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Drug Discovery

Overcoming the Unexpected Functional Inversion of a PqsR Antagonist in *Pseudomonas aeruginosa*: An In Vivo Potent Antivirulence Agent Targeting *pqs* Quorum Sensing**

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Abstract: *The virulence regulator PqsR of Pseudomonas aeruginosa is considered as an attractive target for attenuating the bacterial pathogenicity without eliciting resistance. However, despite efforts and desires, no promising PqsR antagonist has been discovered thus far. Now, a surprising functionality change of a highly affine PqsR antagonist in P. aeruginosa is revealed, which is mediated by a bacterial signal molecule synthase and responsible for low cellular potency. Blockade of the susceptible position led to the discovery of the first antivirulence compound that is potent in vivo and targets PqsR, thus providing a proof of concept for this novel antivirulence therapy.*

Nowadays, human beings are confronted with an alarming situation in view of the lack of effective therapies against antibiotic-resistant bacterial infections.[1] The predicament is attributed to the mode of action of marketed antibiotics, which is based on interference with bacterial growth, which results in an inevitable selection of resistant strains.[2] Consequently, the discovery of novel anti-infectives that are less prone to resistance is challenging. However, the interest of the pharmaceutical industry to develop new antibiotics is decreasing.[3] Furthermore, progress is hampered by a high attrition rate of compounds that are active in cell-free assays, but inefficient in bacteria.[4] A promising strategy to overcome the growing and challenging resistance problem is to selectively target non-vital functions that are associated with the pathogenicity of a bug, such as the production of virulence factors.[5–8] The human opportunistic pathogen *P. aeruginosa* causes severe and fatal infections in cystic fibrosis patients. Aside from an extensive inflammatory response that is dominated by polymorphonuclear neutrophils,[9] virulence factors play a critical role in progressive lung deterioration during infection. Their production is controlled by a cell-density-dependent extraordinary cell-to-cell communication system, which is known as quorum sensing (QS) and uses signal molecules.[10,11] With a focus on developing anti-infectives with novel modes of action, recent contributions from academia[12–14] highlight quorum sensing inhibitors (QSIs) as potential powerful agents for antivirulence therapy. The quorum sensing of the *Pseudomonas* quinolone signal (*pqs*) is a potential target in *P. aeruginosa*. A first attempt to interfere with this system resulted in compounds with low efficiency in an animal model.[15] Herein, we describe an unexpected functional inversion of a QS receptor antagonist (a QSI) into an agonist by *P. aeruginosa* and report the first in vivo potent antivirulence agent targeting *pqs* QS.

PqsR is a key DNA-binding receptor of this *pqs* QS system that is specific to *P. aeruginosa* and a critical regulator that fine-tunes a large set of genes that encode for virulence factors, such as pyocyanin, elastase B, and hydrogen cyanide.[16,17] PQS and 2-heptyl-4-hydroxyquinoline (HHQ) are the natural ligands and agonists of the receptor (Figure 1a), and function as the signal molecules of *pqs* QS.[18,19] The biosynthesis of HHQ is conducted by the enzymes Pqs ABCD, which are encoded by genes located in the *pqs* operon. The transcription of this operon is in turn positively regulated by PqsR. The synthase PqsH finally hydroxylates HHQ to form PQS (Figure 1a).[20–22] A *pqsR* knock-out mutant of *P. aeruginosa* that is deficient in *pqs* QS does not produce any pyocyanin, and displays reduced pathogenicity.[19,21] Thus, we considered PqsR as an attractive target for the development of QSIs. Based on the scaffold of HHQ, we recently reported compound **1** (Figure 1a), which is, to the best of our knowledge, the only PqsR antagonist described to date. It showed an IC_{50} of 51 nM in an *E. coli* reporter gene assay.[23] Considering its high activity towards PqsR, **1** only moderately reduced the production of pyocyanin (Table 1). Therefore, we decided to further characterize the behavior of the antagonist in *P. aeruginosa*. Most interestingly, **1**, which showed a purely antagonistic activity in *E. coli* reporter gene assays (Figure 1b and c), displayed a dose-dependent agonistic activity in *P. aeruginosa* (Figure 1c), which could be the reason for the marked loss of antagonistic activity of **1** (IC_{50} = 51 nM in *E. coli*, 60% inhibition at 10 μM in *P. aeruginosa*; Table 1). The opposite nature of the functional properties in the two bacterial species suggests that a biotransformation of the compound may have occurred in *P. aeruginosa*. We turned our attention to the enzymes that are involved in the *pqs* QS signaling pathway and speculated that a biochemical modification of **1** into **2** by PqsH occurs (Figure 1a) for the following reasons: 1) Compound **1** is structurally very similar

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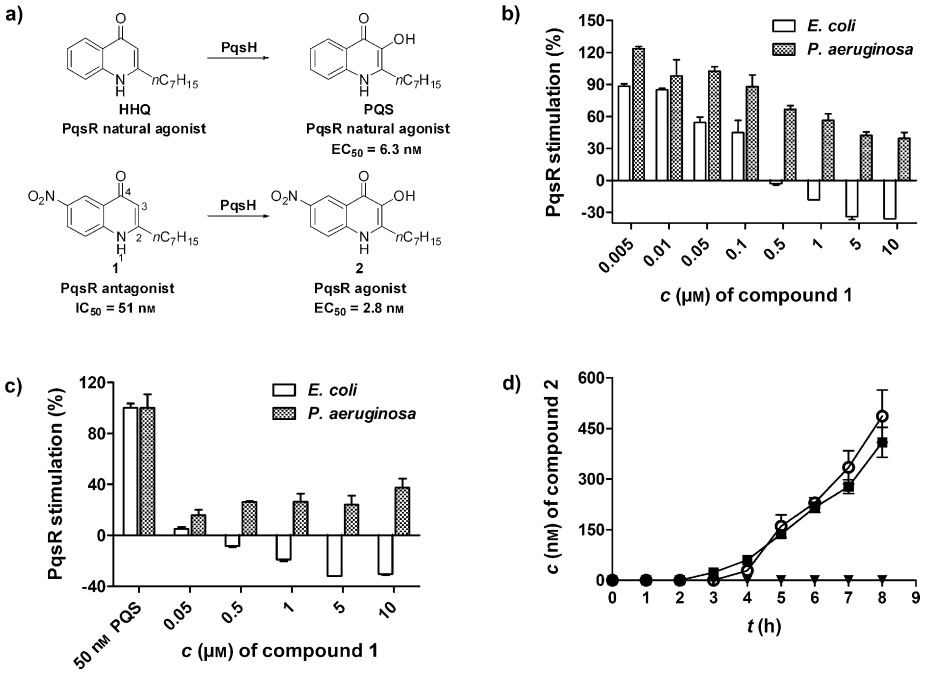
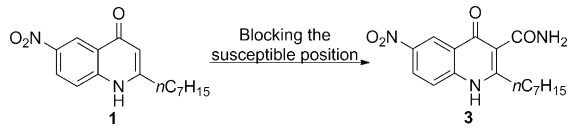


Figure 1. Characterization of the PqsR antagonist **1** in *E. coli* and *P. aeruginosa* and time-dependent formation of **2**. a) Chemical structures of HHQ, PQS, **1**, and **2**. b) Antagonist test of **1** in β-galactosidase reporter gene assays based on *E. coli* or *P. aeruginosa*. The assays were performed in the presence of PQS (50 nM). For the *y* axis, 0% is defined as the basal PqsR stimulation without ligands, and 100% is defined as the PqsR stimulation by PQS (50 nM). Mean values of two independent experiments with $n=4$ are given, error bars represent standard deviation. c) Agonist test of **1** in β-galactosidase reporter gene assays based on *E. coli* or *P. aeruginosa*. For the *y* axis, 0% and 100% stimulation are defined as above. Mean values of two independent experiments with $n=4$ are given, error bars represent standard deviation. Significance of agonistic activity for **1** in *P. aeruginosa* compared to basal level: $p<0.05$. d) Time-course studies of the production of **2** in PA14 (○), *pqsH* (▼), and *pqsA* (■) mutants. Strains were incubated with **1** (5 μM). Mean values of one experiment with $n=3$ are given, error bars represent standard deviation.

Table 1: Determination of antagonistic activity and effects on PQS, HHQ, and the virulence factor pyocyanin.



Parameter	**1**	**3**
IC_{50} in *E. coli* [nM]	51	35
IC_{50} in *P. aeruginosa* [nM]	60% inhibition[a]	404
Reduction of PQS levels[b]	14%*	37%*
Reduction of HHQ levels[b]	1%	54%*
Reduction of pyocyanin levels[b]	44%*	81%* (IC_{50}: 2 μM)

PQS, HHQ, and pyocyanin assays were performed in PA14. Mean values of at least two independent experiments with $n=3$ are given, standard deviation less than 25%. Significance: * $p<0.05$. [a] Tested at 10 μM. [b] Tested at 15 μM.

to the natural ligand HHQ, which is converted into PQS by PqsH; 2) Many PQS analogues show agonistic activity towards PqsR;[23,24] 3) **1** revealed an agonistic activity in *P. aeruginosa* as mentioned above. To prove this hypothesis, ultrahigh-performance liquid chromatography (UHPLC) tandem mass spectrometry was applied to observe the supposed product of biotransformation **2** in *P. aeruginosa* (wild type; PA14). Interestingly, after incubation of PA14 with **1**, the chromatogram of selected reaction monitoring (SRM) transition m/z 303 > 218 revealed a signal peak with identical retention times for the proposed product and the chemically synthesized compound **2** (Supporting Information, Figure S1). To further validate bacterial-cell-mediated conversion of **1** into **2**, and to examine the involvement of PqsH in this process, a time-course study was conducted. The production of **2** in PA14 and in the native-ligand-free mutant *pqsA*[21] with functional PqsH was time-dependent, whereas the *pqsH* mutant failed to synthesize **2** (Figure 1d; for the results after 16 h, see Figure S2). This result clearly identifies PqsH as the enzyme responsible for the biotransformation. Subsequently, we examined the activity of product **2**. In a competition experiment performed in *E. coli*, **2** efficiently restored the PqsR stimulation that was repressed by antagonist **1** (in the antagonist test, **2** restored almost 50% of the PqsR stimulation, even in competition with **1** at ten times higher concentrations; Figure S3). Compound **2** (EC_{50} = 2.8 nM; EC_{50}: ligand concentration to achieve a half-maximal degree of PqsR receptor stimulation) is even more active than the strongest natural PqsR agonist PQS (EC_{50} = 6.3 nM). Taken together, these findings explain that the unexpected agonistic activity that was observed for **1** in *P. aeruginosa* is due to PqsH-mediated functional inversion (Figure 1a).

For a rational development of potent and stable PqsR antagonists, the susceptible 3-position had to be blocked by substitution of the hydrogen atom with an appropriate functional group. A small library of 3-substituted compounds ($CONH_2$, COOH, COOEt, and CONHOH) were synthesized (data not shown). In terms of their agonistic/antagonistic profiles, the carboxamide **3** (for its synthesis, see Figure S4) turned out to be the most promising derivative. Accordingly, **3** was used for further biological evaluation. Most interestingly, **3** showed high potency in the *E. coli* reporter gene assay, but retained its antagonistic activity in *P. aeruginosa* without displaying any agonistic activity up to 15 μM (Table 1).

Next, the effects of the antagonists on the production of the signal molecules were examined in PA14.[25] Compared with **1**, the improved antagonist **3** was able to strongly reduce the HHQ and PQS levels by over 50% and 30%, respectively, at a concentration of 15 μM (Table 1). We then investigated the production of pyocyanin, an important virulence factor

and a major contributor to the pathogenicity of *P. aeruginosa*.[26] Strikingly, **3** efficiently decreased the pyocyanin levels with an IC_{50} of 2 μM, whereas **1** revealed a strongly reduced potency (44% at 15 μM). A growth-inhibition effect could be excluded based on growth curves of PA14 that were measured in the presence of antagonist (15 μM) in minimal medium (Figure S5). Overall, optimization of the antagonist led to enhanced effects on the reduction of signal-molecule and pyocyanin levels, which is in agreement with the improved antagonistic activity that was observed in the *P. aeruginosa* reporter gene assay. To the best of our knowledge, **3** is the compound that most effectively interferes with the *pqs* QS system in *P. aeruginosa*.

Encouraged by these results, we validated the PqsR antagonistic properties of **3** in appropriate animal experiments.[27] As *Caenorhabditis elegans* is sensitive towards a *P. aeruginosa* infection and its virulence factor pyocyanin,[28] **3** was evaluated in a *C. elegans* fast killing assay. The survival rate of *C. elegans* that were incubated on agar plates containing PA14 and **3** (15 μM) remained at 94%; in the absence of the antagonist, however, the survival rate continuously decreased to 47% (control) within six hours (Figure 2a). These results highlight a protective effect of **3** against *P. aeruginosa* infection in the nematode assay.

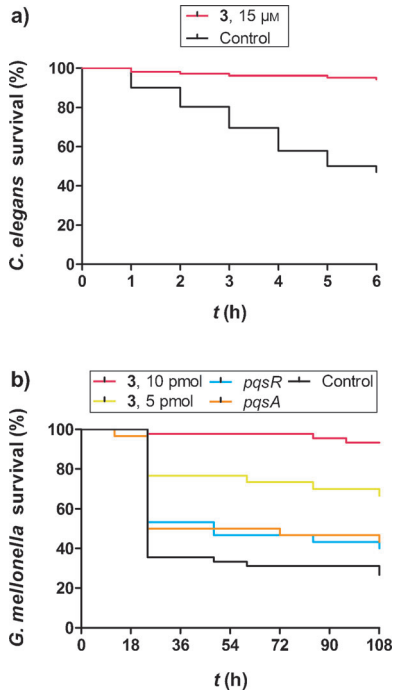


Figure 2. Evaluation of the PqsR antagonist **3** in animal infection models. a) Kaplan–Meier survival curves of *C. elegans* incubated on agar plates containing PA14 and DMSO (control) or **3** (15 μM). Results represent cumulative data from three independent experiments. The survival rate was significantly larger for treated nematodes than for those in the control experiment ($p < 0.0001$; log-rank test). b) Survival curves of *G. mellonella* larvae infected with PA14 receiving no treatment (control) or receiving treatment with compound **3** (5 pmol or 10 pmol), and of larvae infected with the PA14 *pqsR* or *pqsA* mutants. Results represent combined data from at least two independent experiments. The survival rate was significantly larger for treated larvae than for larvae in the control experiment (5 pmol of **3**: $p < 0.001$, 10 pmol of **3**: $p < 0.0001$; log-rank test).

We further challenged a more complex animal infection model with *Galleria mellonella*. This insect model displays a significant positive correlation with a mouse model and is therefore considered as a powerful tool to investigate pathogenicity causing mammalian infections. The larvae of the greater wax moth are susceptible to PA14, with a 50% lethal dose of one bacterium.[29] *G. mellonella* larvae were infected with PA14 in the absence and presence of antagonist **3**. Most interestingly, the treatment of the PA14-infected larvae with antagonist **3** (10 pmol) led to a survival rate of 93% (Figure 2b), whereas only 36% of the infected larvae survived the first 24 h in the absence of **3** (control). Treatment with only 5 pmol of antagonist provided partial protection with a survival rate of 67%. It should be noted that **3** is intensively diluted by the hemolymph after injection. Given an average weight of 450 mg and assuming a total hemolymph volume of 450 μL for each larva, the antagonist exerted its therapeutic effect at a final concentration of 22 nM (corresponding to 7.3 $ng\,g^{-1}$ body weight) in the larva. Most interestingly, PA14-infected larvae receiving treatment with antagonist **3** showed much higher survival rates than those infected with the mutants *pqsA* and *pqsR*, which are deficient in *pqs* QS (Figure 2b). This implies that disruption of QS with small molecules, rather than genetic deletion, can be advantageous. Overall, the results from the two animal studies clearly show that **3** is a strong antivirulence agent.

In summary, we have revealed that the synthase PqsH converts the potent PqsR antagonist into a strong agonist; this process is responsible for the low efficacy of compound **1**. Surprisingly, such a slight structural modification (hydroxylation) leads to complete loss of the antagonistic activity of compound **1** and dramatically imparts the opposite functionality (agonism) to the ligand. A high percentage of anti-infectives suffer from ineffectiveness in cell-based assays or under in vivo conditions, which is generally considered to be due to penetration problems or efflux-pump-mediated excretion. As an optimization addressing these drawbacks is regarded as highly challenging, these compounds are usually discarded. Herein, we suggested that a rational consideration of other potential factors that impair the activity is rewarding. As shown in this case study, ineffective compounds can be rescued by medicinal-chemistry strategies, which decreases the attrition rate during the drug development process. Moreover, our research identified the PqsR antagonist **3** as an antivirulence agent that is highly potent in vivo, which provides the first proof of concept that PqsR antagonists reduce the mortality caused by *P. aeruginosa* in two animal models. This finding provides a promising starting point for further in vivo investigations using mammalian organisms and may open new avenues for the development of anti-infectives that are less prone to resistance. Furthermore, species-selective targeting of specific regulatory pathways might help to minimize adverse effects that are observed with broad-spectrum antibiotics.

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