds. Here, we report the differential effects of alkyl gallates on virulence factors and biofilms, the *in vivo* antivirulence activity of PG against *P. aeruginosa*, and the differential effects of alkyl gallates on QS systems.

**Results**

**Effect of alkyl gallates on the production of virulence factors.** The effects of alkyl gallates on the production of elastase, pyocyanin, and rhamnolipid by *P. aeruginosa* PAO1 cells were evaluated. Six alkyl gallates (MG, ethyl gallate (EG), PG, butyl gallate (BG), hexyl gallate (HG), and OG) and GA were tested. The antibacterial activity of the alkyl gallates was also examined against each virulence factor using an optical-density-based assay. MG, EG, and PG inhibited elastase production in PAO1 cells in a dose-dependent manner without affecting cell viability (Figs 1a and S1). PG exhibited the strongest inhibition of elastase production. The elastase activity of the PAO1 cells was significantly inhibited by 27.5% and 92.1% in the presence of 30 and 300 μM PG for 24 h, respectively, compared with that of untreated cells. BG weakly inhibited elastase production by 20.2% at only 300 μM. However, HG and OG exhibited antibacterial activity within the range required for inhibition of elastase production (Fig. 1a). The specific inhibitory activity (enzyme activity per unit of cell mass) of HG and OG on elastase production suggested that OG enhanced elastase production whereas HG did not affect elastase production (Fig. S2). As a control, furanone C-30 (FC) exhibited similar inhibition as PG, while GA exhibited no inhibition.

In the rhamnolipid and pyocyanin assays, all the alkyl gallates tested dose-dependently reduced the production of rhamnolipid and pyocyanin in PAO1 cells without inhibiting cell growth, whereas GA exhibited no activity (Fig. 1b,c). MG, EG, and PG exhibited increasing inhibition of rhamnolipid and pyocyanin production with increasing alkyl chain length. Pyocyanin production by PAO1 cells was significantly inhibited by 26.8%, 51.9%, and 90.7% in the presence of 1, 10, and 100 μM PG, respectively, compared with that of untreated cells. PG weakly inhibited rhamnolipid production by 15.7%, 46.0%, and 78.9%, respectively. However, HG and FG exhibited weak inhibitory activity against the production of rhamnolipid and pyocyanin. OG again strongly inhibited the production of elastase, pyocyanin, and rhamnolipid by PAO1 cells evaluated. Six alkyl gallates (MG, ethyl gallate (EG), PG, butyl gallate (BG), hexyl gallate (HG), and OG) and GA were tested. The antibacterial activity of the PAO1 cells was significantly inhibited by 27.5% and 92.1% in the presence of 30 and 300 μM PG for 24 h, respectively, compared with that of untreated cells. BG weakly inhibited elastase production by 20.2% at only 300 μM. However, HG and OG exhibited antibacterial activity within the range required for inhibition of elastase production (Fig. 1a). The specific inhibitory activity (enzyme activity per unit of cell mass) of HG and OG on elastase production suggested that OG enhanced elastase production whereas HG did not affect elastase production (Fig. S2). As a control, furanone C-30 (FC) exhibited similar inhibition as PG, while GA exhibited no inhibition.

**Effect of alkyl gallates on biofilm formation.** It is reported that rhamnolipid and pyocyanin play a role in biofilm formation of *P. aeruginosa* PAO1. Because the alkyl gallates differentially inhibited the production of rhamnolipid and pyocyanin, biofilm formation assays were carried out by staining the biofilm biomass to determine whether alkyl gallates affect the development of growing *P. aeruginosa* PAO1 biofilms. The effects of alkyl gallates on the growth of planktonic cells were also examined via an optical-density-based assay. MG exhibited biofilm inhibition activity at 100 and 300 μM but also inhibited the growth of planktonic cells at the same concentrations, suggesting that the antibiofilm activity of MG might be due to its antibacterial activity (Fig. 1d). EG and PG exhibited dose-dependent inhibition of biofilm formation at 30–300 μM without affecting planktonic cell viability, while GA did not exhibit the same effect. PG exhibited weak antibiofilm activity at only 300 μM, similar to the result of the elastase production assay. However, HG did not inhibit biofilm formation even at 300 μM. Interestingly, OG enhanced biofilm formation at 100 and 300 μM with a decrease in planktonic cell density, suggesting that OG stimulated biofilm formation. To confirm the enhancement of biofilm formation by OG, the effects of OG on the production of extracellular polymeric substances (EPS) in biofilms were investigated. Indeed, OG enhanced the production of extracellular polysaccharides and proteins in biofilms, while PG inhibited EPS production and HG did not affect EPS production, which was consistent with the effects of these compounds on biofilm formation (Fig. S3). These different (inhibitory, non-inhibitory, and enhancing) effects of PG, HG, and OG on biofilm formation were consistent with the effects of these compounds on elastase production, suggesting that PG, HG, and OG might differentially regulate the Las system.

The antibiofilm activity of PG was confirmed by confocal laser scanning microscopy. Static grown of PAO1 cells for 6 h led to the formation of a biofilm with a thickness of 18.9 ± 1.34 μm. The treatment of 100 μM PG to the
cells, however, resulted in a substantial decrease in the biofilm thickness to 10.8 ± 1.25 μm, and the live bacterial cell density also decreased (Fig. S4).

The antivirulence and antibiofilm effects of the alkyl gallates against clinical isolates were investigated (Figs S5, S6). The alkyl gallates showed the differential effects on virulence factor production and biofilm formation in six clinical \( P. \) aeruginosa isolates including drug-resistant strains, similar to in the PAO1 strain.

**Inhibitory effects of alkyl gallates on QS.** The effects of alkyl gallates on QS receptors were carried out by an AHL-based \textit{in vitro} QS competition assay using two reporter strains, namely, \textit{Chromobacterium violaceum} CV026 and \textit{Agrobacterium tumefaciens} NT1\textsuperscript{27}, as reported previously\textsuperscript{28}. Briefly, \textit{A. tumefaciens} NT1 carries the \textit{lacZ} reporter gene fused with the TraR receptor gene. The TraR receptor can detect AHLs with a long carbon chain, such as OdDHL, resulting in the production of a cyan pigment\textsuperscript{29}. The \textit{C. violaceum} CV026 strain contains the CviR receptor, which can sense AHLs with a short carbon chain, such as BHL, leading to the synthesis of a purple pigment called violacein\textsuperscript{30}. The TraR and CviR receptors are homologs of the LasR and RhlR receptors,
respectively. Thus, competitive binding assays of the LasR and RhlR receptors is conducted using these reporter strains31,32.

Pigment was produced in NT1 cultures only when OdDHL was added, as expected (Fig. 2a). MG, EG, and PG reduced pigment production at 3–30 μM without affecting cell viability. PG exhibited the most potent activity. BG weakly inhibited pigment production at 10–30 μM. HG and OG inhibited pigment production at 10–30 μM, but also exhibited antibacterial activity, suggesting that the inhibition of pigment production might be due to the antibacterial activity of these compounds. Similarly, the production of the violacin pigment was inhibited by MG, EG, PG, and BG in CV026 cultures without any effect on cell viability (Fig. 2b). However, HG and OG also exhibited antibacterial activity against CV026 cells. The inhibition potency (BG < MG < EG < PG) of MG, EG, PG, and BG against pigment production in both reporter strains was consistent with the inhibition of virulence factor production and biofilm formation. Additionally, consistent with the absence of any effects on virulent factors and biofilm formation, GA exhibited no inhibition of pigment production in either reporter strain, as expected. This finding indicated that MG, EG, PG, and BG antagonized the binding of OdDHL and BHL to the cognate receptors LasR and RhlR, respectively. However, the effects of HG and OG on QS receptors could not be determined using these reporter strains due to the antibacterial activity of these compounds. FC, a well-known QS inhibitor, inhibited the binding of OdDHL to TraR at 1–30 μM (Fig. 3a) but antagonized CviR at only 100 μM (Fig. 2b).

**Effects of alkyl gallates on QS signaling molecule production and QS gene expression.** To examine whether HG and OG inhibit QS, the effects of these compounds on the production of QS signaling molecules were investigated in the culture conditions used for rhamnolipid and pyocyanin assays, in which these compounds did not exhibit antibacterial activity. The production of OdDHL, BHL, and PQS molecules in PAO1 cells was quantitatively determined by LC-MS/MS (Fig. 3). The untreated PAO1 cultures in the 12-h culture supernatants provided yields of 59.4 ± 5.7 ng/mL, 12.0 ± 0.8 ng/mL, and 2803 ± 77.7 ng/mL for OdDHL, BHL, and PQS, respectively. Interestingly, HG inhibited only BHL production at 100 and 300 μM without affecting OdDHL and PQS production. Notably, OG increased OdDHL production in a dose-dependent manner but decreased PQS production at 30–300 μM without affecting on BHL production. PG reduced the production of the three QS molecules at 10 and 30 μM, similar to the positive control FC, and BG exhibited weak inhibition, which was consistent with the weak inhibition of virulence factor production. GA had no activity, as expected.
The effects of alkyl gallates on QS gene expression were measured by real-time quantitative PCR (RT-qPCR). The transcription of QS-regulatory genes (lasI, IasR, rhlI, rhlR, pqsA, phnB, pqsH, and pqsR) and key QS-controlled genes (lasA, lasB, aprE, rhlB, and phzE1) in PAO1 strains was investigated (Fig. 4). Consistent with the selective and weak inhibition of BHL production by HG, this compound weakly repressed the transcription of the rhlI and rhlR genes and the genes associated with the biosynthesis of rhamnolipid and pyocyanin (rhlB and phzE1, respectively) with almost no effect on the other genes. In contrast, OG dramatically activated the transcription of las genes, while repressing the transcription of the pqs, rhlB, and phzE1 genes, with almost no effect on the transcription of the rhlI and rhlR genes (Fig. 4a). Notably, the OG-induced increase in the transcription of the elastase genes lasA and lasB confirmed the enhancement of elastase production by OG (Figs 1a and S2).

PG dose-dependently repressed the transcriptional levels of the QS-related genes at 10 and 30 μM like FC, similar to the positive control FC, as expected (Fig. 4b). BG exhibited weak inhibition, while GA had no inhibitory activity (Fig. S7), as expected. These results suggested that HG inhibited the Rhl system, while OG activated the Las system but inhibited the Pqs system. Additionally, the results demonstrated the antagonistic effects of PG on the QS receptors LasR and RhlR.

The effects of alkyl gallates on QS signaling molecule production and QS gene expression in clinical isolates were investigated. The alkyl gallates showed the differential effects on QS signaling molecule production (Fig. S8) and QS gene expression (Figs S9, S10) in the clinical P. aeruginosa isolates, similar to in the PAO1 strain.

Additionally, the effects of the alkyl gallates on IQS signaling system were investigated by RT-PCR of the biosynthesis gene of IQS (ambB). As a control experiment, in low phosphate Pseudomonas medium supplemented with 4 mM K2HPO4 in which IQS is regulated by lasI (11), the transcription level of ambB was significantly reduced in the LasI and LasR-deficient strains compared with the levels in the wild-type strain (PA14) and the alkyl gallates inhibited the transcription levels of ambB in the three strains as expected (Fig. S11). In contrast, in low phosphate Pseudomonas medium in which IQS is not controlled by lasI, the transcription of ambB was not reduced in the Las-deficient strains compared with the levels in the wild-type strain as expected. The alkyl gallates did not inhibit the transcription of ambB in the three strains (Fig. S12). These results indicated that the alkyl gallates did not affect IQS signaling.

**Agonistic effects of OG on LasR.** The LasR-OddHDL complex regulates elastase production and biofilm formation (10). To investigate how OG activates the Las system, the agonistic effects of OG on LasR in P. aeruginosa were tested by analyzing the effect of OG on elastase production and biofilm formation in a ΔlasI mutant (10) of the PA14 strain, lacking the ability to produce the LasR ligand OddHDL. First, the effects of the alkyl gallates on virulence factor production, biofilm formation, QS signal molecule production, and QS-related genes were
confirmed in wild-type PA14 cells (Figs S13, S14, and S15). Elastase production and biofilm formation were reduced by 91.9% and 87.6%, respectively, in the ΔlasI mutant compared with the levels in the wild-type strain (Fig. 5a,b). The addition of OdDHL as a positive control increased elastase production and biofilm formation in a dose-dependent manner at 1 and 10 μM in the ΔlasI mutant, while BHL, as a negative control, did not affect elastase production and biofilm formation. Similarly, elastase production in the ΔlasI mutant in the presence of 100 and 300 μM OG increased 3.6 and 6.6 times, respectively, in a dose-dependent manner and biofilm formation

Figure 4. Effects of alkyl gallates on QS gene expression. PAO1 cells were cultured in LB medium in the presence of alkyl gallates for 12 h. The effects of the alkyl gallates on QS gene expression were measured by RT-qPCR. The experiment shown is representative of three independent experiments performed in triplicate and the mean ± SD values are presented in each bar. *P < 0.01; **P < 0.001; ***P < 0.0001 versus DMSO treatment.

Figure 5. Effects of OG on elastase production and biofilm formation in the ΔlasI mutant. The ΔlasI mutant of PA14 was cultured in a medium containing different concentrations of alkyl gallates and QS ligands for elastase production and biofilm formation. Data are representative of three independent experiments performed in triplicate and expressed as the mean ± SD values in each bar. *P < 0.01; **P < 0.001; ***P < 0.0001 versus DMSO treatment.
also increased 2.2 and 5.3 times at 10 and 100 μM OG, respectively, compared with the levels in untreated cells. In contrast, FC, PG, and HG did not exhibit these agonistic effects at all. This result indicated the agonistic effect of OG on LasR. This agonistic effect of OG on LasR was verified by the expression of Las-related genes in the ΔlasI mutant (Fig. S16). OG initiated the transcription of lasR, lasA, lasB, and aprE in the ΔlasI mutant, similar to OdDHL, while the other compounds did not affect the transcription of these genes. These results indicated the agonistic effect of OG on LasR.

Effects of HG and OG on RhlR and PqsR. To determine whether HG and OG inhibited virulence factor production via inhibition of the Rhl and Pqs QS systems, respectively, the effects of exogenous QS ligands on the HG- or OG-induced reduction of pyocyanin and rhamnolipid were investigated in PA14 cells. As shown in Fig. S17, exogenous supplementation of BHL (0.1–10 μM) but not PQS reversed the inhibitory activity of HG (100 μM) on the production of rhamnolipid and pyocyanin in a dose-dependent manner (Fig. S17a,c). In contrast, exogenous supplementation of PQS (0.1–10 μM) but not BHL reversed the inhibitory activity of OG (10 μM) on the production of rhamnolipid and pyocyanin in a dose-dependent manner (Fig. S17b,d). As controls, FC- and PG-induced inhibition of pyocyanin and rhamnolipid were reversed by supplementation with both PQS and BHL. This result confirmed that HG and OG inhibit virulence factor production by inhibiting the Rhl and Pqs systems, respectively.

To demonstrate that HG and OG inhibited the production of rhamnolipid and pyocyanin by antagonizing RhlR and PqsR, respectively, the effects of these compounds on the production of rhamnolipid and pyocyanin in rhlR and pqsR mutants were investigated. HG barely inhibited the production of rhamnolipid and pyocyanin in the rhlR mutant, and this inhibition was not affected by exogenous PQS and BHL (Figs 6a,b, S18a,b). However, HG inhibited rhamnolipid production in the pqsR mutant, and this inhibition was reversed by exogenous BHL but not PQS (Fig. 6c,d). This result indicated that HG inhibited the production of rhamnolipid and pyocyanin by inhibiting RhlR. Similarly, OG did not inhibit rhamnolipid production in the pqsR mutant, and this effect was not influenced by exogenous PQS and BHL (Fig. 6c,d). However, OG inhibited rhamnolipid production in the rhlR mutant, and this inhibition was reversed by exogenous PQS but not BHL (Fig. 6a,b). This result indicated that OG inhibited the production of rhamnolipid and pyocyanin by inhibiting PqsR. The effects on pyocyanin production in the pqsR mutant could not be studied because almost no pyocyanin was produced in the pqsR mutant. As controls, FC and PG inhibited the virulence factor production in both the rhlR and pqsR mutants, and this inhibition was reversed by exogenous PQS and BHL (Figs 6 and S18), respectively, confirming that FC and PG inhibited the production of rhamnolipid and pyocyanin by inhibiting RhlR and PqsR.

Antivirulence effects of PG in a C. elegans infection model. The effects of PG on in vivo virulence of P. aeruginosa were investigated using a C. elegans fast-kill infection assay. When C. elegans were fed with P. aeruginosa PAO1, 75% of the nematodes died after 30 h (Fig. 7a). In the presence of PG, the death of the nematodes was substantially reduced in a dose-dependent manner. The percentage of nematode death significantly decreased after PG treatment at 30–300 μM. These results clearly indicated that PG protected C. elegans against infection with P. aeruginosa. Other alkyl gallates also reduced the virulence of P. aeruginosa in C. elegans, while GA did not, consistent with their in vitro antivirulence effects (Fig. S19).

Antivirulence effects of PG in a murine airway infection model. To further assess the protective effect of PG against PAO1 infection, a murine airway infection model was employed. A total of fifteen mice were used in each group for a survival experiment. PAO1-infected mice began to expire at 12 h postinfection, and completely expired at 72 h. However, six of the fifteen mice treated with PG were alive until the termination of the experiment at 72 h (Fig. 7b). This result strongly suggests that PG attenuated the virulence of P. aeruginosa in the mouse airway.

Discussion

EG, PG, and BG inhibited virulence factor production and biofilm formation in the P. aeruginosa PAO1 and PA14 strains by antagonizing the QS receptors LasR and RhlR without affecting cell viability. Additionally, the effects of these compounds on QS signaling molecule production and QS gene expression were verified. PG exhibited the most potent inhibition in both PAO1 and PA14 cells, while BG exhibited the weakest activity. Interestingly, HG inhibited the production of rhamnolipid and pyocyanin, but did not affect elastase production or biofilm formation. Notably, OG inhibited the production of rhamnolipid and pyocyanin, but stimulated elastase production and biofilm formation. Because the antagonizing effects of HG and OG on QS receptors could not be determined using the reporter strains due to the antibacterial activity of these compounds, their effects on QS signaling molecule production and QS gene expression were investigated. HG exhibited selective inhibition of BHL molecule production and rhl gene expression. OG increased OdDHL molecule production and las gene expression but decreased PQS molecule production and pqs gene expression. These results, together with the results of the QS ligand supplementation experiments, suggested that HG might selectively inhibit RhlR, while OG activates LasR and inhibits PqsR; this mechanism was verified using QS mutants of PA14. This is the first report of the anti-QS effects of PG, selective inhibition of RhlR by HG, and differential effects of OG on LasR and PqsR. The highest levels (300 μM) of PG and OG used in all experiments were at least two-times lower than those (0.7 mM and 1.4 mM, respectively) used in food.

The three QS systems in P. aeruginosa are organized hierarchically, with LasR at the top of the signaling cascade and are also interconnected. When activated by OdDHL, the LasR-OdDHL complex triggers the onset of both the rhl and pqs systems by activating the transcription of rhlI, rhlR, pqsR, and pqsH. PqsR (also known as MvfR) is the transcriptional regulator of the Pqs biosynthesis operon pqsABCD, and PqsH completes the final step of Pqs synthesis. The PqsR-PqsS complex enhances the transcription of rhlI, thus influencing the
overall expression of the rhl QS system. However, the RhlR-BHL complex represses PQS signal production by inhibiting the expression of pqsR and pqsABCD. Thus, it has been suggested that the ratio of OdDHL to BHL determines the activation of PQS synthesis. LasR-OdDHL induces the production of virulence factors, such as the LasA and LasB elastases and alkaline protease, and biofilm formation. RhlR-BHL and PqsR-PQS direct gene expression of several virulence factors, such as rhamnolipid, pyocyanin, and hydrogen cyanide. In this study, similar to FC, PG inhibited virulence factor production (elastase, rhamnolipid, and pyocyanin) and biofilm formation by antagonizing the QS receptors LasR and RhlR. Additionally, as shown by using the rhlR mutant (Figs 6a,b, S18a,b), FC and PG also inhibited Pqs-dependent production of rhamnolipid and pyocyanin, which occurred via inhibition of LasR and subsequent downregulation of the Pqs system. HG inhibited only Rhl-dependent production of rhamnolipid and pyocyanin by inhibiting RhlR, without affecting elastase production and biofilm formation which are regulated by the Las system. Because the Rhl system negatively regulates the expression of pqsABCD and subsequent PQS production, HG, an inhibitor of RhlR, is expected to increase the production of PQS molecules and the expression of Pqs-related genes. Because HG did not affect the production of PQS molecules or the expression of Pqs-related genes at 10–300 μM, the effect of higher concentrations

Figure 6. Effects of exogenous QS ligands on HG- and OG-induced inhibition of rhamnolipid production in ΔrhlR and ΔpqsR mutants. (a,b) Rhamnolipid production in the ΔrhlR mutant (a,b) and ΔpqsR mutant (c,d) cultured with alkyl gallates in the presence or absence of different concentrations of PQS (a,c) or BHL (b,d) for 18 h. Data are representative of three independent experiments performed in triplicate and expressed as the mean ± SD values in each bar. *P < 0.01; **P < 0.001; ***P < 0.0001 versus DMSO treatment.
of HG on the Pqs system was tested. Indeed, HG enhanced the production of PQS molecules and the expression of Pqs-related genes at 500 μM and higher concentration (Fig. S20a,b). Additionally, pyocyanin production increased at the higher concentrations of HG, and this effect was also reversed by exogenous BHL (Fig. S20c). This result suggested that BHL strongly down-regulates the Pqs system such that strong inhibition of Rhl QS is necessary for the BHL-induced repression of Pqs QS to be alleviated. This is the first report demonstrating BHL-induced repression of Pqs QS using a RhlR inhibitor. On the other hand, OG activated LasR and inhibited PqsR. Las-OdDHL has been reported to positively regulate the Pqs system. Because OG inhibited the expression of Pqs-related genes, PQS production, and subsequent Pqs-dependent production of rhamnolipid and pyocyanin, OG is hypothesized to inhibit PqsR more potently than activated LasR.

Alkyl gallates have been reported to exhibit various biological activities, including cardiovascular protection39, antihyperglycemic12, antibacterial19,20,40, antifungal18,21, antiviral41, anticancer15, and anti-inflammatory activities42. The chain lengths of alkyl gallates differentially affects the pharmacological activity of these compounds depending on the specific pharmacological activity17,18,43. These compounds act as antioxidants in a variety of ways including differential reacting with radicals15. Alkyl gallates exhibit antibacterial activity against Gram-positive bacteria by disrupting membrane permeability, which occurs due to the membrane binding properties of the hydrophobic alkyl chains of these compounds17. The antibacterial activity of alkyl gallates on S. aureus and B. subtilis increased with alkyl chain length for side chains of 3–10 carbons40. However, the correlation between the chain length of the alkyl gallates and membrane binding activity differs depending on the microorganism17. Although the correlation is high for S. aureus and B. subtilis, intermediate correlation was observed for S. mutans and low correlation was observed for fungi such as Trichophyton rubrum and Microsporum gypseum18. Alkyl gallates have almost no antibacterial activity against Gram-negative bacteria. Alkyl gallates with long chains (C8 and C12)22 and MG44 were reported to have antibacterial activity against Salmonella strains but not against other Gram-negative bacteria including P. aeruginosa, E. coli etc.

The antibacterial activity of antimicrobial compounds varies with inoculum size, growth medium, incubation conditions and endpoint determination method45. Because each assay for biofilm formation and virulence factor production used different media and incubation conditions, the antibacterial activity of the alkyl gallates in each assay was measured in parallel to determine whether the inhibitory activity of these compounds on biofilm formation and virulence factor production is due to growth inhibition activity. Indeed, the alkyl gallates tested exhibited differences in antibacterial activity among the various assays performed in this study. Interestingly, PG had no antibacterial activity in all the assays, even at 2000 μM, but alkyl gallates with shorter or longer alkyl chains than PG exhibited antibacterial activity against P. aeruginosa in this study (Table S2).
PG, propyl 3,4,5-trihydroxybenzoate, is an ester formed by the condensation of GA and propanol. Since 1948, PG has been added to foods containing oils and fats to protect fats, oils, and fat-containing food from oxidation[13,14]. PG also exhibits potential anti-inflammatory effects by inhibiting LPS-induced iNOS expression and nitric oxide production[15]. PG has been reported to weakly inhibit the growth of mainly Gram-positive bacteria such as Staphylococcus aureus, but not Gram-negative bacteria such as P. aeruginosa[22–24]. The antibiofilm and antivirulence activity of PG against Gram-negative bacteria is reported for the first time in this study.

In conclusion, among the alkyl gallates tested, PG most potently reduces P. aeruginosa biofilm formation and virulence factor production without affecting cell viability by antagonizing LasR and RhlR. Additionally, PG exhibited in vivo antivirulence activity in C. elegans and mouse models. However, OG activated the Las system and the subsequent production of elastase and biofilm formation, although this compound inhibited the Pqs system and the subsequent production of rhamnolipid and pyocyanin. Thus, although both PG and OG have been used as food additives, only PG presents the possibility for use as a safe material to treat biofilm-associated infections problem in healthcare facilities or as a medication for treating drug-resistant P. aeruginosa infections.

Methods

Elastase assay. Elastase activity in the supernatant of P. aeruginosa PAO1 and PA14 was determined using an elastin–Congo red assay as previously described[16]. Overnight cultures of P. aeruginosa were grown in LB medium at 37 °C with shaking. The cultures were then diluted 1:100 into fresh LB medium and dispensed in a 96-well microtiter plate. Test compounds dissolved in DMSO were treated to the cultures for 24 h. After cell viability was determined by measuring the optical density (OD) at 600 nm, the cultures were centrifuged at 4000 rpm for 10 min and the elastase activity of the culture supernatants was measured.

Pyocyanin, rhamnolipid, and biofilm assays. Pyocyanin and rhamnolipid production and biofilm formation in P. aeruginosa PAO1 and PA14 were measured as previously described (Supporting Information). After incubation for 24 h, cell viability was assayed by measuring the OD at 600 nm, and pyocyanin and rhamnolipid were then extracted and determined at 520 and 421 nm, respectively. Biofilm cells were measured using crystal violet staining.

QS competition assay. An AHL-based QS competition assay was carried out using two reporter strains, namely, A. tumefaciens NT1 and C. violaceum CV026[17]. Overnight cultures of each strain were grown in LB medium at 30 °C. One mL of the 20-fold-diluted overnight culture was transferred into a 15-mL conical tube and 5μL of X-gal (20 g/L) were added to A. tumefaciens NT1. Ten microliters of 100μM OdDHL (Sigma) and 50 mM BHL (Cayman, Ann Arbor, MI, USA) were then added to A. tumefaciens NT1 and C. violaceum CV026, respectively. Finally, 10μL of each test compound dissolved in DMSO were treated to the cultures, followed by incubation at 30 °C for 48 h. After cell viability was assayed by measuring the OD at 600 nm using a microtiter ELISA reader, color changes in C. violaceum CV026 and A. tumefaciens NT1 were measured at 590 nm and 545 nm, respectively.

Measurement of QS signaling molecules and QS gene expression. The effects of alkyl gallates on QS signaling molecule production[18] and QS gene expression[19] were determined as previously described. The culture method for P. aeruginosa PAO1 and PA14 was the same as that used for rhamnolipid and pyocyanin assays. For the measurement of three QS signaling molecules, 3 mL of the culture supernatants were extracted with 3 mL of ethyl acetate acidified with 0.1% acetic acid, and the organic layer was then evaporated and measured by LC-MS/MS in MRM mode (Supporting Information). For the determination of QS gene expression, total RNA was extracted using TRIzol reagent (Invitrogen), followed by synthesis of cDNA for RT-qPCR detection of the expression of target genes and measurement by RT-qPCR using a Bio-Rad CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA) with the primers listed in Table S1 (Supporting Information). mRNA expression was normalized to the expression of the endogenous rpoD gene.

C. elegans life span assay. A P. aeruginosa-C. elegans infection assay was conducted as previously described[20]. C. elegans nematodes were grown on NGM plates containing an ample lawn of E. coli OP50 as a food source at 20 °C for 48 h. Killing and control plates were prepared by spreading 5μL of overnight cultures of PAO1 and OP50 cells, respectively, on a 35-mm petri plate with 4 mL of PGS agar. Test compounds dissolved in DMSO were treated to the killing plates. The plates were then incubated at 37 °C for 24 h to make a bacterial lawn and then shifted to 23 °C for 24 h. Thirty L4-stage worms were placed on each plates and incubated at room temperature. Nematodes were counted for survival every 5 h for 30 h.

Murine airway infection. Twenty 5-week-old BALB/C inbred female mice (Orient, Korea) were infected with 2.5 × 107 PAO1 cells. Bacteria was precultured for 20 h and diluted 5-fold in fresh MHA medium or in MHA supplemented with 10 or 100μM PG. After shaking incubation for 7 h, the PAO1 cultures were centrifuged, and the pellets were washed with saline, and then resuspended to 5 × 108 cfu/mL. Anesthesia was induced prior to infection by intraperitoneal injection with Zoletil 50 (50 g/L) + Rompun (23.32 g/L) at 0.006 mL/10 g + 0.004 mL/10 g. Mice were infected with a dose of 2.5 × 107 cfu/mouse in 50μL PBS via the intranasal inhalation (n = 15 for each group). Infected mice were observed for survival for 72 h.

Statistical analysis. Data are presented as mean ± standard deviation (SD) values. P-values for testing statistical differences between measurements were estimated by the unpaired Student’s t-test (Excel software, Microsoft, Redmond, WA, USA). A p-value < 0.05 was considered statistically significant.
**Ethics.** All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Handong Global University (South Korea) (protocol #HGU-20170921-006). All experiments were performed in accordance with the approved guidelines of the Institutional Ethical Committee, adhered to Guide for the Care and Use of Laboratory Animals of National Research Council (USA).

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Acknowledgements
This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute, funded by the Ministry of Health & Welfare, Republic of Korea (Grant Number HI17C1807) and the KRIBB Research Initiative Program, Republic of Korea. We thank Prof. Dongeun Yong, Yonsei University College of Medicine, Seoul, South Korea, for providing clinical isolates of P. aeruginosa.

Author Contributions
W.G.K. conceived and designed the experiments. B.K. and J.S.P. performed most of the experiments. H.Y.C. performed the LC-MS/MS analysis. J.H.K. conducted the mouse experiments. B.K., J.S.P., J.H.K. and W.G.K. conceived and designed the experiments. B.K. and J.S.P. performed most of the experiments. H.Y.C. performed the LC-MS/MS analysis. J.H.K. conducted the mouse experiments. B.K., J.S.P., J.H.K. and W.G.K. wrote the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-44236-w.

Competing Interests: The authors declare no competing interests.

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