In-depth Profiling of MvfR-Regulated Small Molecules in Pseudomonas aeruginosa after Quorum Sensing Inhibitor Treatment

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In-depth Profiling of MvfR-Regulated Small Molecules in *Pseudomonas aeruginosa* after Quorum Sensing Inhibitor Treatment

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*Pseudomonas aeruginosa* is a Gram-negative bacterium, which causes opportunistic infections in immuno-compromised individuals. Due to its multiple resistances toward antibiotics, the development of new drugs is required. Interfering with Quorum Sensing (QS), a cell-to-cell communication system, has shown to be highly efficient in reducing *P. aeruginosa* pathogenicity. One of its QS systems employs *Pseudomonas Quinolone Signal* (PQS) and 4-hydroxy-2-heptylquinoline (HHQ) as signal molecules. Both activate the transcriptional regulator MvfR (Multiple Virulence Factor Regulator), also called PqsR, driving the production of QS molecules as well as toxins and biofilm formation. The aim of this work was to elucidate the effects of QS inhibitors (QSIs), such as MvfR antagonists and PqsBC inhibitors, on the biosynthesis of the MvfR-regulated small molecules 2′-aminoacetophenone (2-AA), dihydroxyquinoline (DHO), HHQ, PQS, and 4-hydroxy-2-heptylquinoline-N-oxide (HQNO). The employed synthetic MvfR antagonist fully inhibited pqs small molecule formation showing expected sigmoidal dose-response curves for 2-AA, HQNO, HHQ and PQS. Surprisingly, DHQ levels were enhanced at lower antagonist concentrations followed by a full suppression at higher QSI amounts. This particular bi-phasic profile hinted at the accumulation of a biosynthetic intermediate resulting in the observed overproduction of the shunt product DHO. Additionally, investigations on PqsBC inhibitors showed a reduction of MvfR natural ligands, while increased 2-AA, DHQ and HQNO levels compared to the untreated cells were detected. Moreover, PqsBC inhibitors did not show any significant effect in PA14 pqsC mutant demonstrating their target selectivity. As 2-AA is important for antibacterial tolerance, the QSIs were evaluated in their capability to attenuate persistence. Indeed, persister cells were reduced along with 2-AA inhibition resulting from MvfR antagonism, but not from PqsBC inhibition. In conclusion, antagonizing MvfR using a dosage capable of fully suppressing this QS system will lead to a favorable therapeutic outcome as DHQ overproduction is avoided and bacterial persistence is reduced.

Keywords: *Pseudomonas aeruginosa*, quinolones, 2′-aminoacetophenone, dihydroxyquinoline, persistence, Quorum Sensing Inhibitors, MvfR
INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium able to cause severe chronic infections in immuno-compromised patients, for example in people affected by cystic fibrosis (Gómez and Prince, 2007) or thermally injured individuals (Tredget et al., 2004). The eradication of this pathogen with antibiotic treatments is becoming more and more difficult because of its intrinsic and acquired resistance (Hancock and Speert, 2000; Aloush et al., 2006) and tolerance (Mulcahy et al., 2010) toward these drugs. A new promising strategy for treating P. aeruginosa infections is blocking its pathogenicity without killing the bacteria targeting a cell-to-cell communication system called Quorum Sensing (QS) (Hurley et al., 2012).

This bacterium employs four QS systems interconnected to each other, namely las, iqs, pqs, and rhl, for regulating the expression of several toxins needed for adjusting its metabolism and virulence during the course of infection (Lee and Zhang, 2015). The pqs QS system is selectively expressed by P. aeruginosa and utilizes the signal molecule Pseudomonas Quinolone Signal (PQS) and its precursor 4-hydroxy-2-heptylquinoline (HHQ) for activating the transcriptional regulator MvfR (Multiple Virulence Factor Regulator), also called PqsR. This protein induces the production of different toxins, such as lectins, pyocyanin, and hydrogen cyanide. It also regulates the expression of enzymes needed for the biosynthesis of its natural ligands encoded by the pqsABCDE operon (Xiao et al., 2006) and has been shown to be essential for persister cells development (Que et al., 2013).

Briefly, the synthesis of HHQ and PQS starts with the conversion of anthranilic acid (AA) into its Coenzyme A (CoA) thioester derivative by the action of CoA-ligase PqsA. The activated molecule is then condensed with malonyl-CoA by PqsD leading to the formation of 2′-aminobenzoylethyl-CoA (2-ABA-CoA), which is subsequently hydrolyzed by the thioesterase PqsE or TesB into 2′-aminobenzoylacetate (2-ABA) (Dulcey et al., 2013; Drees and Fetzner, 2015). This reactive intermediate is transformed into HHQ by the heterodimer PqsBC bearing an octanoyl chain (Dulcey et al., 2013). Finally, PqsH oxidizes HHQ into PQS (Schartzer et al., 2010) (Figure 1).

Furthermore, 2-ABA-CoA and 2-ABA are intermediates for the biosynthesis of other important metabolites. Actually, both compounds can cyclize leading to the formation of dihydroxyquinoline (DHQ), which has been shown to be fundamental in P. aeruginosa pathogenicity (Gruber et al., 2016), and in reducing the growth of epithelial cells (Zhang et al., 2008). Moreover, after decarboxylation, 2-ABA is converted into 2′-aminoacetophenone (2-AA), an important signal molecule that coordinates the transition from acute to chronic infection and the development of persister cells (Kesarwani et al., 2011; Que et al., 2013). In addition, 2-ABA could be converted into its hydroxylamine form by the oxidase PqsL and, then, transformed into 4-hydroxy-2-heptylquinoline-N-oxide (HQNO) by the complex octanoyl-PqsBC (Dulcey et al., 2013). HQNO is essential for biofilm formation because it favors extracellular DNA release by programmed cell lyses of the bacteria (Hazan et al., 2016).

Among the potential targets for blocking the pqs system, we herein discuss the transcriptional regulator MvfR and the biosynthetic enzyme PqsBC. So far, a number of MvfR antagonists and PqsBC inhibitors have been developed that efficiently reduced HHQ and PQS production in P. aeruginosa (Zender et al., 2013; Lu et al., 2014a; Starkey et al., 2014). The aim of this work was to gather detailed information about the effects of these QS Inhibitors (QSIs) on the production of MvfR-related small molecules including 2-AA, DHQ, HQNO, HHQ, and PQS. Furthermore, we monitored the expression of the pqs operon in a time-dependent manner upon treatment with the aforementioned QSIs.

Among the QS molecules, 2-AA was proven to be important in the development of P. aeruginosa persister cells (Que et al., 2013), which are individuals within the bacterial population characterized by having a reduced metabolism (Lewis, 2010). Due to their dormant state, antibiotic efficacy is severely impaired in this bacterial sub-group. Targeting persistence by blocking 2-AA production through QS inhibition was shown to be a promising strategy (Starkey et al., 2014). Consequently, an additional goal of the work was to quantify persister phenotype of P. aeruginosa after treatment with QSIs.

MATERIALS AND METHODS

Chemicals and Growth Media

The benzimidazole 1 and the nitrophenylmethanol 2 (Figure 2) were synthesized as described in literature (Rahme et al., 2012; Storz et al., 2014). d5-HHQ was synthesized following the procedure of HHQ using d5-aniline (Lu et al., 2012). The tricyclic 3 (Figure 2) and amitriptyline were purchased from ChemDiv (United States) and Alfa Aesar (Germany), respectively.

Water (Th. Geyer, Germany), acetonitrile (VWR, Germany) methanol (Sigma–Aldrich, United States) and formic acid (Fluka, United States) were LC-MS grade and used for HPLC-MS/MS experiments.

Yeast extract (Fluka, Germany), sodium chloride (VWR, Germany) and peptone from casein (Merck, Germany) were used for the preparation of Luria Bertani (LB) broth needed for performing the quantification experiments of pqs-related signal molecules. Ready-made mixture of LB broth (Fisher, United States) and Tryptic Soy Broth (TSB) 1% [w/v] (BD, United States) were used for pqsA-GFPASV and persister cells experiments.

Bacterial Strains

P. aeruginosa PA14 and its isogenic transposon mutants pqsA, pqsC, pqsH and pqsE kindly provided by Susanne Häussler (Twincore, Hannover, Germany) were used in the experiments for the quantification of MvfR-related small molecules with and without QSIs. P. aeruginosa PA14, its isogenic pqsBC transposon mutant, and its mvfR single mutant were employed for performing persister cells assays. P. aeruginosa PA14 transformed with pAC37 carrying pqsA-GFPASV and gentamicin resistance cassette was used in pqsA expression experiments. The bacterial strains were maintained at −80°C in 25% [v/v] glycerol stocks.
**FIGURE 1** | Current model of the biosynthetic pathway of MvfR-related small molecules. AA, anthranilic acid; CoASH, Coenzyme A; MCoA, malonyl-CoA; 2-ABA-CoA, 2′-aminobenzoylacetetyl-CoA; 2-ABA, 2′-aminobenzoylacetate; DHQ, dihydroxyquinoline; 2-AA, 2′-aminoacetophenone; 2-HABA, 2′-hydroxylaminobenzoylacetate; HHQ, 4-hydroxy-2-heptylquinoline; HQNO, 4-hydroxy-2-heptylquinoline-N-oxide; PQS, Pseudomonas Quinolone Signal.

**FIGURE 2** | Structures of the compounds evaluated in this work. MvfR antagonist 1, PqsBC inhibitors 2 and 3.

**MvfR-Related Small Molecule Quantification**

*Pseudomonas aeruginosa* strains were cultivated as previously described (Maurer et al., 2013). After letting PA14 strains grow for 6 h in LB, an aliquot of bacterial culture was centrifuged for 10 min at 4835 × g and 25°C using a Rotina 380R Centrifuge (Hettich, Germany). The supernatant was discarded, the pellet was resuspended in fresh LB, and the bacterial cells were spun down using the previous centrifugation settings. After repeating the wash step a second time, the OD$_{600}$ of the washed cells was measured using a BioPhotometer plus Spectrophotometer (Eppendorf, Germany) in order to prepare
TABLE 1 | Effects of the MvfR antagonist 1 on production of MvfR-related small molecules in PA14 wt and PA14 pqsH mutant.

<table>
<thead>
<tr>
<th>Strains</th>
<th>2-AA IC₅₀ [µM]</th>
<th>Maximal DHQ production [%]</th>
<th>HQNO IC₅₀ [µM]</th>
<th>Signal molecules IC₅₀ [µM]</th>
<th>Overall IC₅₀ [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA14 wt</td>
<td>1.3 (1.1-1.2)</td>
<td>330 ± 12 ± 1 µM</td>
<td>1.2 (1.1-1.3)</td>
<td>1.1 (1.0-1.2)</td>
<td>1.2 (1.1-1.3)</td>
</tr>
<tr>
<td>PA14 pqsH mutant</td>
<td>0.19 (0.16-0.22)</td>
<td>304 ± 2 ± 0.2 µM</td>
<td>0.27 (0.25-0.30)</td>
<td>0.27 (0.25-0.30)</td>
<td>0.32 (0.30-0.34)</td>
</tr>
</tbody>
</table>

*a In PA14 wt referred to sum of HHQ and PQS concentrations; in PA14 pqsH mutant referred to HHQ concentration. b 95% Confidence Intervals. c Standard Error of the Mean intervals.

FIGURE 3 | Dose-response curves of MvfR antagonist 1 acting on MvfR-related small molecules production in PA14 wt (A) and PA14 pqsH mutant (B).

TABLE 2 | Effects of MvfR antagonist 1 on 2-AA and DHQ production in PA14 pqsC mutant with and without external addition of PQS.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Exogenous PQS [µM]</th>
<th>2-AA IC₅₀ [µM]</th>
<th>DHQ IC₅₀ [µM]</th>
<th>2-AA + DHQ IC₅₀ [µM]</th>
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<tr>
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<td>0</td>
<td>0.06 (0.04-0.06)</td>
<td>0.03 (0.03-0.04)</td>
<td>0.03 (0.02-0.04)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.33 (0.28-0.38)</td>
<td>0.09 (0.05-0.18)</td>
<td>0.11 (0.07-0.19)</td>
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<td></td>
<td>10</td>
<td>4.2 (3.0-5.8)</td>
<td>1.7 (1.3-2.3)</td>
<td>2.0 (1.4-2.8)</td>
</tr>
</tbody>
</table>

The data are reported with 95% Confidence Interval.
Allegretta et al. Profiling pqs QS Signal Molecules

FIGURE 4 | Dose-response curves of MvfR antagonist on 2-AA (A), DHQ (B) and overall (C) production in PA14 pqsC mutant with and without external addition of PQS. The “x” axes indicate the logarithm of the concentration of the antagonists in molar units (M). The error bars indicate Standard Error of the Mean.

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pqsa Expression Assay

The assays were performed following the protocol previously published (Kesarwani et al., 2011) with few modifications. PA14 wt cells transformed with pAC37 were grown overnight in LB with 60 µg/mL of gentamicin, then an aliquot of bacterial culture was centrifuged for 5 min at 8000g at 25°C. The supernatant was discarded, the pellet was resuspended in fresh LB with the same antibiotic, and the bacterial cells were spun down using the previous centrifugation settings. After repeating the wash step a second time, the OD600nm of the washed cells was measured using a Spectronic Unicam Genesys 10 UV spectrophotometer (Thermo Fisher, United States) in order to prepare a final bacterial suspension with OD600nm = 0.02 in LB with 60 µg/mL of gentamicin. Hundred microliters of the prepared culture was poured in each well of a 96-well plate and the compounds were added in triplicates. The final concentration of DMSO was 1% v/v. The plates were incubated at 37°C under static condition in Infinite F200 plate reader (Tecan Group Ltd, Männedorf, Switzerland) monitoring GFP fluorescence (λex = 485 nm; λem = 535 nm) and OD600nm after a short shaking every 15 min. The assays were repeated at least three times.

Persister Cells Assay

The effects of QSI on persistence were evaluated following the published protocol (Starkey et al., 2014) with some modifications. After streaking the bacteria on LB agar and overnight incubation at 37°C, one colony was dispersed in 5 mL of LB and the bacteria were grown at 37°C up to OD600nm = 0.5. Thirty microliters of the culture were transferred into glass tubes with 5 mL of TSB 1% [w/v] and incubated overnight at 37°C under shaking condition. Then, an aliquot of P. aeruginosa culture was centrifuged for 5 min at 8,000 × g and 25°C using a 5810R Centrifuge (Eppendorf, United States). The supernatant was discarded, the pellet was resuspended in fresh LB, and the bacterial cells were spun down using the previous centrifugation settings. After repeating the wash step a second time, the OD600nm of the washed cells was measured using a Spectronic Unicam Genesys 10 UV spectrophotometer (Thermo Fisher, United States) in order to prepare a final bacterial suspension with OD600nm = 0.02 in 5 mL of TSB 1% [w/v] in each glass tube. The target compounds were added in the tubes and the final concentration of DMSO was 0.5% [v/v]. The bacterial suspension was incubated at 37°C under shaking condition for 4 h. An aliquot of 100 µL of culture from each tube was used for dilution plating on LB agar plates and colony forming units (CFU) quantification (normalizers). Fifty microliters of meropenem 1 mg/mL were added in each tube and the cultures were incubated at 37°C for 24 h under shaking condition. Aliquots of 600 µL of bacterial suspension (244.050→158.944; collision energy: 31 V; tube lens: 100 V), HQNO (260.036→158.908; collision energy: 28 V; tube lens: 110 V), PQS (260.048→174.927; collision energy: 30 V; tube lens: 110 V), d4-HHQ (248.081→162.965; collision energy: 32 V; tube lens: 100 V) and amitriptyline (278.061→232.932; collision energy: 16 V; tube lens: 90 V) employing a scan width of 0.010 m/z, a scan time of 0.100 s, and a peak width of 0.70. Calibration curves were prepared following the same protocol and using PA14 pqsA mutant as matrix without compounds and spiked with known concentrations of analytes and IS after the overnight growth. The assays were repeated at least three times.
RESULTS AND DISCUSSION

The QSIs evaluated in this study were the most potent developed MvfR antagonist \(1\) (Starkey et al., 2014), and the two PqsBC inhibitors \(2\) (Starkey et al., 2014) and \(3\) (this study) shown in Figure 2. As previously published, these compounds were able to inhibit dose-dependently the production of the signal molecules HHQ and PQS in PA14 \(pqsH\) mutant and \(wt\), respectively. However, a complete profiling of all MvfR-related small molecules was not performed using different concentrations of QSIs. To ensure a convenient analytic procedure, we developed an “all-in-one” method for the simultaneous evaluation of these bacterial metabolites. Following the protocol by Lépine et al. (2003) with some optimizations, a single assay for quantification of the relevant \(pqs\)-related small molecules was established allowing medium throughput, easy sample processing, low material consumption, without relevant interference between analytes.

**Effects of MvfR Antagonists on \(pqs\)-Related Small Molecules**

As previously shown (Starkey et al., 2014), the MvfR antagonist \(1\) was able to reduce dose-dependently the production of the alkyl-quinolones (AQs) and 2-AA in PA14 \(wt\) with an IC\(_{50}\) (inhibitor concentration causing half maximum inhibition) of 1.1 \(\mu M\) (Table 1; Figure 3A). Interestingly, the sigmoidal curves of each of these metabolites were very steep with a Hill coefficient over 1 giving the idea of a possible exponential effect of the MvfR natural ligands on the \(pqs\) regulon expression. Moreover, while DHQ production was inhibited at high concentrations, its biosynthesis was enhanced up to 330% after incubation of \(P.\ aeruginosa\) with \(1\) at a concentration close to the compound’ IC\(_{50}\). The basal levels of DHQ were reached at lower doses of the QSI. Nevertheless, \(1\) was able to reduce dose-dependently the overall biosynthesis of these metabolites with an IC\(_{50}\) of 1.2 \(\mu M\).

For confirming this characteristic profile, the compound was also evaluated in PA14 \(pqsH\) mutant strain, which does not convert HHQ into PQS. Notably, in a clinical setting, it has been observed that \(P.\ aeruginosa\) tends to produce much...
TABLE 3 | Production of pqs signal molecules in PA14 wt after the treatment with PqsBC inhibitors.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration [µM]</th>
<th>2-AA [%]</th>
<th>DHQ [%]</th>
<th>HQNO [%]</th>
<th>HHQ + PQS [%]</th>
<th>Overall [%]</th>
</tr>
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<tr>
<td>2</td>
<td>250</td>
<td>188 ± 4</td>
<td>389 ± 33</td>
<td>198 ± 12</td>
<td>34 ± 1</td>
<td>98 ± 8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>152 ± 4</td>
<td>249 ± 15</td>
<td>201 ± 10</td>
<td>57 ± 1</td>
<td>101 ± 5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>111 ± 8</td>
<td>141 ± 31</td>
<td>148 ± 19</td>
<td>81 ± 6</td>
<td>100 ± 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>107 ± 2</td>
<td>115 ± 7</td>
<td>128 ± 1</td>
<td>93 ± 4</td>
<td>102 ± 2</td>
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<tr>
<td>3</td>
<td>10</td>
<td>415 ± 39</td>
<td>654 ± 49</td>
<td>218 ± 16</td>
<td>35 ± 3</td>
<td>136 ± 5</td>
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<tr>
<td></td>
<td>1</td>
<td>134 ± 7</td>
<td>131 ± 12</td>
<td>181 ± 39</td>
<td>86 ± 2</td>
<td>100 ± 5</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>99 ± 4</td>
<td>102 ± 1</td>
<td>103 ± 2</td>
<td>96 ± 3</td>
<td>98 ± 3</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>99 ± 4</td>
<td>100 ± 4</td>
<td>97 ± 2</td>
<td>103 ± 5</td>
<td>99 ± 3</td>
</tr>
</tbody>
</table>

100% is the level of metabolite produced in the untreated PA14 wt. The data are reported with Standard Error of the Mean intervals.

![Figure 7](image-url)  
**FIGURE 7** | Effects of PqsBC inhibitors on *pqs* related signal molecules production in PA14 strains. (A) 2 and 3 in PA14 wt. 2-AA: green. DHQ: orange. PQS + HHQ: gray. HQNO: red. Sum of all anthranilic acid derivatives: black. (B) 2 and 3 in PA14 *pqsH* mutant. 2-AA: green. DHQ: orange. HHQ: gray. HQNO: red. Sum of all anthranilic acid derivatives: black. The error bars indicate Standard Error of the Mean. Statistical analysis performed with one-way ANOVA ($\alpha = 0.05$).

more HHQ than the hydroxylated analog (Que et al., 2011). Our experiments with the PQS-deficient *pqsH* mutant revealed that the QSIIs showed similar profiles on the other *pqs*-related molecules production compared to PA14 wt. Indeed, I efficiently inhibited AQs and 2-AA production displaying an IC$_{50}$ of circa 0.30 µM, and very steep inhibitory curves as in PA14 wt (Table 1; Figure 3B). Likewise, they enhanced DHQ formation up to 300% at AQs IC$_{50}$ concentration. Considering that PQS is more active than HHQ in inducing *pqs* expression (Xiao et al., 2006), it is not surprising that the MvfR antagonists were more potent in repressing the biosynthesis of *pqs*-related signal molecules in PA14 *pqsH* mutant than in wt.

The characteristic profiles in *wt* and *pqsH* mutant of this class of QSIIs, such as the steep inhibitory dose-dependent curves
and the overproduction of DHQ close to the IC$_{50}$ for AQ inhibition, suggest the $pqs$ autoloop as a reason. Actually, PQS and HHQ have high activity toward MvfR in the nanomolar range (Xiao et al., 2006; Lu et al., 2014b) and the actual QS signal is amplified through expression of enzymes, which produce again a multitude of additional MvfR natural ligands. Antagonizing the transcriptional regulator would thus have a higher-order effect on the downstream products resulting from pseudo-cooperative effects. Each decrease in signal molecule synthesis achieved by MvfR antagonism would have an additional impact on MvfR deactivation due to less competing autoinducers. In the concentration range close to the antagonist IC$_{50}$, it would be plausible that the biosynthetic pathway cannot convert the major amount of AA into HHQ and PQS maybe because of a slow kinetic step in the biosynthesis. Considering the low affinity of 2-ABA toward PqsBC (Drees et al., 2016), it is feasible to claim that the slow step is the condensation and cyclization reaction performed by PqsBC. This would lead to an accumulation

### TABLE 4 | Production of $pqs$ signal molecules in PA14 $pqsH$ mutant after the treatment with PqsBC inhibitors.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration [µM]</th>
<th>2-AA [%]</th>
<th>DHQ [%]</th>
<th>HQNO [%]</th>
<th>HHQ [%]</th>
<th>Overall [%]</th>
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<tr>
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<td>250</td>
<td>157 ± 8</td>
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<td>99 ± 1</td>
<td>99 ± 1</td>
<td>95 ± 1</td>
<td>95 ± 1</td>
</tr>
</tbody>
</table>

100% is the level of metabolite produced in the untreated PA14 $pqsH$ mutant. The data are reported with Standard Error of the Mean intervals.

**FIGURE 8** | Effects of PqsBC inhibitors on 2-AA and DHQ production in PA14 $pqsC$ mutant. (A) 2 and (B) 3. 2-AA: green. DHQ: orange. Sum of all anthranilic acid derivatives: black. The error bars indicate Standard Error of the Mean. Statistical analysis performed with one-way ANOVA ($\alpha = 0.05$).
of the reactive intermediate 2-ABA that quickly cyclizes into DHQ.

For confirming these hypotheses, the compound was evaluated in PA14 pqsC mutant, which synthesizes only 2-AA and DHQ, in an experimental setup with and without exogenous addition of the signal molecule PQS. Since these bacteria do not produce any MvfR natural ligands, the pqs autoloop is consequently absent and it was possible to control its expression with external administration of PQS. Exogenous addition of the quinolone at 1 and 10 µM reduced the potency of the QSI on MvfR-related small molecules synthesis of circa one and two orders of magnitude, respectively, and increased the steepness of the inhibitory curves (Table 2; Figure 4). Actually, the IC₅₀ of 1 on 2-AA production worsened from 50 nM (without PQS) over 0.3 µM (with 1 µM PQS) to 4.2 µM (10 µM PQS). In addition, the concentration of compound needed to reduce DHQ production to 50% shifted from 30 nM (without PQS) over 90 nM (1 µM PQS) to 1.7 µM (10 µM PQS). Consequently, the overall effect of the QSI on the production of pqs-related molecules within the PqsADE biosynthetic pipeline present in the pqsC mutant was also affected by PQS administration. Hence, an increase of IC₅₀ from 30 nM (no PQS) over 0.11 µM (1 µM PQS) to 2.0 µM (10 µM PQS) was observed.

These findings confirmed that the presence of PQS in the bacterial culture plays an important role in controlling the biosynthesis of pqs-related molecules. Indeed, when the natural ligand is present, the efficiency of the compounds was strongly reduced as the shifts in IC₅₀ confirmed. In addition, the increased steepness of the inhibitory curves after addition of PQS to the PA14 pqsC mutant culture displayed that, in case of a small reduction in concentration of QSI, the MvfR-related compounds were quickly restored to the basal production level.

Furthermore, the expression levels of pqsA were monitored under MvfR antagonist treatment using the PA14 wt transformed with the plasmid carrying the construct pqsA-GFP₅S (Yang et al., 2007). As previously reported (Kesarwani et al., 2011), the kinetic studies showed that the expression of the pqsA promoter present in the pqsA-GFP₅S plasmid occurred at the exponential phase of bacterial growth as it was analyzed for the genomic pqs operon (Déziel et al., 2004). As formerly evaluated (Starkey et al., 2014), 1 efficiently inhibited the promoter expression displaying an IC₅₀ of 16 nM (Figure 5).

Combining these findings with the results in PA14 wt and pqsH mutant indirectly confirmed that the action of autoinducers HHQ and PQS within this positive feedback loop is the
should be the rate-limiting step in this biosynthetic cascade shown that the condensation reaction mediated by PqsBC takes place (as in the initial accumulation of 2-ABA-CoA rather than 2-ABA.

MvfR-antagonist-induced down-regulation of the operon results in an increased DHQ levels. We corroborated and extended Fetzner (2015) regarding the profile of PQS-related molecules to both increased DHQ and 2-AA levels.

The fact that down-regulation of the pqs operon results in an initial increase in DHQ, while the other investigated components show the expected sigmoidal dose-response curves for 2-AA, HHQ, PQS, and HQNO levels. The initial increase in DHQ, while the other investigated components show the expected sigmoidal dose-response curves for 2-AA, HHQ, PQS, and HQNO levels.

As expected (vide supra), the levels of 2-AA and DHQ strongly increased after the treatment with such inhibitors up to 188 and 389% after incubation with 250 µM of 2 and 415 and 654% with 10 µM of 3. Surprisingly, the synthesis of HQNO was also enhanced up to two times compared to the untreated bacteria. Interestingly, the overall production of the AQ signal molecules was not significantly affected. Reducing the concentration of these QSIs led to a reduced inhibitory activity on HHQ and PQS production and a relapse of 2-AA, DHQ and HQNO to the respective basal levels.

For confirming this characteristic profile, the compounds were also evaluated in PA14 pqsH mutant and similar results were obtained compared to the isogenic wt (Table 4; Figure 7B). Here, 2 and 3 reduced at their highest dosage the formation of HHQ down to 62 and 73%, respectively. Moreover, the production of 2-AA, DHQ, and HQNO was decreased by 157, 581, and 265% after incubation with 250 µM of 2 and 150, 264, and 141% at their highest concentration.

Effects of PqsBC Inhibitors on mvfR-Related Small Molecules

The PqsBC inhibitors 2 and 3 reduced the production of the MvfR natural ligands in PA14 wt down to 34 and 35%, respectively, at their highest concentration (Table 3; Figure 7A). As expected (vide supra), the levels of 2-AA and DHQ strongly increased after the treatment with such inhibitors up to 188 and 389% after incubation with 250 µM of 2 and 415 and 654% with 10 µM of 3. Surprisingly, the synthesis of HQNO was also enhanced up to two times compared to the untreated bacteria. Interestingly, the overall production of the AQ signal molecules was not significantly affected. Reducing the concentration of these QSIs led to a reduced inhibitory activity on HHQ and PQS production and a relapse of 2-AA, DHQ and HQNO to the respective basal levels.

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In addition, as in PA14 wt, the overall amount of the MvfR-related compounds was not affected by the addition of these QSIs. Additionally, 2 and 3 were examined in PA14 pqsC mutant for confirming their target selectivity. Both compounds turned out to be inactive in reducing 2-AA and DHQ production independently from the concentration of PQS added into the

FIGURE 10 | Persister cells survival of PA14 wt with and without PqsBC inhibitors 2 and 3, or 2-AA and PA14 pqsBC mutant after the treatment with 10 µg/mL of meropenem for 24 h. The error bars indicate 95% Confidence Interval of the geometric mean. Statistical analysis performed with non-parametric one-way ANOVA (α = 0.05; **p < 0.003; ***p < 0.01; *p < 0.05).

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culture (Figure 8) supporting the in vitro characterization of the two inhibitors (unpublished results; Starkey et al., 2014).

Considering their efficiency in reducing the production of the MvfR natural ligands in PA14 wt and pqsH mutant, these inhibitors were analyzed in the pqsA-GFP_ASV construct for monitoring their potential ability for reducing the expression of the pqs operon. Actually, 2 and 3 reduced the operon transcription down to 36 and 15%, respectively, at their highest dosage (Figure 9). However, it is not clear in this setting why no autolysis is observed despite the presence of HQNO, which is responsible for the programmed cell autolysis observed in liquid cultures of P. aeruginosa (Hazan et al., 2016).

Taking into consideration the obtained results, the effects of the PqsBC inhibitors on the MvfR natural ligands, 2-AA, and DHQ production fitted to the expected behavior of blocking the hetero-dimer PqsBC. Actually, its inhibition would lead to a reduced conversion of the reactive 2-ABA into HHQ with consequently reduced expression of the pqs operon. The excess of this intermediate would be consequently transformed more into the stable molecules 2-AA and DHQ, conversions that do not require PqsBC. Notably, as previously reported, PqsBC into the stable molecules 2-AA and DHQ, conversions that of this intermediate would be consequently transformed more to the expected behavior of blocking the hetero-dimer PqsBC. Actually, its inhibition would lead to a reduced conversion of the reactive 2-ABA into HHQ with consequently reduced expression of the pqs operon. This corresponds to the levels of PA14 wt and pqsH mutant showing persister rates of 1.2 × 10−6 (untreated) up to 4.6 × 10−6 cells. This corresponds to the levels of PA14 wt treated with 2-AA as well as the untreated PA14 pqsBC mutant showing persisters rates of 7.2 × 10−6 and 4.1 × 10−6, respectively (Figure 10). These findings confirmed that targeting PqsBC led to survival of the bacteria in the presence of antibiotic. This might ultimately result in a reduced efficiency of antibiotic therapy.

CONCLUSION

The profiling of P. aeruginosa MvfR antagonist and PqsBC inhibitors emphasized the importance in selecting the target for the development of new anti-infectives, and confirmed that MvfR is an excellent target for a global AQ and 2-AA inhibition. Actually, antagonizing the transcriptional regulator led to an efficient inhibition of the pqs operon expression and, consequently, of the mvfR-small molecules production. As it was found an overproduction of the toxic DHQ at AQs IC50 doses, the current study highlighted that an efficient therapy would be obtained only choosing a concentration of QSI capable to fully suppress the MvfR activity and, in the end, the biosynthetic pathway. The PqsBC inhibitors showed to be less efficient in reducing the MvfR natural ligands synthesis and, moreover, lead to an increased production of 2-AA and DHQ. Actually, they mainly affected the distribution of QS molecules generated within a bacterial population without modifying the overall production. The importance of reducing 2-AA production through MvfR antagonism is critical in preventing the formation of antibiotic tolerant persister cells. In case of PqsBC inhibitors, a combination with other QSIs (e.g., PqsA, PqsD, or MvfR) might still be a valid route toward the use of a novel anti-infective approach.

AUTHOR CONTRIBUTIONS

GA: Performed experiments and wrote the paper. CM and JE: Supervised LC-MS experiments and wrote the paper. DM and LR: Supervised cellular experiments. RH and ME: Supervised experiments and wrote the paper.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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