Design and Evaluation of New Quinazolin-4(3H)-one Derived PqsR Antagonists as Quorum Sensing Quenchers in *Pseudomonas aeruginosa*


**ABSTRACT:** *P. aeruginosa* (PA) continues to pose a threat to global public health due to its high levels of antimicrobial resistance (AMR). The ongoing AMR crisis has led to an alarming shortage of effective treatments for resistant microbes, and hence there is a pressing demand for the development of novel antimicrobial interventions. The potential use of antivirulence therapeutics to tackle bacterial infections has attracted considerable attention over the past decades as they hamper the pathogenicity of target microbes with reduced selective pressure, minimizing the emergence of resistance. One such approach is to interfere with the PA *pqs* quorum sensing system which upon the interaction of PqsR, a Lys-R type transcriptional regulator, with its cognate signal molecules 4-hydroxy-2-heptylquinoline (HHQ) and 2-heptyl-3-hydroxy-4-quinolone (PQS), governs multiple virulence traits and host–microbe interactions. In this study, we report the hit identification and optimization of PqsR antagonists using virtual screening coupled with whole cell assay validation. The optimized hit compound 61 ((R)-2-(4-(3-(6-chloro-4-oxoquinazolin-3(4H)-yl)-2-hydroxypropoxy)phenyl)acetanilide) was found to inhibit the expression of the PA P*pqs* promoter controlled by PqsR with an IC₅₀ of 1 μM. Using isothermal titration calorimetry, a K₅₀ of 10 nM for the PqsR ligand binding domain (PqsR-LBD) was determined for 61. Furthermore, the crystal structure of 61 with PqsR-LBD was attained with a resolution of 2.65 Å. Compound 61 significantly reduced levels of pyocyanin, PQS, and HHQ in PAO1-L, PA14 lab strains and PAK6085 clinical isolate. Furthermore, this compound potentiated the effect of ciprofloxacin in early stages of biofilm treatment and in *Galleria mellonella* infected with PA. Altogether, this data shows 61 as a potent PqsR inhibitor with potential for hit to lead optimization toward the identification of a PA QS inhibitor which can be advanced into preclinical development.

**KEYWORDS:** *Pseudomonas aeruginosa*, PqsR, *Pseudomonas* quinoline signal (PQS), biofilms, quorum sensing inhibition

The rising, wide spread of antimicrobial and multidrug-resistant bacterial strains at an unprecedented rate places a major burden on global public health predominantly with regard to the shortage of effective therapeutic options.¹ Alarming, within the antibacterial pipelines currently under clinical development, there is no novel antibacterial class that could actively treat any of the WHO top priority pathogens also known as ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudo- monas aeruginosa, and Enterobacter spp.*).²,³ *P. aeruginosa* (PA) is a Gram-negative opportunistic bacterium and a common cause for nosocomial infections particularly in cystic fibrosis and immunocompromised patients.⁴ Consequently, there is an urgent need for the discovery of novel approaches to combat bacterial infections with mechanisms of action that do not impose strong selective pressure on bacteria.⁵ In fact, over the past decades, the focus of the preclinical antibacterial projects was shifted toward new targets, mostly in Gram-negative pathogens, as in the case of antivirulence approaches, adjuvant therapies, and antibiotic potentiators.⁶ One of the extensively investigated approaches to target virulence is the development of drugs which can interfere with bacterial quorum sensing systems (QSSs). These are cell-to-cell communication mechanisms used by many bacterial pathogens to control the...
PA possesses three types of QSSs that employ various diffusable chemical signals called quorum sensing signaling molecules (QSSMs) or autoinducers (AIs). The las and rhl systems use N-acylhomoserine lactone derived signals while the pqs system relies on alkyl quinolone (AQ) derived molecules. Using these systems, PA can coordinate the production of virulence determinants in a coordinated population-dependent manner in response to external stimuli or threats. While the las and rhl systems have been studied extensively, the pqs system has been shown in recent years to play a substantial role in the virulence of PA. The biosynthesis of the AQ signals 4-hydroxy-2-heptylquinoline (HHQ) and 2-heptyl-3-hydroxy-4-quinolone (PQS) is initiated from anthranilate through a sequence of enzymes (PqsA, PqsBC, PqsD, PqsH) encoded by pqsABCDEphnAB operon and the pqsH gene. The resulting AQS then interact with the C-terminal ligand binding domain of the LysR type regulator PqsR (also known as MvfR) resulting in a conformational change leading to the activation of the pqs operon, likely by binding to the pqsA promoter (P\textsubscript{pqsA}). To date, there have been several attempts to pharmacologically inhibit the pqs system by either interfering with the biosynthesis of AQS targeting the biosynthetic enzymes (such as PqsD\textsuperscript{15} and PqsA\textsuperscript{16}) or preventing the signal reception (through antagonists of PqsR). Several chemical series have been described as PqsR/pqs inhibitors including quinazolinone, quinolinone, thioacetamide, and hydroxybenzamide derived molecules, but none have yet been taken forward to clinical trials. In this study, we report a new series of quinazolinone derived pqs inhibitors with low micromolar potency in PA PAO1-L. Compound 61, one of the most potent inhibitors in this series, was evaluated using PA phenotypic analysis including pyocyanin and determination of AQ signal levels in various clinical and lab strains. The study also investigated the effect of 61 on PA biofilms, as a sole treatment or in combination with ciprofloxacin. Moreover, the cocrystal structure of two inhibitors (61, 40) with the PqsR ligand binding domain was obtained revealing the main interactions governing binding. To conclude this study, 61 was evaluated in a G. mellonella PA infection model alone and in conjunction with ciprofloxacin.

### RESULTS

**Identification of PqsR Hits through “in silico” Docking and Bioreporter Assays.** A virtual screening of an in-house drug-like compound library composed of 85,061 curated molecules was performed using the PqsR ligand binding domain (PDB 4JVI) employing the Schrödinger Suite for molecular docking. The 583 top scored compounds were selected after introduction of a Glide docking score threshold of −9.0. The selected compounds were then validated for their ability to inhibit the pqs QS system at a concentration of 10 μM using the bioluminescence-based bioreporter strain PAO1-L harboring the chromosomal transcriptional fusion mCTX::P\textsubscript{pqsA-lux}. For a molecule to score as active, a reduction of at least 50% of bioluminescence had to be achieved without affecting bacterial growth. This process led to the discovery of multiple hits, among them was 2-(4-((3-(6-chloro -4-oxoquinazolin-3(4H)-yl)-2-hydroxypropyl)amino)phenyl)acetonitrile with an IC\textsubscript{50} of 13.1 ± 0.6 μM (Figure 1).

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Figure 1. (A) Chemical structure of pqs hit with site of modifications and (B) improved hit molecule 22, with improved activity.

Scheme 1. Synthetic Route for 22 and its Analogues 23−41

![Scheme 1](https://doi.org/10.1021/acsinfecdis.1c00175)
substituted aniline tail, and the β-amino alcohol linker (Figure 1, A). To expedite the synthesis, 22 (Figure 1, B) was designed with a modified tail group bearing a phenyl ether linker in place of the substituted aniline present in 1. Compound 22 showed a 4-fold increase in potency compared to 1 (IC₅₀ 3.2 ± 1.0 μM) and was therefore considered as a new starting point for development of this series. A robust synthetic strategy was employed to synthesize a series of analogues (22−41) with modified head or tail groups through an epoxide ring opening reaction between substituted quinazolin-4(3H)-ones 2−12 and 2-(methylphenoxy)oxirane derivatives 13−21 to afford compounds 22−41 (Scheme 1).

Subsequently to further explore the SAR, the secondary alcohol present in 22 was modified with isosteric replacements (Scheme 2). The amino derivative 42 was obtained from a Mitsunobu conversion of the hydroxyl group to azide which was subsequently reduced to the primary amine using the Staudinger reaction. Compound 42 was further functionalized either through a direct alkylation with iodoethane to give 45 or through conventional amide coupling reaction with either the corresponding anhydrides (46−48) or using PyBrop and triethylamine to afford 49−51. Oxidation of the secondary alcohol by the Dess–Martin reagent afforded ketone 43. Finally, the fluorine derivative 44 was obtained from 22 using dimethylamino-sulfur trifluoride.

To further explore the pharmacological requirement of the secondary alcohol, the alcohol was either removed or replaced with a methyl group.

The cyanomethyl group on the aromatic tail was also subject to replacement with 5-membered ring heterocycles. The nitrile group was reacted with hydroxylamine which was cyclized in the presence of acetic anhydride to yield 5-methyl-1,2,4-oxadiazole derivative 56. While the triazole derivative 57 was obtained by reacting 22 with acetylhydrazine and sodium methoxide. The tetrazole derivative 58 was prepared through the reaction of 22 with NaN₃ in DMF. Acid hydrolysis of the

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**Scheme 2. Synthetic Route of Compounds 42−51**

**Scheme 3. Synthetic Route of Compounds 54 and 55 to Explore the Influence of the Secondary Alcohol Present in 22**

**Scheme 4. Synthetic Route for 22 Derivatives with Various Nitrile Replacements**
nitrile to the corresponding carboxylic acid gave 60 and the carboxamide derivative 59 was prepared through hydrolysis by aqueous hydrogen peroxide in the presence of cesium carbonate (Scheme 4).30

Finally, the single enantiomers of 22 were prepared through reacting commercially available (R)- and (S)-oxiran-2-ylmethyl 3-nitrobenzenesulfonates with 2-(4-hydroxyphenyl)acetonitrile to provide the corresponding epoxides that were subsequently reacted with 2 to afford (R)- and (S)-enantiomers 61 and 62, respectively (Scheme 5).31 The percentages of enantiomeric excess (ee) of the products were 93.5% and 92.1% for the (R)-isomer 61 and (S)-isomer 62 respectively, as determined through chiral phase HPLC analysis.

**Pharmacological Evaluation and Structure Activity Relationship. Screening of Commercial Compound Library.**

To provide initial insights into the exploration of the structure activity relationship within this new hit series, a SAR by activity relationship within this new hit series, a SAR by

Table 1. Structures and Activities of 22 Analogues with Modified Head Groups 23–32

<table>
<thead>
<tr>
<th>ID</th>
<th>R1</th>
<th>R2</th>
<th>X1</th>
<th>remaining activity in PAO1-L at 10 μM (RA%)</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>7-Cl</td>
<td>H</td>
<td>CH</td>
<td>41.2 ± 1.1</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>24</td>
<td>6,7-Cl</td>
<td>H</td>
<td>CH</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>6,8-Cl</td>
<td>H</td>
<td>CH</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>8-Cl</td>
<td>H</td>
<td>CH</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>5-Cl</td>
<td>H</td>
<td>CH</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>6-Br</td>
<td>H</td>
<td>CH</td>
<td>22.3 ± 4.7</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>29</td>
<td>6-NO2</td>
<td>H</td>
<td>CH</td>
<td>17.2 ± 0.6</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>30</td>
<td>7-NO2</td>
<td>H</td>
<td>CH</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>6-CF3</td>
<td>H</td>
<td>CH</td>
<td>14.0 ± 0.9</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>32</td>
<td>6-Cl</td>
<td>Me</td>
<td>CH</td>
<td>84.1 ± 3.2</td>
<td></td>
</tr>
</tbody>
</table>

“NA denotes no activity at a 10 μM concentration.

**Compounds with Modified Tail Groups.** As the manipulation of the headgroup of 22 provided a slight improvement for potency, a series of modifications were introduced at the level of the aromatic phenyl ring of 22 to explore the SAR of this region (Table 2). The cyanomethylene group proved important for biological activity at the para-position while the meta-substituted analogue 33 lacked biological activity. Moreover, aromatic nitriles, e.g. compounds 34 and 35 with para- and meta-benzonitrile respectively, exhibited significant loss of activity. Further, modification to the cyanomethylene group included the introduction of a cyclopropyl moiety 37 resulted in reduced activity, while extending the chain length by an extra methylene group 36 preserved the potency. Introduction of an ether bond between the phenyl and the cyanomethylene group 40 caused a slight reduction of activity. In some cases, substitution of the phenyl ring retained activity, in particular, ortho-substituted fluorine analogue 39 (IC50 2.2 μM) proved active when compared to meta-position 38, which suffered a 2-fold loss in potency (IC50 6.4 μM). Extending the para-position of the phenyl ring with a bulky phenoxy group 41 led to a 30% inhibition of pqs activity at 1 μM test concentration. One of the advantages of having the aliphatic nitrile is the versatility to convert it to various heterocycles to further explore SAR. While the oxadiazole replacement 56 retained activity, demonstrating the limited substitution possibility around the headgroup.
activity, triazole containing compound 57 demonstrated significant loss in activity and the tetrazole derivative 58 was totally inactive. Finally, hydrolysis of the nitrile group to its corresponding carboxamide led to inactive compound 59 while the carboxylic acid derivative 60 reduced activity (35% inhibition at 10 μM). Despite all these modifications on the tail of 22, the para-substituted acetonitrile group proved to be the optimal substituent with a fluorine atom substituted at the ortho-position 39 having a comparable level of activity.

Analogues with Modified Linker Groups. It became apparent from the initial SAR investigation of the head and tail groups of 22 was limited to conservative modifications, therefore the focus of the evaluation moved to the linker group and particularly the secondary alcohol (Table 3). In this series of analogues, the head and tail moieties were fixed corresponding to those of 22, while the linker was varied and substituted. Oxidation of the alcohol to ketone 43 retained biological activity with IC50 of 4.3 μM. Conversely, substitution of hydroxyl with a fluorine atom 44 significantly reduced biological activity. Interestingly, replacing the alcohol with an amine 42 gave a compound with comparable activity (IC50 2.4 μM). However, alkylating the amine with an ethyl group 45 or acylation with small carboxylic acids (46−50) Table 3. Modification of the Linker Group

<table>
<thead>
<tr>
<th>ID</th>
<th>R</th>
<th>Remaining activity in PAO1-L at 10 μM (RA%)</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>NH₂</td>
<td>10.5 ± 1.2</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>43</td>
<td>–O</td>
<td>16.6 ± 1.7</td>
<td>4.3 ± 1.3</td>
</tr>
<tr>
<td>44</td>
<td>F</td>
<td>67.8 ± 11.9</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>NHCH₂CH₃</td>
<td>69.2 ± 15.0</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>NHCOCH₃</td>
<td>86.6 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>NHCOEt</td>
<td>81.6 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>NHCOPr</td>
<td>89.5 ± 6.3</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>NHCOCH₃N(CH₃)₂</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>NHCO(CH₂)₂N(CH₃)₂</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>NH(CH₂)₂N(CH₃)₂</td>
<td>87.40 ± 5.3</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>H</td>
<td>88.6 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>CH₃</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>(K)-OH</td>
<td>11.0 ± 0.8</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>55</td>
<td>(S)-OH</td>
<td>43.5 ± 4.9</td>
<td>7.3 ± 1.4</td>
</tr>
</tbody>
</table>

“NA denotes no activity at a 10 μM concentration.

Table 2. Exploration of the Tail Group of 22

<table>
<thead>
<tr>
<th>ID</th>
<th>R</th>
<th>Remaining activity in PAO1-L at 10 μM (RA%)</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>m-CH₃CN</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>p-CN</td>
<td>76.4 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>m-CN</td>
<td>82.9 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>p-CH₃CH₂CN</td>
<td>16.9 ± 0.8</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>37</td>
<td>p-CN</td>
<td>76.5 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>m-F, p-CH₃CN</td>
<td>37.0 ± 3.3</td>
<td>6.4 ± 0.7</td>
</tr>
<tr>
<td>39</td>
<td>o-F, p-CH₃CN</td>
<td>17.7 ± 1.0</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>40</td>
<td>p-OCH₃CN</td>
<td>27.6 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>p-phenoxy</td>
<td>63.8 ± 8.6</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>F</td>
<td>8.2 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>57</td>
<td>F</td>
<td>54.5 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>F</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>CH₂CONH₂</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>p-CH₃COOH</td>
<td>64.3 ± 6.5</td>
<td></td>
</tr>
</tbody>
</table>

“NA denotes no activity at a 10 μM concentration.
resulted in a loss of activity. In a similar fashion, removing the hydroxyl group or substituting it with a methyl group removed all biological activity.

To conclude the SAR study, the chiral resolution of the alcohol was examined through the synthesis of the (R)- (61) and (S)-enantiomers (62) (Scheme 4). The (R)-enantiomer 61 proved more active (IC₅₀ of 1 μM) over the (S)-enantiomer, which was 7-fold less active (IC₅₀ of 7 μM).

In conclusion, the extensive SAR study demonstrated the very tight structural requirements for activity within this hit series. It is noteworthy that 61, the (R)-enantiomer of 22, proved to be one of the most potent inhibitors and the remainder of our study centered on this compound.
**Isothermal Titration Calorimetry Assay.** As the bioreporter assay provides data about the inhibition levels of the pqs system but does not provide direct evidence that the compounds are binding to PqsR, it was paramount to confirm the binding using an orthogonal approach. For this purpose, we used isothermal titration calorimetry (ITC) which also enables the measurement the binding thermodynamics parameters between PqsR\(^{BD}\) and 61. This analysis revealed that 61 has strong PqsR\(^{BD}\) affinity with dissociation constant \((K_d \text{ of } 10 \text{ nM})\) (Figure 2) and a stoichiometry of 1:1.

**Crystal Structure of PqsR Antagonists with PqsR\(^{BD}\).** To gain a detailed insight into the molecular interaction of the antagonists with PqsR, the X-ray cocrystal structures complexed with 61 and 40 were obtained by ligand soaking (Figure 3a, b, c, and f). As previously reported,\(^{32}\) PqsR\(^{BD}\) compromises a deep pocket (B pocket) which usually accommodates the quinolone part of the autoinducer, as exemplified in the case of 2-nonyl-4-quinolone (NHQ), while the superficial pocket (A pocket) contains the aliphatic side chain (Figure 3d, e).\(^{32}\) The structures revealed that the quinazolinoizine moiety of 61 and 40 reside in the B pocket, in a similar manner to NHQ, surrounded by aliphatic residues (Ala\(^{102}\), Ala\(^{164}\), Ile\(^{207}\), Ile\(^{208}\)) while the hydroxyl group establishes hydrogen bonding with glutamine (Glu\(^{194}\)) and arginine (Arg\(^{209}\)) (Figure 3b and c). The aromatic tail adopted a similar conformation to NHQ sustaining hydrophobic interaction while the nitrile group is oriented toward the aromatic ring of the quinazolinone moiety of 61.\(^{32}\)

**Pharmacological Evaluation of PqsR Inhibitors. Pyocyanin Quantification Assay.** PA produces a range of redox active phenazine derived molecules, among these pyocyanin is one of the most studied compounds which plays various roles in virulence, gene expression, biofilm formation as well as host-tissue damage.\(^{33}\) Pyocyanin biosynthesis is achieved through the activation of the phenazine \(phz\) operons which are under the regulation of the pqs system.\(^{33,34,35}\) Consequently, the attenuation of pqs system leads to a reduction of pyocyanin production, and hence quantification of pyocyanin levels serves as an indirect readout for the inhibition caused by PqsR antagonists. For this purpose, the expression of pyocyanin in the presence of 61 at 3 μM (3-fold the IC\(_{50}\) value) was measured on PAO1-L and PA14 lab strains showing a reduction of 80% and 95% of pyocyanin levels respectively compared to untreated controls (Figure 4). The study was further extended to validate the effect of 61 on a selection of clinical isolates with different genomic backgrounds where PALESB58, PAK6085, and PA748 clinical isolates were selected as representatives (Figure 4).\(^{36}\) The study concluded that these isolates were less sensitive to the effect of 61 with only the PAK6085 strain showing a significant reduction of 27%.

**Alkyl Quinoline Quantification Assay.** To further investigate the impact of the PqsR inhibitor 61 on the expression of the pqsABCDE operon, production of HHQ and PQS signals was quantified using LCMS/MS analysis. Similar to the pyocyanin assay, the effect of 61 on alkylquinoline signal production was quantified in lab strains as well the three clinical isolates mentioned previously using a concentration that equals 3 times the corresponding IC\(_{50}\). The results demonstrated that 61 significantly inhibited HHQ and PQS production in PAO1-L (>90%) and PA14 (~50%) in agreement with the pyocyanin data, although to a lesser extent in PA14 (Figure 5). Once more the PAK6085 clinical isolate was the only one to respond to 61 although to a lesser extent than the lab strains whereas PALESB58 and PA748 did not show a significant response.

**Effect of 61 on PA Biofilm Formation.** Biofilms are highly resilient to antibiotic treatments. Qorum sensing has been shown to play a critical role in biofilm development.\(^{37}\) To examine whether 61 could affect biofilm formation and potentiate antibiotic activity, pre-established PAO1-L biofilms (48 h) grown in the presence of 61 were treated with the broad-spectrum antibiotic Ciprofloxacin (Cip) and their viability investigated using LIVE/DEAD BacLight bacterial viability staining and confocal laser scanning microscopy (CLSM). The effect of 61 on biofilm viability as a single treatment or in combination with Cip was measured at two different time points (5, 16 h) to establish whether this PqsR antagonist could enhance the effect of the antibiotic. It was evident that 61 on its own had no significant effect on biofilm viability, however, when combined with Cip it showed significant potentiation of the antibiotic effect at 5h of incubation (Figure 6). However, this was not observed at 16

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**Figure 4.** Pyocyanin quantification assay. Bar chart representing pyocyanin production in laboratory and clinical strains: blue bars refer to untreated controls, gray bars represent treatment with 3 μM of 61. The data was normalized to a negative control of DMSO in each strain. The error bars represent the standard deviation of biological triplicates. The statistical significance was performed using paired t test analysis (Graphpad 9.0).
as a single treatment and in combination with Cip was performed using paired median survival time of 19 and 20.5 h when treated with e 21 larvae surviving at the end point. Therefore, was further reduced with addition of Cip compared to PAO1-L, and this rate with the addition of Cip compared to PAO1-L, and this showed a median survival time of 12 h, and addition of Cip with a median survival time of 37 h; this effect of stain variation PAO1-N was utilized, and the e ...Cytotoxicity Study of 61 on Lung Epithelial Cells. The effect of 61 on A549 lung epithelial cell cytotoxicity assay was examined to evaluate the suitability of this inhibitor for further development. This study concluded that PqsR inhibitor 61 did not exhibit any cytotoxic effect up to a concentration of 300 μM (LD₅₀ > 300 μM) which is around 300-fold greater than the corresponding IC₅₀ in PAO1-L (1 μM).

**DISCUSSION**

In this study, we reported the discovery of (R)-2-(4-(3-(6-chloro-4-oxoquinazolin-3(4H)-yl)-2-hydroxypropoxy)phenyl)-acetonitrile 61 as a novel PqsR inhibitor. The starting hit 1, which originated from a hit identification campaign, demonstrated weak pqs inhibition in the bioreporter whole cell assay with an IC₅₀ of 13 μM. To facilitate the synthesis of analogues, compound 22 was proposed and shown to be with 4-fold more active than 1 (IC₅₀ = 3.1 μM). To progress this hit to a lead stage, an extensive structure activity relationship was performed on 22 and concluded that even minor structural alterations could lead to obliteration of potency. The key findings of SAR can be summarized as follows: (I) The acetonitrile group at the para-position of the phenyl ring is important for activity and the overall potency can be marginally improved with a meta-fluorine atom, (II) substitution at the 6-position of the quinazolin-4(3H) was preferred for a halogen or an electron withdrawing group, (III) the hydroxyl group of the linking group is preferred and the (R)-enantiomer 61 possesses the improved activity compared to the (S)-enantiomer. Nevertheless, inhibitors of the P₅₃₄lux reporter could potentially be targeting the AQ biosynthetic enzymes rather than PqsR. To further confirm that 61 is acting as a PqsR inhibitor, an ITC analysis was used to determine the thermodynamic parameters of binding and to aid the understanding of the biomolecular interactions. This showed that 61 was able to bind to PqsR₅₃₄ with a Kᵥ of 10 nM. These outcomes were further confirmed by the X-ray crystal structures of 61 and 40 with PqsR₅₃₄. These structures revealed that the binding is mainly governed by hydrophobic...
interactions and supported with electrostatic interactions at the level of the side chains of Gln 194 and Arg209. Despite the interaction with Gln 194 being reported previously in the literature, the interaction with Arg209 was new and unique to these structures. It is worth mentioning that the quinazolinone head groups in 61 and 40 reside in different orientation to our previously reported quinazolinone derived pqS inhibitors.19 The latter compounds were positioned in a conformation that allows the carbonyl group to be in a close proximity to Thr265. Further investigation is needed to pinpoint whether the different ligand conformation in the receptor contributed to the enhanced potency.

These findings along with previously reported structures can be employed to fine-tune potency further.18,19,32,38 Subsequently, the work moved to examine the effect of 61 in a series of phenotypic assays, such as pyocyanin and AQ signal quantification. Most importantly, these assays were performed, unlike the majority of published work in relation to pqS inhibition, in a selection of PA isolates with different genetic backgrounds including lab and clinical strains. This is of exceptional importance in antibacterial drug discovery as the potency of inhibitors may vary significantly in a strain dependent manner which could lead to an inevitable and costly pitfall if not discovered early.7 Indeed, while 61 showed superior activity in suppressing PA phenotypes in lab strains (PAO1-L and PA14), it failed to maintain this effect in clinical isolates. The sequence alignment of PqsR in these strains was performed (see the Supporting Information) and suggested that the sequence is mostly preserved apart from Ala314 to Val mutation in PA14 which is distant from the ligand binding site. The reasons behind the difference in activity of strains was not pursued in this study, but it could be due to variation in the level of activity of pqS operon in these strains or variation in permeability issues through the bacterial membrane and the presence of efflux pumps on the bacterial cell membrane.40 It is noteworthy that all the clinical strains originated from cystic fibrosis patients which reflects the multidrug resistant nature of the microbiome associated with this disease.36,41

Another important phenotype of PA is the ability to form antimicrobial resistant biofilms. To this extent, PA biofilms were treated with 61 as sole agent and also in combination with ciprofloxacin. While 61 demonstrated a slight effect as single treatment, it was able to potentiate ciprofloxacin treatment at 5 h but not at 16 h time points. The reason behind the difference in efficacy was not investigated, but it could be potentially due to ciprofloxacin reaching maximal efficacy at the experimental end point (16 h) shadowing the effect of 61. This suggests that this PqsR inhibitor may be
more effective as an adjuvant for the initial stages of treatment. Further work is needed to establish the impact of treatment at different time points and concentrations, but this is beyond the scope of the current study. Previous reports have shown that PqsR inhibition can increase the sensitivity of mature PA biofilms to antibiotics; however, the biofilm extracellular matrix is a major barrier to the transport of these inhibitors. As an analogy, we previously published thioacetamide derived pqs inhibitors failed to show any inhibitory effect on PA biofilms despite the high potency in planktonic growth. Small molecule penetration to biofilms can be improved applying drug delivery approaches such as bioresponsive nanoparticles. Indeed, the use of pH responsive nanoparticles has been shown to increase the penetration and efficacy of QS inhibitors combined with ciprofloxacin in mature biofilms. Despite no measures being applied in our study to improve penetration through PA biofilms, our results indicate that this QS inhibitor could improve conventional antibiotic therapy against recalcitrant bacterial-biofilm infected centers.

To conclude this work, a G. mellonella infection model was employed to investigate the effect of 61 on mortality rate due to PA infections. In agreement with the previous observation in biofilms, 61 showed no effect on mortality rate as a sole agent; however, when combined with ciprofloxacin, 61 was able to improve the survival compared to ciprofloxacin alone in PAO1-L and PAO1-N inoculated wax worms. In this study the quorum sensing inhibitor 61 was unable to significantly alter the survival of wax worms in both PAO1-L and PAO1-N, this is in contrast to the study performed by D’angelo and colleagues who showed that an approved FDA drug Clofocotol that is a probable PqsR inhibitor was able to reduce the mortality of PAO1 infected wax worms. It must be noted that the wax worms used in the D’angelo study (500 mg) were significantly larger than those used in this study (250 mg) and both PA strains used in this study caused 100% mortality. It is noteworthy that 61 showed a low cytotoxic profile when tested on human lung carcinoma epithelial cells A549 (LD50 > 300 μM) supporting its suitability for further preclinical development. In summary, this study delivered a robust evaluation of 61 as a PqsR antagonist using chemical, biophysical, structural and biological means and provided a solid foundation to enhance the potency and spectrum of this series in future lead optimization studies.

**METHODS**

**Molecular Docking. Preparation of Protein and Receptor Grid Generation.** The X-ray crystal structure of PqsR ligand binding domain in complex with the QZN inhibitor (PDB ID 4JVI) was used as a protein template. The protein was prepared using the protein preparation wizard (Small-Molecule Drug Discovery Suite 2017-4, Schrödinger, LLC, New York, NY, USA), where hydrogen atoms were added via the protein preparation wizard. The inner grid box was set to 10 Å, while the outer box was set to 20 Å.

**Ligand Preparation.** The chemical structures of the inhibitors were sketched using ChemDraw (Version 16.0.1.4, PerkinElmer informatics) via an SDF file. The LigPrep module (Small-Molecule Drug Discovery Suite 2017-4, Schrödinger, LLC, New York, NY, USA) was then used for final preparation of ligands into their lowest energy 3D conformations. The partial atomic charges were assigned to the molecular structures, using the 2005 implementation of the OPLS-AA force field. These energy-minimized structures were used for Glide docking.

**Molecular Docking.** The “Extra Precision” (XP) mode of Glide docking (Small-Molecule Drug Discovery Suite 2017-4, Schrödinger, LLC, New York, NY, USA) was used to perform all docking calculations, using the OPLS-AA 2005 force field. The scale factor of 1.0 for van der Waals radii was applied to atoms of protein with absolute partial charges of less than or equal to 0.25. The number of positions per ligand was set to five, after energy minimization. The best docked structures were chosen using an XP Glide Score (XP Gscore) function as well as visual observations.

**Chemistry Procedures and Compound Characterization.** Reagents and solvents were purchased from Sigma-Aldrich, Alfa Aesar, Fluorochem, and Fisher Scientific. Nuclear magnetic resonance was performed at ambient temperature using a Bruker AV400 spectrometer (400 MHz for H, 101 MHz for C). The samples were prepared in deuterated solvent: DMSO-d₆ and CDCl₃. Chemical shifts (δ) were recorded in ppm, and coupling constants (J) were recorded in Hz. The spectra were analyzed using MestReNova 12.0.1 software. Mass spectrometry: Analytical HPLC were performed on a Shimadzu UFLCXR system coupled to an Applied Biosystems API2000. Three columns thermostated at 40 °C were used. Column one: Phenomenex Gemini-NX 3 μm C18, 50 × 2 mm. Column two: Phenomenex Luna 3 μm (PFP2) 110A, 50 × 2 mm. Column three: Waters Xterra MS C8 2.5 m, 4.6 × 30 mm. Flow rate: 0.5 mL/min. UV detection at 220 nm (channel 2) and 254 nm (channel 1). Analytical HPLC for final compounds were performed on a Shimadzu UFLCXR system coupled to a mass spectrometer (Applied Biosystems API2000). Three columns thermostated at 40 °C were used (column 1: C8-110A, 50 × 2 mm (method 1); column 2: Luna 3 μm (PFP2) 110A, 50 × 2 mm (method 2); column 3: C18 50 × 2 mm (method 3)). The flow rate was set to 0.5 mL-min⁻¹. UV detection was performed at 254 (channel 1) and 220 nm (channel 2). The eluting gradient was as follows: pre-equilibration run for 1 min at 10% B, 10 to 98% B in 2 min, 98% for 2 min, 98 to 10% B in 0.5 min, then 10% B for 1 min (where solvent A is 0.1% formic acid in water and solvent B is 0.1% formic acid in MeCN). All compounds reported here are with purity over 95% unless otherwise mentioned. Chromatography: Thin-layer chromatography (TLC) was performed, UV light and standard TLC stains were used to visualize the Merck Silica gel 60 Å F254 plates. Compounds were purified via column chromatography using either a Thompson pump or normal phase Interchim Puriflash prepacked cartridges filled with 50 μM silica, or a glass column using Sigma-Aldrich silica gel 60 Å (230–240 μm).

Chiral HPLC was performed on Dionex ICS-3000 with an Ultimate-3000 detector equipped with Lux 5 μm Cellulose-4, LC column 250 × 4.6 mm column.

**General Procedure (1) for preparation of epoxides 12, 18, 19, 21.** To a stirred solution of different phenols (1 equiv) in MeCN (50 mL) was added Cs₂CO₃ (2 equiv) and then epichlorohydrin (1.2 equiv). The reaction mixture was refluxed at 85 °C for 16 h. The mixture was then filtered and purified.
using column chromatography with PE:EtOAc 8:2 as the eluent.

2-(4-Oxiran-2-ylmethoxy)phenyl)acetonitrile 12. The title compound was prepared according to general procedure (1) using 4-hydroxyphenylacetonitrile (20.0 g, 0.15 mol) as the starting material. White solid (7.6 g, 25%).

\[ \text{CIMS} \text{ calculated for } C_{9}H_{17}ClN_{3}O_{3}^{+} [M + H]^+: 370.1 \text{ found 370.3, } \text{HRMS calculated for } C_{10}H_{17}ClN_{3}O_{3}^{+} [M + H]^+: 370.0953 \text{ found 370.0956.} \]

2-(4-(3-(7-Chloro-4-oxoquinazolin-3(4H)-yl)-2-hydroxypropoxy)phenyl)acetonitrile 23. The title compound was prepared according to general procedure (2) using 3 (1g, 5.5 mmol) and 12 (1.25 g, 6.6 mmol). Off white solid (0.45 g, 24%).

\[ \text{CIMS} \text{ calculated for } C_{19}H_{17}ClN_{3}O_{3}^{+} [M + H]^+: 370.1 \text{ found 370.5, HRMS calculated for } C_{19}H_{17}ClN_{3}O_{3}^{+} [M + H]^+: 370.0953 \text{ found 370.0957.} \]

2-(4-(6,7-Dichloro-4-oxoquinazolin-3(4H)-yl)-2-hydroxypropoxy)phenyl)acetonitrile 24. The title compound was prepared according to general procedure (2) using 4 (1g, 4.6 mmol) and 12 (1.05 g, 5.5 mmol). Off white solid (0.63 g, 28%).

\[ \text{CIMS} \text{ calculated for } C_{19}H_{17}ClN_{3}O_{3}^{+} [M + H]^+: 370.1 \text{ found 404.5} \]

2-(4-(6,8-Dichloro-4-oxoquinazolin-3(4H)-yl)-2-hydroxypropoxy)phenyl)acetonitrile 25. The title compound was prepared according to general procedure (2) using 5 (1g, 4.6 mmol) and 12 (1.05 g, 5.5 mmol). Off white solid (0.78 g, 35%).

\[ \text{CIMS} \text{ calculated for } C_{19}H_{17}ClN_{3}O_{3}^{+} [M + H]^+: 370.1 \text{ found 404.7} \]

2-(4-(6-Chloro-4-oxoquinazolin-3(4H)-yl)-2-hydroxypropoxy)phenyl)acetonitrile 26. The title compound was prepared according to general procedure (2) using 6 (1g, 5.5 mmol) and 12 (1.25 g, 6.6 mmol). Off white solid (0.84 g, 41%).

<table>
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<th>Chemical</th>
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<tr>
<td>CIMS calculated for C_{19}H_{17}ClN_{3}O_{3}^{+} [M + H]^+: 370.1</td>
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The title compound was prepared according to general procedure (2) using 2 (1g, 5.5 mmol) and 13 (1.26 g, 6.6 mmol). Off white solid (1.4 g, 69%). 1H NMR (400 MHz, DMSO-d$_6$) δ 8.32 (s, 1H), 8.23 (d, J = 2.0 Hz, 1H), 7.72 (dd, J = 7.5, 1.9 Hz, 1H), 7.39–7.27 (m, 1H), 7.07 (t, J = 7.4 Hz, 1H), 7.01–6.87 (m, 3H), 5.12 (d, J = 5.0 Hz, 1H), 4.61 (dd, J = 9.9, 5.0, 1.9 Hz, 1H), 4.22 (dd, J = 12.4, 1.8 Hz, 1H), 4.10 (dd, J = 12.4, 4.9 Hz, 1H), 3.94–3.81 (m, 2H), 3.77–3.72 (m, 1H), 3.49 (dd, J = 12.3, 4.9 Hz, 1H). 13C NMR (100 MHz, DMSO-d$_6$) δ 159.06, 157.99, 152.16, 149.43, 132.59, 128.09, 126.11, 125.59, 125.50, 123.69, 114.52, 113.80, 112.42, 71.34, 67.18, 50.16, 24.86.

4-(2-Hydroxy-4-oxoquinazolin-3(4H)-yl)-1H,10H-phenanthroline 34. The title compound was prepared according to general procedure (2) using 2 (1g, 5.5 mmol) and 14 (1.15 g, 6.6 mmol). Off white solid (0.95 g, 48%). 1H NMR (400 MHz, DMSO-d$_6$) δ 8.29 (s, 1H), 8.09 (d, J = 2.5 Hz, 1H), 7.90–7.78 (m, 3H), 7.71 (d, J = 8.7 Hz, 1H), 7.54 (d, J = 8.3 Hz, 2H), 5.33 (d, J = 5.7 Hz, 1H), 4.62 (s, 2H), 4.28 (dd, J = 13.4, 3.6 Hz, 1H), 3.82 (dd, J = 13.4, 8.6 Hz, 1H). 13C NMR (100 MHz, DMSO-d$_6$) δ 159.93, 149.70, 147.22, 144.74, 134.81, 132.65, 131.60, 129.89, 128.31, 125.45, 123.30, 119.32, 110.53, 71.87, 66.97, 50.17. LCMS calculated for C$_{16}$H$_{10}$C$_{2}$N$_{2}$O$_{4}$ [M + H$^+$]: 356.1 found 356.6.

3-(3-(6-Chloro-4-oxoquinazolin-3(4H)-yl)-2-hydroxypropoxy)phenyl)propanenitrile 36. The title compound was prepared according to general procedure (2) using 2 (1g, 5.5 mmol) and 16 (1.3 g, 6.6 mmol). Off white solid (1.7 g, 80%). 1H NMR (400 MHz, DMSO-d$_6$) δ 8.31 (s, 1H), 8.12 (d, J = 2.5 Hz, 1H), 7.90–7.78 (m, 3H), 7.69 (m, 1H), 7.57 (d, J = 8.3 Hz, 2H), 5.33 (d, J = 5.7 Hz, 1H), 4.62 (s, 2H), 4.28 (dd, J = 13.4, 3.6 Hz, 1H), 3.82 (dd, J = 13.4, 8.6 Hz, 1H). 13C NMR (100 MHz, DMSO-d$_6$) δ 159.68, 159.48, 151.16, 148.43, 129.12, 127.11, 126.59, 126.50, 123.78, 120.61, 118.49, 110.75, 70.22, 67.18, 50.16. LCMS calculated for C$_{16}$H$_{12}$C$_{2}$N$_{2}$O$_{4}$ [M + H$^+$]: 356.1 found 356.2.

2-(3-(6-Chloro-4-oxoquinazolin-3(4H)-yl)-2-hydroxypropoxy)phenyl)cyclopropane-1-carbonitrile 37. The title compound was prepared according to general procedure (2) using 1 (1g, 5.5 mmol) and 16 (1.3 g, 6.6 mmol). Off white solid (1.7 g, 80%). 1H NMR (400 MHz, DMSO-d$_6$) δ 8.30 (s, 1H), 8.09 (d, J = 2.5 Hz, 1H), 7.86 (dd, J = 8.7, 2.5 Hz, 1H), 7.71 (d, J = 8.7 Hz, 1H), 7.24–7.15 (m, 2H), 6.93–6.84 (m, 2H), 5.48 (d, J = 5.7 Hz, 1H), 4.33 (dd, J = 13.4, 3.7 Hz, 1H), 4.16 (tt, J = 8.9, 5.2 Hz, 1H), 3.97 (dt, J = 7.0, 3.2 Hz, 2H), 3.89 (dd, J = 13.4, 8.6 Hz, 1H), 2.85–2.70 (m, 4H). 13C NMR (100 MHz, DMSO-d$_6$) δ 159.97, 157.72, 149.72, 147.23, 134.86, 131.63, 131.58, 129.97, 129.91, 125.49, 123.34, 120.75, 115.01, 110.75, 66.59, 50.07, 30.22, 18.95. LCMS calculated for C$_{16}$H$_{12}$C$_{2}$N$_{2}$O$_{4}$ [M + H$^+$]: 384.1 found 383.9, HRMS calculated for C$_{16}$H$_{12}$C$_{2}$N$_{2}$O$_{4}$ [M + H$^+$]: 384.1109 found 384.1109.
The title compound was prepared according to general procedure (2) using 2 (0.15 g, 0.8 mmol) and 18 (0.2 g, 1 mmol). White solid (93 mg, 25%).

The title compound was prepared according to general procedure (2) using 2 (0.13 g, 0.7 mmol) and 19 (0.18 g, 0.8 mmol). White solid (90 mg, 24%).

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The title compound was prepared according to general procedure (2) using 2 (0.13 g, 0.7 mmol) and 19 (0.18 g, 0.8 mmol). White solid (90 mg, 24%).
C6H2CO3 (13 mg, 0.10 mmol) was added. The mixture was stirred at 60 °C for 16 h. The mixture was purified using column chromatography eluting the desired compound with 95:5 EtOAc:MeOH containing NH4 (0.7 N). White solid (13 mg, 65%). 

1H NMR (400 MHz, DMSO-6) δ 8.34 (s, 1H), 8.10 (d, J = 2.5 Hz, 1H), 7.87 (dd, J = 8.7, 2.5 Hz, 1H), 7.72 (d, J = 8.7 Hz, 1H), 7.26 (d, J = 8.4 Hz, 2H), 6.92 (d, J = 8.4 Hz, 2H), 4.23 (dd, J = 13.5, 5.5 Hz, 1H), 4.00 (dd, J = 10.0, 5.2 Hz, 3H), 3.94 (s, 2H), 3.23 (s, 1H), 2.69 (dq, J = 14.1, 7.1 Hz, 1H), 1.90 (s, 1H), 0.91 (t, J = 7.1 Hz, 3H). 

13C NMR (101 MHz, DMSO-6) δ 160.03, 158.27, 154.86, 152.24, 149.76, 147.20, 134.86, 131.60, 129.91, 129.75, 129.75, 125.51, 123.79, 123.22, 120.00, 115.44, 114.54, 68.72, 55.03, 48.62, 41.25, 21.99, 15.96. LCMS m/z calc for C22H22ClN4O3 [M + H]+: 439.1, found 438.9.

**General Procedure (4)** for **Compounds 46–49**. To a stirred solution of the corresponding acid anhydride (1 equiv) in DMF (20 mL) was added a mixture of 42 (1.35 equiv), PyBrop (2 equiv), and trimethylamine (3 equiv). The mixture was stirred for 4 h at rt. The mixture was purified using column chromatography eluting the desired compound with DC: MeOH:9:1 with 0.1% NH4 (0.7 N).

**Preparation of N-(1-(6-Chloro-4-oxoquinazolin-3(4H)-yl)-3-(4-(cyanomethyl)phenoxy)propan-2-yl)-2-(dimethylamino)acetamide 49.** The title compound was prepared according to general procedure (4) using (dimethyl amino) acetic acid (12 mg, 0.12 mmol), 42 (60 mg, 0.16 mmol), PyBrop (0.1 g, 0.24 mmol), and trimethylamine (46 mg, 0.36 mmol). White solid (28 mg, 54%). 

1H NMR (400 MHz, DMSO-6) δ 8.32 (s, 1H), 8.10 (d, J = 2.5 Hz, 1H), 8.02 (d, J = 9.2 Hz, 2H), 7.87 (dd, J = 8.7, 2.5 Hz, 1H), 7.71 (d, J = 8.7 Hz, 1H), 7.29 (d, J = 8.7 Hz, 2H), 7.03–6.95 (m, 2H), 4.63 (d, J = 9.2 Hz, 1H), 4.39 (dd, J = 13.5, 4.1 Hz, 1H), 4.16–4.04 (m, 3H), 3.96 (s, 2H), 2.76 (d, J = 1.3 Hz, 2H), 2.12 (s, 6H). 

13C NMR (101 MHz, DMSO-6) δ 170.53, 159.96, 158.10, 149.02, 147.07, 134.93, 131.77, 129.95, 129.84, 125.50, 125.40, 123.18, 119.99, 115.57, 115.53, 68.45, 47.87, 47.33, 22.96, 22.00. LCMS m/z calc for C22H22ClN4O3 [M + H]+: 454.1, found 453.9.

**N-(1-(6-Chloro-4-oxoquinazolin-3(4H)-yl)-3-(4-(cyanomethyl)phenoxy)propan-2-yl)propanamide 50.** The title compound was prepared according general procedure (4) using 3-(dimethylamino) propionic acid (15 mg, 0.09 mmol), 42 (44 mg, 0.12 mmol), PyBrop (0.08 g, 0.18 mmol), and trimethylamine (35 mg, 0.27 mmol). White solid (27 mg, 59.67%). 

1H NMR (400 MHz, DMSO-6) δ 8.41–8.29 (m, 2H), 8.11 (dd, J = 11.0, 2.5 Hz, 1H), 7.89 (td, J = 9.0, 2.6 Hz, 1H), 7.73 (dd, J = 10.9, 8.7 Hz, 1H), 7.30 (dd, J = 7.8, 5.7 Hz, 2H), 7.03–6.94 (m, 2H), 4.59 (dq, J = 9.2, 4.5 Hz, 1H), 4.48–4.31 (m, 1H), 4.20 (s, 1H), 4.15–3.94 (m, 3H), 3.96 (s, 2H), 2.65–2.53 (m, 2H), 2.28 (t, J = 7.1 Hz, 1H), 2.25 (s, 4H), 2.10 (s, 1H), 1.19 (t, J = 7.1 Hz, 1H). 

13C NMR (101 MHz, DMSO-6) δ 170.31, 161.94, 158.04, 149.09, 147.04, 135.06, 131.77, 130.06, 129.82 125.53, 124.41, 123.17, 120.00, 115.71, 68.32, 53.49, 47.56, 45.94, 43.09, 30.92, 29.16, 25.92, 22.02. LCMS m/z calc for C22H22ClN4O3 [M + H]+: 468.1, found 467.9.

**N-(1-(6-Chloro-4-oxoquinazolin-3(4H)-yl)-3-(4-(cyanomethyl)phenoxy)propan-2-yl)-4-(dimethylamino)butanamide 51.** The title compound was prepared according general procedure (4) using 4-(dimethylamino) butyric acid (16 mg, 0.09 mmol) 42 (44 mg, 0.12 mmol), PyBrop (0.08 g, 0.18 mmol), and trimethylamine (35 mg, 0.27 mmol). White solid (27 mg, 58.76%). 

1H NMR (400 MHz, DMSO-6) δ 8.38–8.25 (m, 2H), 8.11 (d, J = 2.6 Hz, 1H), 7.88 (dd, J = 8.7, 2.5 Hz, 1H), 7.72 (d, J = 8.6 Hz, 1H), 7.34–7.25 (m, 2H), 7.04–6.95 (m, 2H), 4.58 (tt, J = 9.7, 4.8 Hz, 1H), 4.41 (dd, J = 13.5, 4.2 Hz, 1H), 4.10 (d, J = 5.4 Hz, 2H), 4.04–3.93 (m, 3H), 3.18 (d, J = 4.5 Hz, 1H), 2.71 (d, J = 14.3 Hz, 2H), 2.56 (s, 5H), 2.13 (td, J = 7.3, 3.1 Hz, 2H), 1.75–1.57 (m, 1H). 

13C NMR (101 MHz, DMSO-6) δ 172.03, 159.96, 158.08, 149.13, 147.03, 134.96, 131.79, 129.94, 128.94, 125.57, 124.88, 123.19, 120.01, 115.57, 75.48, 68.41, 56.92, 47.84, 43.29, 39.70, 39.49, 32.42, 22.01. LCMS m/z calc for C22H22ClN4O5 [M + H]+: 482.2, found 481.8.

https://doi.org/10.1021/acsinfecdis.0c00175

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6-Chloro-3-(3-chloropropyl)quinazolin-4(3H)-one 52. To a stirred solution of 2 (0.3 g, 1.6 mmol) in MeCN (50 mL), 1-bromo-3-chloropropane (0.3 g, 1.9 mmol), Cs₂CO₃ (1 g, 3.2 mmol), and TBAI (59 mg, 0.16 mmol) were added. The formed mixture was refluxed overnight. Subsequently, the reaction was cooled down, filtered, and reduced. The residue was chromatographed using PE:EtOAc 1:1 as an eluent. White solid (0.3 g, 73%). ¹H NMR (400 MHz, DMSO-d₆) δ 8.41 (s, 1H), 8.09 (d, J = 2.5 Hz, 1H), 7.86 (dd, J = 8.7, 2.5 Hz, 1H), 7.71 (d, J = 8.7 Hz, 1H), 4.11 (t, J = 6.6 Hz, 2H), 3.72 (t, J = 6.6 Hz, 2H), 2.19 (p, J = 6.6 Hz, 2H). ¹³C NMR (101 MHz, DMSO-d₆) δ 159.84, 148.92, 147.15, 134.84, 131.75, 129.94, 125.46, 123.33, 44.69, 43.04, 31.59. LCMS m/z calc for C₁₁H₁₁Cl₂N₂O⁺ [M + H]⁺: 257.0, found 259.1.

6-Chloro-3-(3-chloro-2-methylpropyl)quinazolin-4(3H)-one 53. To a stirred solution of 2 (0.3 g, 1.6 mmol) in MeCN (50 mL), 1-bromo-3-chloro-2-methylpropene (0.3 g, 1.9 mmol), Cs₂CO₃ (1 g, 3.2 mmol), and TBAI (59 mg, 0.16 mmol) were added. The formed mixture was refluxed overnight. Subsequently, the reaction was cooled down, filtered, and reduced. The residue was chromatographed using PE:EtOAc 1:1 as an eluent. White solid (0.3 g, 71%). ¹H NMR (400 MHz, DMSO-d₆) δ 8.38 (s, 1H), 8.10 (d, J = 2.5 Hz, 1H), 7.86 (dd, J = 8.8, 2.5 Hz, 1H), 7.72 (d, J = 8.8 Hz, 1H), 4.08–3.88 (m, 2H), 3.73–3.60 (m, 2H), 2.49–2.41 (m, 1H), 0.99 (d, J = 6.8 Hz, 3H). ¹³C NMR (101 MHz, DMSO-d₆) δ 160.70, 149.05, 147.08, 134.98, 131.81, 130.05, 125.63, 123.32, 50.79, 49.03, 34.73, 15.55. LCMS m/z calc for C₁₁H₁₂Cl₂N₂O⁺ [M + H]⁺: 271.0, found 270.8.

General Procedure (5) for Preparation of 54–55. To a stirred solution of 52 or 53 (0.3 g, 1 mmol) and TBAI (37 mg, 0.1 mmol) and Cs₂CO₃ (1 g, 1.3 mmol) in MeCN (100 mL) and 4-hydroxyphenyl acetonitrile (0.15 g, 1.1 mmol) was added. The mixture was heated at 100 °C overnight. The mixture was filtered and purified using column chromatography eluting the desired compound with 40:60 PE:EtOAc.

2-(4-(3-(6-Chloro-4-oxoquinazolin-3(4H)-yl)-2-methylpropoxy)phenyl)acetonitrile 54. The title compound was prepared according to general procedure (5). White solid (200 mg, 49%). ¹H NMR (400 MHz, DMSO-d₆) δ 8.39 (s, 1H CH¼), 8.09 (d, J = 2.5 Hz, 1H), 7.87 (dd, J = 8.7, 2.5 Hz, 1H), 7.72 (d, J = 8.7 Hz, 1H), 7.24–7.20 (m, 2H), 6.86–6.79 (m, 2H), 4.12 (m, 2H), 4.01 (m, 1H), 3.92 (d, J = 5.5 Hz, 4H), 1.02 (d, J = 6.8 Hz, 3H). ¹³C NMR (101 MHz, DMSO-d₆) δ 160.02, 158.23, 149.28, 147.13, 134.87, 131.74, 129.95, 129.69, 129.69, 125.53, 123.63, 123.30, 119.99, 112.55, 112.75, 70.95, 50.05, 21.97, 14.96. LCMS m/z calc for C₂₅H₂₁Cl₂N₄O₂⁺ [M + H]⁺: 368.1, found 367.7.

2-(4-(3-(6-Chloro-4-oxoquinazolin-3(4H)-yl)-2-hydroxypropoxy)phenyl)acetonitrile 55. The title compound was prepared according to general procedure (5). White solid (100 mg, 40%). ¹H NMR (400 MHz, DMSO-d₆) δ 8.41 (s, 1H CH¼), 8.10 (d, J = 2.6 Hz, 1H), 7.87 (dd, J = 8.7, 2.6 Hz, 1H), 7.72 (d, J = 8.7 Hz, 1H), 7.28–7.19 (m, 2H), 6.91–6.83 (m, 2H), 4.18 (s, J = 6.4 Hz, 2H), 4.05 (t, J = 6.4 Hz, 2H), 3.93 (s, 1H), 2.18 (p, J = 6.4 Hz, 2H). ¹³C NMR (101 MHz, DMSO-d₆) δ 159.85, 158.18, 149.12, 147.20, 134.84, 131.72, 129.95, 129.72, 129.72, 125.47, 123.63, 123.33, 120.00, 115.31, 115.31, 65.72, 44.57, 28.25, 21.98. LCMS m/z calc for C₂₅H₂₁Cl₂N₄O₂⁺ [M + H]⁺: 354.1, found 354.0.
MHZ, DMSO-d$_6$ $\delta$ 8.32 (s, 1H), 8.12 (d, $J$ = 2.5 Hz, 1H), 7.88 (dd, $J$ = 8.6, 2.5 Hz, 1H), 7.73 (d, $J$ = 8.6 Hz, 1H), 7.40 (s, 1H), 7.18 (d, $J$ = 8.2 Hz, 2H), 6.91–6.86 (dd, 2H), 6.83 (s, 1H), 5.50 (d, $J$ = 5.6 Hz, 1H), 4.35 (dd, $J$ = 13.4, 3.6 Hz, 1H), 4.18 (m, 1H), 4.02–3.84 (m, 3H), 3.30 (s, 2H). 13C NMR (101 MHz, DMSO-d$_6$) $\delta$ 173.00, 159.98, 157.46, 149.74, 147.24, 134.87, 131.64, 130.52, 130.52, 129.92, 129.26, 125.50, 123.33, 114.76, 114.76, 70.68, 66.51, 50.07, 41.82. LCMS m/z calc for C$_{16}$H$_{12}$ClN$_3$O$_3$ $^+$ [M + H]$^+$: 388.1, found 387.9.

2-(4-(3-(6-Chloro-4-oxoquinazolin-3(4H)-yl)-2-hydroxypropoxy)phenyl)acetic acid 60. A suspension of 22 (0.1 g, 0.27 mmol) in water (5 mL) and HCl (1 mL, 10.8) was stirred at 75 °C for 18 h. Afterward, the reaction was treated with saturated NaHCO$_3$ and then extracted with EtOAc (20 mL). The aqueous layer was then acidified with HCl (36%), and the resulting solid was filtered and used without further purification. Off white solid (46 mg, 44%). 1H NMR (400 MHz, DMSO-d$_6$) $\delta$ 12.2 (br s, 1H), 8.32 (s, 1H), 8.23 (d, $J$ = 2.2 Hz, 1H), 7.71 (dd, $J$ = 7.4, 2.1 Hz, 1H), 7.52 (d, $J$ = 7.5 Hz, 1H), 7.21–7.13 (m, 2H), 6.89–6.81 (m, 2H), 5.07 (d, $J$ = 5.0 Hz, 1H), 4.91–4.81 (m, 1H), 4.23 (dd, $J$ = 12.4, 1.7 Hz, 1H), 4.10 (dd, $J$ = 12.4, 4.9 Hz, 1H), 3.82 (dd, $J$ = 12.5, 9.9 Hz, 1H), 3.77–3.67 (m, 1H), 3.60–3.43 (m, 2H). 13C NMR (101 MHz, DMSO-d$_6$) $\delta$ 174.89, 159.87, 156.72, 150.16, 148.43, 133.56, 128.83, 127.11, 114.64, 71.22, 67.18, 50.16, 42.50. LCMS m/z calc for C$_{16}$H$_{12}$ClN$_3$O$_3$ $^+$ [M + H]$^+$: 389.1, found 388.8.

**General Procedure (6) for Preparation of 61–62.** To a stirred solution of 2-(4-(hydroxypropyl)acetanilide (1 g, 5.3 mmol) in 100 mL MeCN was added Cs$_2$CO$_3$ (3.3 g, 10.6 mmol) and either (R)-2-(4-oxo-2-ylmethyl 3-nitrobenzensulfonate or (S)-oxiran-2-ylmethyl 3-nitrobenzensulfonate (1.7 g, 6.4 mmol), and the mixture was refluxed for 18 h. Subsequently, the reaction mixture was filtered and concentrated. The residue was purified using PE:EtOAc 9:1 as an eluent. Either (R)-2-(4-(oxiran-2-ylmethoxy)phenyl)-acetanilide or (S)-2-(4-(oxiran-2-ylmethoxy)phenyl)-acetanilide (0.5 g, 3 mmol) was further reacted with 2 (0.47 g, 2.6 mmol) in the presence of Cs$_2$CO$_3$ (1.7 g, 5.2 mmol) and TBAI (110 mg, 0.3 mmol) according to general procedure 2. (R)-2-(4-(3-(6-Chloro-4-oxoquinazolin-3(4H)-yl)-2-hydroxypropoxy)phenyl)acetanilide 61. White solid (0.56 g, 59%). 1H NMR (400 MHz, DMSO-d$_6$) $\delta$ 8.32 (s, 1H), 8.08 (d, $J$ = 2.3 Hz, 1H), 7.91 (dd, $J$ = 8.7, 2.3 Hz, 1H), 7.71 (d, $J$ = 8.7 Hz, 1H), 7.24 (d, $J$ = 8.2 Hz, 2H), 6.89 (d, $J$ = 8.3 Hz, 2H), 5.51 (d, $J$ = 5.6 Hz, 1H), 4.33 (dd, $J$ = 13.5, 3.6 Hz, 1H), 4.17 (m, 1H), 4.12–3.94 (m, 2H), 3.94 (s, 2H), 3.94–3.86 (m, 1H). 13C NMR (101 MHz, DMSO-d$_6$) $\delta$ 159.48, 158.50, 149.61, 147.28, 134.89, 131.63, 129.91, 129.78, 125.57, 123.78, 123.39, 119.99, 115.48, 70.72, 66.98, 50.32, 22.01. LCMS calculated for C$_{19}$H$_{17}$ClN$_3$O$_4$ $^+$ [M + H]$^+$: 370.1 found 370.5. HRMS calculated for C$_{19}$H$_{17}$ClN$_3$O$_4$ $^+$ [M + H]$^+$: 370.0953 found 370.0950.

**Bacterial Strains and Growth Conditions.** The *P. aeruginosa* strains and plasmids used in this study are shown in Table 4. Bacteria were grown in lysogeny broth (LB) at 37 °C, unless stated otherwise. Where required, tetracycline (Tc) was added to the media at 125 µg/mL, to select for recombinants. Synthetic alkylquinolones were added at the concentrations indicated.

** Biosensor Reporter Assay.** Strains PA14 mCTX::p$_{pqsA}$-lux and PAO1-L mCTX::p$_{pqsA}$-lux were constructed using plasmid mini-CTX::p$_{pqsA}$-lux as previously described, and the assay was performed according to a published method. For initial screening, the compounds were tested at a concentration of 10 µM, which was prepared from a 10 mM stock, in DMSO.

**Isothermal Titration Calorimetry (ITC).** PqsR (PqsR$^{RUB}$) was prepared by Ni-NTA (HisTrap HP) followed by gel filtration with a Superdex 75 16/60 and flash frozen in liquid nitrogen until required. For ITC, the sample was thawed, clarified by centrifugation, and dialyzed overnight against a buffer of 50 mM tricine-NaOH (pH = 8.5), 250 mM NaCl, 5% glycerol. The sample was quantified by A280 using an extinction coefficient of 22920 M$^{-1}$ cm$^{-1}$. The sample was diluted to 10 µM. Compound 61 was dissolved in DMSO and diluted into assay buffer to 100 µM. An appropriate amount of DMSO was added to the sample to prevent mismatch. ITC measurements were conducted on a MicroCal PEAK ITC.
instrument using a sample volume of 280 μL with 19 ligand injections of 2 μL. The cell temperature was kept at 25 °C and stirring was carried out at 750 rpm. Reference power was set to 6 DP with spacing kept at 180 s. The same procedure was carried out for the T265A mutant. Mutants were prepared using the site-directed mutagenesis protocol from Agilent and the primer sequences: 5′-GGCGATCCGACCGAAGGTT-GATTTGC-3′ and 5′-GCAATACACCTTCGGCTGA-TGCCGC-3′.

**Protein Expression and Crystallization.** Crystallization and structural solution was performed as previously described.10,19

**Pyrroloquinoline Quinone Quantification.** The experiment was performed following a published protocol with minor modifications.33 PA strains were cultured into 5 mL fresh medium overnight. Compounds were assayed at 3 × IC_{50} concentration, for 16 h, at 37 °C (Kuhner LT W Shaker, Adler Kühner AG, Basel, Switzerland). Cells were centrifuged at 10,000 RCF for 10 min (Allegra 64R centrifuge, Beckman Coulter, High Wycombe, UK), and the supernatant was transferred to 15 mL falcon tubes with a HSW 10 mL Soft-Ject Syringe and a 0.22 μM Sartorius syringe-driven filter (Fisher Brand, Loughborough, UK). Pyroloquinoline was extracted into chloroform by mixing 7.5 mL of supernatant with 4.5 mL of chloroform. Pyroloquinoline was further extracted into 1.5 mL of 0.2 M HCl, which gave a pink/red solution, and the absorbance was measured at 520 nm.

**LCMS-MS Alkyl Quinoline Quantification.** For each test sample, 100 μL of sterile filtered supernatant (the same preparation as for pyroloquinoline using the following concentrations: PAO1-L 3 μM, PA14 3 μM, PA7−48 15 μM, LESB58 5 μM, and PAK6085 9 μM) was spiked with 10 μL of an internal standard solution (10 μM d4-PQS in MeOH) and diluted with water, to a total volume of 500 μL. Samples were then extracted three times with an 0.5 mL aliquot of ethyl acetate, vortex mixing the aqueous/organic mix for 2 min, then stirring was carried out as 750 rpm. Reference power was set to 10,000 RCF for 10 min (Allegra 64R centrifuge, Beckman Coulter, High Wycombe, UK), and the supernatant was filtered supernatant (the same sample). An inoculum was prepared separately for each treatment at the indicated concentrations were washed in PBS and the viability of attached cells evaluated by fluorescent staining with the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Life Technologies) according to manufacturer instructions. Following staining, coverslips were rinsed with distilled water and imaged using a LSM700 AxioObserver (Carl Zeiss, Germany) confocal laser scanning microscope (CLSM). Viable and nonviable biofilm biomass quantification from image stacks of biofilms was done with Fiji-ImageJ software. Live/dead ratios were established for each treatment and compared to untreated controls.

**Galleria mellonella Survival Assay.** Antibiotic free Galleria mellonella were purchased from Livefoods Direct Ltd. (Sheffield, UK) maintained at room temperature in the dark and used within 3 days. Larvae were selected in the final (7th) instar and were randomly assigned to groups after weighing ~250 mg. Ten larvae were assigned to each grouping in 9 cm Petri dishes: Sham (injection, no infection), injection (PBS), DMSO only (DMSO) inoculation only (PA), inoculation plus ciprofloxacin (PA + CIP), inoculation plus 61 (PA + 61) and inoculation plus ciprofloxacin and 61 (PA + CIP + 61). The PA strains PAO1-N and PAO1-L were selected as these give differing rates of mortality, chronic-like and acute-like respectively. An inoculum was prepared separately for each grouping at a final size of 1 × 10^4 CFU/mL. The addition of the quorum sensing inhibitor 61 was performed prior to inoculation to a final concentration of 10 μM. Inoculation of wax worms was performed using a 50 μL Hamilton syringe (Fisher, Leicestershire, UK) with 29 G 0.5 mm needle attachment (Becton Dickinson U.K. Ltd, Oxford, UK) briefly 10 μL of inoculum was injected into the rear most left pro-leg of the larve. To the rear most right pro-leg 10 μL of 0.025 μg/mL CIP treatment was injected post inoculum for Cip treated wax worms. Wax worm larvae were incubated at 37 °C in the dark. Larvae were monitored hourly and death event recorded when no movement was observed and no response to mechanical stimulus was elicited.

**Cytotoxicity Assay.** The human carcinoma lung epithelial cells A549 (ATCC CCL-185) are grown at 37 °C in 5% CO_2 for 24 h in RPMI medium. A cell concentration of 1 × 10^5 cells/mL was used and dispensed in 96 well plates (100 μL in each well). A serial dilution of 61 was then prepared in growth media and added to the plate in triplicate. Potassium dichromate at 100 μM was used as positive control, and a solvent vehicle control was prepared as negative control. Cells were then incubated for a further 16 h, and 20 μL of Alamar blue added to each well. After 5 h incubation, fluorescence was
measured with excitation at 510 nm and emission at 590 nm. Values were then normalized against the untreated control.55


Sigmoidal dose–response curves and the representation of all data were prepared using GraphPad Prism 9.0.2.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00175.

Structures and activities of commercial analogues of 22, representative isothem generated from injection of 61 into the T265A PqsR mutant construct, and crystallographic data collection and refinement statistics (PDF)

Special Issue Paper
Published as part of the Antibiotic Alternatives special issue.

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Author Contributions
F.S. and A.M. contributed equally to this work. F.S. performed in silico and in vitro screening, designed and performed syntheses directed the microbiology experiments. A.M. performed syntheses, microbiology experiments, and contributed to writing. F.S., M.S., P.W., and M.C. designed and supervised the study and led the writing of the manuscript. E.V.O., M.R., S.N.R., S.G., R.L., and N.H. performed the experimental microbiology. W.R. and J.E. performed and designed crystallography and ITC experiments. B.K., R.L., S.H., T.S., C.A.S.B., I.K.-I., and R.C.L. contributed to experimental design. We also thank Jeff Gauthier for his support in performing sequence alignment.

Funding
This work was supported by the Engineering and Physical Sciences Research Council [grant numbers EP/N006615/1 and EP/K005138/1, EP/N03371X/1] and JPI-AMR and MRC for funding the SENBIOTAR program (ref. MR/N501852/1). F.S., M.R., S.N.R., P.W., M.J.S., J.E., and M.C. are funded by the National Biofilms Innovation Centre (NBIC) which is an Innovation and Knowledge Centre funded by the Biotechnology and Biological Sciences Research Council, InnovateUK and Hartree Centre [Award Number BB/R012415/1]. S.G. and W.R. are funded via the Wellcome
Trust doctoral training program in antimicrobials and antimalarial resistance (ref: 108876/B/15/Z).

Notes
The authors declare no competing financial interest.

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