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Potent and Selective Modulation of the RhlR Quorum Sensing Receptor using Non-Native Ligands – An Emerging Target for Virulence Control in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa uses *N*-acylated L-homoserine lactone signals and a triumvirate of LuxR-type receptor proteins – LasR, RhlR, and QscR – for quorum sensing (QS). Each of these receptors can contribute to QS activation or repression, and thereby, the control of myriad virulence phenotypes in this pathogen. LasR has traditionally been considered at the top of the QS receptor hierarchy in *P. aeruginosa*; however, recent reports suggest that RhlR plays a more prominent role in infection than originally predicted, in some circumstances superseding LasR. Herein, we report the characterization of a set of synthetic, small molecule agonists and antagonists of RhlR. Using *E. coli* reporter strains, we demonstrate that many of these compounds can selectively activate or inhibit RhlR instead of LasR and QscR. Moreover, several molecules maintain their activities in *P. aeruginosa* at concentrations analogous to native RhlR-signal levels. These compounds represent useful chemical probes to study the role of RhlR in the complex QS circuitry of *P. aeruginosa*, its direct (and indirect) effects on virulence, and its overall merit as a target for anti-infective therapy.

Keywords

N-acylated L-homoserine lactone; LuxR-type receptor; RhlR; virulence; quorum sensing

Introduction

Bacteria use an intercellular signaling process called quorum sensing (QS) to control group behaviors in a population density-dependent manner.^[1] QS is based on the production and detection of small molecule signals, or autoinducers. Many bacteria use QS to initiate relationships with their eukaryotic hosts, allowing for the establishment of host-beneficial symbioses or host-deleterious infections. In fact, QS controls virulence in numerous common pathogens;^[2] for example, this regulatory system allows bacteria to produce

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extracellular proteases and toxins and/or initiate biofilm formation only once they have amassed sufficient population to overwhelm the host response. Prominent pathogens in humans that use QS to control virulence include *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus*.^[3] As these bacteria (perhaps most notably the Gram-negative bacteria)^[4] are gradually becoming resistant to traditional antibiotic therapies,^[4a, 5] their QS systems have attracted considerable interest as possible targets for novel anti-infective strategies. Recent studies indicate that blocking QS pathways, as opposed to growth, may not provide sufficient selective pressure for resistant mutants to overtake a population.^[6] Accordingly, such an anti-virulence strategy could be advantageous over standard bactericidal approaches.^[7]

The QS systems of Gram-negative bacteria are largely driven by the production and detection of *N*-acylated L-homoserine lactones (AHLs).^[1b, 8] The AHL signals are produced by LuxI-type synthases, and in dense bacterial populations, these diffusible signals reach concentrations sufficient to effectively bind and activate their cognate, intracellular LuxR-type receptors. Once activated, the LuxR-type receptors typically dimerize and regulate the transcription of a multitude of genes related to group-related behaviors, including virulence factor production and biofilm formation in many pathogens.

P. aeruginosa is a Gram-negative opportunistic pathogen that often causes lethal infections in immunocompromised patients (e.g., those suffering from cystic fibrosis, HIV, and chronic wounds),^[9] and is a leading cause of hospital-acquired infections in the US.^[10] This pathogen utilizes two LuxI/LuxR-type protein pairs to regulate many aspects of virulence (Figure 1).^[11] These two pairs—the LasI/LasR and RhlI/RhlR systems—produce and respond to *N*-(3-oxo)-dodecanoyl L-homoserine lactone (OdDHL) and *N*-butanoyl L-homoserine lactone (BHL), respectively. In addition, *P. aeruginosa* produces the “orphan” LuxR-type receptor QscR that lacks an associated LuxI-type synthase and native AHL signal. In lieu of binding a cognate signal, QscR primarily recognizes OdDHL, the native ligand for LasR.^[12] Adding further complexity to this QS network is the extent to which the LuxR-type receptor regulation is interconnected. For example, LasR controls the expression of the Rhl system through transcriptional activation of both *rhlI* and *rhlR*.^[11] Further, QscR is a negative regulator of *P. aeruginosa* QS, and while the precise mechanism of this repression is not well understood, it has been proposed that QscR may sequester both LasR and RhlR through the formation of inactive heterodimers.^[13]

Because of the elevated position of LasR, and to some extent QscR, in this hierarchical QS cascade,^[14] efforts to inhibit *P. aeruginosa* QS have primarily involved the identification of small molecules (and macromolecules) capable of modulating the activities of these two receptors.^[15] Our laboratory has contributed to these efforts by generating and screening libraries of synthetic AHL analogues with various acyl chain compositions for modulation of LasR and QscR activity.^[15a, 16] These studies have resulted in the discovery of several AHL agonists that strongly activate LasR or QscR, along with a set of competitive antagonists that inhibit LasR or QscR in the presence of their native ligand, OdDHL.

That said, far fewer efforts have focused on the development of synthetic modulators of RhlR activity,^[17] presumably due to the lower position of RhlR in the generally accepted

LuxR-type receptor hierarchy in *P. aeruginosa* (Figure 1). However, a suite of recent reports has cast doubt upon this proposed hierarchy and has bolstered the appeal of RhlR as a target for potential anti-virulence strategies. First, the production of RhlR-dependent virulence factors has been observed in *P. aeruginosa lasR* mutants both grown under phosphate-limiting conditions and isolated from clinical samples.^[18] These findings indicate that, under some growth conditions, the *rhl* system is not subordinate to the *las* system. Second, RhlR appears to play a role in the regulation of LasR-dependent virulence factor production. Notably, Dekimpe and co-workers^[19] showed that *P. aeruginosa* mutant strains lacking *lasR* are able to produce elastase B, a virulence factor commonly thought to be fully dependent on LasR for production.^[20] However, only strains lacking both the *las* and *rhl* circuits are incapable of producing this enzyme. This result indicates that elastase B production may be influenced by changes in RhlR activity, and as such, the effects of RhlR modulation may extend beyond the generally accepted RhlR regulon. Finally, Bassler and co-workers have recently shown that small-molecule modulation of RhlR activity can inhibit the production of *P. aeruginosa* virulence factors and protect both the model nematode *Caenorhabditis elegans* and human lung cells from killing by *P. aeruginosa*.^[17d] These collective data indicate that RhlR can play a significant role in the expression of *P. aeruginosa* virulence phenotypes.

Small molecules can represent useful tools to study many biological phenomena with both temporal and spatial control,^[21] and we contend that the identification of compounds that selectively modulate RhlR over LasR and QscR would provide a set of valuable chemical probes to better delineate the precise roles of RhlR in *P. aeruginosa* virulence. Such insights could prove instrumental in the development of new therapies targeting QS in *P. aeruginosa* infections.^[22] We recently reported a small set of synthetic RhlR ligands that strongly modulate virulence phenotypes in *P. aeruginosa*.^[23] These compounds were all AHL-derived and capable of either strong RhlR agonism or antagonism. In the current study, we sought to follow up on this past work and (i) further explore the chemical space for RhlR agonism and antagonism by non-native AHLs, (ii) determine their relative efficacies and potencies, and (iii) examine the selectivities of lead compounds for RhlR over LasR and QscR.

Herein, we report a comparative analysis of the RhlR activation and inhibition data for a ~100-member synthetic AHL library. Using heterologous *E. coli* reporter strains of LuxR-type receptor activity, we demonstrate that a subset of these compounds selectively activate or inhibit RhlR over LasR and QscR. Several of these RhlR-selective agonists and antagonists also modulate the activity of RhlR in *P. aeruginosa*. Structure-activity relationships (SARs) garnered from this study should provide a pathway for the design of yet more potent and selective ligands for RhlR. Notably, the non-native AHLs with short, aliphatic tails in this study are exquisitely selective for RhlR (as agonists) over the other native LuxR-type receptors in *P. aeruginosa*. Given that partial agonism of RhlR has been recently shown to decrease virulence in *P. aeruginosa* infection models,^[17d] these agonists could provide a pathway toward the development of novel antivirulence strategies.

Results and Discussion

Library selection

We have previously reported the design and synthesis of several focused libraries of non-native AHLs.^[15a, 16a-c, 24] Well represented within these libraries are AHLs with substituted aryl tails. Prior research in our lab has revealed these libraries to include potent activators or inhibitors of LuxR-type receptors produced by a variety of Gram-negative bacteria, including *P. aeruginosa* (LasR and QscR),^[15a, 16] *A. baumannii* (AbaR),^[25] *Agrobacterium tumefaciens* (TraR),^[16a-c] *Vibrio fischeri* (LuxR),^[16a-c, 26] *Chromobacterium violaceum* (CviR),^[24a] *Pectobacterium carotovora* (ExpR1 and ExpR2),^[27] *Pseudomonas syringae* (AhIR),^[28] and *Rhodopseudomonas palustris* (RpaR).^[24b] The breadth of activities and high potencies (many with EC₅₀ and IC₅₀ values equal to if not lower than the EC₅₀ values of the native AHL ligands) displayed by these AHLs on such a variety of LuxR-type receptors made them a reasonable starting point for the identification of non-native AHL modulators of RhlR activity. We therefore selected ~100 compounds from these AHL libraries (containing a range of acyl groups; shown in Figure S1) for the current study. This library contains the 52 AHLs reported in our initial screen for RhlR modulators,^[23] and is now expanded to include an additional ~50 related analogs.

Biological assay methods

To screen the AHLs for RhlR agonism or antagonism, we first utilized a recombinant *E. coli* strain (JLD271) that reports RhlR activity via the production of β -galactosidase from a transcriptional fusion with the *rhII* promoter (see Experimental Section).^[23] We used this heterologous reporter strain at the outset, as opposed to a reporter in the native *P. aeruginosa* background, to examine the activity of RhlR in isolation from LasR and QscR. Notably, this strain lacks the gene encoding the *E. coli* LuxR-type receptor SdiA. This receptor has been shown to strongly activate transcription from the *rhII* promoter in certain *E. coli* RhlR reporter strains.^[29] To avoid this possible interference, we used a *sdiA* mutant as our host strain for screening. AHLs were screened for both RhlR agonism and antagonism at 100 μ M, with RhlR's native ligand, BHL, serving as a control in these assays. Agonism assays were performed by testing non-native AHLs alone, while antagonism assays were performed by testing AHLs in competition against BHL (at its EC₅₀ value). Because the RhlR dose–response agonism curve for BHL is particularly shallow in this *E. coli* reporter, relatively high (900 μ M) concentrations of BHL were required to fully activate RhlR. The full set of primary agonism and antagonism data is reported in Table S1; we focus on the most active lead compounds herein (shown in Figure 2).

RhlR agonism assay data

Eighteen AHLs displayed greater than 60% RhlR agonism in the *E. coli* reporter assay (Table 1; Figure 2A). To better gauge the relative potencies of these AHLs, we performed dose–response analyses in the *E. coli* reporter and calculated their EC₅₀ values for RhlR agonism (Table 1; for full dose–response curves of potent compounds, see Figure S2). The bulk of these EC₅₀ values were comparable to, or lower than, that for BHL (11.1 μ M). Most of the non-native RhlR agonists fell into two structural classes: (i) short-chain aliphatic AHLs and (ii) phenylacetanoyl homoserine lactones (PHLs). RhlR agonism by the former

structural class was perhaps not surprising, as each of these compounds (i.e., **B12**, **S4**, and **D8**) harbor aliphatic chains of similar length to that of BHL. As AHL analogs similar in structure to native AHL signals have been shown to bind in the native ligand binding site of related LuxR-type proteins (e.g., CviR),^[30] it is likely that these aliphatic AHLs target the BHL-binding site in RhIR. Notably, **B12** and **S4** were both as (if not more) potent RhIR agonists as BHL, with EC₅₀ values of 3.1 μ M and 2.8 μ M, respectively (vs. 11.1 μ M for BHL). While the maximum RhIR activity induced by **B12** was only 79% of that induced by BHL, **S4** and BHL induced similar maximum RhIR activities. The cyclopentyl tails found in both **B12** and **S4** suggest that rigidifying the butanoyl tail of BHL can increase its potency.

With the exception of three moderately agonistic phenylpropionyl homoserine lactones (PPHLs **E30**, **E31**, and **E37**), the remaining RhIR agonists identified from the screen belonged to the PHL structural class of AHLs. The PHL has been a common lead scaffold identified in our prior studies of LuxR-type receptors. Thus, our results with RhIR further underscore the “privileged” nature of the PHL motif for LuxR-type receptor modulation.^[31] Several of these PHLs (**C11**, **E1**, and **E5**) were able to activate RhIR at lower concentrations than that of BHL (Table 1); however, the highest RhIR activity induced by these compounds was only ~70% of maximum possible RhIR activity. The large majority of the PHL-derived RhIR agonists contained *meta*-substituted aromatic rings. The nature of the substituent at this position ranged from small and electron-withdrawing (–F, **C3**) to larger and electron-donating (–SCH₃, **E7**), which made it difficult to develop more specific SARs beyond *meta*-substitution for PHL-based agonism of RhIR.

RhIR antagonism assay data

Eighteen AHLs, distinct from the 18 RhIR agonists above, displayed greater than 60% RhIR *inhibition* in the *E. coli* reporter (Table 2; Figure 2B). Dose–response analyses were performed on these most active RhIR antagonists to determine their IC₅₀ values (Table 2; for full dose–response curves of potent compounds, see Figure S3). With one exception (the cyclohexyl-derived AHL **S6**), all of the RhIR antagonists contained aromatic acyl groups. Specifically, these RhIR antagonists belonged to the PHL, PPHL, or phenoxyacetyl homoserine lactone (POHL) classes. The most defining characteristic of these AHLs was substitution at the *para*-position of their aromatic groups. This trend was most obvious for RhIR antagonists in the POHL and PPHL structural classes, as aromatic substitution for these compounds was exclusively at the *para*-position. Three PHL-derived antagonists of RhIR (**C16**, **E11**, and **E12**) bore substitution in the *para*-position plus at least one other position. Intriguingly, one PHL antagonist (**B4**) was the D-lactone stereoisomer; the activity of this compound, albeit moderate, suggests that L-lactone stereochemistry is not a requirement for RhIR antagonism by AHLs.

As was observed for the RhIR agonists, the RhIR antagonists harboured aromatic substituents that were relatively diverse in size and electronic nature (ranging from 2,3,4,5,6-pentafluoro; **C16** to 2-naphthyl; **E12**). The PHLs and POHLs with halogen-substituted aromatic groups (**C10**, **E11**, and **E21**) tended to display the strongest RhIR antagonism, with IC₅₀ values ranging from 3.4 to 8.1 μ M. Notably, these IC₅₀ values were determined against BHL at 10 μ M, suggesting that PHLs **C10**, **E11**, and **E21** can compete 1:1 with BHL for

RhlR in the *E. coli* reporter. However, most of the AHLs listed in Table 2 did not inhibit RhlR completely (i.e., to 100% antagonism) at 100 μ M. Maximum inhibition values ranged from 55-85%, regardless of aromatic ring substitution. In fact, instead of displaying increasing RhlR inhibition at increasing concentrations, the majority of these AHLs yielded antagonism dose curves of non-monotonic shape (see Figure S3 for examples), a phenomenon that we have described previously for AHL-derived antagonists of LasR, QscR, and other LuxR-type receptors in similar reporter systems.^[15a, 16a, 16b, 16d] Non-monotonic antagonism dose curves are characterized by inhibition that eventually upturns to activation at high concentrations of antagonist relative to the native ligand, indicative of receptor activation.^[32] Mechanistic studies into the origins of these dose curves are on-going in our laboratory and will be reported in due course. Nevertheless, these AHLs can still be defined as moderate to strong RhlR antagonists within a specific concentration range, and we shall refer to them herein as such. We note that three of the RhlR antagonists did yield typical, sigmoidal dose-response curves (PHLs **C10**, **C19**, and **C20**). Furthermore, these compounds all displayed near complete RhlR inhibition at the highest concentrations tested. Of this trio, 4-iodo PHL **C10** is particularly noteworthy, being one of the three most potent RhlR antagonists identified in this study ($IC_{50} = 8.1 \mu$ M).

RhlR-selective QS modulators

Synthetic RhlR modulators, such as those identified above, are certainly interesting in their own right. However, as we introduced at the outset, RhlR agonists and antagonists that selectively modulate RhlR activity over that of LasR and/or QscR could be instrumental as chemical probes of RhlR in *P. aeruginosa*. The successful identification of such RhlR-selective AHLs requires the comparison of compound activities in each of the three *P. aeruginosa* LuxR-type receptors. We thus sought to test the lead RhlR modulators identified above for their activities in LasR and QscR. It is important to highlight that our lab has previously examined the activity of several of these compounds in heterologous (*E. coli*) LasR^[16a-c] and QscR^[15a, 16d] reporters; however, the resulting data are difficult to compare to the RhlR screening data above, as (i) the AHLs were tested at different concentrations and (ii) the previous reporter strains differ greatly from the *E. coli* RhlR reporter strain used herein.

Therefore, to ensure that assay data could be confidently compared between the three receptors, we utilized *E. coli* LasR^[33] and QscR reporter strains that were as similar as possible to the RhlR reporter strain used above (for construction of the QscR reporter strain, see Experimental Section). First, the LasR and QscR receptor expression vectors only varied from that in the RhlR reporter by the receptor encoded. Second, like the RhlR reporter, both the LasR and QscR reporter strains produced β -galactosidase when their respective receptor proteins were activated. Third, the reporter vectors only varied in the receptor-regulated promoter sequence to which *lacZ* was fused: The LasR reporter vector contains a transcriptional fusion of *lacZ* with the *lasI* promoter, while the QscR reporter vector contains a transcriptional fusion of *lacZ* with the *PA1897* promoter. Fourth, while the presence of *sdhA* has not been shown to affect the activity of either LasR or QscR,^[29] a *sdhA* mutant of *E. coli* was used as the host strain for the LasR and QscR reporters to maintain full consistency with the RhlR reporter strain.

Using these two *E. coli* reporter strains, the lead RhlR modulators from Tables 1 and 2 were evaluated at 100 μ M for agonism and antagonism of LasR and QscR. The native ligand for these two receptors, OdDHL, was used for assay controls. Analogous to the RhlR assays above, agonism assays were performed by testing library members alone, while antagonism assays were performed by testing library members in competition with OdDHL. Agonists were deemed RhlR selective if they were at least 2.5-fold more active in RhlR agonism assays than they were in LasR and QscR agonism assays. Likewise, RhlR antagonists were defined as selective if they were at least 2.5-fold more active in RhlR antagonism assays relative to LasR and QscR antagonism assays.^[34]

Of the 18 RhlR agonists identified in the primary screen, 10 met the criterion for RhlR-agonistic selectivity (highlighted in Table 3). Perhaps not surprisingly, all of the short chain aliphatic AHLs (**B12**, **S4**, and **D8**) were selective RhlR agonists. The same structural features that enhance the agonistic activities of these compounds toward RhlR (i.e., a short, BHL-like aliphatic chain) also diminish their agonistic activities toward LasR and QscR, as these receptors are typically activated by AHLs with larger and more hydrophobic acyl chains (i.e., OdDHL-like). In addition, over half of the PHL agonists of RhlR displayed selectivity; these PHLs contained *para*-, *meta*-, or *ortho*-fluorines (**C2**, **C3**, and **C4**), a *meta*-chlorine (**C6**), an *ortho*-iodine (**C12**) or no substituents (**C1**) on the phenyl ring. The presence of few (or no) aryl substituents on these agonist scaffolds suggests that the presumably small BHL binding site of RhlR may select for sterically small PHLs (assuming these compounds can target the native ligand binding site).

Fewer AHLs displayed RhlR-*antagonistic* selectivity relative to the number of agonists, as **D15**, **E22**, and **E34** were the only three compounds of the 18 RhlR antagonists to meet the receptor selectivity requirement (Table 4). These compounds belonged to the POHL and PPHL structural classes. Of these three, we note that **D15** also exhibited appreciable agonism of LasR, and **E34** agonized both LasR and QscR in these reporter assays. Thus, **E22** is the only fully selective RhlR antagonist at 100 μ M that we identified (i.e., it does not appreciably inhibit or activate LasR or QscR at that concentration). We hypothesize that *selective* inhibition of RhlR by AHLs may be more difficult to achieve than selective activation by AHLs—namely, the structural requirements for non-native AHLs to bind *and* *activate* distinct receptors are likely specific to each receptor (e.g., short acyl chain AHLs activate RhlR vs. long acyl chain AHLs activate LasR or QscR), while the structural requirements for non-native AHLs to inhibit a LuxR-type receptor may be less stringent since the AHLs could inhibit their respective receptors via a multitude of different pathways (e.g., receptor destabilization, inhibition of receptor dimerization, and/or reduction of receptor affinity for DNA or transcription factors, etc.).

Activity of RhlR-selective agonists and antagonists in *P. aeruginosa*

With selective activators and inhibitors of RhlR in heterologous *E. coli* reporter strains in hand, we sought to determine if these compounds maintained their RhlR agonism or antagonism in the receptor's native background, *P. aeruginosa*.^[35] Molecules active in *P. aeruginosa* would be the most useful as chemical probes; however, *P. aeruginosa* can present challenges for small molecule approaches to QS modulation. This pathogen has a thicker,

less permeable outer membrane than does *E. coli*, and it uses active efflux pathways to export small molecules that successfully penetrate the cell.^[36] Indeed, we recently found that many non-native AHLs are susceptible to active efflux by the *P. aeruginosa* MexAB-OprM pump.^[33] This finding further motivated us to examine the extent of activity preservation for our RhlR-selective ligands in *P. aeruginosa*. To do so, we selected a *P. aeruginosa* strain that reports on the activity of RhlR via the production of GFP from a transcriptional fusion with the *rhlI* promoter (see Experimental Section). This reporter strain lacks functional RhlI and LasI synthases, and is therefore incapable of synthesizing BHL or OdDHL, respectively. RhlR-selective modulators from Tables 3 and 4 were evaluated at 100 μ M in the presence of 100 nM OdDHL to stimulate the production of RhlR (via activation of LasR). Similar to the *E. coli* agonism assays, the *P. aeruginosa* agonism assays were performed by testing compounds in the absence of BHL, while antagonism assays were performed in the presence of BHL. We note that these reporter experiments in *P. aeruginosa* are similar to those in our initial study of RhlR modulators;^[23] however, here we sought to scrutinize the reporter assay data to delineate relative compound potencies and SARs for RhlR modulation in *P. aeruginosa*.

The activities of our RhlR-selective agonists in the *P. aeruginosa* reporter are listed in Table 5. In general, the RhlR activities of AHLs with short aliphatic acyl chains (**D8**, **S4**) were maintained in *P. aeruginosa*, while PHL agonists of RhlR (**C1**, **C2**, **C3**, **C4**, **C6**, **C12**, and **E2**) displayed significantly less activity in the *P. aeruginosa* RhlR reporter strain relative to the *E. coli* strain. This diminution in activity for PHLs in *P. aeruginosa*—likely due to the aforementioned efflux challenges—is congruent with our prior observations.^[33] BHL is known to readily diffuse both in and out of *P. aeruginosa*,^[37] explaining the maintenance of agonistic activity by closely related analogues **D8** and **S4**. The other short-chain AHL **B12**, in contrast to **D8** and **S4**, displayed agonistic activity in *P. aeruginosa* that was over a third less than that observed in the *E. coli* RhlR reporter. As both the substrate specificity of MexAB-OprM and the rate of cell membrane diffusion are known to be strongly dependent on acyl chain length in *P. aeruginosa*,^[37–38] we speculate that the extra methylene carbon in **B12** (as compared to **S4**) may make it more susceptible to these natural defences. Dose–response analyses were performed for RhlR agonism by **D8** and **S4**, and EC₅₀ values of 7.2 μ M and 4.6 μ M were calculated, respectively. Notably, these values indicate that **D8** and **S4** are ~5-fold more potent agonists of RhlR in the *P. aeruginosa* reporter than BHL (with an EC₅₀ = 30.6 μ M), which marks them (to our knowledge) as the most potent non-native agonists of RhlR reported to date in *P. aeruginosa*.

The sole selective RhlR inhibitor in *E. coli*, 4-iodo POHL **E22**, retained high activity on RhlR in *P. aeruginosa* when dosed at 100 μ M (Table 5). However, upon performing antagonism dose–response analysis, we still saw an expected (~4-fold) reduction in potency, with it displaying an IC₅₀ of 67.2 μ M in *P. aeruginosa* (Figure S6; vs. an IC₅₀ of 17.3 μ M in *E. coli*, Figure S3). Nevertheless, this compound retains sufficient efficacy to serve as the selective-antagonistic complement of the selective RhlR agonists **D8** and **S4** described herein.

Conclusions

In summary, we have characterized a suite of non-native AHLs that are able to activate and inhibit the *P. aeruginosa* quorum receptor, RhlR. Short chain, aliphatic AHLs were found to strongly activate RhlR, while aryl HLs were found to strongly inhibit this receptor in cell-based reporter assays. In this regard, the AHLs **D8** and **S4** are particularly notable, as they are capable of activating RhlR in *P. aeruginosa* at ~5-fold lower concentrations than its native ligand BHL. The identification of small molecule modulators of RhlR is significant given the scarcity of such ligands and, perhaps more importantly, the recent emergence of RhlR as a potential new target for *P. aeruginosa* antivirulence strategies. Indeed, agonism of RhlR has been shown to suppress the production of the *P. aeruginosa* virulence factor pyocyanin^[23] and decrease virulence in *P. aeruginosa* infection models;^[17d] thus, potent and selective RhlR agonists may be advantageous in some infection control scenarios.

Several of the RhlR modulators identified possess attributes that make them particularly suited to application as RhlR chemical probes. The agonists **S4** and **D8** and the antagonist **E22** are potent enough to modulate RhlR in a *P. aeruginosa* background. Our prior study demonstrating their ability to modulate virulence factor production linked to RhlR in wild-type *P. aeruginosa* corroborates these data.^[23] This activity profile indicates that these compounds are, at most, only modestly affected by mechanisms (active efflux, membrane permeability, etc.) that have historically made *P. aeruginosa* a difficult target for small molecule approaches. Furthermore, the current study has revealed that these modulators *selectively* modulate RhlR instead of LasR and QscR, which should allow for the activation or inhibition of only RhlR on demand in its native background, with an intact triumvirate of LuxR-type receptors. As such, the RhlR chemical probes evaluated here represent new tools in the chemical arsenal to further define the role of RhlR in the regulation of *P. aeruginosa* virulence.

With these lead compounds in hand, on-going studies are focused on (i) biochemical experiments to uncover the mechanism by which these compounds interact with and activate or inhibit RhlR, (ii) the development of second-generation RhlR modulators with enhanced potencies and selectivities in *P. aeruginosa*, and (iii) the application of the current leads as tools to study QS and virulence in *P. aeruginosa*. The results of these studies will be reported in due course.

Experimental Section

Bacterial strains and growth conditions

The strains and plasmids used in this study are summarized in Table 6. All biological media and reagents were obtained from commercial sources and used according to the manufacturer's instructions. All strains were grown in lysogeny broth (LB) at 37 °C with shaking (at 200 rpm) unless specified otherwise. Bacterial growth was assessed by measuring the culture cell density according to absorbance at 600 nm (OD₆₀₀). To prepare the *E. coli* RhlR, LasR, and QscR reporter strains, the corresponding receptor expression plasmids and reporter plasmids were transformed iteratively into *E. coli* JLD271 by electroporation (listed sequentially in Table 6). The *E. coli* reporters were grown in LB

containing 100- μ g/mL ampicillin and 10- μ g/mL gentamicin. The *P. aeruginosa* PAO-JP2 reporter strain harbouring the *prhII*-LVAgfp reporter plasmid was grown in LB containing 300- μ g/mL carbenicillin. Freezer stocks of bacterial strains were maintained at -80°C in 1:1 LB:glycerol.

Chemicals and compound handling

The non-native AHLs examined in this study were synthesized and purified as described previously.^[15a, 16a-c] A full listing of these compound structures is shown in Figure S1. OddHL was purchased from Sigma–Aldrich, and BHL was purchased from Cayman Chemical Company. Chlorophenol red- β -D-galactopyranoside (CPRG), the substrate for the β -galactosidase assays, was purchased from Roche. Stock solutions of compounds were prepared in DMSO (at 100 mM) and stored at -20°C in sealed vials. The amount of DMSO used in small molecule screens did not exceed 1% (v/v). All synthetic compounds were tested in triplicate, and 3 separate trials were performed using unique cultures. No compound had an effect on bacterial growth over the concentrations tested as gauged by monitoring OD₆₀₀ over the time course of the assays. IC₅₀ and EC₅₀ values were calculated with GraphPad Prism software (v. 4.0) by using a variable slope sigmoidal curve fit.

Construction of pSC11-Q

A 348 base pair (bp) fragment spanning the upstream region of *PA1897* (–304 to +43 relative to the *PA1897* translational start codon) was amplified from plasmid pJL101* using primers 5'-GAATAT GGATCC TCTCTC CGCAGA TACCTG-3' (BamHI site underlined) and 5'-GCTATT GAATTC TGAAGA TGAATA GCGCCA C-3' (EcoRI site underlined). The PCR-generated fragment was digested with EcoRI and BamHI and subsequently ligated to EcoRI/BamHI-digested pPROBE-KT to generate pPROBE-KQ2. From this plasmid, a 340 bp fragment spanning the upstream region of *PA1897* (–300 to +39 relative to the *PA1897* translational start site) was amplified using 5'-CAGATT GTCGAC TCTCTC CGCAGA TACC-3' (SalI site underlined) and 5'-CAGCTA GGATCC GAAGAT GAATAG CGCC-3' (BamHI site underlined). The PCR-generated fragment was digested with SalI and BamHI and subsequently ligated to SalI/BamHI-digested pSC11 to generate pSC11-Q.

E. coli reporter gene assays

Primary assays for RhlR, LasR, and QscR activity in *E. coli* JLD271 reporter (β -galactosidase) strains were performed as previously reported,^[23] with the following modifications: For all primary and dose–response antagonism assays, the concentration of native ligand utilized was approximately equal to its EC₅₀ value in each bacterial reporter strain. For RhlR, LasR, and QscR primary antagonism assays, synthetic ligand (100 μ M) was screened against BHL (10 μ M), OdDHL (2 nM), and OdDHL (15 nM), respectively. For RhlR, LasR, and QscR primary agonism assays, synthetic compound (100 μ M) was screened alongside and compared to the natural ligand (900 μ M BHL or 100 μ M OdDHL) for the receptor. As part of the β -galactosidase assay protocol, plates to which CPRG substrate had been added were incubated at either 25°C (LasR assay) or 30°C (RhlR and QscR assays) until positive control wells developed a deep red colour (10 min for LasR, 30 min for RhlR, and 60 min for QscR). Dose–response reporter gene assays were performed according to these protocols by using varying concentrations of compound.

***P. aeruginosa* reporter assays**

Primary assays for RhlR activity in *P. aeruginosa* were performed as previously reported for LasR,^[33] with the following modifications: OdDHL was added to subculture to a final concentration of 100 nM immediately before it was dispensed into plates to induce the expression of RhlR. For RhlR antagonism assays, synthetic ligand (100 μ M) was screened against BHL at its EC₅₀ (30 μ M). For RhlR agonism assays, synthetic ligand (100 μ M) was screened alongside and compared to BHL (at 900 μ M) for the system. Dose–response reporter gene assays were performed according to these protocols by using varying concentrations of compound.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.
Schematic of the LuxR-type QS receptor network in *P. aeruginosa*. Pointed-head arrows indicate positive regulation; flat-head arrows indicate negative regulation.

Figure 2.

Structures of RhlR modulators identified in the *E. coli* reporter strain (JLD271/pJN105R2/pSC11-rhlI*). (A) AHLs displaying greater than 60% RhlR agonism. OdDHL and BHL are shown at left for reference. (B) AHLs displaying greater than 60% RhlR antagonism. **PHL **B4** is the D-homoserine lactone stereoisomer.

Table 1Percent activation data and EC₅₀ values for RhlR agonists.^[a]

Compound	Activation (%) ^[b]	EC ₅₀ (μM) ^[c]	95% CI (μM) ^[d]
BHL	100	11.1	6.4 – 19.3
B12	79	3.1	1.3 – 7.7
C1	84	14.7	8.4 – 25.8
C2	71	9.0	4.3 – 19.1
C3	79	--	
C4	66	--	
C6	82	5.5	2.5 – 12.3
C8	80	--	
C11	63	5.8	4.1 – 8.3
C12	70	--	
D8	81	--	
E1	69	2.0	0.6 – 6.9
E2	86	4.7	0.6 – 37.9
E5	67	1.7	0.7 – 4.0
E7	70	6.6	3.8 – 11.4
E30	64	6.6	3.3 – 13.5
E31	65	11.1	6.3 – 19.4
E37	69	27.1	12.5 – 58.5
S4	91	2.8	0.8 – 10.1

^[a] Compounds were evaluated in the *E. coli* β-galactosidase reporter strain JLD271/pJN105R2/pSC11-rhlI*. See Experimental Section for full details of strains and assay methods.

^[b] Compounds were evaluated at 100 μM. Activity for 900 μM BHL set to 100%. All assays performed in triplicate. Error = ±10%. Selected percent activation data reproduced from ref. 23.

^[c] -- = Compound was not sufficiently potent to display a full sigmoidal curve over the concentration range tested (100 nM – 1 mM), preventing accurate calculation of an EC₅₀ value; compound solubility limited testing at higher concentrations.

^[d] CI = confidence interval for EC₅₀ value.

Table 2Percent inhibition data and IC₅₀ values for RhlR antagonists.^[a]

Compound	Inhibition (%) ^b	IC ₅₀ (μM) ^[c]	95% CI (μM) ^[d]
B4	66	--	
C10	84	8.1	6.4 – 10.4
C13	60	17.9 ^[e]	14.8 – 21.6
C16	66	--	
C19	87	20.0	14.3 – 28.1
C20	86	24.4	17.4 – 34.3
D15	61	--	
E6	78	--	
E8	65	--	
E11	62	3.4 ^[e]	1.7 – 6.9
E12	77	20.7	14.9 – 28.7
E15	75	10.7 ^[e]	6.8 – 16.8
E17	66	12.0 ^[e]	7.7 – 18.7
E21	66	5.9 ^[e]	4.2 – 8.3
E22	74	17.3 ^[e]	12.1 – 24.6
E23	64	--	
E34	62	21.8 ^[e]	12.8 – 37.1
S6	69	--	

^[a]Compounds were evaluated in *E. coli* β-galactosidase reporter strain JLD271/pJN105R2/pSC11-rhlI*. See Experimental Section for full details of strains and assay methods.

^[b]Compounds were evaluated at 100 μM in the presence of 10 μM BHL. Untreated culture set to 100%. All assays performed in triplicate. Error = ±10%. Selected percent inhibition data reproduced from ref. 23.

^[c]-- = Compound was not sufficiently potent to display full sigmoidal curve over the concentration range tested (100 nM – 1 mM), preventing accurate calculation of an IC₅₀ value; compound solubility limited testing at higher concentrations.

^[d]CI = confidence interval for IC₅₀ value.

^[e]Compound displayed non-monotonic dose–response behavior, increasing to agonism at high concentrations.

Table 3

Percent LasR, RhIR, and QscR activation data for RhIR agonists in Table 1.

Compound	% LasR activation ^[a]	% RhIR activation ^[b]	% QscR activation ^[c]
B12	−10	79	12
C1	−3	84	3
C2	12	71	10
C3	−2	79	13
C4	−4	66	0
C6	30	82	30
C8	44	80	33
C11	56	63	34
C12	−4	70	5
D8	26	81	13
E1	21	69	29
E2	23	86	26
E5	62	67	34
E7	32	70	24
E30	53	64	79
E31	71	65	58
E37	22	69	65
S4	−4	91	4

^[a]Compounds were evaluated at 100 μ M in *E. coli* β -galactosidase reporter strain JLD271/pJN105L/pSC11. Activity for 100 μ M OdDHL set to 100%. All assays performed in triplicate. Error = $\pm$ 10%. See Experimental Section for full details of strains and assay methods.

^[b]Values reported in Table 1. Bolded values indicate activities that confer RhIR-selective agonism.

^[c]Compounds were evaluated at 100 μ M in *E. coli* β -galactosidase reporter strain JLD271/pJN105Q/pSC11-Q. Activity for 100 μ M OdDHL set to 100%. All assays performed in triplicate. Error = $\pm$ 10%.

Table 4

Percent LasR, RhIR, and QscR inhibition data for RhIR antagonists in Table 2.

Compound	% LasR inhibition ^[a]	% RhIR inhibition ^[b]	% QscR inhibition ^[c]
B4	15	66	37
C10	58	84	−79
C13	91	60	14
C16	76	66	74
C19	81	87	64
C20	93	86	26
D15	−80	61	4
E6	85	78	24
E8	23	65	40
E11	28	62	15
E12	81	77	60
E15	84	75	33
E17	83	66	36
E21	31	66	20
E22	−9	74	18
E23	−49	64	22
E34	−52	62	−190
S6	56	69	34

^[a]Compounds were evaluated at 100 μ M against 2 nM OdDHL in *E. coli* β -galactosidase reporter strain JLD271/pJN105L/pSC11. Activity for untreated culture set to 100%. All assays performed in triplicate. Error = $\pm$ 10%. Negative values indicate receptor agonism. See Experimental Section for full details of strains and assay methods.

^[b]Values reported in Table 2. Bolded values indicate activities that confer RhIR-selective antagonism.

^[c]Compounds were evaluated at 100 μ M against 15 nM OdDHL in *E. coli* β -galactosidase reporter strain JLD271/pJN105Q/pSC11-Q. Activity for untreated culture set to 100%. All assays performed in triplicate. Error = $\pm$ 10%.

Table 5

Activation and inhibition data for selective RhIR modulators (and potency data for the most active compounds) in *P. aeruginosa*.^[a]

Compound	% RhIR activation (EC ₅₀) ^[b]	% RhIR inhibition (IC ₅₀) ^[c]
B12	51	--
D8	90 (7.2 μM)	--
S4	105 (4.5 μM)	--
C1	53	--
C2	28	--
C3	28	--
C4	24	--
C6	26	--
C12	11	--
E2	42	--
E22	--	92 (67.2 μM)

^[a] Compounds were evaluated in *P. aeruginosa* GFP reporter strain PAO-JP2/*prhII*LVAGFP. See Experimental Section for full details of strains and assay methods. Selected percent activity data reproduced from ref. 23.

^[b] For percent activation data, compounds were evaluated at 100 μM in the presence of 100 nM OdDHL. Activity for 900 μM BHL set to 100% activation. Numbers in parentheses are EC₅₀ values of tested compounds. For 95% confidence intervals, see Figure S5. All assays performed in triplicate. Error = ±10%.

^[c] For percent inhibition data, compounds were evaluated at 100 μM against 30 μM BHL in the presence of 100 nM OdDHL. Activity for cells treated with 100 nM OdDHL alone were set to 100% inhibition. Number in parentheses is IC₅₀ value of tested compound. For 95% confidence intervals, see Figure S6. All assays performed in triplicate. Error = ±10%.

Table 6

Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant properties ^[a]	Source or ref.
<i>Strains</i>		
<i>E. coli</i> JLD271	K-12 <i>lacX74 sdiA271::Cam</i> ; Cl ^R	[29]
<i>P. aeruginosa</i> PAO-JP2	PAO1 <i>lasI::Tet rhlI::Tn501-2</i> ; Hg ^R Tc ^R	[20]
<i>Plasmids</i>		
pJN105R2	arabinose-inducible RhlR expression vector; Gm ^R	[23]
pSC11-rhlI*	<i>rhlI'-lacZ</i> transcriptional fusion; RhlR reporter vector; Ap ^R	[23]
pJN105L	arabinose-inducible LasR expression vector; Gm ^R	[39]
pSC11	<i>lasI'-lacZ</i> transcriptional fusion; LasR reporter vector; Ap ^R	[40]
pJN105Q	arabinose-inducible QscR expression vector; Gm ^R	[39]
pSC11-Q	<i>PA1897'-lacZ</i> transcriptional fusion; QscR reporter vector; Ap ^R	This study
p <i>rhlI</i> -LVAgfp	<i>rhlI'-gfp</i> [LVA] transcriptional fusion; RhlR reporter vector; Cb ^R	[41]
pJL101	<i>PA1897-lacZ</i> reporter; source of <i>PA1897</i> promoter DNA; Ap ^R	[39]
pPROBE-KT	broad host range promotorless <i>gfp</i> transcriptional fusion vector; Km ^R	[42]
pPROBE-KQ2	<i>PA1897-gfp</i> transcriptional fusion; subcloning vector; Km ^R broad host range promotorless <i>gfp</i> transcriptional fusion vector; Km ^R	This study, ^[42]

^[a] Abbreviations: Cl^R, chloramphenicol resistance; Hg^R, mercury resistance; Tc^R, tetracycline resistance; Gm^R, gentamicin resistance; Ap^R, ampicillin resistance; Cb^R, carbenicillin resistance; Km^R, kanamycin resistance.