From *in vitro* to *in vivo*: Establishment of a Test System for the Biological Evaluation of Novel Quorum Sensing Inhibitors as Anti-infectives Against *Pseudomonas aeruginosa*

Dissertation

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„Wer all seine Ziele erreicht,

hat sie wahrscheinlich zu niedrig gewählt.“

Herbert von Karajan

PAPERS INCLUDED IN THIS THESIS

This thesis is divided into four publications, which are referred to in the text by their letter.

A **Validation of PqsD as an anti-biofilm target in Pseudomonas aeruginosa by development of small-molecule inhibitors**

Michael P. Storz, Christine K. Maurer, Christina Zimmer, Nathalie Wagner, Christian Brengel, Johannes C. de Jong, Simon Lucas, Mathias Müsken, Susanne Häussler, Anke Steinbach, and Rolf W. Hartmann

J Am Chem Soc 2012, 134:16143-16146

B **Development and validation of a UHPLC–MS/MS procedure for quantification of the Pseudomonas Quinolone Signal in bacterial culture after acetylation for characterization of new quorum sensing inhibitors**

Christine K. Maurer, Anke Steinbach, and Rolf W. Hartmann

J Pharm Biomed Anal 2013, 86:127-134

C **Overcoming the unexpected functional inversion of a PqsR antagonist in Pseudomonas aeruginosa: an in vivo potent antivirulence agent targeting pqs quorum sensing**

Cenbin Lu*, Christine K. Maurer*, Benjamin Kirsch, Anke Steinbach, and Rolf W. Hartmann

Angew Chem Int Ed Engl 2014, 53:1109-1112

* These authors contributed equally to this work.

D **Optimization of anti-virulence PqsR antagonists regarding aqueous solubility and biological properties resulting in new insights in structure-activity relationships**

Cenbin Lu, Benjamin Kirsch, Christine K. Maurer, Johannes C. de Jong, Andrea Braunshausen, Anke Steinbach, and Rolf W. Hartmann

Eur J Med Chem 2014, 79:173-183

CONTRIBUTION REPORT

The author would like to declare her contributions to the papers A-D included in this thesis.

A The author contributed to the *in vitro* assay development. She developed and performed the HHQ, PQS, and DHQ inhibition experiments.

B The author designed the study and performed all biological and analytical experiments. She conceived and wrote the manuscript.

C The author developed and performed assays to measure growth curves of *P. aeruginosa*, pyocyanin and HAQ levels. She developed and performed *G. mellonella* infection assay. She contributed to composition of manuscript.

D The author developed and performed assays to determine pyocyanin and HAQ levels.

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1 Introduction

The discovery of penicillin by Alexander Fleming in the early twentieth century was a milestone in the treatment of bacterial infections [1]. It initiated the so-called golden era of antibacterial drug discovery that was characterized by an explosive development of several new classes of antibiotics [2, 3]. By its successful and global use, antibacterial chemotherapy became a significant contributor to health of modern society [3, 4]. This led to the widespread opinion that “it is time to close the book on infectious diseases”, which the US Surgeon General, Dr. William Stewart, is supposed to have stated in the 1960s [5]. However, Alexander Fleming should have been right after all with his warning that “it is not difficult to make microbes resistant” [1]. Owing to a misusage of antibiotics in public health care and animal feed, resistant bacterial strains readily developed and, in the more and more globalized world, rapidly spread leading to failure of formerly effective drug therapies [6-10]. Paradoxically, while resistances were increasingly emerging, the number of newly discovered antibiotic classes and of approved antibacterial drugs tremendously decreased [11]. Since the 1980s, many pharmaceutical companies left the field of antibiotic research assuming that there was no need for further antibiotics [12]. Even today, the short duration of an antibiotic therapy, the restricted application, and the high probability of resistance evolution render the development of new antibiotics financially unattractive [12, 13]. This trend provoked a growing fear of the occurrence of non-treatable superbugs [14] and of the return to the pre-antibiotic era [15, 16]. Indeed, ineffectiveness of antibiotic treatments has not only led to a dramatic increase in morbidity and mortality, but also posed an economic burden on the public health [17]. Therefore, an urgent need exists to develop novel anti-infectives that overcome existing resistances and, ideally, do not provoke new ones [18].

Several approaches to override emerged antibacterial resistance have been followed so far. For example, antibiotics susceptible to increased efflux, reduced uptake, or inactivation by modifying enzymes have been applied in combination with an ancillary drug acting as efflux pump inhibitor [19], penetration enhancer [20], or enzyme inhibitor [21, 22]. Furthermore, structural modification of an existing class of antibiotics has been an often followed strategy [21]. Apart from the possibility to interfere with resistance-related mechanisms, addressing new binding sites within an established antibacterial target has proven to be a promising way to develop novel and efficient antibiotics [23-26]. Moreover, the identification of novel antibiotic targets has been an option to develop effective treatments [13].

However, these strategies attacking bacterial functions that are essential for growth or survival and thus exerting a selection pressure on the bacteria bear the risk to provoke, earlier or later, new resistances [27]. Furthermore, another form of resistance, namely the self-organization and -protection of bacteria within a biofilm is not necessarily overcome by these conventional treatments [28, 29]. Moreover, persistent infections as source for latent, chronic, or recurring infections may not be eradicated by common antibiotic therapy [30, 31]. Therefore, innovative anti-infective strategies able to override these disadvantages are urgently needed. Recently, anti-pathogenicity approach has become a new paradigm for antimicrobial therapy [27].

1.1 Anti-pathogenicity Concept

With the growing effort in understanding the way bacteria can cause disease, a new concept arose aiming at interference with bacterial pathogenesis rather than inhibiting cell viability [27, 32, 33]. Multiple pathogenic mechanisms are involved in bacterial pathogenesis that might be targeted with so-called anti-pathogenic drugs [32, 33]. An important contributor to pathogenesis of acute infections is the production of virulence factors, which enables a bacterium to invade a host and to survive within it [34, 35]. Accordingly, anti-virulence strategies have been proposed that aim at ‘disarming’ the pathogen instead of ‘killing’ it and, finally, rely on the host immune system to clear the infection. This might bear the advantage of reduced selective pressure and risk of resistance development [27, 36]. Pathogenesis of chronic infections is mainly governed by formation of biofilms that confer to the bacteria resistance against antibiotics and host defenses [28, 35]. Accordingly, anti-biofilm approaches might deprive the bacteria of their protective shield and increase their susceptibility to antimicrobial treatment [32, 37]. Although biofilms are sometimes categorized as cell-associated virulence determinants [35, 38], the terms ‘biofilm’ and ‘virulence’ will be differentiated in the following with the latter referring to virulence in acute infections according to Lu et al. [39, 40].

Taken together, the anti-pathogenicity concept promises not only a reduced provocation of new resistances [27, 36] but also an overcoming of existing antibiotic resistances [37]. A further advantage of this concept over antibiotic therapy might be preservation of the beneficial bacterial consortia (e.g. the gut flora [41]) in the host avoiding adverse effects [27, 42].

1.2 *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous, highly adaptive Gram-negative bacterium [43] that is able to infect virtually every mammalian tissue [44-46]. It is a major originator of nosocomial infections, especially of such acquired in intensive care units [47, 48]. As an opportunistic pathogen, it infects individuals exhibiting a substantial break in first-line defenses such as burn victims and immunocompromised patients (e.g. patients suffering from cystic fibrosis (CF) or receiving chemotherapy) [35, 46]. According to the immune status of the patient, it can act as quiescent colonizer, as highly virulent invader during acute infections, or as originator of chronic/persistent infections [31, 35, 38, 46]. For instance, it can colonize individuals suffering from chronic obstructive pulmonary disease, cause fulminant acute ventilator-associated pneumonia, or initiate chronic infection in CF patients [38, 49]. Suchlike *P. aeruginosa* infections are commonly associated with high incidence, severity, recalcitrance, and mortality making *P. aeruginosa* the ‘superbug’ [33, 50].

The severity of acute infections is mainly governed by production of a large arsenal of extracellular virulence factors responsible for tissue invasion, toxicogenesis, and dissemination finally leading to multiple organ failure and death [35]. For example, the protease elastase contributes to tissue invasion and resistance to host immune defenses by degrading elastin and immune components, respectively [35]. The hemolytic rhamnolipids interfere with lung surfactant and contribute to host immune resistance by inhibiting mucociliary transport [35] and by lysing polymorphonuclear leukocytes [51]. The sugar-binding lectins function as adhesins and cytotoxins for the respiratory epithelium and contribute to biofilm formation [35, 52, 53]. The phenazine pyocyanin is a redox-active cytotoxin that interferes with several cellular functions by production of reactive oxygen species [54]. It is required for full virulence in acute infection models and seems to play an important role in chronic infections too [54, 55].

The antibiotic treatment of *P. aeruginosa* infections is challenged by a variety of intrinsic and acquired resistances, which can be developed simultaneously (multi-drug resistance) and within short time intervals [56]. Notably, *P. aeruginosa* possesses a large network of multi-drug efflux pumps that can actively export drugs from the cell [56]. Additionally, drug uptake is hampered by highly restricted outer membrane permeability combined with orthogonal sieving properties of the two (inner and outer) membranes [2]. These factors especially contribute to *P. aeruginosa* resistance against current drugs and impede the development of new ones [57].

Besides such forms of resistance that are related to the single bacterial cell, the pathogen can activate protective functions that are associated with multicellular lifestyle. To be precise, *P. aeruginosa* can build biofilms, highly structured bacterial communities attached to a surface and encapsulated in an extracellular matrix [58]. Thereby, impaired diffusion, reduced metabolism and growth as well as increased efflux activity are important mechanisms believed to contribute to biofilm-specific antibiotic tolerance [28, 59]. Moreover, within biofilms, the bacteria are protected against the immune system of the host [60]. Biofilm formation and low production of virulence factors are characteristic of chronic *P. aeruginosa* infections [35]. In suchlike infections, progressive tissue damage is caused by a chronic inflammation process rather than by the direct action of virulence factors [35, 61]. Furthermore, full eradication of chronic infections with *P. aeruginosa* is often hampered by the survival of a small sub-population of cells exhibiting multi-drug tolerance to antibiotics without undergoing genetic change [31, 62]. Critically, these so-called dormant or persister cells can serve as source for latent, chronic or relapsing infections [30, 63, 64].

1.3 Inhibition of Quorum Sensing (QS) as Anti-pathogenicity Approach

As introduced above, interference with bacterial pathogenicity is believed to be a forward-looking concept for creating efficient anti-infectives. Recently, interruption of quorum sensing (QS) has been proposed as promising anti-pathogenicity strategy [32, 65-70]. The term ‘quorum’ originates from the Roman Empire, where it denoted the minimum number of votes necessary to come to a collective decision in the Roman Senate [71]. Translated to the bacterial world, ‘quorum sensing’ terms the bacterial strategy to coordinate collective behaviors in response to cell population density [72]. Thereby, bacteria communicate via signal molecules, small diffusible molecules that are produced, secreted, and sensed by the bacteria. The concentration of the signal molecules increases dependently on the cell density. When a minimal threshold concentration, the ‘quorum’, has been reached, the bacteria concertedly initiate changes in gene expression acting as quasi-multicellular organism [73, 74]. QS enables the bacteria to coordinately regulate diverse physiological processes including virulence and biofilm formation [73]. Moreover, it increases the survival prospects of bacteria in a host since it allows the single bacterium to be undetected by the immune defense until a collective, success-promising attack is possible [68].

The pathogenicity of *P. aeruginosa* is strongly associated with its QS systems. These control the expression of genes involved e.g. in virulence factor production during acute infections or

in biofilm formation and persister cell accumulation during chronic infections [35, 75, 76]. Thus, inhibition of QS seems to be an attractive strategy to combat a variety of infections caused by *P. aeruginosa* [32, 64].

1.4 The QS Network of *P. aeruginosa*

P. aeruginosa possesses an intricate QS network that comprises at least four interconnected QS circuits and is organized in a multi-layered hierarchy [77], as shown in Fig. 1. Two of these QS systems, the *las* and the *rhl* system, are based on *N*-acyl-homoserine lactones (AHLs), the major class of signal molecules employed by Gram-negative bacteria [78]. Thereby, the *las* system uses *N*-(3-oxododecanoyl)-*L*-homoserine lactones and the *rhl* system *N*-butyryl-*L*-homoserine lactones (BHLs) as signal molecules [79, 80]. Their biosynthesis is mediated by the AHL synthases LasI and RhlI, respectively [81-83]. The signals can activate the corresponding receptors LasR and RhlR inducing the expression of target genes including those encoding their own synthases [77, 84, 85]. This autoinduction process enables the bacteria to rapidly augment the amount of signal molecules thus called autoinducers [74]. The third QS system makes use of 2-alkyl-4-(1*H*)-quinolones (AQs) as signal molecules and hence is termed the *Pseudomonas* quinolone signal (PQS) system, in short the *pqs* system [77, 86]. In contrast to the widespread AHL signaling, quinolone-based QS is restricted to particular *Pseudomonas* and *Burkholderia* species [87-89]. While 2-heptyl-4-(1*H*)-quinolone (HHQ) is produced by both, *Pseudomonas* and *Burkholderia*, 2-heptyl-3-hydroxy-4-(1*H*)-quinolone, also known as PQS, is uniquely biosynthesized by *Pseudomonas* [86, 90]. The signal molecules PQS and its biosynthetic precursor HHQ serve as the natural agonists of the receptor PqsR, a transcriptional regulator often referred to as multiple virulence factor regulator (MvfR) [91-93]. Thereby, PQS binds to PqsR with 100-fold higher affinity than HHQ does [91, 94]. Comparably to the positive feedback loop of the AHL-based QS systems, HHQ and PQS can stimulate the expression of their own biosynthetic operon *pqsABCDE* by agonizing PqsR [95-98]. Balancing of *pqs* QS activity is achieved by negative feedback loops mediated via PqsE [99], HHQ, PQS [100], and 2-aminoacetophenone (2-AA) [101]. Whereas the gene products of *pqsABCD* are primarily involved in AQ biosynthesis [97], PqsE seems to act additionally as ‘PQS response protein’ regulating the expression of several *pqs* QS-controlled virulence genes [99, 102-104]. Recently, a fourth QS system has been discovered using 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde as signal molecule [77, 105]. Its biosynthesis relies on the *ambBCDE* gene cluster. Due to its role in integrating environmental stress conditions with the QS network, the signal has been named IQS.

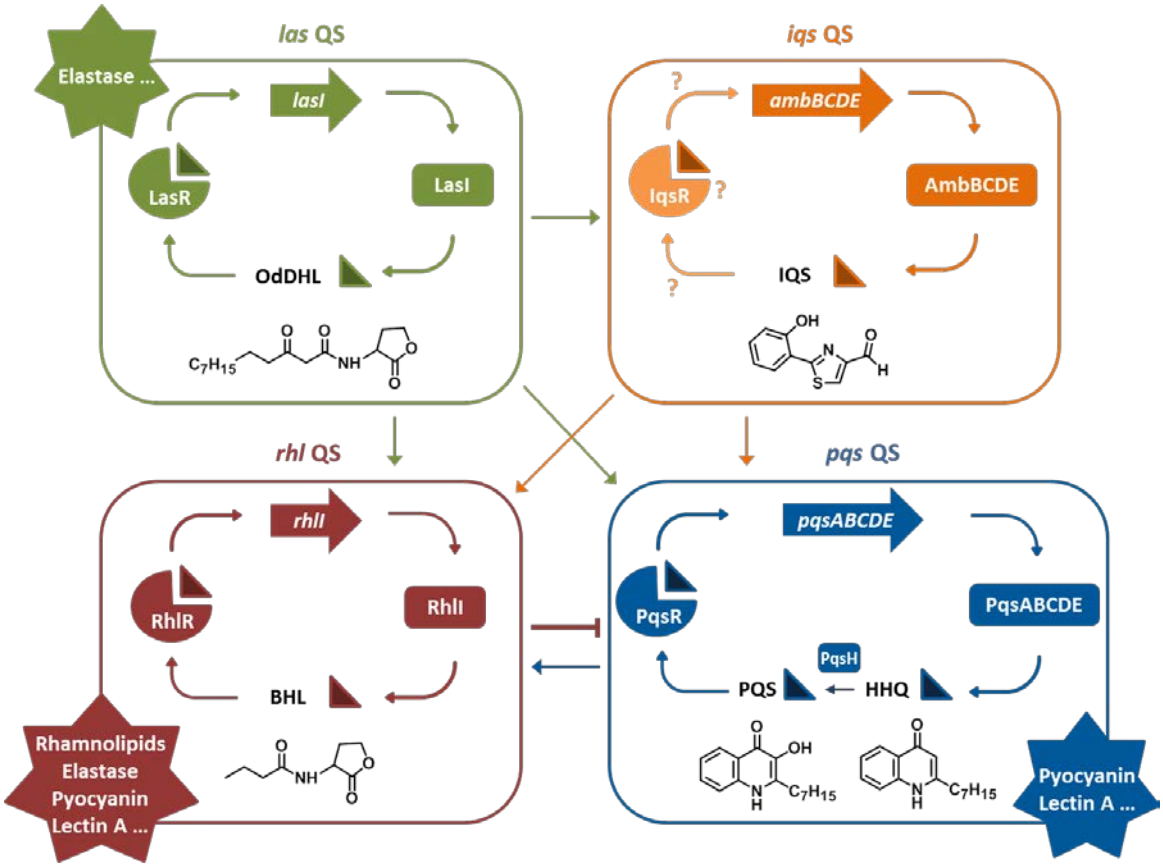

Figure 1. Schematic of the quorum sensing (QS) network in *P. aeruginosa* comprising the four QS circuits *las, iqs, rhl,* and *pqs*. Abbreviations: QS, quorum sensing; OdDHL, *N*-(3-oxo-dodecanoyl)-homoserine lactone; IQS, integrating QS signal; BHL, *N*-butyryl-*L*-homoserine lactone; PQS, *Pseudomonas* quinolone signal; HHQ, 2-heptyl-4-(1*H*)-quinolone. Straight thin arrows represent positive regulation and straight thick lines negative regulation. Modified from ref. [77].

The four QS networks of *P. aeruginosa* are hierarchically interconnected [77]. The superordinate system in the QS hierarchy is the *las* system. It controls the activation of the *rhl* and *pqs* circuits by positively regulating RhlR, PqsR and PqsH (the enzyme converting HHQ into PQS) [90, 96, 106]. Moreover, IQS production is tightly controlled by the *las* system [105]. The *rhl* system negatively regulates the expression of the *pqs* QS biosynthetic operon [107], whereas PQS was found to cause an induction of *rhlI* transcription and RhlR production [108, 109]. Moreover, disruption of IQS biosynthesis led to a decrease in BHL and PQS levels suggesting a positive regulatory role of *iqs* QS on *rhl* and *pqs* QS [105]. Given the predominant role of *rhl* in virulence gene activation and its dependency on all other QS systems, it has been proposed to function as a 'workhorse for the QS command' [77]. However, under certain circumstances, this hierarchy can be overridden. In the absence of functional LasR, PQS can be produced belatedly and stimulate *rhl*-dependent phenotypes

[109] probably mediated via RhlR [110]. Similarly, the dominance of the *las* over the *iqs* system can be reversed under phosphate depletion conditions, under which IQS was able to upregulate the *rhl* and *pqs* systems in a *lasR* mutant [105]. Similarly, several other environmental (e.g. iron, oxygen, nutrients) and host factors have been found to modulate the QS hierarchy [77, 111, 112]. These observations hint at a high flexibility of the QS network in adapting to external influences in favor of bacterial pathogenicity. Thus, they should be taken into consideration during development of QS inhibitors (QSIs) [77].

The QS network controls a large array of virulence factors. Whereas elastase and rhamnolipids are controlled by *las* and *rhl*, lectin A and pyocyanin are among the primarily *rhl*-dependent virulence factors [109]. All of these virulence factors are co-regulated by the *pqs* QS system (see section 1.5.2). The influence of *iqs* QS on the virulence factors pyocyanin and elastase seems to be mediated indirectly via *pqs* QS and *rhl* QS modulation [105].

1.5 The *pqs* QS System

The *pqs* QS system of *P. aeruginosa* uses AQs to regulate a variety of genes including its own biosynthetic operon and genes involved in virulence and biofilm formation [86, 99].

1.5.1 Biosynthesis of 2-Alkyl-4-(1*H*)-quinolones (AQs)

The current model for biosynthesis of AQs is depicted in Fig. 2. The precursor anthranilate [90, 113] can be obtained either from tryptophan via the kynurenine pathway or from chorismic acid via an anthranilate synthase encoded by the PqsR-regulated *phnAB* operon [114]. This unique QS-related pathway serves as additional source of anthranilate supporting efficient signal molecule production [91, 114-116]. The biosynthetic gene cluster *pqsABCDE*, which is under the control of PqsR, encodes the enzymes involved in the synthesis of HHQ [97, 98]. In the first step, the ligase PqsA catalyzes the formation of anthraniloyl-coenzyme A (ACoA) from anthranilate, adenosine triphosphate, and CoA [117]. The activated anthranilate is then able to build a covalent adduct with the active site cysteine of PqsD, a *β*-ketoacyl-ACP synthase III (FabH)-type condensing enzyme [118-121]. In the presence of *β*-ketodecanoic acid as second substrate, the anthranilate-PqsD complex has been shown to release HHQ *in vitro* following a ping-pong kinetic mechanism [120-123]. However, recent investigations elucidated that, in the cellular context of *P. aeruginosa*, PqsD most likely uses malonyl-CoA as second substrate to give 2-aminobenzoylacetyl-CoA (2-ABA-CoA) [97, 98, 119]. A very recent study showed that PqsE can act *in vitro* as thioesterase hydrolyzing 2-ABA-CoA to 2-aminobenzoylacetate (2-ABA) [98]. This function, however, can be partially taken over by

the broad-specificity thioesterase TesB in accordance with the AQ production found in a *pqsE* mutant [98, 102]. The condensation of 2-ABA with octanoic acid seems to be accomplished by PqsC in complex with PqsB yielding HHQ [97, 124]. Finally, the conversion of HHQ into PQS is performed by the NADH-dependent flavin mono-oxygenase PqsH, the expression of which is positively regulated by LasR, but not by PqsR [90, 125]. Notably, *P. aeruginosa* is able to synthesize besides HHQ and PQS over 55 distinct AQs classifiable into five different structural series [90, 126]. Two other bioactive secondary metabolites, 2,4-dihydroxyquinoline (DHQ) and 2-AA, also derive from the AQ biosynthetic pathway [97, 98]. Whereas DHQ is supposed to be the result of spontaneous intramolecular cyclization of the intermediates 2-ABA-CoA or 2-ABA, 2-AA is most likely formed by decarboxylation of 2-ABA [97, 98, 119].

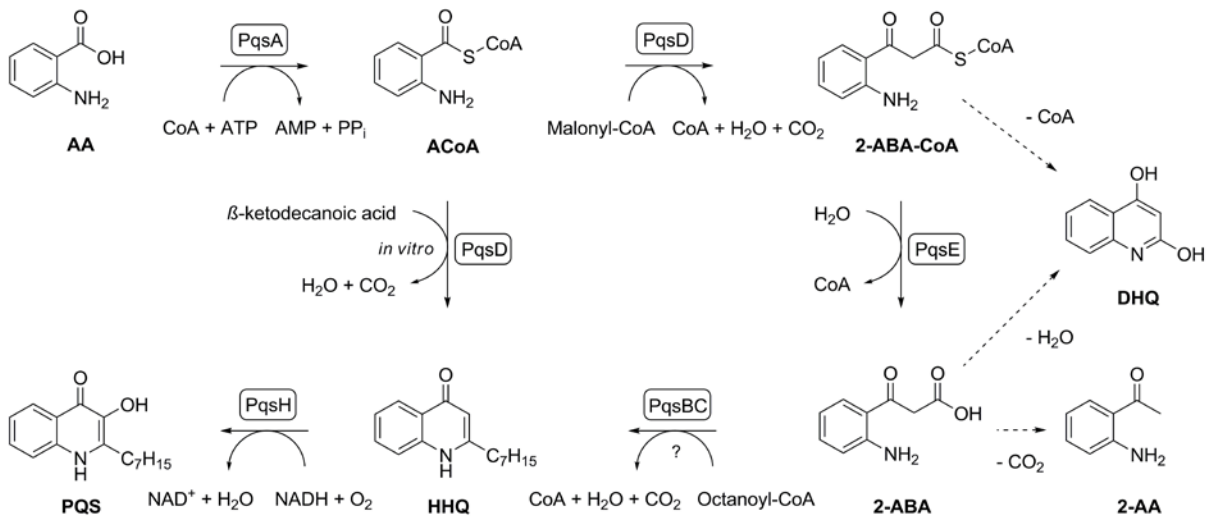

Figure 2. Current model for biosynthesis of 2-alkyl-4-(1*H*)-quinolones and related secondary metabolites by *P. aeruginosa*. Abbreviations: AA, anthranilic acid; CoA, coenzyme A; ATP, adenosine triphosphate; AMP, adenosine monophosphate; PP_i, pyrophosphate; ACoA, anthraniloyl-CoA; 2-ABA-CoA, 2-aminobenzoylacetyl-CoA; DHQ, 2,4-dihydroxyquinoline; 2-ABA, 2-aminobenzoylacetate; 2-AA, 2-aminoacetophenone; HHQ, 2-heptyl-4-(1*H*)-quinolone; NADH/NAD^+, reduced/oxidized form of nicotinamide adenine dinucleotide; PQS, *Pseudomonas* quinolone signal. Solid arrows represent enzyme-catalyzed and dashed arrows spontaneously occurring reactions. Adapted from refs. [97, 98, 122].

1.5.2 AQ-dependent Regulation of Pathogenicity

As described above, *pqs* QS signaling works via the AQs HHQ and PQS that drive the expression of their biosynthetic operon *pqsABCDE* by activating their receptor PqsR [95, 96]. AQ signaling controls pathogenicity via both, PqsE-dependent and PqsE-independent mechanisms [99]. In contrast to its minor impact on *in cellulo* AQ biosynthesis, PqsE is

important for mediating the cellular PQS response [102]. Thereby, it regulates a major subset of the AQ-controlled genes acting in an AQ-independent manner [99, 100, 103]. Thus, the primary function of AQ signaling in regulating PqsE-dependent pathogenicity seems to be driving PqsE expression [100]. In addition, AQs act on pathogenicity via PqsE-independent mechanisms. Some pathogenicity traits, however, require the direct action of both, AQs and PqsE [99].

PqsE is fully required for production of the primarily *rhl*-dependent virulence factors pyocyanin and lectin A as well as partially required for production of the *las*- and *rhl*-dependent virulence factors elastase and rhamnolipids [99, 100, 103, 109]. Notably, PqsE requires functional RhlR to regulate these virulence factors, but neither AQs nor PqsR [100, 103]. Moreover, PqsE is in part involved in upregulation of siderophore (e.g. pyochelin) production [99, 100] and fully required for swarming motility [99]. Decisively, PqsE is able to fully restore pathogenicity in four different acute infection models even in the absence of AQs [99, 100]. In contrast, PqsE is only partially needed for biofilm development [99], a function that might be at least in part mediated by contribution of lectin A [52], pyocyanin [127, 128], and rhamnolipids [129].

Besides their role as autoinducers, multiple non-signaling functions have been ascribed to AQs [130]. First, PQS is involved in induction of iron acquisition systems by chelating iron (III) thereby complementing the respective action by PqsE [99, 100, 131, 132]. Accordingly, PQS elevated the production of the siderophores pyochelin and pyoverdine [131, 132]. Second, the pro- and anti-oxidant activities of PQS suggest a role in balancing life and death in *P. aeruginosa* populations to select the fittest, shape the population structure, and contribute to multi-cellular development processes in bacterial biofilms [133]. Third, PQS promotes biofilm formation [109] possibly via induction of bacteriolytic membrane vesicles [134] and subsequent DNA release [129, 135]. Fourth, PQS and HHQ can suppress innate immune responses facilitating bacterial adaptation to the host [136]. Moreover, HHQ is involved in swarming repression, a phenotype that is inversely related to biofilm formation [99, 137]. Many non-signaling AQs exhibit cytochrome inhibitory or antimicrobial activities conferring *P. aeruginosa* a growth advantage in competitive situations [90, 138, 139]. Although the physiological role of DHQ, one side-product of AQ biosynthesis, is not yet fully understood, it has been suggested to contribute to pathogenicity by reducing the viability of murine lung epithelial cells [119]. The other side-product, 2-AA, induces chronic and persistent infection phenotypes of *P. aeruginosa*. This is achieved by silencing the PqsR regulon and thus acute virulence [101] as well as by promoting persister cell accumulation

and host tolerance [75, 140]. Considering the respective described functions of PqsE and AQs, it seems as if PqsE is essential for acute infections, while AQs might play a more important role in chronic/persistent infections, as speculated by Rampioni et al. [99].

1.6 Interruption of *pqs* QS in *P. aeruginosa*

Although *pqs* QS has been by far less investigated and exploited for anti-infective research than AHL-based signaling [141], it represents an attractive target that bears advantages over the AHL-related systems. Due to the widespread occurrence of AHLs in Gram-negative bacteria [73], inhibitors of the respective QS systems might affect multiple bacterial species including the beneficial microbiota. Targeting the *pqs* system, in contrast, provides an option for selective therapy of pathogenic *Pseudomonas* and *Burkholderia* sometimes coexisting in chronic lung infections [142]. This selective intervention might provide the microbiota an advantage in availability of nutrients and habitat and thus let them keep the pathogens in check [143]. Moreover, the *las* system can become ineffective due to mutations in the *lasR* gene and its functions can be taken over partially by other QS systems [77, 105, 109, 110, 144]. The *pqs* system, however, can operate independently on *las* [109, 110] and suchlike mutations have not been discovered yet [64]. Furthermore, an *rhlR* mutant did not display reduced virulence in an acute infection model in contrast to the *pqs* QS mutants [92]. Therefore, disruption of *pqs* QS might be the more suitable anti-pathogenicity approach.

The *pqs* system provides several potential drug targets involved either in signal biosynthesis (PhnAB, PqsA-E, PqsH), signal reception (PqsR), or signal response (PqsE) [102]. As already described in section 1.5.2, the ‘PQS response protein’ PqsE controls numerous downstream virulence genes [103]. However, the exact mechanism of action regarding this function has not yet been elucidated [98, 99], which might complicate drug discovery. Moreover, addressing terminal effectors within a regulatory cascade might miss effects mediated by a higher level of regulation [99]. In contrast, inhibition of AQ biosynthesis or reception might allow disruption of the full profile of *pqs* QS-controlled phenotypes [99] and thus represents a promising concept for development of novel anti-infectives.

1.6.1 Blocking AQ Biosynthesis by Inhibition of PqsD

As described in section 1.5, the AQ biosynthesis machinery is responsible for a large panel of effector molecules fulfilling diverse functions related to *P. aeruginosa* pathogenicity. Hence, blocking this cascade with small molecule inhibitors should result in an efficient reduction of pathogenicity. However, not all biosynthetic enzymes are equally suited as drug targets.

Blocking *pqs* QS-specific anthranilate synthesis might be inefficient due to redundancy of anthranilate-supplying machineries [102, 115]. Although PqsB and PqsC are essential for AQ biosynthesis, they are not required for DHQ and 2-AA production [97]. Notably, their inhibition could even lead to accumulation of these metabolites [64, 119]. Although the thioesterase activity of PqsE contributes to AQ synthesis *in vitro*, its role might be taken over in part by 'housekeeping' thioesterases *in cellulo* [98]. Moreover, inhibition of thioesterase function should lead to accumulation of DHQ [97, 98]. Finally, PqsH is not a valid target, as a *pqsH* mutant overproduces HHQ and displays wild-type virulence in a murine infection model [91]. On the contrary, PqsA and PqsD seem to be essential and efficient enzymes in AQ biosynthesis [97].

Indeed, genetic mutation of *pqsA* or *pqsD* led to reduced pathogenicity in acute and chronic infection scenarios. On the one side, a mutation in the *pqsA* or *pqsD* gene resulted in reduced pyoverdine levels [132] and abolished pyocyanin production [102]. Accordingly, a *pqsD* and a *pqsA* mutant exhibited attenuated acute virulence in *C. elegans* nematodes and murine burn injury models, respectively [91, 92, 99, 102]. On the other side, a *pqsA* mutant was classified as poor biofilm producer forming flat biofilms and lacking the typical mushroom-shaped structure associated with antibiotic tolerance [112, 129, 135, 145]. The mutant biofilm exhibited enhanced sensitivity towards detergent treatment [135]. This might be at least in part due to the reduced production of extracellular DNA (eDNA), an intercellular connector and stabilizer in biofilms [112, 135]. Due to its role in biofilm tolerance by trapping e.g. aminoglycosides and antimicrobial peptides, a reduction of its formation should render biofilms more susceptible to antibiotics and host defenses [146]. Notably, a *pqsA* mutant also formed less biofilm and exhibited enhanced susceptibility to ciprofloxacin in a murine *in vivo* biofilm model [147]. Similarly, increased susceptibility towards ciprofloxacin could be demonstrated in wild-type biofilms, in which *pqs* QS was repressed by addition of an excess of iron [112]. Although *pqs* QS seems to be important for establishment of biofilms [112, 135, 148], the *pqsABCDE* operon was found to be downregulated in mature *in vivo* biofilms [149] suggesting a role of *pqs* QS in biofilm formation rather than maintenance [112, 149]. However, evidence suggests that *pqs* QS might also be involved in biofilm dispersal, which was delayed in a *pqsA* mutant [150].

A few small-molecule inhibitors of PQS production with unknown molecular target or mechanism and low *in cellulo* potency have been described, but not further optimized [117, 141, 151]. Nevertheless, interruption of *pqs* QS with high doses of PqsA substrates led to restricted systemic dissemination of *P. aeruginosa* and reduced mortality in an acute murine

infection model [152]. This supported that AQ biosynthesis might be a useful pharmacological target for therapy of acute infections. However, these PqsA substrates seemed to additionally affect tryptophan biosynthesis and interference with downstream targets by the formed CoA thioesters could not be excluded [117, 152].

Given that the described beneficial effects of AQ biosynthesis disruption by PqsA knockout or inhibition should also be achievable through blockade of PqsD activity, both, PqsA and PqsD, seem to be promising anti-pathogenicity targets. However, as no structural information about PqsA is available, while the X-ray structure of PqsD is known, we considered PqsD as the most promising biosynthetic enzyme to be addressed for drug discovery. The first reported *in vitro* PqsD inhibitors were only moderately affine and were not tested in cellular assays due to potential antibiotic activity [122]. This motivated us to start a drug discovery program aiming at novel potent and non-bactericidal PqsD inhibitors (Publication A in 3.1 [153]). Meanwhile, numerous drug discovery approaches addressing PqsD have been undertaken yielding highly potent, selective, and non-bactericidal PqsD inhibitors [141, 154-157].

1.6.2 Blocking AQ Reception by Antagonism of PqsR

As stated in chapter 1.6, blockade of signal reception should affect the full panel of *pqs* QS-controlled pathogenicity phenotypes. Indeed, transcriptome analysis of a *pqsR* mutant revealed downregulation of multiple virulence genes [92]. Accordingly, a *pqsR* mutant did not form any pyocyanin or lectin A and produced substantially reduced levels of elastase and rhamnolipids [102, 109]. Decisively, in *C. elegans* nematodes and different acute infection models including murine burn injury models, reduced virulence was attributed to a *pqsR* mutant [91-93, 102]. Furthermore, PqsR might play a role in biofilm formation, as a *pqsR* mutant showed reduced lectin A and eDNA levels, both important biofilm matrix components, and lacked PQS, a stimulator of biofilm formation [109, 112]. Moreover, it is required for production of the pro-persistent molecule 2-AA, which was absent in a *pqsR* mutant [101].

Thus, we considered PqsR as highly attractive target for the development of novel anti-infectives. Previous studies by us and others had discovered fragments [158, 159] and HHQ-derived compounds [160, 161] as first PqsR antagonists. However, they all shared low potency in reducing *P. aeruginosa* virulence. This prompted us to reveal the reasons for the low *in cellulo* efficacy of our highly potent HHQ-based antagonists [160] in order to develop effective anti-infectives (Publication C in 3.3 [39]). Meanwhile, PqsR has been addressed as drug target [162] with great success even in advanced acute infection models [64]. This

confirms the suitability of PqsR as therapeutic target and highlights the importance of publication C [39].

2 Aim of the Thesis

Infections with *P. aeruginosa* are especially difficult to eradicate with current antibiotics due to intrinsic, acquired, and biofilm-mediated resistance and persistence. Thus, novel anti-infectives are urgently needed that can overcome existing resistances and do not provoke new ones. Interference with bacterial pathogenicity by inhibition of QS promises these advantages. This intercellular communication system operates via signal molecules that control virulence and biofilm formation. Thereby, the signal-synthesizing enzyme PqsD and the signal-receiving receptor PqsR are considered as promising targets due to their key role in *P. aeruginosa* QS. Target validation, however, relied primarily on mutant analyses. Moreover, first inhibitors suffered from low potency *in vitro* and *in cellulo*. Therefore, the general objective of this thesis was to biologically evaluate novel QSIs targeting PqsD or PqsR in order to contribute to their development to potent anti-infectives and to the validation of their drug targets.

The first part of this thesis aimed at the development of PqsD inhibitors. For discovery of first hits and guidance of their optimization, an *in vitro* assay based on the isolated target had to be developed. The most promising inhibitors should then be characterized *in cellulo* for their ability to inhibit production of signal molecules such as PQS without inhibiting bacterial growth. Evaluation in a biofilm formation assay should assess the suitability of PqsD as anti-biofilm target. In the second part of this thesis, irreproducible results occurring during routine quantification of PQS should be overcome by development and validation of a novel LC-MS/MS method for quantification of PQS after derivatization. The third part of this thesis aimed at circumventing the ineffectiveness of the first PqsR antagonist *in cellulo*. The optimized compound should be characterized *in cellulo* and in appropriate *in vivo* models to judge its potential as anti-virulence agent. Based on these studies, the proof-of-concept (POC) for anti-infective therapy targeting PqsR should be provided. In the fourth part of this thesis, biological evaluation should guide the optimization of PqsR antagonists regarding aqueous solubility.

3 Results

3.1 Publication A: Validation of PqsD as an anti-biofilm target in Pseudomonas aeruginosa by development of small-molecule inhibitors [153]

(DOI: 10.1021/ja3072397)

3.2 Publication B: Development and validation of a UHPLC–MS/MS procedure for quantification of the Pseudomonas Quinolone Signal in bacterial culture after acetylation for characterization of new quorum sensing inhibitors [163]

(DOI: 10.1016/j.jpba.2013.07.047)

3.3 Publication C: Overcoming the unexpected functional inversion of a PqsR antagonist in Pseudomonas aeruginosa: an in vivo potent antivirulence agent targeting pqs quorum sensing [39]

(DOI: 10.1002/anie.201307547)

3.4 Publication D: Optimization of anti-virulence PqsR antagonists regarding aqueous solubility and biological properties resulting in new insights in structure-activity relationships [40]

(DOI: 10.1016/j.ejmech.2014.04.016)

4 Final Discussion

The general objective of this thesis was to biologically evaluate QSIs blocking biosynthesis or reception of *pqs* QS signal molecules in order to contribute to the validation of their drug targets and to their development as anti-infectives. Biological evaluation was based on a test system comprising *in vitro*, *in cellulo*, and *in vivo* assays. Therefore, in the following, the composition of the test system, the biological results, the target validation, and the potential of the novel QSIs will be discussed.

For the sake of lucidity, the denomination of the compounds mentioned in chapter 4 is composed of a letter indicating the corresponding manuscript and the Arabic compound number used in the latter (e.g. A1 denotes compound 1 from publication A).

4.1 Biological Evaluation

4.1.1 *In vitro* Evaluation of QS inhibitors (QSIs)

The discovery of initial hits, their optimization, and the derivation of structure-activity relationships rely on the availability of a suitable bioassay. In case of the PqsD inhibitors, we decided to start the drug discovery process with an *in vitro* assay based on the isolated target for several reasons. First, such an assay unambiguously reflects the inhibitory potency of compounds towards the target irrespectively of pharmacokinetic (PK) issues or aspects of system biology encountered in *P. aeruginosa* whole cell assays [122, 164]. Second, compounds with intrinsic antibacterial activity can also be evaluated [122, 165]. The successful development of potent and selective PqsD inhibitors derived from known inhibitors of the antibacterial target RNA polymerase underlines the importance of this aspect [157, 166, 167]. Third, performing an *in vitro* assay is less time-consuming and hazardous than cultivating opportunistically pathogenic bacteria. The observation that purified PqsD catalyzes *in vitro* the production of HHQ from the substrates ACoA and *β*-ketodecanoic acid [122] provided the basis for establishment of a 96-well format-based PqsD inhibition assay [121, 123]. Recently, malonyl-CoA has been proposed to serve as the second substrate of PqsD in *P. aeruginosa* cells [97]. However, the identity of the second substrate is not of relevance for the PqsD inhibitors developed in publication A [153] since they have been designed and confirmed to interfere with the first substrate ACoA [168]. Overall, the developed *in vitro* assay provided the basis for discovery and optimization of the PqsD inhibitors developed in publication A [153].

The first described PqsD inhibitors only weakly inhibited PqsD with IC_{50} values of 35 or 65 µM (IC_{50} is the inhibitor concentration to achieve half-maximal degree of inhibition) and were likely to be antimicrobially active [122]. Therefore, we decided to follow a ligand-guided strategy (for a comprehensive discussion regarding inhibitor design and optimization see section 4.1 of the thesis of my colleague Dr. Michael Storz [143]). Thereby, an ACoA-derived nitro-substituted transition state mimic, **A3**, proved to be the first PqsD inhibitor identified in this study that was more potent (IC_{50} of 7.9 µM) than the first reported inhibitors. However, given a nanomolar median affinity for marketed small molecule drugs [169], this is only a moderate activity. Suspecting *inter alia* the high conformational flexibility of the alkyl chain as reason for both, weak affinity [170] and insufficient drug-likeness [171], the molecule was systematically simplified and rigidized. The resulting inhibitor, **A19**, was slightly more active (IC_{50} of 3.2 µM) than the initial hit **A3** and the most potent *in vitro* PqsD inhibitor reported at that time. Noteworthy, **A19** provided the starting point for follow-up publications dealing with the elucidation of its binding mode [168], its use as tool compound to study the binding mode of other PqsD inhibitors [167], and its further optimization [155].

Despite the above-mentioned benefits, an *in vitro* assay based on the purified target was not considered as the most suitable option for the discovery of PqsR antagonists. One reason is the insolubility and thus the unavailability of the purified full length PqsR receptor [91, 94, 161]. Using a truncated version of PqsR including only the co-inducer binding domain might miss potential inhibitors [94], e.g. such targeting the DNA binding domain or preventing receptor oligomerization [161, 172]. Moreover, a standard *in vitro* binding assay could not provide any information about the functionality of PqsR ligands. To circumvent these drawbacks, we decided to use a *ß*-galactosidase reporter gene assay in *E. coli* monitoring PqsR-mediated transcription of the lacZ reporter gene being under the control of the *pqsA* promoter [160, 164, 173]. Although in this assay, PqsR activity is a function of both, target affinity and PK properties, the *E. coli* system represents a more sensitive way to monitor PqsR activity than the *P. aeruginosa*-based counterpart [164] for reasons already discussed above. Overall, the reporter gene assay represented the fundament for evaluation of PqsR antagonists.

Based on this assay, we recently identified the first PqsR antagonists using a ligand-based approach. By introducing electron-withdrawing groups into the 6-position of HHQ, potent antagonists were obtained with nanomolar IC_{50} values [160]. The most potent antagonist, **C1** (IC_{50} of 51 nM), was the starting point for the investigations undertaken in publication C [39],

as discussed in section 4.1.2.1. Interestingly, compound **C2** was a strong agonist (effector concentration to achieve half-maximal degree of effect (EC_{50}) of 2.8 nM) in the *E. coli* reporter gene assay that was even more potent than the natural agonist PQS (EC_{50} of 6.3 nM). Thus, it can be classified as superagonist [68]. This explains why even low concentrations of **C2**, as produced during the time frame of reporter gene experiments, efficiently restored PqsR stimulation suppressed by **C1**. The resulting antagonist **C3** showed improved antagonistic activity (IC_{50} of 35 nM) compared to **C1** and is the most potent HHQ-derived PqsR antagonist reported to date [39, 40, 160, 161].

The promising results prompted us to further optimize this compound class regarding aqueous solubility in publication D [40]. The synthesized compounds were evaluated in the *E. coli* reporter gene assay to monitor maintenance of activity and functionality while improving solubility. This is important since introduction of a single functional group, e.g. a polar substituent to improve solubility, concomitantly changes also the electronic and steric properties of a molecule with possible impact on affinity and functionality [174]. Unfortunately, most structural changes towards enhanced solubility resulted in moderate to weak antagonists or agonists. Notably, a significant negative correlation between solubility and activity was observed for the nine precisely characterized antagonists from Table 2 in publication D (see Fig. 3A). Accordingly, their lipophilicity as judged by the calculated octanol-water partition coefficient positively correlated with activity with exception of compound **D26** (i.e. **C3**) that was more active than expected (see Fig. 3B). Suchlike property-activity relationships are not unexpected given the hydrophobic ligand binding pocket of PqsR and the fact that a natural HHQ analog is stabilized therein entirely by hydrophobic interactions [161]. Nevertheless, the activity measured in this assay is also a function of pharmacokinetics, as mentioned above. Thus, these observations could also be the result of e.g. restricted cell permeability for hydrophilic compounds in case of passive diffusion [2]. Overall, both factors might have impaired the compound optimization. Moreover, the rather sharp structure-functionality relationships, especially for substituents in 3-position also observed for quinazoline-based PqsR antagonists [161] might have limited the scope of structural modifications. Nevertheless, one promising antagonist, **D16**, resulted from this publication exhibiting similar potency (IC_{50} value of 72 nM) and slightly improved solubility as compared to **C1** and **C3**. In addition, structure-activity/functionality relationships could be derived (see section 2.3 of publication D [40]). These gave new insights into ligand-receptor interactions complementing the information provided by the co-crystal structure [161].

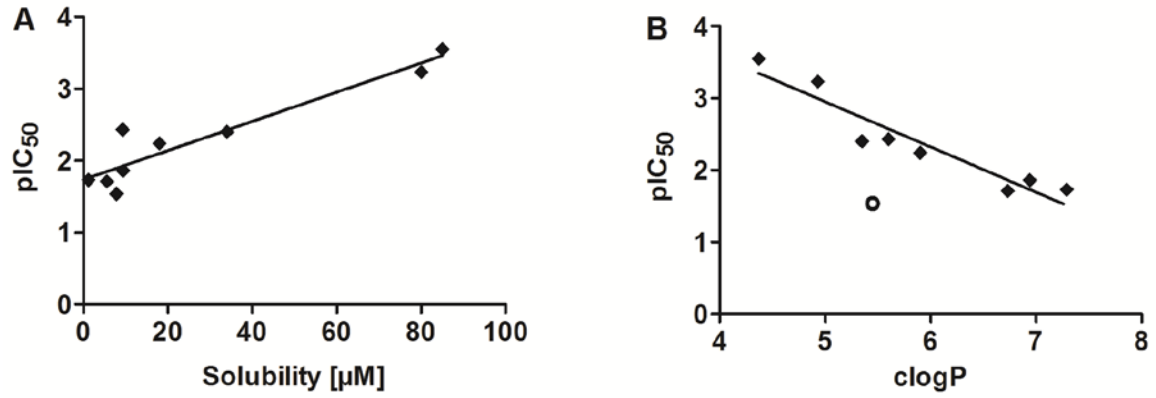

Figure 3. Property-activity relationships of novel PqsR antagonists. For the nine fully characterized PqsR antagonists from Table 2 of publication D [40], significant correlations were found between their micromolar aqueous solubility (A) or their calculated octanol-water coefficients (clogPs) obtained with ACD Percepta logP Classic software (B) and their PqsR antagonistic activity as expressed by the negative common logarithm of the half-maximum nanomolar inhibitory concentration (pIC_{50}) determined in the *E. coli* reporter gene assay. Compound **D26** (i.e. **C3**) was excluded from correlation B (empty circle). Statistics of the correlations: solubility versus pIC_{50}, $p < 0.001$, Pearson coefficient $r = 0.94$; clogP versus pIC_{50}, $p < 0.001$, Pearson coefficient $r = -0.95$.

4.1.2 *In cellulo* Characterization of QSIs

One strength of target-based drug discovery is the applicability of rationality-guided drug development programs [175]. Nevertheless, the pharmacological relevance of the investigated drug-target interaction remains to be proven [175, 176]. One reason might be the potential artificiality of the used screening assays resulting from e.g. heterologous protein expression or irrelevance of the used substrates. Moreover, initial target validation mainly relies on studies with knockout mutants. As discussed in detail in section 4.2.3, a genetic knockout might affect the system biology in a different way from a temporally and quantitatively tunable pharmacological intervention [177] and thus might not necessarily reflect the pharmacological relevance of a target [175]. Moreover, microbe-specific PK issues might restrict the availability of a drug at the target site and thus the expected pharmacological effect (see section 1.2 and ref. [2]). Consequently, it is advisable to check the *in cellulo* activity of the developed QSIs in the target pathogen *P. aeruginosa* as early as possible.

4.1.2.1 *P. aeruginosa* Reporter Gene Assay

For evaluation whether low efficacy of a drug in the target pathogen is due to pharmacodynamic or PK issues, it is desirable to have an assay system that can distinguish between both factors. Therefore, for evaluation of the PqsR antagonists, we used a *P. aeruginosa*-based ß-galactosidase reporter gene assay that functioned analogously to the *E. coli*-based counterpart. This assay was based on a *P. aeruginosa pqsA* mutant devoid of the

intrinsic *pqs* QS network [119] and was comparably sensitive to stimulation by PQS. Consequently, the bioactivity monitored in this assay should be a function of *P. aeruginosa*-specific pharmacokinetics. This provided the basis for the investigations undertaken in publication C [39].

Recently, the highly potent PqsR antagonist **C1** (IC_{50} of 51 nM in the *E. coli* assay) had shown an unexpectedly low activity in a *P. aeruginosa* virulence assay (44% inhibition of pyocyanin formation at 15 µM) [160]. To explain the result, we tested the compound in the *P. aeruginosa*-based reporter gene assay for the above-mentioned reasons. Therein, the compound displayed about 200-fold reduced PqsR antagonistic activity (60% inhibition at 10 µM) revealing *Pseudomonas*-specific PK issues as reason for the low anti-virulence activity. Moreover, **C1** showed dose-dependent agonism in the *P. aeruginosa* reporter gene assay in the absence of PQS. Since **C1** exhibited pure antagonism in *E. coli*, partial agonism could be excluded. This let us suspect a biotransformation as the responsible PK issue in the target pathogen. This hypothesis was confirmed by rationality-guided systematic investigations as discussed in detail in publication C [39]. The biotransformation was accompanied by a functional inversion catalyzed by PqsH. This was the reason for the low anti-virulence efficacy of **C1** in *P. aeruginosa*. Based on this knowledge, **C1** could be rescued by blocking its metabolic hotspot. Decisively, the resulting highly potent PqsR antagonist **C3** (IC_{50} of 35 nM) maintained its antagonistic functionality and nanomolar activity (IC_{50} of 404 nM) in *P. aeruginosa*.

Taken together, the synergistic interplay between the two reporter gene assays had provided the incentive for the metabolism studies for **C1** and thus contributed to the rescue of the HHQ-derived class of PqsR antagonists.

4.1.2.2 Effects on Signal Molecule Production

According to the current model of *pqs* QS, the targets PqsD and PqsR are essential for the biosynthesis of the *pqs* QS signal molecules HHQ and PQS [102]. Therefore, we investigated the effects of the PqsD inhibitors and PqsR antagonists on signal production *in cellulo*. Although HHQ can be produced by PqsD *in vitro*, the current model of biosynthesis suggests 2-ABA-CoA as its direct product [97, 98]. Since this is unstable and readily degrades to DHQ, we also included DHQ in the analysis as the most direct read-out for PqsD activity *in cellulo*. For this purpose, assays were developed to directly quantify HHQ, PQS, and DHQ levels in the supernatant of *P. aeruginosa* PA14 cultures. The PA14 strain was chosen since it is a well-studied [178], highly virulent, and clinically relevant isolate [179]. Its broad host promiscuity promises the use of a large variety of *in vivo* infection models to study the effects

of QSIs [179]. As reliable analytical method, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was chosen enabling selective detection and quantification of the metabolites [163]. The use of deuterated internal standards promised a ready adaptation of HHQ and PQS analysis to other relevant matrices [180] such as biofilms [109] or sputum of CF patients [181]. Applicability in routine inhibitor testing was facilitated by 24-well format of the bioassay, simple sample work-up, and time-efficient analytical method. However, during routine application of LC-MS/MS, similar problems regarding PQS quantification arose as described by Ortori et al. [182]: poor peak shapes resulting in unquantifiable peaks and irreproducible results. Thus, the aim of publication B [163] was to develop a reliable analytical procedure for accurate and precise quantification of PQS in *P. aeruginosa* cultures. Since the hydroxy group in 3-position of PQS was discussed to be the reason for its poor chromatographic properties [131, 182], we applied chemical derivatization to overcome this problem [183, 184]. The developed microwave-assisted acetylation procedure was fast, reproducible, and allowed full removal of the reagents avoiding contamination of the LC-MS/MS system in contrast to the use of non-volatile EDTA or citric acid described by others [182, 185]. Decisively, the chromatographic behavior of acetylated PQS was greatly improved and led to reproducible quantification results. Fulfillment of the criteria of a validated method according to international guidelines [186] finally demonstrated the reliability of the developed bioanalytical procedure. Based on the full calibration results, time- and cost-efficient two-point calibration was shown to be sufficient. In summary, publication B [163] provided the basis for *in cellulo* characterization of the developed QSIs regarding inhibition of PQS biosynthesis.

PqsD inhibitor **A16** as well as PqsR antagonist **C3** significantly inhibited HHQ and PQS production *in cellulo* in accordance with the current model of *pqs* QS biosynthesis. This supported the physiological relevance of the drug-target interplay monitored in the initial assays [175]. Moreover, since PqsD and PqsR are intracellular targets, the results also demonstrated that **A16** and **C3** were able to cross the Gram-negative cell barrier, which could not be taken for granted, as already discussed [2]. The fact that relatively high QSI concentrations were needed to provoke an effect in *P. aeruginosa* was not surprising considering the complexity of the *in cellulo* system. Besides PK challenges, higher substrate (ACoA) and ligand (PQS) concentrations are encountered *in cellulo* due to the autoinductive loop that might compete with the QSIs for their binding site on PqsD and PqsR, respectively [159]. For PqsR antagonists, the sensitivity of the wild-type system is about 100-fold reduced compared to the reporter system in the *pqsA* mutant (IC_{50} of ~ 40 µM versus ~ 400 nM). The

discrepancy corresponds to differences in ligand concentrations (5-50 µM versus 50 nM PQS) [126, 163, 187]. This implies that PqsR antagonists require at least a single-digit nanomolar target affinity to exert a satisfying effect on signal synthesis. Similarly, higher ACoA concentrations [152, 163] might explain the decreased sensitivity of the cellular system to PqsD inhibitors (IC_{50} of ~ 300 µM versus ~ 3 µM). The PqsA substrate 2-amino-6-fluorobenzoate exhibited a similarly high IC_{50} (109 µM) towards PQS production [117, 163]. In accordance with the findings from publication C [39], the PqsR antagonists un-substituted in 3-position did not significantly inhibit PQS production irrespectively of their antagonistic potency.

In contrast, the production of DHQ was strongly enhanced in the presence of the PqsD inhibitor **A16**. This finding was in accordance with the former hypothesis that PqsA was sufficient for DHQ production [89]. However, according to the revised model of *pqs* QS biosynthesis [97], inhibition of PqsD should lead to decreased DHQ production. The same scenario of increased DHQ levels was observed for PqsR antagonist **C3** and a structurally distinct PqsR antagonist (unpublished data). Possible explanations for this phenomenon and its implications on target validity will be discussed in detail in section 4.2.

4.1.2.3 Effects on Virulence Factor Pyocyanin

According to the current model of the *pqs* QS system, reduction of signal molecule production should lead to attenuated acute virulence, i.e. reduced formation of virulence factors [92, 102]. Consequently, the next step was to prove this link by pharmacological intervention to evaluate *pqs* QS as target for anti-virulence therapy. Although *P. aeruginosa* produces a large arsenal of virulence determinants, we decided to focus on one relevant, representative virulence factor to facilitate straightforward compound evaluation. This should be under the control of the major *pqs* QS response pathway [99, 100], efficiently influenced by *pqs* QS [109], and easily quantifiable for future routine testing [115]. Therefore, we developed an assay assessing the effect of QSIs on virulence factor pyocyanin [54, 55, 100]. The reliability of the developed assay was confirmed by comparable IC_{50} values (unpublished data) with those published for the PqsR antagonists 3-NH_2-7-Cl-C9-QZN and M64 [64, 161].

As expected, the PqsR antagonists inhibiting HHQ/PQS production also reduced pyocyanin formation. As observed for HHQ/PQS inhibition, micromolar inhibitor concentrations as well as substitution in 3-position were needed for efficient inhibition. The most potent PqsR antagonists **C3** and **D16** were also most efficient in inhibiting pyocyanin formation with IC_{50} values of 2 µM and 4 µM, respectively. Importantly, as pyocyanin is representative of PqsE-dependent virulence, these results implied that PqsR antagonists might also repress the

production of further virulence factors such as lectin A, elastase, or rhamnolipids [100, 103, 109]. In contrast, PqsD inhibitor **A16** did not change pyocyanin levels at a concentration sufficient to inhibit HHQ and PQS levels by around 50%. The reasons and impact on target suitability will be discussed comprehensively in section 4.2.

4.1.2.4 Effects on Biofilm Formation

According to the actual concept, the *pqs* QS system is involved in biofilm formation [147]. Since AQs, especially PQS, have been shown to promote biofilm formation (see section 1.5.2 and refs. [99, 109]), we analyzed the effect of PqsD inhibitor **A16** on this phenotype. In the corresponding biofilm formation assay, **A16** was added to a 24 h-grown biofilm and the biovolume was quantified after further 24 h using live-dead (DNA) staining and confocal laser scanning microscopy [188].

The PqsD inhibitor **A16** reduced the biovolume of a *P. aeruginosa* PA14 biofilm. This is in accordance with the current model of *pqs* QS and with the results obtained with a *pqsA* mutant in the same biofilm assay [145]. The fact, that a rather high concentration (~ twofold IC_{50} towards HHQ/PQS inhibition) was needed to observe an effect is not surprising, given the higher complexity and resistance of a biofilm system as compared to planktonic cultures [28]. However, a final assessment of the effect (38% inhibition at 500 µM) was difficult since no quantitative information was available for a *pqsD* mutant, other AQ biosynthesis mutants, or any *pqs* QSI [161]. Moreover, comparison to any biofilm inhibitors is complicated due to low comparability of biofilm assay results obtained in different laboratories. One the one side, different assay protocols are used, which is especially critical given the high sensitivity of biofilm formation towards assay parameters [189, 190]. For example, the conditions applied in the present assay produce biofilms lacking the typical mushroom-shaped structures [112, 145] making an assessment of the PqsD inhibitor regarding this feature impossible. On the other side, varying read-outs (e.g. biovolume, biofilm thickness) or staining dyes might impact the outcome and impede a comparison of the results. For example, using DNA stains allows detection of eDNA [188], but not of other *pqs* QS-controlled biofilm components such as lectins [52]. This high adaptability of *in vitro* biofilm results generally questions the *in vivo* relevance of results obtained in suchlike *in vitro* biofilm assays [190]. Thus, the PqsD inhibitor should be further evaluated in appropriate *in vivo* biofilm models [147] to confirm the relevance of the observed effect.

4.1.2.5 Effects on Bacterial Growth

For exclusion of antimicrobial activity as reason for the observed *in cellulo* effects not desired for anti-virulence compounds [27], their toxicity against *P. aeruginosa* had to be analyzed. This was especially important for the QSIs developed during this thesis. First, the PqsD inhibitors might also inhibit the structurally related antimicrobial target FabH [191]. Second, the PqsR antagonists were derived from AQs, some of which showed antibiotic activity [138, 192, 193]. Therefore, an end point measurement of the optical density at 600 nm as a function of bacterial growth was regularly performed within each assay. However, one should not solely rely on end point measurements since they cannot detect growth delay [194]. Assessment of growth kinetics is one of the most sensitive methods to characterize a compound's toxicity against bacteria [194]. Therefore, *P. aeruginosa* PA14 growth curves were measured in the presence of the highest compound concentrations used in *P. aeruginosa* bioassays.

According to the anti-pathogenicity concept, neither the PqsD inhibitor **A16** nor the PqsR antagonists **C3** and **D16** affected the growth of *P. aeruginosa* under standard assay conditions. These results gave a hint on the selectivity of the PqsD inhibitor towards FabH. Moreover, the same results were obtained for PqsR antagonist **C3** intended to be used *in vivo*, when tested in minimal medium mimicking the nutrient-limited conditions encountered *in vivo* [194].

4.1.3 *In vivo* Validation of QSIs

The encouraging *in cellulo* results obtained with the PqsR antagonist **C3** prompted us to expand the test system from *in cellulo* to *in vivo* to provide the POC for PqsR-targeting anti-infective therapy. Due to the favorable anti-virulence properties of **C3**, we decided on models for acute infection as test systems. For provision of the POC at this stage of drug development, it was reasonable to choose models with reduced risk of PK issues, easy handling, facile read-out, rapid result generation, and independency of ethical considerations. Thus, we opted for invertebrate models [195].

4.1.3.1 *Caenorhabditis elegans* Fast-Killing Assay

The nematode *Caenorhabditis elegans* is susceptible to killing by *P. aeruginosa*, which is mediated via different mechanisms dependent on the assay conditions [196, 197]. The *C. elegans* fast-killing assay was chosen for several reasons. First, it has been associated with phenazine-mediated virulence by *P. aeruginosa* PA14 [196]. Second, host-related PK issues are irrelevant since killing is mediated by diffusible toxins rather than by infectious processes

[196]. Third, different *pqs* QS mutants of another strain, PAO1, including a *pqsR* mutant displayed reduced pathogenicity in this assay [102].

Strikingly, 94% of *C. elegans* worms survived in the presence of *P. aeruginosa* PA14 on agar plates containing 15 µM of **C3**, whereas 53% of the worms died in the absence of **C3** within 6 h. The results showed that a PqsR antagonist is able to protect *C. elegans* from *P. aeruginosa*-mediated killing, which was in accordance with the reduced pyocyanin production by **C3** and the reduced pathogenicity of a *pqsR* mutant in this assay [102]. Given a relative survival rate of 88% in presence of a *pqsR* mutant [102], this was an impressive effect. However, since drug action occurs only *in vitro* and simply the *in vivo* consequences for the host (host-virulence factor interactions) are monitored, this assay is to be regarded as a predictive pre-test rather than a real *in vivo* model.

4.1.3.2 *Galleria mellonella* Infection Model

For the reasons discussed above, we went on with the more advanced *in vivo* infection model based on *Galleria mellonella* allowing the administration of defined doses of bacteria and drug [195]. Importantly, a significant positive correlation has been found between the virulence of *P. aeruginosa* PA14 mutants in *G. mellonella* and mice. This attributed to this model an excellent predictive power for pathogenicity in mammalian infections [198]. Moreover, a phenazine mutant displayed reduced pathogenicity in *G. mellonella* [198].

Interestingly, 93% of the *G. mellonella* larvae survived an infection with *P. aeruginosa* PA14 when treated with the PqsR antagonist **C3**, while 64% of the non-treated larvae died within the first 24 h. This was in accordance with the results obtained with the *C. elegans* assay. Administration of half of the dose yielded a survival rate of 67% demonstrating dose-dependency of the observed effect. Assuming a distribution volume of 450 µL in the hemolymph of a larva, the final concentration of **C3** in a larva was only 22 nM. This is equivalent to a dose of 7 ng/g body weight classifying **C3** as unusually potent drug (≤ 100 ng/g) according to Lipinski et al. [199]. This is also an unexpectedly low concentration given an IC_{50} towards pyocyanin of 2 µM and the complexity of the host system. Several reasons might account for that. First, additional relevant virulence factors might be hit by the PqsR antagonist. Second, fewer colony-forming units (CFUs) were injected into *Galleria* (2-10 CFUs per larva) than encountered in the pyocyanin assay (~ 10^5 CFUs), which was necessary due to the high sensitivity of the larvae towards *P. aeruginosa* PA14 (one bacterium is the estimated 50% lethal dose) [198]. Third, bacteria and drug were co-administered to avoid double injections implicating a short pre-incubation period. Overall, these results proved for the first time the concept of anti-virulence therapy targeting PqsR. Meanwhile, these findings

have been confirmed in mammalian models of acute infection using a different PqsR-targeting QSI [64].

Noteworthy, the survival rate of *P. aeruginosa* PA14-infected larvae treated with **C3** was higher than that of larvae infected with the *pqs* QS-deficient mutants *pqsA* and *pqsR*. A growth inhibitory effect of the compound was excluded before (see section 4.1.2.5). Furthermore, the QS mutants and the wild-type exhibited comparable *in vitro* growth rates [99, 200]. Therefore, we discussed the following possible explanation: 'deletion' of a QS function on the genetic level might irreversibly paralyze the respective QS system disposing the bacterium to bypass the lost QS function [175], as encountered in *lasR* mutants [144]. In contrast, 'disruption' with a small molecule might modulate QS activity reversibly avoiding an induction of a bypassing mechanism [177], which seemed to be advantageous.

4.2 PqsD and PqsR - Valid Targets for Anti-infective Therapy?

One goal of this thesis was to evaluate the potential of PqsD and PqsR as targets for anti-infective therapy of *P. aeruginosa* infections, i.e. acute, chronic and persistent infections (see section 1.4).

4.2.1 Acute Infections

Using the PqsR antagonist **C3**, we proved for the first time that pharmacological interference with PqsR led to reduced mortality in acute *in vivo* infection models by selective attenuation of *P. aeruginosa* virulence without influence on growth. This validated PqsR as anti-virulence target for combating acute infections. Successful therapy of such infections with a different PqsR antagonist in mammalian *in vivo* models meanwhile confirmed these results [64].

During evaluation of PqsD as anti-virulence target, several problems occurred. Against expectation, the PqsD inhibitor **A16** provoked a strong increase in levels of DHQ [97], a potential pathogenicity factor [119]. One the one side, a bypass mechanism to rescue PqsD activity is a thinkable reason, as it is not unusual for *P. aeruginosa* to compensate lost virulence functions [109, 144, 201]. On the other side, the rather low *in vitro-in cellulo* correlation of the **A16**-derived compound class [155] and the increased DHQ levels observed for a putative PqsB/C inhibitor [64] or a *pqsE* mutant [98] might suggest that **A16** affected additional targets in *cellulo*. However, PqsR antagonist **C3** fully attenuated *P. aeruginosa* virulence in acute infection despite increased DHQ levels. This argued against a relevant contribution of DHQ to pathogenesis of such infections. Accordingly, a DHQ-deficient *pqsA* mutant [119] and a DHQ-overproducing *pqsE* mutant [98] exhibited comparable virulence attenuation in acute infection models [92, 100].

Unexpectedly, the PqsD inhibitor **A16** did not influence pyocyanin levels at a concentration sufficient to significantly inhibit HHQ and PQS production. This was surprising regarding the lack of pyocyanin production in a *pqsD* mutant and mutants of other AQ biosynthetic enzymes [102]. However, poor effects on pyocyanin levels have also been observed for other AQ biosynthesis inhibitors including PqsA substrates [152], a putative PqsB/C inhibitor [64], and inhibitors of unknown target [64]. Thereby, over 90% inhibition of PQS production was required to affect and complete inhibition to abolish pyocyanin formation [152]. This showed that traces of signal molecules are sufficient to activate transcription of the *pqsE* gene, i.e. to enable pyocyanin production. Overall, these findings do not basically preclude PqsD or other AQ biosynthetic enzymes as anti-virulence targets, but make high demands on their inhibitors, i.e. to quasi knock-out the target chemically.

In contrast, PqsR antagonist **C3** inhibited pyocyanin production even at concentrations, at which HHQ/PQS production was only moderately reduced. The same was observed for structurally different PqsR antagonists developed by us [158, 159] and others [64]. This implies that PqsR, in contrast to PqsD, might act on pyocyanin via an additional mechanism besides driving *pqsE* transcription [99].

Overall, the findings from this thesis suggest PqsR as more suitable anti-virulence target than PqsD. This might be supported by the observation that the most efficient *pqs* QSIs identified in a phenotypic screening were such targeting PqsR rather than such interfering with AQ biosynthetic enzymes [64].

4.2.2 Chronic and Persistent Infections

Chronic *P. aeruginosa* infections are associated with biofilm formation. The PqsD inhibitor **A16** was able to reduce the biovolume of a *P. aeruginosa* biofilm by 38% at approximately twice its IC_{50} for HHQ/PQS production. Although at this concentration, the PqsE pathway as represented by pyocyanin was not affected (unpublished data), this result corresponded to the observation that biofilm formation required also primarily AQ-dependent processes [99], as described in section 1.5.2. Due to its low aqueous solubility, efficacy of PqsR antagonist **C3** could not be demonstrated in the same biofilm assay (unpublished data). However, another more soluble member of the same structural class was able to reduce biofilm formation [162]. So did another quinazoline-derived PqsR antagonist reported by Ilangovan et al. [161]. Overall, these finding show, that both, PqsD and PqsR, are suitable anti-biofilm targets independently on the strict requirements for anti-virulence efficacy. However, their validity for therapy of chronic *P. aeruginosa* infections remains to be finally proven using suitable *in*

vivo models for reasons discussed in section 4.1.2.4. Such investigations are currently on-going.

Recently, PqsR antagonists have been shown to reduce persister cell formation in a murine persistence model validating PqsR as anti-persistence target [64]. Since the pro-persistent properties of PqsR have been related to a side-product of AQ biosynthesis, namely 2-AA, the biosynthetic enzyme PqsD should be a suitable anti-persistence target as well. The final POC *in cellulo* and *in vivo*, however, remains to be provided.

Taken together, the findings from this thesis and recent literature discussed above argue for PqsR as more favorable drug target than PqsD due to efficacy in a broader spectrum of clinically relevant infections. Nevertheless, PqsD has chances as drug target for chronic/persistent infections. Moreover, combination therapy with PqsR antagonists and PqsD inhibitors might bear great potential for efficient therapy due to synergistic interruption of the auto-inductive loop and due to reduced risk of resistance development [202]. Perfection of this concept might be achieved with dual target (PqsD-PqsR) inhibitors promising reduced probability of drug-drug interactions and improved compliance during long-term therapy of chronic infections [203].

4.2.3 Target Validation with Small Molecules vs. Mutants

Mostly, initial target validation relies on bacterial mutant studies. However, a suchlike target validation might not necessarily reflect the suitability of a target for pharmacological intervention and therapy [175]. The results from this thesis confirm this. First, mutant analyses might not judge the sensitivity of a certain phenotype towards target inhibition, which is possible with small molecules whose effect is quantitatively tunable [177]. Accordingly, *pqsD* mutant analyses did not reveal the issues associated with the low sensitivity of the PqsE pathway to AQ biosynthesis inhibition. Second, irreversible knock-out of the target might induce compensatory mechanisms [144], which does not necessarily happen upon reversible target modulation with small molecules [175]. Accordingly, PqsR might not have been identified as anti-virulence target in the *G. mellonella* infection model. Third, complete inactivation of a complex regulatory circuit might fail to dissect individual functions of targets [98]. Accordingly, mutant analyses might have never suggested a role for PqsR in direct control of pyocyanin production. Similarly, *pqsE* mutant studies might have never dissected PqsE regulatory and biosynthetic functions [98]. Fourth, in the future, small molecule inhibitors can be used to study time-dependent scenarios such as prophylactic versus therapeutic drug application, which might not be possible with mutants [177]. Taken together,

these results warn of solely relying on mutant analysis to judge the therapeutic suitability of a target.

4.3 (2-Nitrophenyl)methanols and 6-Nitro-HHQs as QSIs

One aim of this thesis was to contribute to the development of novel potent *pqs* QSIs by their biological evaluation. During this thesis, two classes of *pqs* QSIs were developed: the (2-nitrophenyl)methanols (NPMs) represented by PqsD inhibitor **A16** and the 6-nitro-HHQs with substitution in 3-position (SNHHQs) represented by PqsR antagonist **C3**. In the following section, their potential and drawbacks will be discussed.

Although they do not belong to the first reported class of PqsD inhibitors [122, 165], the NPMs are the first reported cell-active PqsD inhibitors. They constitute the only class of PqsD inhibitors that shows significant inhibition of signal molecules in the *P. aeruginosa* wild-type in contrast to the meanwhile developed more potent *in vitro* PqsD inhibitors [157]. The NPMs might owe this unique feature their low molecular weight and rather low lipophilicity in line with proposed rules for intracellular activity in Gram-negative bacteria [204]. Moreover, as ACoA mimics they can inhibit PqsD *in cellulo* irrespectively of the nature of the second substrate used *in vitro* [97], which might be critical for compounds not interacting with the ACoA binding site [165]. Moreover, the NPMs are the only class of AQ biosynthesis inhibitors, for which an anti-biofilm activity has been described. The biofilm-surrounding matrix is mainly negatively charged [51] e.g. due to eDNA that might capture cationic compounds [146] or repulse negatively charged ones. Thus, the neutral NPMs might have an advantage over charged compounds in penetrating biofilms. Furthermore, the NPMs did not inhibit bacterial growth indicating selectivity over FabH as discussed in section 4.1.2.5. However, increase of DHQ levels might argue for additional targets such as other biosynthetic enzymes (see section 4.2.1).

A major drawback of the NPM inhibitors so far is that none of them has ever reached an inhibition of signal molecule production sufficient to considerably affect pyocyanin production (unpublished data). Given that an approximately tenfold IC_{50} regarding PQS inhibition was necessary for other AQ biosynthesis inhibitors to affect pyocyanin production [152, 163] and that macrophage proliferation was impaired at a twofold IC_{50} of PqsD inhibitor **A16**, its target activity needs to be further improved. Notably, the low sensitivity of the PqsE response pathway towards signal molecule inhibition might be a common hurdle for all AQ biosynthesis inhibitors [152] requiring a knock-out-like inhibition of the target enzymes (see section 4.2.1). However, although the NPMs have turned out to be tight-binding inhibitors characterized by long residence time on the target, this aim could not yet be reached [155].

Nevertheless, the NMPs obey the restrictive ‘rule of three’ established for hit selection in fragment-based discovery and hence bear great potential for activity improvement without losing drug-likeness [205]. This potential is also reflected by high ligand efficiencies [206].

The SNHHQs developed in this thesis belong to the first reported class of PqsR antagonists that exhibited nanomolar target affinity [160]. However, they suffered from low cellular activity due to a functional inversion by an enzyme involved in signal synthesis [39]. This shows that natural ligand-based drug design can be disadvantageous given the high structural similarity to the natural product evolved by nature to fit into the binding pockets of target proteins [125, 207]. Accordingly, AHL-derived QSIs have been found to be substrates of an efflux pump responsible for secretion of AHL signals [57]. Nevertheless, rational biochemistry and medicinal chemistry strategies, as applied in this thesis, can help to overcome such problems and reduce attrition rates. Consequently, this class of PqsR antagonists could be rescued. They even represent the most potent ligand-derived [161] and second-most potent published class of PqsR antagonists regarding antagonistic and anti-pyocyanin activities [64]. Accordingly, the PqsR antagonist **C3** was an excellent anti-virulence agent in an acute infection model. Moreover, it is worth mentioning that the SNHHQs do not interfere with bacterial growth as discussed in section 4.1.2.5. The idea to exploit the immunosuppressive functions of HHQ and PQS therapeutically [208], might also be applicable to the SNHHQs as their derivatives. This might be especially useful in chronic infections associated with destructive chronic inflammation [209]. Notably, during antagonist optimization, agonists were discovered, some of which were even more potent than the natural agonist PQS. Such superagonists might bear some therapeutic potential too [68]. For example, premature activation of QS might enable the immune system to detect the presence of single bacteria before they collectively attack the host [68]. Furthermore, overactivation of the *pqs* QS system has been associated with biofilm dispersal [150] suggesting the superagonists as potential biofilm-dispersing agents.

A major disadvantage of this class of PqsR antagonists was their poor aqueous solubility, which prompted us to optimize this physicochemical property. However, the high lipophilicity of the ligand binding pocket and the sharp structure-functionality relationships might have impeded a suchlike optimization, as discussed in section 4.1.1. Thus, the poor physicochemical properties of this compound class still remain to be optimized. Alternatively, prodrug strategies{Baker, 2004 301 /id} or appropriate formulations, as developed meanwhile [211], might enable a biomedical use of these compounds. Being less demanding regarding aqueous solubility, topical administration might be a possibility to readily use the compounds

for biomedical applications (e.g. in acute lung infections) or provision of the POC in an appropriate murine model [147].

4.4 Outlook

In the following the already discussed perspectives will be summarized:

- Given the strict requirements for anti-virulence efficacy and the better suitability of PqsD as anti-biofilm target, the primary *in cellulo* read-out for PqsD inhibitors should be signal molecule rather than pyocyanin production. In addition, primarily AQ-dependent phenotypes might be investigated such as siderophore production.
- In general, the *in cellulo* effects of the QSIs should be analyzed in relevant (e.g. nutrient- or phosphate-limited) media and in presence of relevant host factors (e.g. antimicrobial peptides) to mimic environmental stress conditions encountered in the host, which might influence QS hierarchy and relevance.
- Time-dependent scenarios such as biofilm formation inhibition (prophylaxis) versus biofilm dispersion (therapy) should be studied in appropriate assay settings, the latter especially with superagonists.
- To validate PqsD as anti-persistence target, the PqsD inhibitors should be evaluated regarding their influence on 2-AA production and persister cell formation.
- Given the comparable effort in performing *C. elegans* and *G. mellonella* assays, the latter should be favored involving infectious process and host-pathogen interactions.
- The POC for anti-biofilm therapy targeting PqsR or PqsD should be provided in appropriate *in vivo* models instead of solely relying on *in vitro* assays.
- The promising PqsR antagonists should be evaluated in more advanced animal models such as murine models for acute lung infection.
- For biomedical application, the novel PqsR antagonists should be further optimized regarding water solubility by medicinal chemistry strategies (e.g. prodrug approaches) or by appropriate formulation (e.g. ultra-small nanoparticles) or, alternatively, used for topical applications (e.g. as an aerosol in lung infections).
- The PqsD inhibitors should be optimized regarding their activity by e.g. fragment growing in order to achieve full inhibition of AQ biosynthesis and thus anti-virulence effects.

5 References

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6 Summary

Innovative, efficient anti-infectives are needed because of increasing antibiotic resistance. Thus, strategies have been proposed interfering with bacterial pathogenicity instead of viability such as inhibition of quorum sensing. This intercellular communication system uses signal molecules to coordinate virulence and biofilm formation. *Pseudomonas aeruginosa* uses unique signal molecules such as 2-heptyl-3-hydroxy-4-(1*H*)-quinolone (PQS). Therefore, compounds should be developed blocking their biosynthesis and reception by inhibiting PqsD and antagonizing PqsR, respectively. In this thesis, novel PqsD inhibitors were studied. The best compound strongly inhibited the production of signal molecules and biofilm without affecting growth. Irreproducibility of routine quantification of PQS in *P. aeruginosa* cultures was overcome by development and validation of a novel LC-MS/MS approach. A functional inversion was identified as reason for ineffectiveness of the first PqsR antagonist in *P. aeruginosa.* Blocking the metabolic hot spot led to a very potent anti-infective fully protecting *Galleria mellonella* larvae from lethal *P. aeruginosa* infection. This was the first proof-of-concept for an anti-infective therapy targeting PqsR. Optimization of the physicochemical properties of the respective compound class resulted in a new compound with improved water solubility and efficient reduction of signal molecules and virulence factor formation.

7 Zusammenfassung

Aufgrund von Antibiotikaresistenzen werden dringend neue Antiinfektiva benötigt, welche idealerweise die Pathogenität der Bakterien reduzieren ohne diese abzutöten, z.B. durch Hemmung von Quorum Sensing. *P. aeruginosa* nutzt dieses Kommunikationssystem zur Koordination von Virulenz und Biofilmbildung unter Verwendung von Signalmolekülen wie 2-Heptyl-3-hydroxy-4-(1*H*)-Chinolon (PQS). Neue Wirkstoffe sollten daher deren Biosynthese oder Wirkung durch Hemmung von PqsD oder PqsR unterbinden. In dieser Arbeit sollten neue PqsD Inhibitoren charakterisiert werden. Der potenteste Inhibitor konnte die Bildung von Signalmolekülen und Biofilm stark reduzieren ohne das Bakterienwachstum zu beeinträchtigen. Die Entwicklung und Validierung eines neuen LC-MS/MS-Verfahrens erlaubte reproduzierbare Routinequantifizierung von PQS. Die schwache Wirksamkeit des ersten PqsR-Antagonisten in *P. aeruginosa* war auf eine Funktionalitätsumkehr zurückzuführen. Diese konnte durch chemische Modifikation vermieden und so schließlich ein potentes Antiinfektivum entwickelt werden, das *Galleria mellonella*-Larven vor tödlichen *P. aeruginosa*-Infektionen schützte. So wurde erstmals gezeigt, dass eine Hemmung von PqsR zur Therapie von Infektionen genutzt werden kann. Optimierung der physikochemischen Eigenschaften der entsprechenden Verbindung lieferte schließlich einen neuen Wirkstoff mit verbesserter Wasserlöslichkeit, der die Bildung von Signalmolekülen und Virulenzfaktoren effizient reduzierte.

8 List of Abbreviations

2-AA	2-aminoacetophenone
2-ABA	2-aminobenzoylacetate
2-ABA-CoA	2-aminobenzoylacetyl-coenzyme A
ACoA	anthraniloyl-coenzyme A
AHL	*N*-acyl-homoserine lactone
AQ	2-alkyl-4-(1*H*)-quinolone
BHL	*N*-butyryl-*L*-homoserine lactone
CF	cystic fibrosis
CFU	colony-forming unit
CoA	coenzyme A
DHQ	2,4-dihydroxyquinoline
EC_{50}	effector concentration to achieve half-maximal degree of effect
eDNA	extracellular DNA
HHQ	2-heptyl-4-(1*H*)-quinolone
IC_{50}	inhibitor concentration to achieve half-maximal degree of inhibition
LC-MS/MS	liquid chromatography-tandem mass spectrometry
NPM	(2-nitrophenyl)methanol
PK	pharmacokinetic
POC	proof-of-concept
PQS	*Pseudomonas* quinolone signal
QS	quorum sensing
QSI	quorum sensing inhibitor
SNHHQ	6-nitro-HHQ with substitution in 3-position

9 Appendix

9.1 List of Publications

1. Storz MP, **Maurer CK**, Zimmer C, Wagner N, Brengel C, de Jong JC, Lucas S, Musken M, Haussler S, Steinbach A, Hartmann RW (2012) Validation of PqsD as an anti-biofilm target in Pseudomonas aeruginosa by development of small-molecule inhibitors. J Am Chem Soc 134:16143-16146
2. Lu C, Kirsch B, Zimmer C, de Jong JC, Henn C, **Maurer CK**, Musken M, Haussler S, Steinbach A, Hartmann RW (2012) Discovery of antagonists of PqsR, a key player in 2-alkyl-4-quinolone-dependent quorum sensing in Pseudomonas aeruginosa. Chem Biol 19:381-390
3. Klein T, Henn C, de Jong JC, Zimmer C, Kirsch B, **Maurer CK**, Pistorius D, Muller R, Steinbach A, Hartmann RW (2012) Identification of small-molecule antagonists of the Pseudomonas aeruginosa transcriptional regulator PqsR: biophysically guided hit discovery and optimization. ACS Chem Biol 7:1496-1501
4. Haupenthal J, Husecken K, Negri M, **Maurer CK**, Hartmann RW (2012) Influence of DNA template choice on transcription and inhibition of Escherichia coli RNA polymerase. Antimicrob Agents Chemother 56:4536-4539
5. Steinbach A, **Maurer CK**, Weidel E, Henn C, Brengel C, Hartmann RW, Negri M (2013) Molecular basis of HHQ biosynthesis: molecular dynamics simulations, enzyme kinetic and surface plasmon resonance studies. BMC Biophys 6:10
6. **Maurer CK**, Steinbach A, Hartmann RW (2013) Development and validation of a UHPLC-MS/MS procedure for quantification of the Pseudomonas Quinolone Signal in bacterial culture after acetylation for characterization of new quorum sensing inhibitors. J Pharm Biomed Anal 86:127-134

7. Zender M, Klein T, Henn C, Kirsch B, **Maurer CK**, Kail D, Ritter C, Dolezal O, Steinbach A, Hartmann RW (2013) Discovery and biophysical characterization of 2-amino-oxadiazoles as novel antagonists of PqsR, an important regulator of Pseudomonas aeruginosa virulence. J Med Chem 56:6761-6774
8. Lu C, **Maurer CK**, Kirsch B, Steinbach A, Hartmann RW (2014) Overcoming the unexpected functional inversion of a PqsR antagonist in Pseudomonas aeruginosa: an in vivo potent antivirulence agent targeting pqs quorum sensing. Angew Chem Int Ed Engl 53:1109-1112
9. Nafee N, Husari A, **Maurer CK**, Lu C, de RC, Steinbach A, Hartmann RW, Lehr CM, Schneider M (2014) Antibiotic-free nanotherapeutics: ultra-small, mucus-penetrating solid lipid nanoparticles enhance the pulmonary delivery and anti-virulence efficacy of novel quorum sensing inhibitors. J Control Release 192:131-140
10. Lu C, Kirsch B, **Maurer CK**, de Jong JC, Braunshausen A, Steinbach A, Hartmann RW (2014) Optimization of anti-virulence PqsR antagonists regarding aqueous solubility and biological properties resulting in new insights in structure-activity relationships. Eur J Med Chem 79:173-183
11. Hartmann RW, Steinbach A, Lu C, **Maurer CK**, Kirsch B, Haussler S, Musken M (2014) Prioritätsbegründende internationale PCT-Anmeldung PCT/EP2014/000893 "PqsR modulators", Anmeldetag 3.4.2014, Anmelder: Helmholtz-Zentrums für Infektionsforschung, Braunschweig
12. **Maurer CK**, Lu C, Empting M, Hartmann RW (2015) Synthetic quorum sensing inhibitors (QSIs) blocking receptor signaling or signal molecule biosynthesis in *P. aeruginosa*. In: Kalia, V. C. Quorum sensing vs quorum quenching: a battle with no end in sight. Springer India, New Delhi, 303-317

9.2 Conference Contributions

Poster presentations:

Henn C, Negri M, **Maurer CK**, Wagner N, Steinbach A, Hartmann RW (2011) Disclosing the kinetic mechanism of PqsD by SPR, enzyme kinetic analysis and MD simulations. 1st International HIPS Symposium, June 2011, Saarbrücken, Germany

Maurer CK, Storz MP, de Jong JC, Weidel E, Zimmer C, Steinbach A, Hartmann RW (2012) Anti-infectives with novel mode of action: Interruption of *P. aeruginosa* cell-to-cell communication by PqsD inhibitors. DPhG Jahrestagung 2012, October 2012, Greifswald, Germany

Lu C, **Maurer CK**, Kirsch B, Steinbach A, Hartmann RW (2013) A Rescue Missin in Drug Discovery: Rebirth of an Ineffective PqsR Antagonist as an *in vivo* Higly Potent Anti-virulence Agent. 3rd International HIPS Symposium, July 2013, Saarbrücken, Germany

Maurer CK, Storz MP, Brengel C, Weidel E, Zimmer C, Müsken M, Häussler S, Steinbach A, Hartmann RW (2013) Validation of PqsD as anti-biofilm target in *Pseudomonas aeruginosa*. EUROBIOFILMS 2013 Third European Congress on Microbial Biofilms - Basic and Clinical Aspects, September 2013, Ghent, Belgium

Oral presentation:

Maurer CK, Lu C, Zender M, Kirsch B, Steinbach A, Hartmann RW (2014) Discovery of antagonists of the *Pseudomonas* quinolone signal receptor as promising anti-infectives. 4th International HIPS Symposium, July 2014, Saarbrücken, Germany

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