

# A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation

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**Quorum sensing is a chemical communication process that bacteria use to regulate collective behaviors. Disabling quorum-sensing circuits with small molecules has been proposed as a potential strategy to prevent bacterial pathogenicity. The human pathogen *Pseudomonas aeruginosa* uses quorum sensing to control virulence and biofilm formation. Here, we analyze synthetic molecules for inhibition of the two *P. aeruginosa* quorum-sensing receptors, LasR and RhlR. Our most effective compound, meta-bromo-thiolactone (mBTL), inhibits both the production of the virulence factor pyocyanin and biofilm formation. mBTL also protects *Caenorhabditis elegans* and human lung epithelial cells from killing by *P. aeruginosa*. Both LasR and RhlR are partially inhibited by mBTL in vivo and in vitro; however, RhlR, not LasR, is the relevant in vivo target. More potent antagonists do not exhibit superior function in impeding virulence. Because LasR and RhlR reciprocally control crucial virulence factors, appropriately tuning rather than completely inhibiting their activities appears to hold the key to blocking pathogenesis in vivo.**

Quorum sensing is a process of bacterial communication used to collectively control group behaviors (1, 2). This process relies on the production, release, and group-wide detection of signal molecules called autoinducers, which in gram-negative bacteria are typically homoserine lactones (HSLs) (1, 2). HSLs are produced by LuxI-type enzymes, and cytoplasmic LuxR-type proteins function as HSL receptors (1, 2). Apo-LuxR-type proteins are insoluble (3, 4). Binding to autoinducers stabilizes LuxR-type receptors, enabling dimerization, DNA binding, and transcription of quorum-sensing target genes (3, 4). LuxI/R-signaling cascades are essential for virulence in many pathogens, and disabling these circuits with small molecules could prevent virulence (2). *Pseudomonas aeruginosa* is a pathogen important in cystic fibrosis, burn units of hospitals, and in implanted medical devices including intubation tubes and stents (5). To orchestrate synchronous production of virulence factors and biofilm formation, *P. aeruginosa* relies on two major LuxI/R quorum-sensing systems, the Las and Rhl systems (6–8). In *P. aeruginosa*, LasI produces and LasR responds to the autoinducer 3OC12-HSL (Fig. 1A) (5, 7, 10, 11). The LasR:3OC12-HSL complex activates transcription of many genes including *rhlR*, which encodes a second quorum-sensing receptor (Fig. 1B) (11, 12). RhlR binds to the autoinducer C4-HSL (Fig. 1A), the product of RhlI (6, 7, 13, 14). RhlR:C4-HSL also directs a large regulon of genes, some of which are also members of the LasR regulon (Fig. 1B) (6, 15, 16). This tandem regulatory arrangement allows LasI/R to control the first wave of quorum-sensing-controlled gene expression and RhlI/R to control the second (6, 11, 16, 17). Because LasR activates expression of *rhlR*, deletion of *lasR* reduces expression of both LasR- and RhlR-regulated target genes (11, 17). Previous screens have identified small-molecule inhibitors of the *P. aeruginosa* quorum-sensing receptors; however, none delivered a molecule capable of influencing virulence in tissue culture or animal models (18–20).

## Results

We previously reported the molecule chlorolactone (CL) (Fig. 1A) as an inhibitor of the LasR/RhlR homolog CviR from

*Chromobacterium violaceum* (21). Based on the CL structure, we designed and synthesized a focused library of ~30 molecules (21) (SI Appendix, Fig. S3A). Here, we present our analyses for CL and three additional molecules [chloro-thiolactone (CTL), meta-chloro-thiolactone (mCTL), and meta-bromo-thiolactone (mBTL); Fig. 1A]. First, we tested the molecules for inhibition of quorum sensing in WT *P. aeruginosa* PA14 using the quorum-sensing-regulated production of the virulence factor pyocyanin as the readout (Fig. 1C). Following activation by LasR:3OC12-HSL, the RhlR:C4-HSL complex activates expression of the pyocyanin operon (15, 22–24) (Fig. 1B). Therefore, *P. aeruginosa* PA14 produces pyocyanin at high cell density, and limited pyocyanin production occurs in a *lasR* or *rhlR* null mutant and in the *lasR*, *rhlR* double mutant (Fig. 1C).

Neither CL nor CTL inhibited pyocyanin production in vivo (Fig. 1C). By contrast, mCTL and mBTL showed potent inhibition of pyocyanin (Fig. 1C) while not affecting *P. aeruginosa* PA14 growth. We calculated 50% inhibitory concentration (IC<sub>50</sub>) values of 8 μM (±2) for mBTL and 9 μM (±2) for mCTL (Fig. 1D). Inhibition was not due to interaction of these molecules with pyocyanin, as incubation of pyocyanin-containing cell-free culture fluids from untreated *P. aeruginosa* PA14 with 100 μM mBTL or mCTL did not alter pyocyanin levels (SI Appendix, Fig. S1). We synthesized four previously reported LasR inhibitors for comparison in our pyocyanin assay (SI Appendix, Fig. S2A) (18–20). Two compounds, itc-13 and V-06-018, inhibited pyocyanin production in *P. aeruginosa* PA14 with higher IC<sub>50</sub> values [56 μM (±10) and 18 μM (±2), respectively] than mBTL and mCTL (SI Appendix, Fig. S2B and C). The other molecules (PD-12 and B7) showed limited or no inhibition in vivo. (SI Appendix, Fig. S2B).

## Significance

In this study, we prepare synthetic molecules and analyze them for inhibition of the *Pseudomonas* quorum-sensing receptors LasR and RhlR. Our most effective compound, meta-bromo-thiolactone, not only prevents virulence factor expression and biofilm formation but also protects *Caenorhabditis elegans* and human A549 lung epithelial cells from quorum-sensing-mediated killing by *Pseudomonas aeruginosa*. This anti-quorum-sensing molecule is capable of influencing *P. aeruginosa* virulence in tissue culture and animal models. Our findings demonstrate the potential for small-molecule modulators of quorum sensing as therapeutics.

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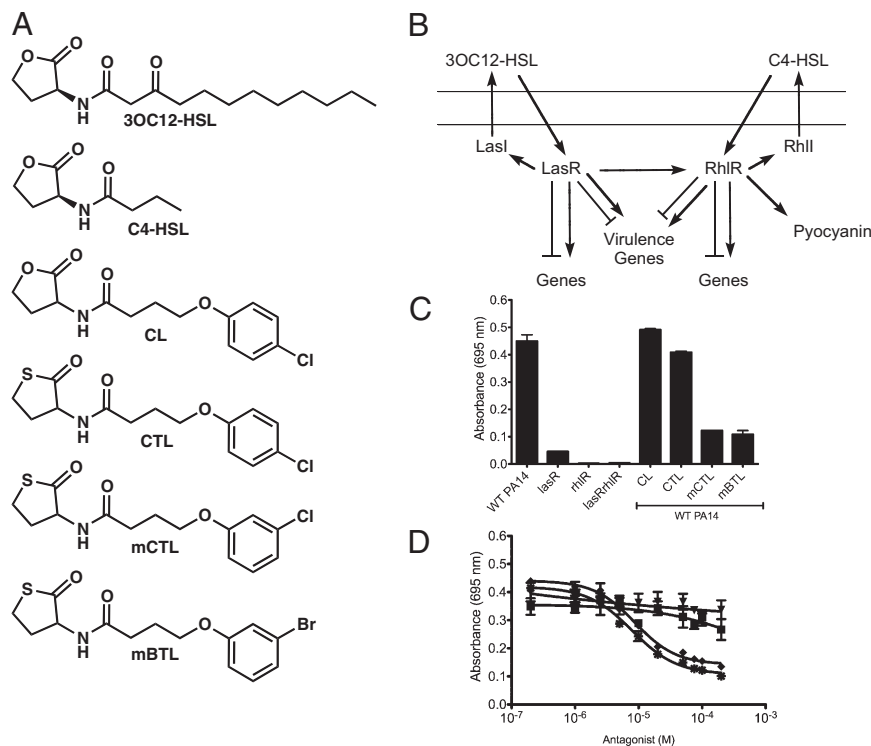
The authors declare no conflict of interest.

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Data deposition: The microarray intensity data have been deposited in the Princeton University MicroArray database, <http://puma.princeton.edu> (PUMA db, [http://puma.princeton.edu/cgi-bin/publication/viewPublication.pl?pub\\_no=558](http://puma.princeton.edu/cgi-bin/publication/viewPublication.pl?pub_no=558)).

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**Fig. 1.** Small-molecule control of pyocyanin production in *P. aeruginosa* PA14. (A) Structures of autoinducers and inhibitors discussed in this study. (B) A simplified schematic of the major components of the *P. aeruginosa* quorum-sensing circuit. (C) Pyocyanin production was measured at OD = 695 nm in cell-free culture fluids prepared from WT *P. aeruginosa* PA14, *lasR*, and *rhlR* single and double mutants and in WT treated with 100  $\mu$ M CL, CTL, mCTL, and mBTL. Error bars represent SD for two replicates. (D) Pyocyanin inhibition titrations were performed with WT *P. aeruginosa* PA14 in triplicate with CL (inverted triangles), CTL (squares), mCTL (diamonds), and mBTL (asterisks). Error bars represent SD of three replicates.

Thus, mBTL is more potent than mCTL and these previously identified inhibitors.

To determine the enantiomer of mBTL responsible for inhibition, we performed a chiral separation. The *S* enantiomer is active ( $IC_{50} = 4 \mu$ M), whereas the *R* enantiomer displays residual activity ( $IC_{50} = 100 \mu$ M) (*SI Appendix*, Fig. S3 A and B). Because the racemic mixture has an  $IC_{50}$  value only twofold higher than the isolated *S* enantiomer, we conclude that the *R* enantiomer does not influence inhibition. For ease of synthesis, we use racemic mixtures for the remainder of the work.

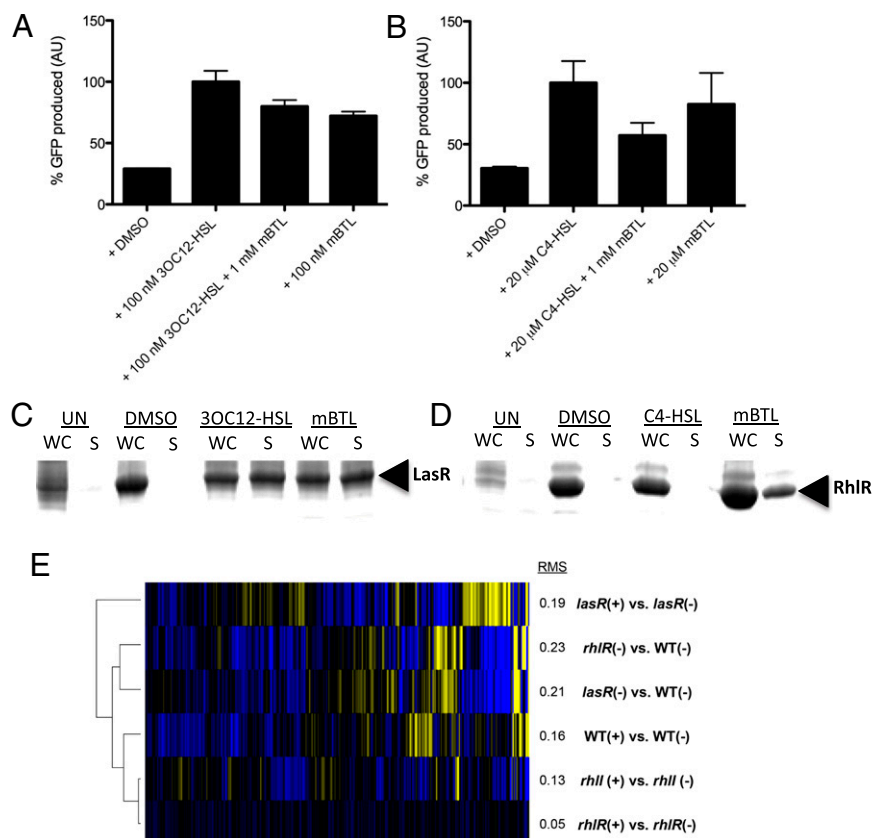
mBTL contains a four-carbon linker (Fig. 1A). We synthesized and tested mBTL derivatives with two- to six-carbon linkers and lactone versions of mBTL harboring four- or six-carbon linkers, containing or lacking a 3-oxo-moiety. None of these compounds exhibited increased potency (*SI Appendix*, Fig. S3 A, C, and D). Thus, mBTL remains the most potent *in vivo* inhibitor in our collection.

We investigated whether mBTL interacts with LasR, RhlR, or both receptors using recombinant *Escherichia coli* strains producing the receptor proteins and containing target *gfp* reporter fusions (*rsaL-gfp* for LasR and *rhlA-gfp* for RhlR). In the absence of ligand, neither receptor activates expression of the target-*gfp* fusion (Fig. 2 A and B). When autoinducer is supplied at 95% maximal effective concentration ( $EC_{95}$ ), LasR and RhlR activate gene expression. mBTL partially antagonizes this effect ( $22 \pm 4\%$  and  $43 \pm 10\%$  inhibition, respectively; Fig. 2 A and B). In the absence of autoinducer, mBTL, when provided at the  $EC_{95}$  of the native ligand for each receptor, acts as a partial agonist of both LasR and RhlR ( $72 \pm 3\%$  and  $80 \pm 25\%$ , respectively). As we have previously reported (21), molecules such as mBTL that resemble autoinducers induce conformational changes in LuxR-type receptors that impair their ability to interact with RNA polymerase which lowers their transcriptional activation potential. In the presence of autoinducer, this reduction in transcription potential manifests as

antagonism. In the absence of autoinducer, some transcription activation can still occur. Thus, without autoinducer present, molecules such as mBTL appear as partial agonists.

LuxR-type proteins require cognate autoinducers to fold (3, 4). Consistent with this, LasR and RhlR are insoluble in the absence of autoinducer and are present in the whole cell (WC) fraction but not the soluble (S) fraction following SDS/PAGE (Fig. 2 C and D). Addition of 100  $\mu$ M 3OC12-HSL or mBTL solubilizes LasR (Fig. 2C). mBTL also solubilizes RhlR; surprisingly, C4-HSL, its native autoinducer, does not (Fig. 2D). We suspect that autoinducer-bound RhlR is not particularly soluble when overproduced in *E. coli*, and the protein likely aggregates. mBTL appears to protect the protein from aggregation. We have previously demonstrated that similar protection occurs with CviR bound to CL (21).

To verify that mBTL functions by inhibiting quorum sensing *in vivo*, we used microarrays to examine the consequences of administering mBTL to WT *P. aeruginosa* and to the *lasR* and *rhlR* mutants. Treatment of WT *P. aeruginosa* PA14 caused alterations in expression of many of the known quorum-sensing targets (*SI Appendix*, Table S1). For example, LasR-regulon genes including *rpoS* and *nor* were down-regulated (*SI Appendix*, Tables S1 and S2). RhlR-controlled virulence genes, for example, those encoding rhamnolipids (*rhlA* and *rhlB*), and phenazines (*phzA2*, *phzB1*, and *phzB2*) were also repressed (*SI Appendix*, Table S1 and S3). Indeed, the profile of WT cells treated with mBTL matches well with the combined profiles of the *lasR* and *rhlR* mutants (*SI Appendix*, Table S1). However, the fold activation and fold repression is not as dramatic in the mBTL-treated WT as in the mutants, confirming that mBTL does not fully inhibit either regulator (*SI Appendix*, Table S1–S3). Thirteen genes are activated twofold or more in WT *P. aeruginosa* PA14 when treated with mBTL (*SI Appendix*, Table S4). By contrast, 213 genes are down-regulated twofold or more when WT is treated with



**Fig. 2.** mBTL binds and inhibits LasR and RhIR, and the primary in vivo target of mBTL is RhIR. (A) LasR activation of expression of *rsaL-gfp* in *E. coli*. (B) RhIR activation of expression of *rhIA-gfp* in *E. coli*. In each panel, *gfp* expression in the presence of the cognate autoinducer (100 nM 3OC12-HSL or 20 μM C4-HSL) is set to 100%. mBTL was tested for inhibition at 1 mM. Agonism was examined at 100 nM for LasR and at 20 μM for RhIR. Error bars represent SD of three replicates. (C) SDS/PAGE analysis of whole cell (WC) and soluble (S) extracts from *E. coli* cultures expressing LasR in the presence of DMSO, 100 μM 3OC12-HSL, or 100 μM mBTL. An uninduced control (UN) is shown for comparison. (D) Same as C with RhIR in the presence of DMSO, 100 μM C4-HSL, or 100 μM mBTL. (E) Hierarchical clustering, heat maps, and the RMS of the fold change ( $\log_{10}$ ) of mBTL-treated (+) or DMSO-treated (-) WT *P. aeruginosa*, *lasR*, *rhIR*, and *rhII* mutants. Dendrogram to the left of the map indicates average Euclidean linkage distances between the gene expression profiles. Blue and yellow indicate decreased and increased expression, respectively. Data are the average of three independent biological experiments, one in which the Cy3 and Cy5 dyes were swapped.

mBTL (*SI Appendix*, Table S1). These data indicate that the major role of mBTL in WT *P. aeruginosa* PA14 is as an antagonist that exerts control over virulence through partial inhibition of LasR and RhIR, not via up-regulation of other genes.

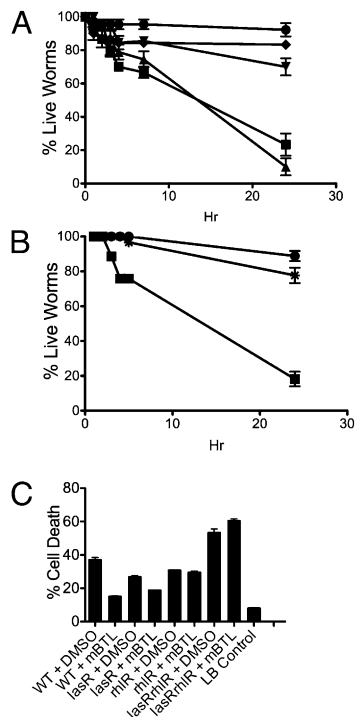
The most important comparisons for defining the target of mBTL are the mBTL-treated and untreated *lasR* and *rhIR* mutants (*SI Appendix*, Tables S5 and S6 and Fig. 2E). In the absence of LasR, mBTL treatment still alters expression of some genes. By contrast, there is essentially no difference between the untreated and mBTL-treated *rhIR* mutant profiles. Thus, although some of the mBTL effects occur through LasR, all of the mBTL effects depend on RhIR (*SI Appendix*, Tables S5 and S6 and Fig. 2E). Thus, mBTL functions in vivo by inhibiting quorum sensing via RhIR.

Given that mBTL acts as a partial agonist of RhIR in recombinant *E. coli* when the cognate autoinducer C4-HSL is not present (Fig. 2B), we explored further the extent of mBTL agonism of RhIR in vivo. To do this, we treated a *P. aeruginosa* *rhII* null strain with mBTL and performed microarrays. mBTL activated twofold or higher expression of 44 genes (*SI Appendix*, Table S7). For comparison, RhIR bound to C4-HSL activated nearly 300 genes (*SI Appendix*, Table S3). In every case in which a gene could be activated by C4-HSL or by mBTL, C4-HSL was a much more potent activator. Importantly, only one gene that was activated by mBTL in the *rhII* mutant was activated by mBTL in WT *P. aeruginosa* (*SI Appendix*, Table S7 and S4). These data indicate that mBTL can act as a weak RhIR agonist in vivo, but only when the native

autoinducer is absent, a situation that is not likely to occur in WT bacteria.

To determine if mBTL can limit virulence, we used a *Caenorhabditis elegans* fast-kill infection assay (8, 25). WT *P. aeruginosa* PA14 and the *lasR* mutant rapidly kill *C. elegans*: 77 and 90% of worms die after 24 h, respectively (Fig. 3A), indicating that LasR is not required for pathogenesis in this assay. Much less killing occurs when the nematodes are exposed to the *rhIR* or the *lasR*, *rhIR* double null strain (31 and 17%, respectively, died in 24 h; Fig. 3A), showing that RhIR is required for virulence in this assay. Treatment of WT and the *lasR* mutant *P. aeruginosa* PA14 strains with 50 μM mBTL protects *C. elegans* from killing (23 and 50% death, respectively; Fig. 3B and *SI Appendix*, Fig. S4). Together, these results confirm that the relevant in vivo target of mBTL is RhIR and, importantly, that inhibiting RhIR could form the basis of an antibacterial therapeutic strategy.

We tested whether mBTL could improve the outcome for mammalian cells during infection using the human lung carcinoma cell line A549. mBTL at 100 μM is not toxic to A549 cells (*SI Appendix*, Fig. S5). WT, *lasR*, and *rhIR* single mutants and the *lasR*, *rhIR* double mutant *P. aeruginosa* are all capable of killing A549 cells (Fig. 3C). Treatment with mBTL reduces killing by the WT and the *lasR* mutant strain but not by the *rhIR* or the *lasR*, *rhIR* double mutant strains (Fig. 3C). These results validate the conclusion reached on the basis of the microarray data presented above. Specifically, the relevant target of mBTL is



**Fig. 3.** mBTL inhibits *P. aeruginosa* PA14 virulence toward *C. elegans* and human A549 lung cells. (A) *C. elegans* were applied to lawns of *E. coli* HB101 (circles), WT *P. aeruginosa* PA14 (squares), *lasR* mutant (triangles), *rhIR* mutant (inverted triangles), and *lasR*, *rhIR* double mutant (diamonds) strains. The percent live worms was calculated every hour for the first 5 h and again at 24 h. Error bars represent SEM of three replicates. (B) Same as A. *E. coli* HB101 (circles), WT *P. aeruginosa* PA14 (squares), and WT *P. aeruginosa* in the presence of 50 μM mBTL (asterisks). (C) The percent cell death was calculated using propidium iodide uptake into A549 lung cells after 8 h and normalized to cells lysed with detergent. Error bars represent SEM of three replicates.

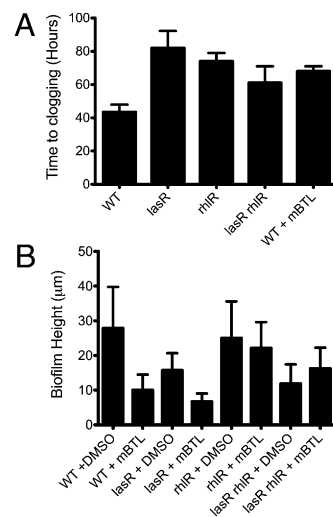
present in the WT and the *lasR* mutant but is not in the *rhIR* single and *lasR*, *rhIR* double mutant strains. Thus, RhIR appears to be the major *in vivo* target.

Beyond being a clinically relevant pathogen, *P. aeruginosa* is an industrial and medical nuisance because it causes blockages in filtration devices and stents. *P. aeruginosa* PA14 also clogs microfluidic chambers that model such devices (26). Clogging is due to biofilms that produce exopolysaccharide-containing streamers that act as sieves to catch passing cells (26). Compared with WT, *lasR* and *rhIR* single and double null mutants exhibit dramatically delayed clogging, demonstrating that quorum sensing is required to form blockages (Fig. 4A). In the presence of 100 μM mBTL, WT *P. aeruginosa* PA14 exhibits a phenotype indistinguishable from the mutants (Fig. 4A). We also examined the ability of mBTL to influence static biofilm formation in *P. aeruginosa* PA14. WT *P. aeruginosa* PA14 forms biofilms with an average height of  $27.5 \pm 11.5$  μm (Fig. 4B). Treatment of WT with 100 μM mBTL results in a decrease in the average height to  $10 \pm 4$  μm (Fig. 4B). Treatment of the *lasR* null strain with mBTL also decreases the average height of the biofilm ( $15.7 \pm 4.9$  μm to  $6.6 \pm 2.3$  μm). However, treatment of the *rhIR* null strain ( $25 \pm 10.6$  μm untreated vs.  $22.1 \pm 7.5$  μm treated) or the *lasR*, *rhIR* double null strain ( $11.9 \pm 5.5$  μm untreated vs.  $16.2 \pm 5.9$  μm treated) did not influence the height of the biofilm, indicating that the action of mBTL relies on the presence of RhIR (Fig. 4B). Taken together these findings show that mBTL inhibits quorum-sensing-controlled biofilm formation in *P. aeruginosa* PA14.

## Discussion

This work exploring interference with quorum sensing demonstrates that mBTL, an analog of the native *P. aeruginosa* auto-inducers, represses expression of the genes encoding the virulence factor pyocyanin, prevents biofilm formation, and protects *C. elegans* and human lung epithelial cells from killing by *P. aeruginosa*. Both the LasR and RhIR quorum-sensing receptors are partially inhibited by mBTL; however, in the contexts that we have examined, RhIR, not LasR, is the relevant *in vivo* target. Much emphasis has been placed on the discovery of LasR inhibitors because LasR is situated at the top of the *P. aeruginosa* quorum-sensing cascade (27). Our results suggest that the critical *in vivo* target for modulation is RhIR. Experiments in *Drosophila melanogaster* also demonstrate that the *P. aeruginosa* PA14 *lasR* mutant strain is virulent, whereas the *rhIR* mutant strain is attenuated (28) suggesting, as do our results, that RhIR is the pertinent *in vivo* quorum-sensing regulator.

It is curious that mBTL, our most potent *in vivo* inhibitor, is a partial agonist/partial antagonist of RhIR and LasR in the recombinant *E. coli* assay (Fig. 2A and B) and according to the *P. aeruginosa* microarrays (SI Appendix, Table S1). This feature of mBTL may be critical for its ability to function *in vivo*. Because LasR and RhIR act reciprocally to control key virulence genes (Fig. 1B), molecules that function as pure antagonists of both receptors might not be superior antivirulence therapies. Consistent with this notion, CL is a more potent inhibitor of both LasR- and RhIR-directed transcription in *E. coli* than is mBTL, and CL does not agonize either receptor (SI Appendix, Fig. S6). Nevertheless, CL exhibits no *in vivo* inhibition of pyocyanin production (Fig. 1B and C), and it does not attenuate killing of A549 lung cells by *P. aeruginosa*. We suspect that a “sweet spot” exists in terms of antagonist potency for inhibitors of LasR and/or RhIR and that mBTL has the appropriate level of potency to be effective *in vivo*. Consider a case in which LasR activates and RhIR represses a particular virulence factor (see Fig. 1B). A highly potent, dual LasR–RhIR antagonist could result in derepression and thus production of the virulence factor. Many examples exist in which LasR and RhIR act reciprocally in their regulation of particular genes, including virulence genes (9, 11, 17).



**Fig. 4.** mBTL inhibits quorum-sensing-regulated clogging of microfluidic chambers and biofilm formation in static cultures. (A) Time to clogging was measured for the designated *P. aeruginosa* PA14 strains and for the WT in the presence of 100 μM mBTL. Error bars represent SD of six replicates. (B) Biofilms were grown in static cultures at the base of a glass-bottom microtiter plate in the presence or absence of 100 μM mBTL. Biofilm thickness was measured using confocal microscopy. Error bars indicate SD of five to eight biological replicates.

It is noteworthy that, in the lung cell assay, the *lasR*, *rhIR* double mutant causes more cell death than does the WT (Fig. 3C). This result may stem from misregulation of virulence factors that are controlled in opposing directions by RhlR and LasR (Fig. 1B). In the preceding paragraph, we argue that potent inhibition of both LasR and RhlR could result in increased pathogenicity. The phenotype of the double mutant in the lung cell assay supports this idea. Complete inhibition of LasR and RhlR would yield the same phenotype as deletion of both receptors. Apparently, that phenotype is high virulence. Consistent with our results and interpretation, the quadruple  $\Delta lasI$ ,  $\Delta lasR$ ,  $\Delta rhII$ ,  $\Delta rhIR$  mutant remains virulent in a mouse lung infection model (29). Lastly, we note that the double *lasR*, *rhIR* null mutant is not hypervirulent in the nematode assay. The quorum-sensing-controlled virulence factors required for pathogenicity in mammalian cells are not precisely identical to those that are essential for virulence in nematodes which presumably accounts for this discrepancy (5, 8). What is critical is that, despite these differences, mBTL attenuates virulence of WT *P. aeruginosa* PA14 in both assays and in a RhlR-dependent manner.

In the nematode and lung cell experiments shown here, we pregrew the bacteria with mBTL and supplied a dose of inhibitor at the start of infection. Virulence was not reduced when *P. aeruginosa* PA14 was pregrown in the absence of inhibitor (SI Appendix, Fig. S7), indicating that mBTL functions as a prophylactic. The use of prophylactics, specifically for *P. aeruginosa*, is an established procedure, so anti-quorum-sensing prophylactic molecules could find use in the clinic. Further optimization of mBTL could tune efficacy and delivery and possibly broaden use beyond prophylaxis. An immediate focus is the head group given that thiolactones are susceptible to hydrolytic ring opening.

Our results showing that mBTL prevents biofilm formation and clogging in microfluidic devices (Fig. 4) suggest that, in addition to the implications of mBTL as an anti-infective, deploying anti-quorum-sensing molecules could prevent the failure of devices that are prone to fouling by biofilms. Thus, such molecules could be useful in industrial settings and in contexts requiring medical implants. Here we administered the inhibitor in the liquid phase; a next goal is to embed mBTL-like molecules in materials used for production of such devices, resulting in products that are innately resistant to biofilms. Taken together, our data concerning mBTL make an excellent case for the effectiveness of quorum-sensing modulators for the attenuation of quorum-sensing-controlled phenotypes in pathogenic bacteria.

## Materials and Methods

**Strains and Media.** *E. coli* strains were grown at 37 °C in Luria broth (LB) (Fisher). Plasmid pET23b (Novagen) was used to express *lasR* and *rhIR* in *E. coli* strain BL21-Gold (DE3) (Stratagene). Plasmids were maintained with 100 µg/mL ampicillin. Plasmid pEV5141 (30) was used for *rhIA-gfp* or *rsaL-gfp* expression and maintained with 50 µg/mL of kanamycin. *P. aeruginosa* strains were grown with shaking at 37 °C in LB. *C. elegans* WT strain N2 was propagated on nematode growth media with an *E. coli* HB101 lawn as the food source at 20 °C. A549 human lung carcinoma cells (American Type Culture Collection #CCL-185) were grown in DMEM (Gibco) plus 20% fetal bovine serum and 1× PenStep (Sigma) at 37 °C.

The *P. aeruginosa* *rhIR* strain (*rhIR::MAR2xT7*) and the *rhII* strain (*rhII::MAR2xT7*) come from the *P. aeruginosa* PA14 ordered transposon library (31). The *P. aeruginosa* *lasR* and *lasR*, *rhIR* double mutant strains were constructed using  $\lambda$  Red recombination as described previously (32–34). See SI Appendix for complete method.

**Pyocyanin Analyses.** Overnight, *P. aeruginosa* cultures were subcultured 1:1,000 into 5 mL fresh medium. CL, CTL, mBTL, and mCTL were assayed at 100 µM for endpoint assays and at concentrations ranging from 200 nM to 200 µM for titrations following 17 h of aerobic growth with shaking at 37 °C. Cells were separated from culture fluids via centrifugation at  $16.3 \times 1000 g$  for 15 min. Culture fluids were passed through 0.22-µm syringe-driven filters (Millipore). Cell-free culture fluids were analyzed for pyocyanin on a Beckman Coulter DU-800 spectrophotometer from 200 to 800 nm; 695 nm was chosen for graphical

representation. Titration data were fit with a variable-slope sigmoidal dose-response curve using GraphPad Prism to determine the IC<sub>50</sub> values.

**LasR and RhlR GFP Assays.** The LasR–GFP assays were performed in *E. coli* strain BL21 DE3 Gold (Agilent) carrying pET23b (Novagen) containing *lasR* (maintained with 100 µg/mL ampicillin) and carrying plasmid pEV5141 (30) containing the *rsaL* promoter-driving expression of *gfp* (maintained with 50 µg/mL of kanamycin.) The RhlR–GFP assays were performed in *E. coli* strain BL21 DE3 Gold (Agilent) carrying pET23b (Novagen) containing *rhIR* (maintained with 100 µg/mL ampicillin) and carrying plasmid pEV5141 (30) containing the *rhIA* promoter-driving expression of *gfp* (maintained with 50 µg/mL of kanamycin.) These *E. coli* strains were grown overnight and subcultured 1:100 into fresh medium with appropriate antibiotics and grown shaking for 8 h at 37 °C for the LasR–GFP strain and 12 h for the RhlR–GFP construct. 3OC12–HSL (50 nM) or C4–HSL (20 µM) was added to the LasR–GFP and RhlR–GFP preparations, respectively. Compounds were tested at 1 mM for antagonism and at 100 nM or 20 µM for agonism. These concentrations were chosen for agonism studies to match the concentrations of auto-inducers used in our experiments. For antagonism studies, we used the EC<sub>95</sub> concentration for each receptor. GFP was measured on an Envision plate reader.

**LasR and RhlR Overexpression.** Overexpression of LasR and RhlR were performed as previously reported for CviR (21). See SI Appendix for complete method.

**RNA Extraction and Microarray Analysis.** Overnight, *P. aeruginosa* PA14 cultures were back-diluted 1:1,000 into 5 mL of fresh LB and 100 µM mBTL, or an equivalent amount of DMSO, was added to cultures which were grown aerobically with shaking at 37 °C for 17 h. Nine ODs of cells were harvested for each treatment. Lysozyme (1 mg/mL in Tris-EDTA buffer) (Sigma) was added for 10 min at RT. Total RNA was prepared using the RNeasy Midi Kit (Qiagen). cDNA was synthesized and normalized as previously reported (35). cDNA was hybridized using the Gene Expression Hybridization Kit (Agilent) to a custom microarray (Agilent design ID 43307), which was designed using the Agilent eArray tool with two probes for most genes. Samples were hybridized for 22 h at 65 °C with continuous rotation at 10 rpm using a rotisserie hybridization oven (Agilent model #G2545A). Microarrays were scanned using an Agilent G2505C scanner and analyzed using Agilent Feature Extract software version 9.5. Resulting microarray intensity data have been deposited in the Princeton University MicroArray database (<http://puma.princeton.edu>) for archiving and analyzed using Matlab R2013a (MathWorks).

**C. elegans Life Span Assays (8, 25).** *C. elegans* fast killing assays were conducted with 90 WT N2 worms for each condition (30 worms per replicate, three replicates performed). *C. elegans* were propagated on NGM plates before eggs being harvested from gravid adults using a standard bleaching protocol (30 mL 5% bleach, 15 mL 5-M KOH, 55 mL dH<sub>2</sub>O). Harvested eggs were placed on lawns of fresh *E. coli* HB101 and allowed to grow for 48 h (to reach the L4 stage) at 20 °C before being moved to lawns of *P. aeruginosa* and placed at 25 °C on sorbitol, glucose, and cholesterol plates. Nematodes were scored for survival every hour for 5 h and again at 24 h. The percent living worms was calculated in triplicate for each time point and 50 µM mBTL or an equivalent volume of DMSO was added to plates and to the bacterial cultures during growth.

**A549 Human Lung Cell Infections.** Human A549 cells were grown in CellStar tissue culture flasks. Before infection, the A549 cells were treated with trypsin–EDTA (CellGro), split, counted, and aliquotted into 96-well plates at 20,000 mammalian cells per well [cell counts were estimated using Trypan Blue (CellGro) exclusion]. Cells were grown for 20 h at 37 °C in DMEM (Invitrogen). Cells were washed 3× with warm PBS (Gibco) before 100 µL of “master mix” was added to each well for each condition. Master mix contained 1 mL prewarmed PBS, 5 µL of 2 mg/mL propidium iodide (Bioprobes), 1 µL of a 100-mM inhibitor stock or DMSO, and 10 µL of OD<sub>600</sub> = 2 *P. aeruginosa* PA14 grown in the presence of mBTL or DMSO. Infections were monitored using a Perkin Elmer Wallac EnVision plate reader every 2 h with the Texas Red FP filter available from Perkin Elmer.

**Biofilm-Induced Clogging of Microfluidic Flow Cells.** Clogging experiments were performed as previously described (26) in the presence or absence of mBTL. See SI Appendix for the detailed method.

**Biofilm Growth in Static Culture.** Overnight, *P. aeruginosa* cultures were back-diluted 1:1,000 into tryptone broth with 100 µM mBTL, or an equivalent concentration of DMSO, and grown to midlogarithmic phase (OD<sub>600</sub> = 0.5). A 96-well plate with glass bottom (Thermo Fisher), which was filled with 200 µL of tryptone broth containing 100 µM mBTL or DMSO, was then inoculated

with 2  $\mu$ L of the midlogarithmic culture. The 96-well plates were incubated for 24 h before adding 5  $\mu$ M SYTO 9 nucleic acid stain (Invitrogen). Biofilm thickness was measured using confocal microscopy (Nikon).

**Chemistry Materials and Methods.** The following compounds were synthesized as previously described: CL, CTL, mBTL, mCTL (21), itc-13 (20), and PD-12 (36). See [SI Appendix](#) for complete synthesis and evaluation methods.

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