Synthetic Quorum Sensing Inhibitors (QSIs) Blocking Receptor Signaling or Signal Molecule Biosynthesis in *Pseudomonas aeruginosa*

Christine K. Maurer, Cenbin Lu, Martin Empting, and Rolf W. Hartmann

Abbreviations

ACP	Acyl carrier protein
AHL	*N*-acyl homoserine lactone
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
C_4-HSL	*N*-butanoyl-l-homoserine lactone
CoA	Coenzyme A
HAQ	4-hydroxy-2-alkylquinoline
HHQ	2-heptyl-4-hydroxyquinoline
IC_{50}	Inhibitor concentration to achieve a half-maximal degree of inhibition
MTA	5-methylthioadenosine
NADH/ NAD^+	Reduced/oxidized form of nicotinamide adenine dinucleotide
3-oxo-C_{12} -HSL	*N*-(3-oxo-dodecanoyl)-l-homoserine lactone
PP_i	Pyrophosphate
PQS	*Pseudomonas* quinolone signal
QS	Quorum sensing
QSI	Quorum sensing inhibitor
QZN	Quinazolinone
RNAP	RNA polymerase
SAM	l-*S*-adenosylmethionine

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Introduction

Pseudomonas aeruginosa masters quorum sensing (QS) communication to coordinately regulate pathogenicity-associated group behaviors including the production of virulence factors and biofilm formation, which facilitate the invasion into the hosts, counteract host immune system, as well as promote the resistance/tolerance toward conventional antibiotics. Three main QS systems are employed by the pathogen, denoted as *las* (Gambello and Iglewski 1991; Passador et al. 1993), *rhl* (Ochsner et al. 1994; Ochsner and Reiser 1995), and *pqs* (Pesci et al. 1999). All the networks are hierarchically interconnected: *las* controls the other two systems; *pqs* positively regulates the *rhl* signaling, whereas *rhl* in turn puts a negative feedback upon *pqs* (Wilder et al. 2011; McGrath et al. 2004). Regarding the central role of QS for the infectious process, the interruption of these pathways by blocking the receptors or inhibiting the signal synthesis via small molecules is an attractive therapeutic strategy to attenuate the bacterial pathogenicity, thereby overcoming intractable *P. aeruginosa* infections (Rasmussen and Givskov 2006).

V.C. Kalia (ed.), *Quorum Sensing vs Quorum Quenching: A Battle with No End in Sight*,
DOI 10.1007/978-81-322-1982-8_24, © Springer India 2015

Synthetic QSIs Blocking Receptor Signaling

The interference with the QS receptors via QSIs is a promising approach to efficiently interrupt the communication networks, thereby decreasing the QS-controlled pathogenicity (Kalia 2013; Rasmussen and Givskov 2006). Generally, such QSIs are derived from the natural ligands of the target receptors, structurally unrelated natural products, or small molecules/fragments. These QSIs could be either pure antagonists or weak agonists, all of which effectively compete with the natural agonists preventing a sufficient stimulation of the receptors.

N-Acyl l-Homoserine Lactone (AHL) Receptors as Targets

In Gram-negative bacteria, *N*-acyl l-homoserine lactones (AHLs) are the most commonly used signal molecules for QS. The innate receptors of these autoinducers belong to the LuxR type which act as transcriptional regulators upon activation by their native agonists. In *P. aeruginosa*, the so-called *las* and *rhl* systems have been identified to be the key AHL-based QS systems. The native ligands of the involved cytoplasmic receptors LasR and RhlR are *N*-(3-oxo-dodecanoyl)-l-homoserine lactone (3-oxo-C_{12}-HSL or OdDHL) and *N*-butanoyl-l-homoserine lactone (C_4-HSL or BHL), respectively (Fig. 1a).

Notably, a higher rank in the regulatory hierarchy is accounted to the *las* system. An additional LuxR-type receptor – the "orphan" receptor QscR also responding to 3-oxo-C_{12}-HSL – has been reported for this particular human pathogen (Chugani et al. 2001). However, the major part of scientific studies concentrates on the development of LasR antagonists to disrupt AHL signaling in *P. aeruginosa*. Desirable cellular effects of LasR- and/or RhlR-targeting QSIs would be the reduction of virulence factor production (e.g., elastase, hydrogen cyanide, pyocyanin, pyoverdine, rhamnolipids, or alkaline protease) as well as the attenuation of biofilm formation. Indeed, several groups have reported on the successful development of synthetic agents with promising *in cellulo* activities. The following section provides a detailed, yet not exhaustive, overview on the structural space covered by synthetic QSIs interfering with AHL signaling. In general, compounds addressing LasR and RhlR can be divided into two categories: (1) structural mimics of AHLs and (2) structurally unrelated substances.

AHL Mimics

Many nonnatural agonists and antagonists of LuxR-type receptors possess structural features very similar to the native ligands or are actually direct synthetic derivatives thereof (Fig. 1b, c).

Quite obviously, the AHL scaffold can be divided into two sections. The head group consists of the five-membered homoserine lactone moiety, while the tail region comprises a linear *N*-acyl residue of varying length. The amide-based linker between both segments facilitates modular approaches for straightforward synthesis and derivatization. Many reports in the literature dealing with synthetic AHL analogues focus on structural modifications in one of these two sections while leaving the other part of the molecule constant. Nevertheless, combinations of nonnatural head and tail modules have also been described.

Noteworthy, the native lactone pentacycles of 3-oxo-C_{12}-HSL and C_4-HSL are easily hydrolyzed under physiological conditions. The resulting linear product is QS inactive (McInnis and Blackwell 2011b). Thus, replacing this moiety by stable bioisosters is a worthwhile endeavor providing access to nonnatural QS modulators with an in vivo half-life superior to the natural ligands. The range of possible substituents is broad and includes penta- and hexacyclic thiolactones, homo- and heteroaromatic residues, as well as saturated ring systems (Fig. 1b). Interestingly, modifications within the head group of AHL-receptor agonists enable to achieve the desirable functional inversion to yield promising synthetic antagonists. Structural differences between agonists and antagonists can be very subtle. For example, Suga and coworkers

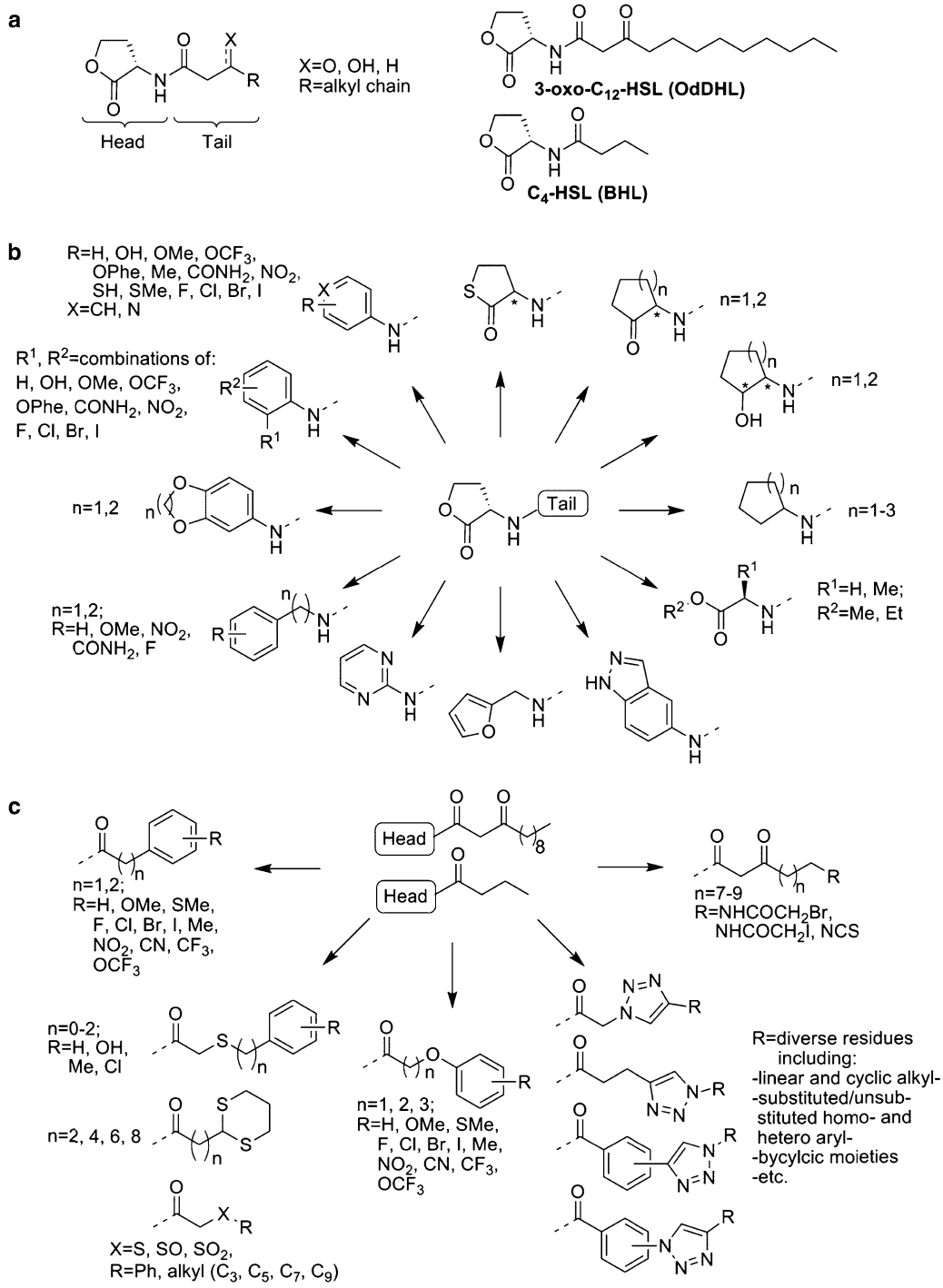

Fig. 1 (**a**) Structure of *N*-acyl l-homoserine lactone-based autoinducers in *P. aeruginosa*. (**b**) A selection of reported substituents as head group replacements found in the literature (McInnis and Blackwell 2011a, b; Jog et al. 2006; Ishida et al. 2007; Smith et al. 2003; Kim et al. 2009; Persson et al. 2005; Morkunas et al. 2012; Hodgkinson et al. 2012). (**c**) A selection of substituents as tail section replacements found in the literature (Persson et al. 2005; O'Loughlin et al. 2013; Geske et al. 2005, 2007, 2008; Stacy et al. 2013; Amara et al. 2009). Abbreviations: *3-oxo-C_{12}-HSL N*-(3-oxo-dodecanoyl)-l-homoserine lactone, *C_4-HSL N*-butanoyl-l-homoserine lactone

have shown that a compound possessing the natural 3-oxo-dodecanoyl tail and a nonnatural 2-aminocyclohexanoyl head group is a potent agonist and can be converted into an antagonist through head group replacement by a 2-aminocyclohexanone substituent (compound **1**) (Jog et al. 2006). A similar effect can be observed for the usage of a 2-aminophenol analogue (**2**) (Smith et al. 2003). The latter derivative has the advantage that it has no stereoisomers which abolishes the need for stereo control and/or racemate separation during synthesis/purification. Moreover, it has been reported that the incorporation of aromatic head groups usually yields an antagonistic functional profile (Hodgkinson et al. 2012). In summary, nonnatural head groups usually contain a cyclic motif which can have diverse electronic and/or chemical properties as well as varied substitution patterns providing control over agonist/antagonist functionality of the desired compound without compromising affinity to the respective LuxR-type receptor.

Reported variations in the tail region of synthetic AHL mimics are also quite numerous (Fig. 1c). Indeed, the difference between the native AHL signal molecules in *P. aeruginosa* lies in the length and chemistry of this section mediating receptor selectivity. The absence of the β-keto motif in the RhlR-selective autoinducer (C_4-HSL) inspired researchers to omit this structure also for the generation of LasR-addressing modulators. Interestingly, also short alkyl chains and even cyclic structures are accepted in this part of the molecule. Hence, substituted homo- and heteroaromatics with varying alkyl linker chains have been successfully incorporated into antagonists of LuxR-type AHL receptors in *P. aeruginosa* (Geske et al. 2008). Introduction of 1,2,3-triazole-based "click" linkers by Blackwell and coworkers allowed for additional synthetic modularity and combinatorial library generation (Stacy et al. 2013). However, antagonists possessing nonnatural tail groups that mimic the linear unbranched structure of the native signal molecules usually demonstrate higher potency than multi-cyclic or angled motifs (Geske et al. 2007). Incorporation of electrophilic reactive groups within such a linear tail section resulted in covalent QSI addressing LasR (e.g., compound **6**) (Amara et al. 2009).

Combining favorable nonnatural head and tail groups with each other may yield rather unexpected results. Spring and coworkers have shown that many of these "chimeric" compounds are of low potency or essentially inactive (Hodgkinson et al. 2012). Hence, conservation of the native 3-oxo-dodecanoyl chain in antagonists with nonnatural head groups can be mandatory for strong QS inhibition. A selection of promising AHL-mimicking LasR and RhlR antagonists and their effects on *P. aeruginosa* is given in Fig. 2.

Structurally Unrelated AHL-Interfering QSI

The application of experimental screening methodologies using compound libraries led to the identification of structurally diverse LasR/RhlR antagonists (Wu et al. 2004; Musthafa et al. 2012; Muh et al. 2006a, b). A selection of respective compounds together with biological activities is given in Fig. 3. One structure showing very promising effects in vitro is referred to as C-30 (**10**). This compound is a synthetic derivative of a marine natural compound found in *Delisea pulchra* and has been investigated for in vivo efficacy in murine infection models. **10** was capable of attenuating QS-mediated virulence in mice and improved bacterial clearance from infected animal lungs (Wu et al. 2004). However, the molecular mode of action by which C-30 (**10**) interacts with its target receptor (LasR) is not yet fully elucidated.

Finally, it has to be stated that means by which the potency of such LuxR-type antagonists can be determined are various and range from recombinant reporter gene assays, over the direct quantification of receptor-regulated downstream products, to the investigation of effects on biofilm formation. Each of these biological evaluation methodologies is highly dependent on experimental parameters like used cell culture media, effective concentration of organic cosolvents like dimethyl sulfoxide, or, importantly, chosen *P. aeruginosa* strain. Hence, a comparison of QSI interfering with AHL signaling developed by separate working groups is difficult and in many cases not practical. Additionally, not in all

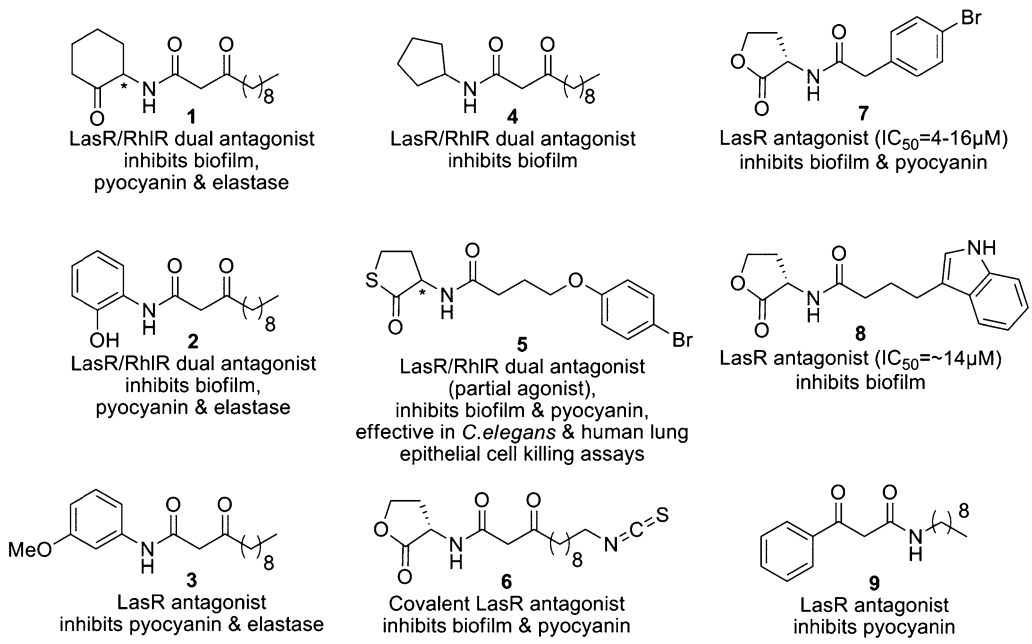

Fig. 2 A selection of LasR and/or RhlR antagonists with reported effects on *P. aeruginosa* cells (Jog et al. 2006; Smith et al. 2003; Hodgkinson et al. 2012; Ishida et al. 2007; O'Loughlin et al. 2013; Amara et al. 2009; Geske et al. 2005; Muh et al. 2006b)

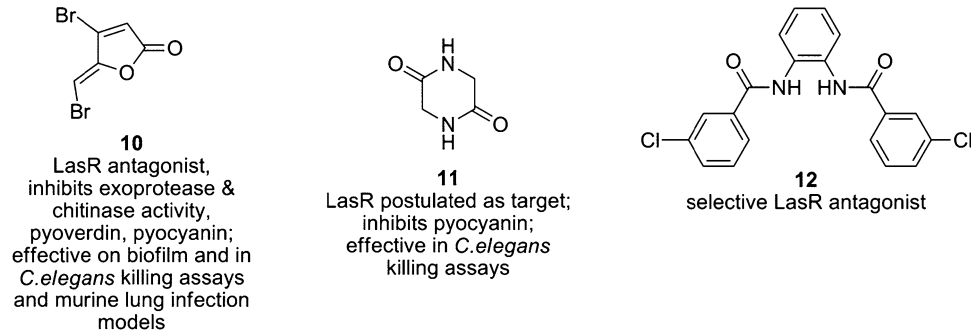

Fig. 3 A selection of LasR and/or RhlR antagonists with reported effects on *P. aeruginosa* cells (Hentzer et al. 2003; Wu et al. 2004; Musthafa et al. 2012; Muh et al. 2006a)

cases it has been analyzed whether a cellular effect (e.g., reduction of virulence factor production) was mediated via antagonism of LasR, RhlR, or both. Indeed, a very strong dual antagonizing agent might prove ineffective in the cellular context as the *las* and the *rhl* systems can act reciprocally on the production of key virulence factors. Thus, detailed biological studies including in vivo experiments will be necessary to guide further developments in the field of AHL-interfering QSI in *P. aeruginosa*.

4-Hydroxy-2-Alkylquinoline (HAQ) Receptor as Target

PqsR is the receptor of the *P. aeruginosa*-specific *pqs* QS circuit and functions as a critical regulator that fine-tunes a large set of pathogenicity-associated genes that encode for virulence factors, such as pyocyanin, elastase B, and hydrogen cyanide. *Pseudomonas* quinolone signal (PQS) and 2-heptyl-4-hydroxyquinoline (HHQ) – the two most predominant members of

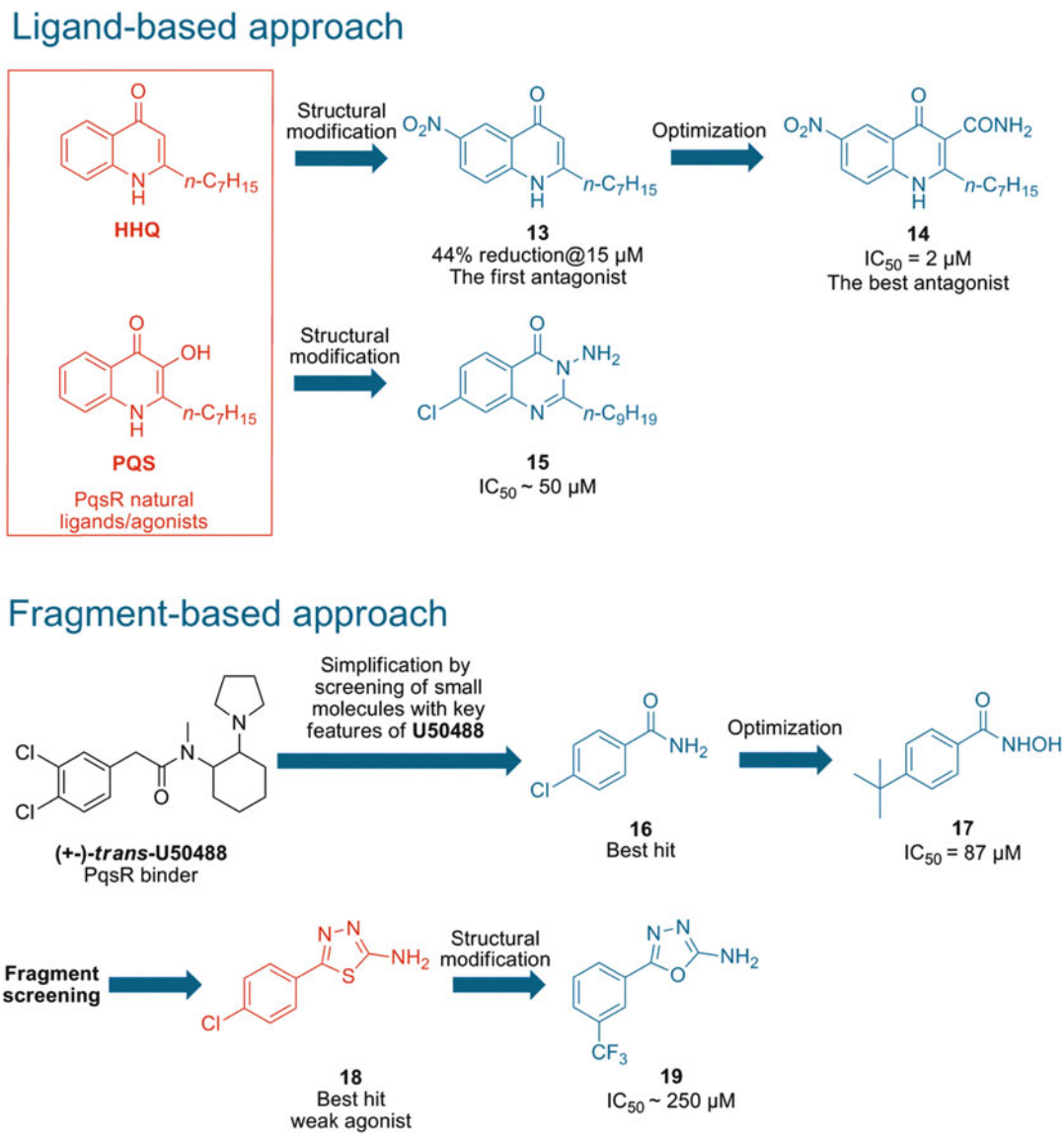

Fig. 4 Discovery of PqsR antagonists following ligand-based and fragment-based approaches and the biological effects on the production of virulence factor pyocyanin in *P. aeruginosa*. Abbreviations: *HHQ* 2-heptyl-4-hydroxyquinoline, *PQS Pseudomonas* quinolone signal, IC_{50} inhibitor concentration to achieve a half-maximal degree of inhibition

the 2-alkyl-4-hydroxyquinoline (HAQ) family – are the agonists of the receptor (Fig. 4) and serve as the signal molecules of the network. While QSIs interfering with receptors of *las* and *rhl* systems are being intensively investigated, only a few compounds targeting PqsR (PqsR antagonists) have been reported.

Following a ligand-based approach, two research groups have individually discovered potent PqsR antagonists. Hartmann and coworkers identified the first PqsR antagonists (e.g., compound **13**, Fig. 4) by means of introducing strong electron-withdrawing groups into the 6-position of HHQ (Lu et al. 2012). Interestingly, such in

vitro highly active antagonists reveal opposite functionality (agonistic activity) in *P. aeruginosa* culture, which is attributed to an unexpected functional inversion mediated by a bacterial enzyme PqsH. Overcoming the problem via further structural optimization resulted in the most potent PqsR antagonist to date (inhibitor concentration to achieve a half-maximal degree of inhibition (IC_{50}) toward pyocyanin: 2 μM, compound **14**), which demonstrated anti-virulence efficacy in vivo, thereby providing the first proof of concept for the PqsR-targeting therapeutic strategy (Lu et al. 2014). Meanwhile, Williams and coworkers reported a series of novel PqsR antagonists based on a quinazolinone (QZN) core mimicking the quinolone scaffold of the natural ligands (compound **15**) (Ilangovan et al. 2013). The QZN compounds do not only strongly inhibit the *pqs* QS and production of pyocyanin but also attenuate the biofilm formation of *P. aeruginosa*. Importantly, this work provided the first co-crystal structures of the PqsR ligand-binding domain with either agonist or antagonist giving a deep insight into the ligand-receptor interactions (Ilangovan et al. 2013).

Application of fragment-based approaches is another promising way to discover potential PqsR antagonists. Based on the knowledge that the κ-opioid receptor agonist (±)-*trans*-U50488 stimulates the transcription of the PqsR-controlled operon, this compound was identified as a PqsR binder via biophysical methods (Klein et al. 2012). To simplify this compound into smaller molecules and modify it into potent antagonists, 106 fragments with key features derived from U50488 were screened and the best hit **16** was further optimized resulting in a hydroxamic acid **17** with both high activity and ligand efficiency. As expected, this antagonist significantly diminished the pyocyanin production. Similarly, attractive hits were discovered via fragment screening of a library collection composed of 720 small molecules (Zender et al. 2013). The most outstanding hit **18** having a 2-phenyl-1,3,4-thiadiazole core was subsequently transformed into potent PqsR antagonist **19**, which successfully suppressed *pqs* QS activity as well as production of virulence factor pyocyanin. Particularly, it is worth to note that **19** is more drug-like than other known PqsR antagonists regarding physicochemical properties.

Overall, PqsR has been attracting attention and the recent contributions highlight QSIs antagonizing PqsR as promising anti-virulence compounds combating *P. aeruginosa*.

Synthetic QSIs Blocking Signal Molecule Biosynthesis

An alternative approach for interference with QS is inhibition of the signal molecule synthesis. Although reports applying this strategy are far less numerous than those about signal reception inhibition, evidence indicates that inhibition of signal synthesis is feasible and effective both in vitro and in vivo (LaSarre and Federle 2013). Usually, signal molecules are generated via a cascade of enzymes, which can be targeted individually for inhibition of signal molecule production.

AHL Biosynthesis

In *P. aeruginosa*, AHL signals C_4-HSL and 3-oxo-C_{12}-HSL are produced by the LuxI-type AHL synthases LasI and RhlI, respectively (Raychaudhuri et al. 2005; Parsek et al. 1999; Gould et al. 2004a). Both enzymes use l-*S*-adenosylmethionine as substrate. The second substrates, butanoyl-acyl carrier protein (ACP) for RhlI and 3-oxo-dodecanoyl-ACP for LasI, are likely derived from fatty acid biosynthesis. Thereby, FabI, a nicotinamide adenine dinucleotide-dependent enoyl-ACP reductase, reduces *trans*-2-enoyl-ACPs to the corresponding acyl-ACPs. FabB, a β-ketoacyl-ACP synthase I, condenses malonyl-ACP with an acyl-ACP resulting in the respective 3-oxo-acyl-ACP (Fig. 5a) (Hoang and Schweizer 1999). So far, only a limited number of reports on AHL biosynthesis inhibition have been published.

Triclosan (**20,** Fig. 5b) was identified as potent inhibitor of FabI (IC_{50} of 0.2 μM) that could suppress the production of C_4-HSL in vitro. However, as *P. aeruginosa* was resistant to triclosan

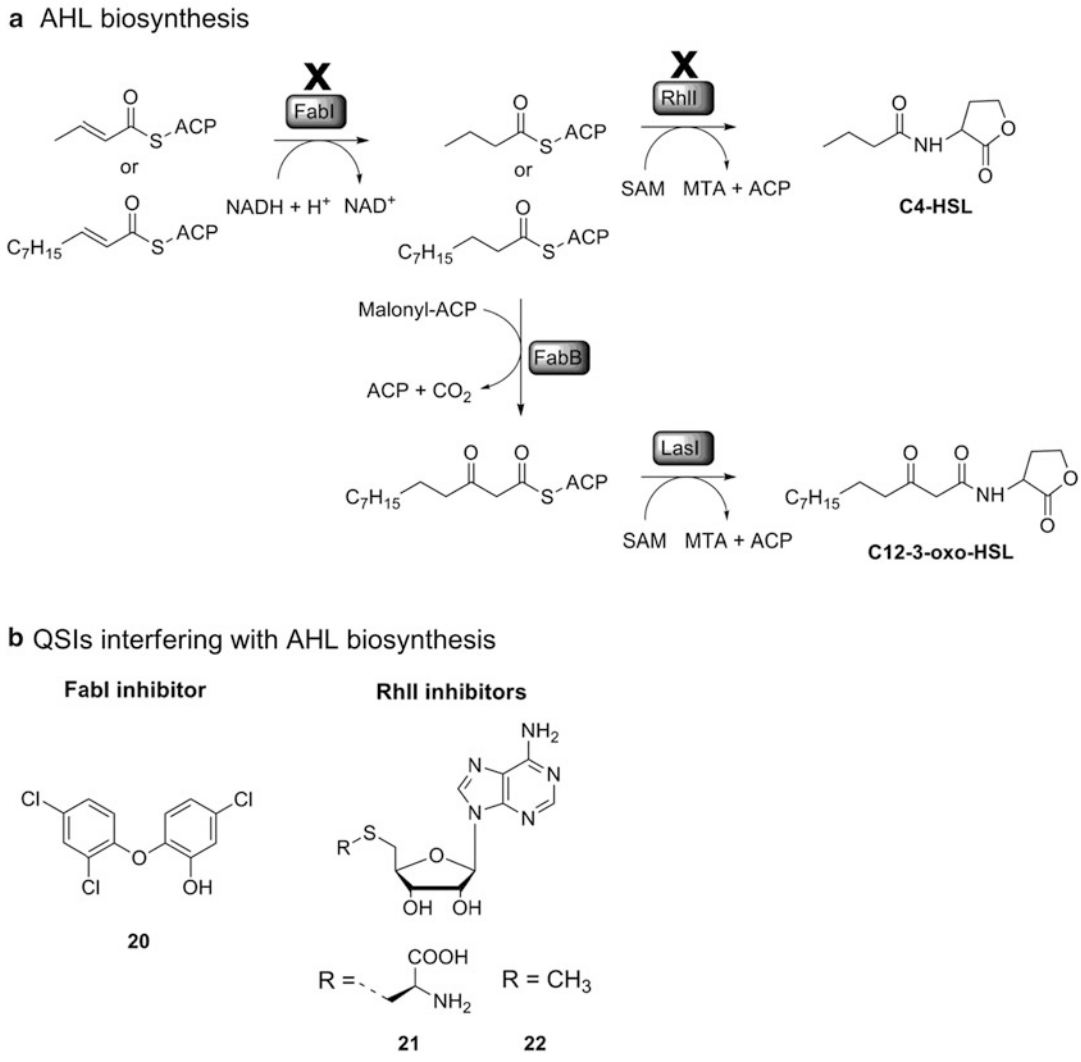

Fig. 5 Current model for *N*-acyl l-homoserine lactone biosynthesis in *P. aeruginosa* (**a**) and representative QSIs (**b**). Abbreviations: *AHL N*-acyl l-homoserine lactone, *ACP* acyl carrier protein, *NADH/NAD*$^+$ reduced/oxidized form of nicotinamide adenine dinucleotide, *SAM* l-*S*-adenosylmethionine, *MTA* 5-methylthioadenosine, *C_4-HSL N*-butanoyl-l-homoserine lactone, *3-oxo-C_{12}-HSL N*-(3-oxo-dodecanoyl)-l-homoserine lactone

due to active efflux, the validity of FabI-targeting QS inhibition could not be demonstrated yet (Hoang and Schweizer 1999).

Interference with C_4-HSL production by inhibiting the synthase RhlI with substrate analogues, reaction intermediates, and final products was investigated by Greenberg and coworkers (Parsek et al. 1999). Inter alia, the substrate analogue l-*S*-adenosylcysteine (**21,** Fig. 5b) and the reaction product 5′-methylthioadenosine (**22,** Fig. 5b) strongly inhibited RhlI activity in vitro at micromolar concentrations (Parsek et al. 1999). However, these homologues have not been evaluated *in cellulo* as they are likely to affect the central pathways of amino acid and fatty acid metabolism (Scutera et al. 2014).

Although the crystal structure of LasI has been elucidated (Gould et al. 2004a, b), no inhibitor targeting the synthase in *P. aeruginosa* has been described so far. However, recent advances in inhibiting LuxI synthases in other bacterial species might pave the way for the development of such inhibitors in *P. aeruginosa* (Chung et al. 2011; Christensen et al. 2013).

HAQ Biosynthesis

The *pqs* QS system, which is unique to *P. aeruginosa*, makes use of HAQs as signal molecules, among which PQS and its precursor HHQ play a major role (Xiao et al. 2006). Their biosynthesis requires the enzymes PqsA-D and PqsH (Fig. 6a). Thereby, PqsA acts as a ligase catalyzing the formation of anthraniloyl coenzyme A (CoA) from anthranilate, adenosine triphosphate, and CoA (Coleman et al. 2008). PqsD, a β-ketoacyl-ACP synthase III (FabH)-type condensing enzyme, has been shown to catalyze the condensation reaction between anthraniloyl-CoA and β-ketodecanoic acid to give HHQ in vitro (Pistorius et al. 2011; Steinbach et al. 2013). However, recent studies revealed that in the cellular context of *P. aeruginosa*, PqsD more likely employs anthraniloyl-CoA and malonyl-CoA as substrates to form 3-(o-aminophenyl)-3-keto-propionic acid. This reactive intermediate is then condensated with octanoyl to yield HHQ by a PqsB/PqsC complex (Dulcey et al. 2013). As the exact mechanism of action of PqsB/PqsC still remains elusive, there have been no attempts so far to develop QSIs targeting PqsB/PqsC. Finally, HHQ is converted into PQS by the monooxygenase PqsH (Schertzer et al. 2010). However, PqsH has not been considered as suitable target as a *pqsH* mutant displayed wild-type virulence in mice (Xiao et al. 2006). Thus, most efforts have been put into the development of small molecule inhibitors blocking PqsA and PqsD.

The first reported inhibitor of PQS production in *P. aeruginosa* was methyl anthranilate (**23**, Fig. 6b), an analogue of the PqsA substrate anthranilate that was able to decrease the levels of PQS-dependent virulence factor elastase. However, concentrations in the millimolar range were necessary to see a pronounced inhibitory effect on PQS formation (Calfee et al. 2001). Although it could be excluded that methyl anthranilate was a substrate or inhibitor of PqsA (Coleman et al. 2008), the exact mechanism of action remained unknown.

Rahme and coworkers aimed at developing more potent substrate analogues based on the anthranilate structure (Lesic et al. 2007). Introduction of electron-withdrawing halogen

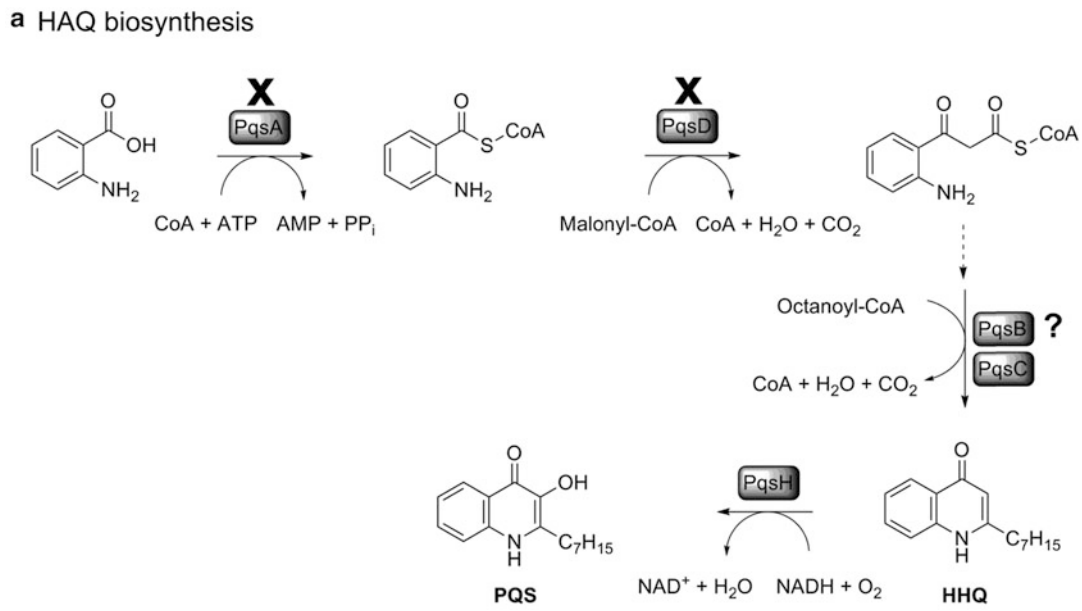

Fig. 6 Current model for 4-hydroxy-2-alkylquinoline biosynthesis in *P. aeruginosa* (**a**) and representative QSIs (**b**). Abbreviations: *HAQ* 4-hydroxy-2-alkylquinoline, *CoA* coenzyme A, *ATP* adenosine triphosphate, *AMP* adenosine monophosphate, *PP_i* pyrophosphate; II, *HHQ* 2-heptyl-4-hydroxyquinoline, *NADH/NAD^+* reduced/oxidized form of nicotinamide adenine dinucleotide, *PQS Pseudomonas* quinolone signal

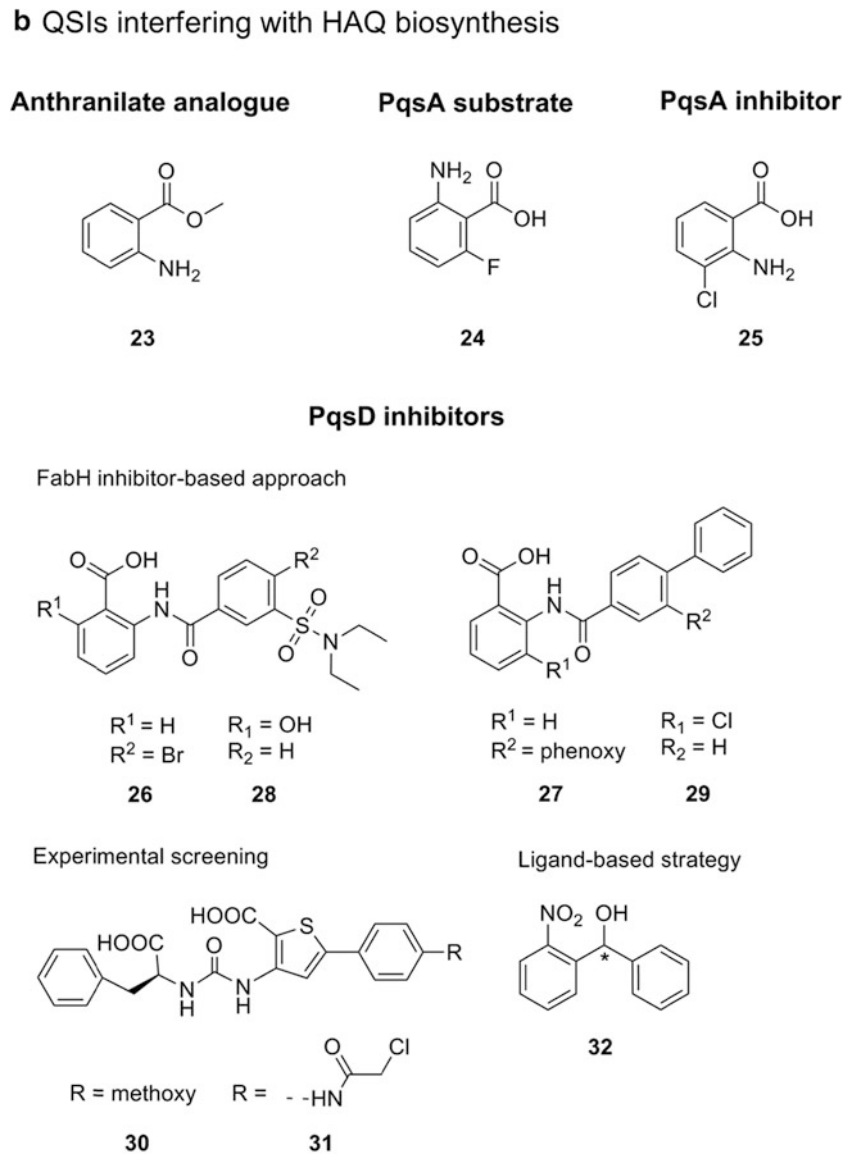

Fig. 6 (continued)

atoms into the phenyl ring should restrict formation of an activated carbonyl. Indeed, these derivatives strongly inhibited HHQ and PQS formation (Lesic et al. 2007). For instance, 6-fluoroanthranilic acid (**24**, Fig. 6b) exhibited an IC_{50} of 109 μM regarding inhibition of PQS synthesis (Maurer et al. 2013). Excitingly, these compounds were shown to have therapeutic benefits in vivo, where they increased survival and limited systemic dissemination of *P. aeruginosa* in a thermal injury mouse model (Lesic et al. 2007). These effects were concluded to be likely due to PqsA inhibition as anthranilic acid accumulated in cultures grown in the presence of the compounds.

Pesci and coworkers purified PqsA for the first time and developed an in vitro assay for identifying substrates and inhibitors of PqsA (Coleman et al. 2008). For example, anthranilate derivatives bearing chloro- and fluoro-substituents in 4- to 6-position of the benzene ring, such as compound **24** (K_m of

11 μM), were found to be substrates of PqsA. In contrast, 3-chloroanthranilic (**25**, Fig. 6b) acid was identified as an inhibitor of PqsA (K_i of 12.9 μM). In general, the extent of inhibition of PQS synthesis achievable with both substrates and inhibitors did not correlate with respective K_m or K_i values. Finally, it remained to be elucidated whether the inhibition of PQS production by the substrates was due to competition with anthranilate or to the inhibition of downstream enzymes by formed CoA thioesters (Coleman et al. 2008).

In 2011, Müller and coworkers developed an in vitro enzyme assay with purified PqsD using anthraniloyl-CoA and β-ketodecanoic acid as substrates and detecting the product HHQ (Pistorius et al. 2011). As a starting point for the identification of PqsD inhibitors, known inhibitors of FabH, a structural and functional homologue of PqsD, were tested. Indeed, compounds **26** (IC_{50} of 65 μM) and **27** (IC_{50} of 35 μM) could be identified as the first inhibitors of PqsD (Fig. 6b). However, their activity was only moderate and they were not tested in cellular assays as they were expected to exhibit antibiotic activity (Pistorius et al. 2011).

Hartmann and coworkers initiated several rational design projects for the development of potent, selective, and non-bactericidal PqsD inhibitors. In the course of these studies, three classes of PqsD inhibitors have been identified following a design approach based on known inhibitors of FabH, an experimental screening, and a ligand-based strategy. For each class, a series of compounds was synthesized and evaluated for its inhibitory potency in an in vitro PqsD assay. Therefrom, structure-activity relationships were derived. Furthermore, molecular docking based on the crystal structure of PqsD (Bera et al. 2009), biochemical assays, and biophysical methods including surface plasmon resonance spectroscopy (Henn et al. 2012) were applied to characterize the compounds regarding binding site, binding mode, or molecular interactions with the target. Based on that knowledge, structural optimizations were performed that led to potent PqsD inhibitors with IC_{50} values in the single-digit micromolar to submicromolar range (Storz et al. 2012; Weidel et al. 2013; Sahner et al. 2013; Hinsberger et al. 2014; Storz et al. 2013).

The design approach based on known FabH inhibitors (Pistorius et al. 2011) resulted in two subclasses of compounds with 2-benzamidobenzoic acid core structure. The 3′-sulfonamide-substituted series was found to reversibly bind to the substrate access channel within PqsD. The most potent inhibitor **28** (Fig. 6b) exhibited an IC_{50} value of 1.2 μM (Weidel et al. 2013). The 3′-phenoxy/4′-phenyl-substituted 2-benzamidobenzoic acids, originally reported as inhibitors of bacterial RNA polymerase (RNAP), were systematically optimized regarding their activity and selectivity profile. The most promising compound **29** (Fig. 6b) strongly inhibited PqsD (IC_{50} of 6.2 μM) while not affecting RNAP (Hinsberger et al. 2014).

From the class of 5-aryl-ureidothiophene-2-carboxylic acids identified by experimental screening, compounds **30** (IC_{50} of 0.5 μM) and **31** (IC_{50} of 2 μM) turned out to be the most potent (Fig. 6b). As the latter binds covalently to the active site, it carries the potential of strong biological effects (Sahner et al. 2013).

The ligand-guided design strategy led to a potent class of PqsD inhibitors, the nitrophenyl-methanols, which showed time-dependent inhibitory activity, tight-binding behavior, and active site binding (Storz et al. 2013). The most promising member of this series, compound **32** (Fig. 6b), exhibited high potency (IC_{50} of 3.2 μM) and high ligand efficiency (0.39). Applied at micromolar concentrations, this compound strongly inhibited HHQ and PQS synthesis as well as biofilm formation in *P. aeruginosa* without affecting growth. Thus, by the use of **32**, it was shown for the first time that inhibition of signal molecule synthesis is feasible with a PqsD inhibitor and that PqsD is a valid anti-biofilm target (Storz et al. 2012).

Conclusion

Numerous highly active blockers of QS receptors as well as inhibitors of QS signal synthases were identified using diverse design strategies during the last decade. Among

the developed compounds, several showed anti-infective activities. However, studies providing detailed in vivo data are rare. Nevertheless, the recent scientific achievements emphasize the potential applicability of QSIs as a weapon to treat the recalcitrant infectious diseases caused by *P. aeruginosa*.

Opinion

Numerous scientific contributions demonstrate that inhibition of QS can be regarded as a promising strategy against *P. aeruginosa* infections, and the discovery of small molecules targeting QS is rapidly progressing. However, there are obstacles that hinder the successful translation of such QSIs into real anti-infective drugs. First, the methodologies used for biological evaluation of QSIs are various and the results are highly dependent on experimental conditions, especially on the chosen *P. aeruginosa* strain. This makes a comparison of QSIs developed by different working groups difficult or even impossible. Second, the molecular target of QSIs discovered by cellular screening approaches is often unknown, which hampers a directed optimization of their activity, selectivity, and pharmacokinetic profile. Third, QSIs derived from target-based drug discovery approaches often fail to exhibit cellular activity. Development of such QSIs overcoming the Gram-negative cell wall and escaping the widespread efflux pumps remains a very challenging task. Fourth, despite exciting in vitro and *in cellulo* activities achieved with QSIs, an in-depth evaluation of such inhibitors in advanced animal models or even clinical trials is still to be performed. Such studies are mainly hampered by lack of drug-like molecules. Many QSIs, especially those derived from natural products, suffer from chemical instability, exhibit toxic effects, or possess inappropriate pharmacokinetic properties. Thus, in our opinion, current QS research should focus on development of drug-like molecules applicable for in-depth in vivo studies providing the proof of concept for QS inhibition-based treatment of *P. aeruginosa* infections.

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