**Pseudomonas aeruginosa** Quorum Sensing Systems as Drug Discovery Targets: Current Position and Future Perspectives

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**ABSTRACT:** Antimicrobial resistance (AMR) is a serious threat to public health globally, manifested by the frequent emergence of multidrug resistant pathogens that render current chemotherapy inadequate. Health organizations worldwide have recognized the severity of this crisis and implemented action plans to contain its adverse consequences and prolong the utility of conventional antibiotics. Hence, there is a pressing need for new classes of antibacterial agents with novel modes of action. Quorum sensing (QS), a communication system employed by bacterial populations to coordinate virulence gene expression, is a potential target that has been intensively investigated over the past decade. This Perspective will focus on recent advances in targeting the three main quorum sensing systems (las, rhl, and pqs) of a major opportunistic human pathogen, *Pseudomonas aeruginosa*, and will specifically evaluate the medicinal chemistry strategies devised to develop QS inhibitors from a drug discovery perspective.

**INTRODUCTION**

Antimicrobial resistance is a global threat that is imposing an ever increasing burden on public health because of the rapid selection of antibiotic resistance associated with the over- and misuse of antibacterial reagents.1,2 The withdrawal of most major pharmaceutical companies from antibiotic discovery and their alternative focus on chronic, noncommunicable diseases reflects the difficulties in developing novel antibacterial agents and the enormous cost of bringing new therapeutics to the clinic. In addition, the increasing complexity of the legislation imposed by regulatory bodies and risks associated with antibacterial drug discovery research has restricted further advances in this field.3,4 Over the past 17 years, only four new classes of antibiotics have been discovered with the majority of FDA-approved drugs being based on alterations to existing structures (Figure 1).4–6

The antibiotic crisis is associated with the appearance of multidrug resistant pathogens, also known as "superbugs" that are capable of surviving antibiotic treatment as in the case of the so-called "ESKAPE" panel pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter species*).7 According to the World Health Organization (WHO), *Pseudomonas aeruginosa* represents one of the "critical priority pathogens" that requires urgent attention because of its multidrug resistance (MDR) to a broad spectrum of antibiotics including carbapenems and third generation cephalosporins.8,9 *P. aeruginosa* is commonly responsible for lung, skin, eye, wound, blood-borne, and urinary tract infections occurring in both hospitals and the community.10,11 This Gram-negative bacterium is a common cause of nosocomial infections and a major pathogen in both cystic fibrosis (CF) and immunocompromised patients and those with burns, open fractures, or implanted medical devices such as catheters.12,13

**VIRULENCE OF P. aeruginosa**

The clinical significance of *P. aeruginosa* arises from its ability to express a plethora of virulence factors that aid invasion of, and cause damage to, host tissues.14 Among these, flagella and pili contribute to tissue surface adhesion as well as to tissue migration via swarming and twitching motility.15,16 *P. aeruginosa* also secretes multiple tissue degrading exoenzymes, exotoxins, and host defense-inactivating effector proteins which play key roles in bacterial virulence.17–19

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**Figure 1.** FDA approved antibiotics for the period 2000–2017 (red) and novel approved antibiotic classes with unprecedented chemical structures and modes of action (green). Data are collected from U.S. Food and Drug Administration (FDA) (www.fda.gov as of March 15, 2018).
toward interference with QS-mediated signaling to disrupt bacterial communication in order to attenuate virulence such that the infecting bacteria can be cleared by the host defenses. Hence, the use of QS inhibitors (QSI) that do not directly compromise bacterial viability should impose less selective pressure with respect to resistance than conventional antibiotics. QS inhibitors (QSI) alone may not be sufficient to eradicate infections especially in immunocompromised individuals but are likely to act synergistically in combination with growth inhibitory antibiotics. QSI may however be very effective as prophylactics. Since 2000, the number of QSI publications has shown a significant upward trajectory mostly with respect the underlying molecular biology with medicinal chemistry related papers and published patent applications representing only a small percentage of the total (Figure 2).

Figure 2. Representation of the number of publications related to QS for the period 2000–2017 as analyzed using the SciFinder Scholar search tool (https://scifinder.cas.org, as of March 15, 2018).

P. aeruginosa AS A “SUPERBUG”

P. aeruginosa is highly resistant to antimicrobials due to intrinsic, acquired, and evolved mechanisms. P. aeruginosa exhibits intrinsic resistance to antibiotics because of the low permeability of its outer membrane and the presence of at least 12 efflux pumps which are able to expel various antibiotics including cephalosporins, carbapenems, fluoroquinolones, and aminoglycosides. In addition, β-lactamase genes are frequently chromosomally encoded making P. aeruginosa resistant to penicillins and cephalosporins. Acquired resistance in P. aeruginosa is mainly driven by horizontal gene transfer whereby genes coding for specific resistance traits are transferred from one bacterium to another. Acquired resistance can also be induced through a mutational change, for example, in DNA gyrase, resulting in lower affinity for fluoroquinolones. A third mechanism for developing resistance is known as evolved resistance, whereby P. aeruginosa responds to numerous stimuli, for instance, subinhibitory concentrations of antibiotics, nutrient deprivation, pH, and temperature dependence and through the expression of genes which enhance specific activities such as efflux pump mechanisms and/or those that modify cell envelope composition. For these reasons, the effectiveness of molecules targeting P. aeruginosa infections can be significantly compromised by these bacterial defense mechanisms. Therefore, it is important that knowledge of existing resistance mechanisms is considered when introducing new molecular scaffolds into the rational design of inhibitors of bacterial QS regulatory pathways.

QUORUM SENSING AS A DRUG DISCOVERY TARGET

QS is a mechanism for cell to cell communication between bacteria that relies on the production and sensing of diffusible quorum sensing signal molecules (QSSMs) that are sometimes referred to as autoinducers (AIs). Once a bacterial population reaches a certain threshold that is reflected by the concentration of QSSMs in the surrounding environment, the transcription of multiple genes is synchronized enabling the population to behave collectively. This diffusible signal-mediated regulation controls a wide range of activities from swarming and swimming motility, biofilm maturation, virulence factor, and secondary metabolite production as well as antibiotic resistance. In recent years, attempts to develop new classes of antimicrobial agents have included the targeting of specific virulence factors or virulence regulatory mechanisms rather than cell viability with a view to minimize the selective pressures that lead to the emergence of resistance. One of these strategies is directed
INHIBITION OF THE las QUORUM SENSING SYSTEM

The las system in P. aeruginosa employs N-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL) as the cognate QSSM that, upon binding the LuxR type transcriptional regulator LasR, activates the expression of multiple genes. These include lasI which codes for the 3OC12-HSL synthase as well as numerous virulence factor genes (e.g., the elastases LasA and LasB, alkaline protease, and exotoxin A) required for biofilm development and the rhl and pqs systems. Recent work has confirmed that pharmacological antagonism of the LasR receptor induces and stabilizes conformational changes that prevent the complex (LasR−antagonist) from binding to DNA so preventing transcription of the target genes.

LasR INHIBITORS

LasR has attracted attention as a drug discovery target driven by its position in the P. aeruginosa QS hierarchy. There is a considerable amount of literature describing inhibitors for LasR from the past 15 years, and these can be classified into four categories: AHL-like antagonists, non-AHL-like antagonists, covalent binders, and natural-product-based inhibitors. It is also noteworthy that several assays for evaluating LasR inhibition have been described. These are mostly bacterial cell-based employing transcriptional fusions to LasR target gene promoters coupled to reporter genes providing bioluminescent or fluorescent readouts. However, these assays have not been standardized and employ different homologous (P. aeruginosa) or heterologous (E. coli) host strains making direct comparisons of inhibitor potencies between studies challenging.

AHL-LIKE INHIBITORS

Inhibitors with Modified Tail Structures. One of the earliest attempts to modify 3OC12-HSL was through the introduction of a sulfur containing tail which had variable effects on LasR antagonism depending on chain length and the oxidation state of the sulfur, with the best inhibitor 5 (Figure 4) displaying 50% LasR inhibition at 6 μM using an E. coli reporter strain. A recent patent described a sulfur-based tail 6 with IC50 of 5.2 μM in a P. aeruginosa reporter strain. Another tail group modification was introduced by Geske et al., who shortened the aliphatic chain and incorporated an aromatic end group (7 and 8). Both compounds showed inhibition in a P. aeruginosa

Figure 3. Schematic diagram of the interconnected las, rhl, and pqs quorum sensing systems in P. aeruginosa. Green arrows and red blocked lines indicate up- or down-regulation, respectively. Oval shapes represent various proteins, color coded circle shapes represent QSSMs, and large colored arrows represent genes. Thin gray arrows represent protein expression, and thin blue arrows indicate QSSMs biosynthesis.

Figure 4. LasR inhibitors: AHL analogs with modified tail structures.
reporter strain with IC\textsubscript{50} values of 16.1 and 14.8 \(\mu M\), respectively.\textsuperscript{36} In a later study, the same group of researchers expanded the SAR of these inhibitors and submicromolar antagonists were identified using an \textit{E. coli} reporter strain (9, 1.72 \(\mu M\); 10, 610 nM; 11, 250 nM; 12, 340 nM).\textsuperscript{37} Similar structures have also been published as LasR antagonists with IC\textsubscript{50} values of 1 and 10 \(\mu M\) for 13 and 14, respectively.\textsuperscript{38} Although Jadhav et al. focused on the structure–activity relationship of 3OC12-HSL analogs as immune modulators, a series of LasR antagonists was found bearing modifications of the hydrophobic tail region. The compounds were screened using a native \textit{P. aeruginosa} reporter assay at 100 \(\mu M\) ligand concentration to reveal that 15 and 16 had reduced the activity of LasR to less than 6%.\textsuperscript{39} Triazole-derived tail structures were designed by Stacy et al. with various linker lengths from the headgroup, and the compounds were assessed using an \textit{E. coli} LasR reporter assay to identify three LasR inhibitors with low micromolar IC\textsubscript{50} (17, 3.27 \(\mu M\); 18, 4.03 \(\mu M\); 19, 2.64 \(\mu M\)).\textsuperscript{40}

**Modified Head Structures.** Suga et al. published a series of compounds with modifications to the headgroup with various saturated and aromatic ring replacements preserving the tail structure which resulted in a number of agonists and antagonists.
with little structural diversity. One of the highlighted agonists in their work was compound 20 (Figure 5) which is based on 2-
aminocyclohexanol. Interestingly, the keto derivative 21 was
shown to be a weak antagonist at ligand concentrations of 50 μM. Replacing the saturated ring with an aromatic ring provided
antagonist 22 that exhibited reduced activity of LasR in PAO-
JPI2 lasI-gfp, a las rhl double mutant transformed with a
plasmid containing the lasI promoter fused to green fluorescent
protein gene (gfp). Possible RhlR antagonism in the reporter
screens was also noted at concentrations of 10 μM or greater.
However, these results did not translate into virulence factor
attenuation with respect to pyocyanin or biofilm reduction.41,42
The latter observation could be interpreted by the recent finding
of Moore et al. that 20 is actually a partial agonist and had no
antagonistic activity.43 Compound 23, containing a p-methoxy-
phenyl group, exhibited inhibitory activity on pyocyanin and
estastase production with no evidence of reporter strain
inhibition.44 Modifications of the headgroup by McLnnis et al.
were shown to be detrimental for activity with a phenyl group 24
being the best replacement among the published compounds.
Nevertheless, it had relatively weak activity when tested in a P.
aeruginosa reporter compared with E. coli.45 Park et al. designed a
series of compounds with a pyrrole headgroup and aliphatic tail
that were validated using a biofilm assay to conclude that 25 had
the strongest effect particularly at 100 μM ligand concentration.
Even though the study presented some molecular docking data
on 25 binding to LasR, no experiments were performed to
validate this in silico modeling.46

- NON-AHL-LIKE STRUCTURES

The design of LasR inhibitors has also focused on addressing the
chemical and enzymatic stabilities associated with the original
lactone-based structure. The AHL lactone ring under alkaline
conditions undergoes a ring opening reaction to the corresponding γ-hydroxyarboxylate.47 Further, the AHL
structure is also prone to enzymatic degradation by lactonases
and amino acylases which render these QSSMs inactive.48

In a search for non-AHL-like LasR inhibitors, Hentzer et al.
disclosed a halogenated furanone 26 (Figure 6), which is a
synthetic analog of a furanone-derived natural product isolated
from the marine alga Delisla pulchra.49 Compound 26 showed a
dose-dependent inhibition of virulence and the development of
antibiotic resistant biofilms. Transcriptomic profiling after
treatment of P. aeruginosa with 26 resulted in the repression of
diverse genes controlled by AHL-dependent QS. Most
importantly, 26 at a dose of 0.7 mg/kg had significant efficacy
in treating P. aeruginosa lung infections in a mouse infection
model.50,51 The mechanism of action of 26 however has not
been elucidated. The compound is toxic at concentrations of
≥100 μM, and although surface enhanced Raman scattering
showed signal-specific structural changes in LasR upon ligand
binding, Moore et al. were unable to demonstrate inhibition of
LasR activity at subgrowth inhibitory concentrations in the lasR
bioreporter PAO-JP2 lasI-gfp.52

An ultrahigh throughput screen (UHTS) was performed on a
library of 200 000 compounds by Müh et al. using a P. aeruginosa
fluorescently tagged reporter strain which gave two LasR
antagonists 27 and 28 with IC50 values of 30 nM and 10 μM,
respectively. The inhibition of LasR correlated with reduced
estastase and pyocyanin production. It is noteworthy that the
hydrophobic tail is still preserved to a certain extent in these two
molecules.53 Moreover, the screen also identified a LasR
inhibitor 29 with a low potency (IC50 = 50 μM). Similar

compounds to 29 have been shown to be activators of the
receptor, and cocryystal structures with LasR have been
obtained.54,55 Borde et al. screened a synthetic compound
library of 16 000 compounds using a recombinant lasR
expressing Pseudomonas putida for both agonists and antago-
nists. LasR inhibitors with a thiocarbamate functionality (30 and
31) were their most active hits showing antagonism of 50–60%
from the marine alga Delisia pulchra. Transcriptomic pro-
systems.61 Utilization of computer-aided virtual screening to
identify new LasR antagonists has also been successful.62

- COVALENT BINDERS AS INHIBITORS FOR LasR

Amara et al. developed a series of covalent antagonists with an
electrophilic warhead, such as isothiocyanate, that is able to form
a covalent bond with cysteine (Cys79) within the LasR ligand
binding site. The study focused on 46 (Figure 7) as it is a

covalent binder with an IC50 of 134 μM in the P. aeruginosa
bioreporter strain PAO-JP2 lasI-gfp. Biofilm and pyocyanin
production were also reduced at a 50 μM concentration.63

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In a follow-on study, 46 was refined by the introduction of an electronwithdrawing halogen at the β-position to increase the electrophilicity of the isothiocyanate. Compound 47 was thoroughly investigated for its binding to LasR Cys79, used in an ex vivo human wound infection model and in a *Caenorhabditis elegans* survival model where it protected the worms from *P. aeruginosa* infections. Nevertheless, these results have been recently contradicted by Moore et al., who reported that 46 is a LasR agonist that induces LasB elastase production. O’Brien et al. developed the irreversible binder 48 based on 29 with substitution of 2-chlorobenzoate by a maleimide linker that was proposed to form a covalent bond with Cys79. In an *E. coli* based reporter assay, 48 showed a concentration-dependent inhibition with an IC$_{50}$ of 4.8 μM and was able to reduce pyocyanin production and biofilm formation in *P. aeruginosa*.

### LasI INHIBITORS

LasI, the 3OC12-HSL synthase, has received less attention as a target for disrupting the *las*-dependent QS. There appears to be only one report describing 49 (Figure 8) as a LasI inhibitor (~40% inhibition at 20 μM) in a heterologous *E. coli*-based reporter strain causing a significant reduction in biofilm and swarming motility. A microarray-based transcriptomic assay revealed that lasI along with the pqsABCDE genes were downregulated after treatment with 49 compared to the untreated cells, a result that would be expected given that LasR/3OC12-HSL is involved in regulating both AHL and AQ biosynthesis in *P. aeruginosa* (Figure 3).

### INHIBITION OF THE rhl QUORUM SENSING SYSTEM

The *P. aeruginosa* rhl system employs 2 N-butanoyl-L-homoserine lactone (C4-HSL) and is responsible for the expression of multiple virulence factors including rhamnolipids, HCN, swarming motility and contributes to biofilm maturation. The expression of the rhl system is controlled by the las system as the LasR/3OC12-HSL complex activates the transcription of both rhlR and rhl leading to more C4-HSL and the activation of a wide range of RhlR/C4-HSL controlled genes. The rhl QS system has received less attention compared with the las system as most published work has focused on the latter probably due to its location at the top of the *P. aeruginosa* QS hierarchy. Hence it remains unclear as to whether RhlI and RhlR both represent a valid drug discovery target especially since a rhlI mutant in contrast to a rhlR mutant retains full virulence in a mouse infection model.

The RhlR modulator 50 (Figure 9) was reported to act as an antagonist in the presence of C4-HSL and an agonist in its absence. 50 reduced pyocyanin production (IC$_{50}$ = 8 μM), inhibited biofilm formation, and enhanced *C. elegans* survival during a *P. aeruginosa* PA14 infection at 50 μM ligand concentration. However, a subsequent study, described 50 as an agonist for RhlR, inhibiting pyocyanin production through down-regulation of the pqs system. Furthermore, 52 and 53 were identified as RhlR agonists with EC$_{50}$ values of 4.5 and 7.2 μM in *P. aeruginosa* PAO-JP2 las-gfp reporter, respectively, significantly reducing pyocyanin but not rhamnolipid production. It is noteworthy that these compounds appeared in a recent patent along with other analogs with the thiocarbazole headgroup. The study concluded that for an RhlR modulator to function as an antivirulence agent, it is required to be an agonist rather than antagonist since it reduces pyocyanin production through pqs down-regulation. However, in such cases RhlR activation would lead to overproduction of rhamnolipids, a virulence factor involved in swarming, biofilm maturation and detachment, and early infiltration of *P. aeruginosa* into human airway epithelia. Hence, RhlR requires further investigation and evaluation as antiquorum sensing target before considering it for any further medicinal chemistry optimization.

### NATURAL PRODUCTS

There is considerable body of literature describing various natural products that interfere with *P. aeruginosa* QS particularly the *las* and *rhl* systems. These efforts are summarized in Table 1.

### PERSPECTIVE VIEW

The *las* QS system has been the most intensively investigated as an antivirulence drug target. Despite the numerous attempts to target signal reception (LasR) or signal synthesis (LasI), most inhibitors lack the lead-like properties required and failed to proceed to preclinical development due to one or more of the following drawbacks: (i) presence of hydrophobic and/or metabolically labile groups such as lactone/thiolactone or reactive species (i.e., isothiocyanate); (ii) unsurmountable physiochemical properties (e.g., high lipophilicity and/or molecular weight); (iii) weak potency and ambiguity of antivirulence profiles and efficacy toward *P. aeruginosa* clinical isolates; (iv) lack of uniformity and standardization of methodology governing the assessment of the compounds, e.g., use of heterologous *E. coli* reporters. It should also be noted that lasR mutants frequently arise in chronic human *P. aeruginosa* infections. Nevertheless, the knowledge gained, along with the availability of crystal structures for LasR and LasI, should facilitate the future discovery and evaluation of more drug-like molecules. In addition, the administration route and indications for such inhibitors plays a major role in...
physiochemical property criteria definition; for instance, transdermal treatment (i.e., for wound infections) will require different compound properties to those desirable for oral administration. With respect to rhl inhibition, it is not yet clear whether antagonizing this system alone would yield therapeutic benefits. Moreover, the lack of structural information for RhlR and RhlI makes compound design more difficult. Therefore, rhl requires further validation as a target.

THE PSEUDOMONAS QUINOLONE SYSTEM (pqs)

The P. aeruginosa pqs QS system relies on 2-alkyl-4-quinolone signal molecules rather than AHLs that interact with their

Table 1. Summary of Natural Product with P. aeruginosa QS Quenching Activities

<table>
<thead>
<tr>
<th>Natural Product</th>
<th>Structure</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narigenin (derived from Cobetrum albitorum)</td>
<td><img src="image" alt="Structure" /></td>
<td>Down regulation of lasI, lasR, rhlR and rhlR genes and reduced C4-HSL and 3OC12-HSL at 4 mM.</td>
</tr>
<tr>
<td>Proanthocyanidins</td>
<td><img src="image" alt="Structure" /></td>
<td>Reduced the expression of lasI, lasR, rhlR and rhlR genes and antagonized LasR and RhlR promoting survival of Drosophila melanogaster at doses of 200 μg/mL.</td>
</tr>
<tr>
<td>(phenylsulfanyl)alanine</td>
<td><img src="image" alt="Structure" /></td>
<td>Inhibited biofilm development at 1 mM through the inhibition of las and rhl systems.</td>
</tr>
<tr>
<td>Benzyl decanoyl-L-serinate</td>
<td><img src="image" alt="Structure" /></td>
<td>Reduced production of elastase and rhamnolipids and potentiated antibiotic activities at 100 μM through partial down regulation of lasR, lasI, rhlR and rhlI.</td>
</tr>
<tr>
<td>Coumarin</td>
<td><img src="image" alt="Structure" /></td>
<td>At 1.36 mM, coumarin slightly inhibited pqsA and rhlR expression, biofilm formation and swarming.</td>
</tr>
<tr>
<td>Eugenol</td>
<td><img src="image" alt="Structure" /></td>
<td>At 400 μM, eugenol inhibited pqs and las in heterologous E. coli transcriptional reporter assays, biofilm formation and swarming motility.</td>
</tr>
<tr>
<td>Sulfuraphane from broccoli</td>
<td><img src="image" alt="Structure" /></td>
<td>~100 μM antagonized LasR in both PAO1 and heterologous E. coli reporters, inhibited pyocyanin and biofilm.</td>
</tr>
<tr>
<td>Trans-cinnamaldehyde</td>
<td><img src="image" alt="Structure" /></td>
<td>At 2.27 mM, inhibited C4-HSL and pyocyanin production (42.06 %) likely to be mediated via RhlR rather than LasI inhibition.</td>
</tr>
<tr>
<td>Baicalin from Scutellaria baicalensis extract.</td>
<td><img src="image" alt="Structure" /></td>
<td>Baicalin at a sub-minimum growth inhibitory concentration (MIC) of 256 μg/ml (equal to 573 μM) inhibited AHL synthesis and biofilm development, enhanced antibiotic efficacy in a mouse foreign body infection model, disrupted motility and promoted survival in C. elegans. Baicalin downregulated the P. aeruginosa QS network genes (lasI, lasR, rhlR, rhlR, pqsA and pqsR). 63 was identified as a PAINS filter hit.</td>
</tr>
<tr>
<td>7,8-dihydroxy flavone</td>
<td><img src="image" alt="Structure" /></td>
<td>Non-competitive inhibition (~70%) of LasR and RhlR in heterologous E.coli reporters, reduced pyocyanin and swimming. 64 identified as a PAINS filter hit.</td>
</tr>
<tr>
<td>Hordenine</td>
<td><img src="image" alt="Structure" /></td>
<td>At 6 mM, 65 inhibited C4-HSL by ~70% and 3OC12-HSL by ~30%, and reduced rhamnolipid, elastase, protease, alginate, pyocyanin and biofilm. Hordenine also downregulated lasI, lasR, rhlR and rhlR expression.</td>
</tr>
<tr>
<td>Ajoene derived from garlic extract</td>
<td><img src="image" alt="Structure" /></td>
<td>LasR (IC50 15 μM) and RhlR (50 μM) using P. aeruginosa reporter strains.</td>
</tr>
</tbody>
</table>
cognate receptor PqsR (also known as MvfR), a LysR family transcriptional regulator that characteristically consists of an N-terminal DNA-binding domain and a C-terminal ligand binding domain (Figure 3 and Figure 10). *P. aeruginosa* produces a diverse range of over 50 AQ molecules of three main classes, 2-alkyl-4-hydroxyquinolines, 2-alkyl-3-hydroxy-4-quinolones, and 2-alkyl-4-hydroxyquinoline-N-oxides including HHQ (2-heptyl-4-hydroxyquinolone), and the *Pseudomonas* quinolone signal (PQS) (2-heptyl-3-hydroxy-4(1H)-quinolone). PQS/HHQ and their C-9 congeners are all able to activate PqsR. In contrast to HHQ, PQS is an iron chelator and regulates the expression of genes involved in the iron-starvation response and virulence factor production via both PqsR-dependent and PqsR-independent pathways.94,95 AQ biosynthesis is achieved via the condensation of anthranilic acid and mediated via PqsA, PqsBC, PqsD, and PqsE to generate HHQ which is converted to PQS via PqsH. Non-AQ side products of this route are highlighted in red.

**Figure 10.** Biosynthesis of alkylquinolones starting from activated anthranilic acid and mediated via PqsA, PqsBC, PqsD, and PqsE to generate HHQ which is converted to PQS via PqsH. Non-AQ side products of this route are highlighted in red.

**Figure 11.** Structures of various PqsR antagonists.

triggering the autoinduction response characteristic of most QS systems.24,95 Recent studies have revealed that although PqsE is a thioesterase that contributes to AQ biosynthesis, the mechanism by which PqsE controls a subset of virulence factors including pyocyanin is still not understood.98,99 Interestingly, the pathogenicity of a *P. aeruginosa pqsE* mutant in contrast to a *pqsA* mutant was not attenuated in a mouse wound infection model. However, pqsE alone in the absence of AQ production can restore the virulence of a *pqsA* mutant.99,100

The AQ system is linked with las and rhl QS as LasR (positively) and RhlR (negatively) regulate PqsR through binding to its promoter.101 Moreover, pqsH is positively regulated by LasR/3OC12-HSL which increases the abundance of PQS.102 The pqs system plays a major role in the virulence of *P. aeruginosa* as demonstrated by the attenuation of *pqsA* and *pqsR* mutants in murine infection models.99,103 In contrast to LasR/3OC12-HSL and RhlR/C4-HSL that bind to the promoters of multiple target genes, transcriptome experiments suggest that PqsR has only a single target promoter, that of *pqsA*.95 However, using ChIPseq, Maura et al. suggested that PqsR bound to 35 locations on the *P. aeruginosa* chromosome, although only 22% of these were to promoter regions.104 Most of...
Figure 12. Overlay poses of the cocrystal structures of PqsR ligand binding domain with a natural agonist NHQ (PDB code 4JVD, green), quinazoline based antagonist 79 (PDB code 4JV1, blue), and thioacetamide based antagonist 80 (PDB code 6B8A, pink). Figure 12A highlights the hydrophobic residues surrounding pocket B. Figure 12B shows the binding pose of heterocyclic headgroup residing in pocket A. The poses were generated using Maestro, Schrödinger, LLC, New York, NY (2018).

The binding sites were located inside a gene or overlapping several genes, and hence their functionality as PqsR binding sites remains to be validated.104

### INHIBITION OF PQS QUORUM SENSING IN P. AERUGINOSA

**Inhibition of Signal Reception by PqsR.** Lu et al. discovered a PqsR antagonist based on HHQ by introducing modifications to the benzene moiety of the quinoline ring and the aliphatic side chain. The highest potencies, as demonstrated using a recombinant E. coli transcriptional reporter, were achieved upon the introduction of a strong electron withdrawing group (Figure 11; IC₅₀ = 51 nM; 74; IC₅₀ = 54 nM) at the 6-position of the HHQ quinoline ring. Despite the fact that these compounds showed good potency using the E. coli reporter, in P. aeruginosa, only a modest reduction in pyocyanin was noted and they failed to reduce elastase, rhamnolipids, or AQ levels.105,106

When 73 and 74 were further tested in P. aeruginosa, it became clear that PqsH-mediated the oxidation at the 3-position of the quinoline ring of these inhibitors converting them to potent agonists. This effect was not observed for compound 75 (IC₅₀ = 35 nM, E. coli reporter, IC₅₀ = 4 μM, P. aeruginosa reporter) where a blocking group (CONH₂) was introduced at the 3-position to preserve the antagonistic activity in P. aeruginosa.107-109 Klein et al. reported another series of weak PqsR inhibitors derived from N-hydroxybenzamides with modifications at the para-position. Only modest activity against PqsR was attained with 76 (IC₅₀ in E. coli of 12.5 μM vs IC₅₀ in P. aeruginosa of 23.6 μM) which weakly affected pyocyanin at IC₅₀ concentrations.110 In a follow-up study, the carboxamide group was replaced by a 1,3,4-oxadiazole moiety to provide a similar antagonist activity to 76 along with marginal activity on pyocyanin and AQ production.111

In a similar effort, the same group published a series of weak PqsR antagonists based on a triazole scaffold (78, IC₅₀ = 26 μM) in a pursuit of dual PqsR/PqsD inhibitors. Compound 79, the first PqsR inhibitor with low micromolar potency in P. aeruginosa, was discovered by Ilangovan et al. based on a quinazoline headgroup with a hydrazide at the 3-position. 79 displayed a clear effect on pyocyanin production, AQ synthesis, and biofilm formation.112 Shortly after this study, Starkey et al. reported a PqsR antagonist 80 with submicromolar potency and a substantial effect on virulence factors at concentrations of >1 μM. In addition, 80 enhanced the effect of tobramycin in clearing P. aeruginosa PA14 biofilms. Moreover, this inhibitor potentiated the effect of ciprofloxacin, reduced persistence, and increased postinfection survival rates in burn and lung infection models in mice.113,114 Spero Therapeutics published a patent for aryloxacytioindoles as PqsR inhibitors detailing an extensive SAR. One of the optimal compounds 81 demonstrated submicromolar potency (50–250 nM) and around 50% reduction of AQs in an in vivo acute thigh infection model in mice after oral administration with 4 doses of 200 mg/kg postinfection.115 No further development of these inhibitors has been reported.

### STRUCTURAL INSIGHTS OF PQS R LIGAND BINDING DOMAIN

To date, there is no report describing the crystal structure for the full length PqsR protein; however, the truncated co-inducer binding domain of PqsR (PqsRcbd) has been described in literature.112,116-118 Ilangovan et al. reported the first crystal structure of PqsRcbd bound to a natural agonist (2-aryloxyacetoindoles as PqsR inhibitors detailing an extensive change at the level of Thr265 in pocket B where the aliphatic chain resides supported by hydrophobic interactions with Tyr 258, Ile189, Ile186, and Val170 (Figure 12). The same group also described the cocrystal structure of PqsRcbd with the quinazoline-derived inhibitor 79 (PDB code 4JV1) which induced a subtle conformational change at the level of Thr265 in pocket A to accommodate the chlorine substituent at the 7-position. The binding of 79 also featured a hydrogen bond between the hydrazide group and the side chain of Leu207.112 Kitao et al. recently reported the crystal structure of 80 (PDB code 6B8A, Figure 12) which adopted a similar binding mode to 79 with the benzimidazole ring residing in place of quinazoline and the thioacetamide linker making a
hydrogen bond to Gln194. It is noteworthy that the bulky phenoxyp phenyl group occupied the cigar shaped hydrophobic pocket B and induced the reorientation of Leu183 and Ile186 residues.

INHIBITION OF AQ BIOSYNTHESES

Inhibitors of PqsA. PqsA, a CoA-ligase enzyme, is the first enzyme in the AQ biosynthetic pathway responsible for the conversion of anthranilic acid into anthraniloyl-CoA mediated via adenylation to give 82 followed by a thioesterification (Figure 13). PqsA represents a valid antivirulence target due to its essential role in AQ biosynthesis. P. aeruginosa pqsA mutants exhibit reduced biofilm formation compared with isogenic wild type strains. Initial attempts to inhibit this enzyme were based on substrate analogs such as halogenated anthranilate derivatives (Figure 14, 83, 84, 85), which showed weak inhibition with millimolar concentrations of the ligands being required to demonstrate an effect. Nevertheless, these PqsA inhibitors increased survival rates in mice infected with P. aeruginosa and reduced bacterial systemic dissemination. Ji et al. recently reported the design of anthraniloyl-AMP analogs as PqsA inhibitors through the replacement of the phosphate bridge with a sulfonamide based linker. Despite the high affinity of 86 and 87 in the enzymatic assay (86, K_i = 88 nM; 87, K_i = 109 nM), the inhibitors only weakly reduced AQ and pyocyanin levels in P. aeruginosa most likely due to their limited bacterial cell permeability. The resolution of the PqsA ligand binding domain crystal structure by Witzgall et al. should aid the design of future enzyme inhibitors with improved potency and permeability.

Inhibitors of PqsD. PqsD is the second enzyme in the HHQ biosynthetic pathway and is responsible for the condensation of anthraniloyl-CoA (Figure 10) with malonyl-CoA to produce 2-aminobenzoylacetate-CoA. The first weak PqsD antagonists (Figure 15, 88, IC_50 = 65 μM; 89, IC_50 = 35 μM using in vitro enzymatic assays) were derived from inhibitors of FabH a structural and functional homolog of PqsD. These compounds were further optimized to low micromolar potencies (90, IC_50 = 1.1 μM; 91, IC_50 = 1.6 μM following the same assay) and were able to compete with anthraniloyl-CoA for the substrate pocket. Strikingly, this benzamide−benzoic acid scaffold was employed by the same group for the search of bacterial polymerase inhibitors (RNAP) as antibacterial agents. Although a follow-up study highlighted the areas of the molecules that contribute to selectivity against PqsD, this was only improved by 50-fold. Moreover, there was a lack of evidence for the effect of these PqsD inhibitors on P. aeruginosa growth particularly given that RNAP inhibition was solely assessed in an E. coli based assay. Following a ligand-based approach, inhibitor 92 was identified with IC_50 of 3.2 μM in PqsD functional assay (ITC: K_d = 13 μM). However, the in vitro effect of 92 on biofilm and AQ production was only achieved using high concentrations (250−500 μM). A follow-on paper described the SAR of 93 with little improvement in cellular activity. Urea-based PqsD inhibitors were also described by Sahner et al. with IC_50 values of 0.5 μM and 0.14 μM for compounds 93 and 94, respectively. Once again, a similar
scaffold was used by the same group to identify RNAP inhibitors in E. coli which questions the selectivity of 93 and 94 against PqsD and their effect on bacterial growth.132,133 Catechol-derived scaffold 95 was reported as a PqsD inhibitor based on the structural similarity between PqsD and CHSD, a chalcone synthase from Medicago sativa. Despite the micromolar potency in the enzymatic assay for 95 (IC_{50} = 7.9 μM), only a slight effect was obtained in cells at concentration of 0.25 mM.134 Moreover, 95 was identified as PAINS structure and therefore could act as a false positive in the enzymatic assay. A sulfonyl pyrimidine scaffold was employed in the discovery of dual PqsD/PqsR inhibitors by Thomann et al., who reported compound 96 with selectivity against PqsD (IC_{50} = 1.7 μM) that caused a 60% reduction in PA14 biofilm at concentration of 100 μM.135

Interestingly, compound 97 which shares the same scaffold with 96 showed weak dual inhibition against both targets (PqsD, IC_{50} = 21 μM; PqsR IC_{50} = 15 μM) with effects on pyocyanin and biofilm at high micromolar concentrations.

**Inhibitors of PqsE.** PqsE is a thioesterase enzyme capable of transforming the PqsD reaction product 69 (2-ABA-CoA) into 2-aminobenzoylacetate 70, the HHQ precursor. Although PqsE mutants produce similar levels of HHQ to the wild type strain they generate more DHQ arising from the intramolecular cyclization of the PqsD product, 2-ABA-CoA.136,137 2-Aminoacetophenone 72 is another metabolite obtained from this pathway through the decarboxylation of 2-ABA-CoA. PqsE therefore plays a central role in AQ biosynthesis and balances the formation of AQs, 71 and 72 from 2-ABA-CoA with the dead-end product, 71 and 72. However, the functions of PqsE are not fully understood since the thioesterase activity does not account for the AQ-independent activities of PqsE in regulating virulence factors including pyocyanin, HCN, and rhamnolipids in the absence of AQ production.95,100 The attempt to inhibit PqsE was achieved through fragment based drug discovery, and three fragments (Figure 16, 98, 99, 100) were identified as inhibitors with submillimolar potencies with their binding confirmed by cocrystallization experiments and isothermal calorimetry. Although these compounds attenuated the thioesterase activity of PqsE, as demonstrated by the accumulation of DHQ and 2-ABA, they failed to modulate pyocyanin production.137 Therefore, the validity of PqsE as a drug target requires further investigation particularly as another broad spectrum thioesterase (TesB) in P. aeruginosa may be able to take over its biological function with respect to AQ biosynthesis.98

**Inhibitors of PqsBC.** PqsBC is a heterodimeric β-keto acyl synthase III enzyme responsible for the condensation of 2-ABA and octanoyl CoA to form HHQ.17 It was reported that 2-aminoacetophenone (2-AA) 72, a secondary metabolite from the AQ biosynthesis pathway, was able to inhibit PqsBC with an IC_{50} of 46 μM in a PqsBC-based biochemical assay16 and reduces virulence in an acute infection model in mice.138 Maura et al. reported the first synthetic inhibitors for PqsBC (Figure 17, 101) which are based on a benzimidazole scaffold, previously described for PqsR inhibitor 80. Through using LCMS/MS to quantify AQ synthesis intermediates, they found that some analogs were able to inhibit PqsBC as evidenced by a reduction in HHQ production concomitant with increased levels of 2- AA and DHQ. The EC_{50} values for 102 (dual PqsR and PqsBC) and 103 (PqsBC) were determined in a PqsBC enzymatic assay to be 13.4 μM and 12.5 μM, respectively. It is intriguing that minor structural changes to 94 (higher activity toward PqsR) enhanced the activity toward PqsBC. It has been shown that selective PqsBC inhibitors induced less tolerance in P. aeruginosa cells toward the β-lactam antibiotic meropenem compared to dual or selective PqsR inhibitors.139 Allegretta et al. re-evaluated previously published pqsR inhibitors and their effect on PqsBC inhibition and showed that compounds 104 and 105 are able to significantly induce an increase in 2- AA and DHQ levels to concentrations of 10 μM and 250 μM.140 It is noteworthy that compound 104 was reported as a weak PqsD inhibitor in a previous study.131 However, the validity of PqsBC as an antivirulence drug target remains doubtful as even though PqsBC inhibition leads to a reduction of AQ signal synthesis, it induces accumulation of 2- AA and DHQ molecules that enhance the persistence of P. aeruginosa and promote chronic infections.138,140,141 Hence, PqsBC inhibitor combination therapy would be advisable with other pqs pathway inhibitors.

**GENERAL AND MULTITARGET pqs INHIBITORS**

In addition to the compounds listed above, there are other reports of pqs inhibitors with no specific, defined targets. For instance, recent work described 4-aminooquinoline derived molecules as inhibitors for pqs signaling with a potency of ~2 μM for compound 106 (Figure 18) against P. aeruginosa PA14.116 The study demonstrated the effect of this class of inhibitors in a series of phenotypic assays including biofilm formation in two different laboratory strains of P. aeruginosa (PAO1-L, PA14).118 Molecular docking studies implicated PqsR as the plausible target, but this was not confirmed experimentally.142 The 7- chloroquinoline scaffold was also presented in another study showing that 107 was able to disrupt biofilm formation and pyocyanin production at a concentration of 138 μM through inhibition of PQS signaling (81%).143 Pyrrolic derivative 108 was reported in a patent as a pqs inhibitor with IC_{50} values of 22 μM and 17 μM in strains PAO1-L and PA14, respectively.145 Reduced pyocyanin and AQ biosynthesis without affecting bacterial growth up to a concentration of 100 μM.146 Fang et al. reported a “pan” QS inhibitor 109 for P. aeruginosa with low-micromolar activity (IC_{50} of 0.56 μM for las, 3.49 μM for rhl, and 5.63 μM for pqs using P. aeruginosa reporters) leading to the down-regulation of multiple virulence factors (pyocyanin, rhamnolipids, elastase).109 Also exhibited high clearance rate of bacteria post foreign body infections in mice.147

**PERSPECTIVE VIEW**

The pqs system in P. aeruginosa is crucial for the full virulence and persistence of this human pathogen as well as some of its immune modulatory effects. Reports describing the occurrence of lasR mutations in clinical P. aeruginosa isolates from chronic infections such as those encountered in cystic fibrosis lend further significance to pqs signaling as a target for antipseudomonal drugs. Now that the mechanistic biochemical basis for pqs biosynthesis and signal transduction have been elucidated and complemented with an understanding of the structural basis for
its essential components including crystal structures for PqsR, PqsA, PqsD, PqsBC, and PqsE, it is clear that certain elements of the pqs system represent attractive drug discovery targets. Indeed, inhibitors 80 and 81 have advanced in preclinical stages and the available data provide a robust proof of concept for targeting PqsR. PqsA, PqsD are emerging as additional new targets that have yet to be fully explored. Despite the fact that the PqsBC heterodimer is critical for AQ biosynthesis, its validity as antivirulence targets remains to be elucidated. The lack of attenuation of pqsE mutants in mouse infection models indicates that PqsE is unlikely to be a good target.

RESISTANCE TO ANTIQUORUM SENSING AGENTS

There is some evidence that QS mutants arise in bacterial populations specifically under conditions where QS is essential for bacterial growth but only where they continue to benefit from the metabolic activities of QS competent cells. QSI resistance could fall into one of the following categories: (i) overexpression of QS signal receptor genes or a receptor homologue to overcome inhibition, (ii) point mutations in the receptor such that it becomes signal independent, and (iii) preference of one particular QS system over others.
There are few reports describing the development of resistance to QSIIs in *P. aeruginosa*. For the furanone-derived compound 27, the underlying resistance mechanism was solely reasoned to the up-regulation of an efflux pump due to a mutation in mexR and ralC regulatory genes. 148,149 However, it is noteworthy that the QSI concentration used for this study was 72 ± 25-fold higher than that previously reported for QS inhibition by 26 in the original literature. At such elevated concentrations, 26 is cytotoxic and growth inhibitory and so will exert selective pressures on the bacteria to drive the evolution of mutations that confer resistance.

Currently, the literature relating to QSI resistance is scarce and limited to certain cases and specific growth conditions. Further investigations of selection for QSI resistance in conditions that mimic in vivo infections will be vital to establish a sound platform for the future design and development of QSIIs with nanomolar potencies.

**CONCLUSION**

Through reviewing the medicinal chemistry related QS literature, it is clear that more effort needs to be directed toward the design of drug-like molecules with favorable physiochemical properties. It becomes evident that the majority of the inhibitors identified to date function as useful probes for mechanistic studies rather than lead-like compounds for further drug development as summarized in Table 2.

In addition, the lack of methodological standardization in assessing QSI candidates including the use of single laboratory-adapted *P. aeruginosa* strains limits the broad validity of any findings such that these may be distant from relevant clinical infections and so constitute a major pitfall in this field. Within the *P. aeruginosa* QS circuitry, the pqs system holds promise for prospective therapeutics particularly at the level of PqsR where inhibitors with nanomolar potencies and lead-like properties have already been developed. However, it is important to note that QS inhibitors are most likely to be beneficial as adjuvants for conventional antibiotics rather than as standalone therapeutic agents, although they may prove useful for prophylaxis. Undoubtedly, polypharmacology through the concurrent use of inhibitors for various targets/QS systems could also prove highly beneficial in combating multiantibiotic bacterial resistance.

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**Notes**

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Fadi Soukari obtained his Ph.D. in Medicinal Chemistry from University of Nottingham (U.K.) in 2013 under supervision of Professor Peter M. Fischer. He was then appointed as a Postdoctoral Research Fellow working on anticanter project targeting CDK9. He then joined Prof. Câmara, Dr. Stocks, and Prof. Williams groups to work on multinational project (SENBIOTAR) for the discovery of new PqsR antagonist as novel antipseudomonal agents. Along his work, he contributed to teaching and supervision of a number of M.Sc. and Ph.D. research students.

Paul Williams is currently Professor of Molecular Microbiology in the School of Life Sciences, Faculty of Medicine and Health Sciences, University of Nottingham, U.K. From 1996 to 2008 he was Director of the Institute of Infections & Immunity after which he became Head of the School of Molecular Medical Sciences, University of Nottingham. His research interests focus primarily on the regulation of gene expression in bacteria through cell–cell communication (quorum sensing) and the development of novel antibacterial agents and bacterial biofilm resistant polymers. He has published over 300 research papers and reviews, has served on the Medical Research Council UK, MRC Infection and Immunity board and the scientific advisory board of the EU Joint Programming Initiative in Antimicrobial Resistance and is a Wellcome Trust Senior Investigator.

Michael J. Stocks was appointed as an Associate Professor in Medicinal Chemistry in 2012 within the School of Pharmacy at The University of Nottingham. He has over 20 years of industrial experience in drug discovery within AstraZeneca, and during his industrial career, he was both the lead scientist and project leader on multiple preclinical research projects as well as the synthetic medicinal chemistry lead of the AstraZeneca compound enhancement initiative. Since joining the School of Pharmacy in 2012, Michael has grown his research group and his research has focused on the medicinal chemistry design of compounds to study and modulate the function of biological targets.

Miguel Câmara is a Professor of Molecular Microbiology in the School of Life Sciences, University of Nottingham since 2009. He has 24 years of expertise studying molecular mechanisms of quorum sensing-based control of gene expression in bacteria with emphasis on biofilms, the influence on the behavior of polymicrobial communities, and the interaction with mammalian hosts and eukaryotic organisms. He has also been working on the identification of novel antimicrobial targets to treat detrimental biofilms and coordinated several international antimicrobial programs mainly focused on quorum sensing inhibition as the main target. He has >120 research papers in peer reviewed journals and is co-director of the National Biofilms Innovation Centre and a member of the UK Cystic Fibrosis Trust Strategic Implementation Board.

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**ABBREVIATIONS USED**

2-ABA, 2-aminobenzoylactic acid; 3OC12-HSL, N-(3-oxododecan-1-yl)-homoserine lactone; AHL, acylhomoserine lactone; AI, autoinducer; AMR, antimicrobial resistance; AQ, alkyl quinolone; C4-HSL, N-butanoyl-1-homoserine lactone; FDA, U.S. Food and Drug Administration; HCN, hydrogen cyanide; HHQ, 2-heptyl-4-hydroxyquinoline; Kc, dissociation constant; Kd association constant; mAb, monoclonal antibody; MDR, multidrug resistance; PQS, *Pseudomonas* quinolone signal; QS, quorum sensing; QSI, quorum sensing inhibitor; QSSM, quorum sensing signal molecule; QZN, quinazolinone; WHO, World Health Organisation.


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