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Competitive Live-Cell Profiling Strategy for Discovering Inhibitors of the Quinolone Biosynthesis of *Pseudomonas aeruginosa*

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Supporting Information

ABSTRACT: Quinolones of the human pathogen *Pseudomonas aeruginosa* serve as antibacterial weapons and quorum sensing signals and coordinate the production of important virulence factors. A central enzyme for the biosynthesis of these quinolones is the synthetase PqsD. We developed an activity-based probe strategy that allows to screen for PqsD inhibitors in a cellular model system of live cells of *Escherichia coli* overexpressing PqsD. This strategy allowed us to determine IC_{50} values for PqsD inhibition directly in live cells. Our most potent inhibitors were derived from the anthranilic acid core of the native substrate and resulted in single-digit micromolar IC_{50} values. The effectiveness of our approach was ultimately demonstrated in *P. aeruginosa* by the complete shutdown of the production of quinolone quorum sensing signals and quinolone *N*-oxides and a considerable inhibition of the production of phenazine virulence factors.

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Screening approaches with purified proteins have yielded numerous potent enzyme inhibitors. Yet, *in vitro* activity does not always go along with biological efficacy in an organism. Reasons are often permeability barriers, active detoxification mechanisms, or promiscuous binding to off-targets.[1,2] Thus, strategies for probing enzyme inhibition in a living cell are highly desirable. Activity-based probes have become powerful tools for the discovery of target proteins of small molecules,[3−5] for diagnostic purposes[6,7] and for dissecting enzyme functions and biochemical pathways.[8−11] Competitive ABPP strategies have been successfully applied to discover selective enzyme inhibitors in the background of complex proteomes.[12−14] We have recently shown that simple electrophilic chemical probes can selectively target the active site cysteine of the *Pseudomonas aeruginosa* quinolone biosynthesis enzyme PqsD *in vitro*.[15] Since this approach failed to generate *in situ* inhibitors of quinolone biosynthesis we now aimed for a live-cell inhibitor screening strategy. The human pathogen *P. aeruginosa* produces more than 50 different quinolones.[16] For instance, 2-heptyl-4-quinolone (HHQ) and the *Pseudomonas* quinolone signal (PQS) serve as quorum sensing signals controlling the production of virulence factors and modulate the host immune response.[17−19] 2-Alkylquinolone *N*-oxides (AQNOs) inhibit growth of competing bacterial species such as *Staphylococcus aureus* and contribute to autolysis of aged *P. aeruginosa* cultures (Figure 1a).[20,21] Since quinolones have many important roles for the virulence of *P. aeruginosa* and its ability to occupy and defend niches in the human body, quinolone biosynthesis is a prime target for potential antivirulence strategies. A common step in the biosynthesis of all quinolones is the decarboxylative coupling reaction of coenzyme A-activated anthranilic acid (ACoA) with malonyl-CoA, which is catalyzed by PqsD.[22,23] Hydrolysis of the resulting thioester by PqsE leads to 2-aminobenzoylacetate (2-ABA), which is the precursor for the subsequent reactions to 2-alky-4-quinolones (AQs), 3-hydroxy-AQs, and AQNOs (Figure 1a).[24] We here report a competitive in-cell probe strategy that allowed to detect a potent mechanism-based PqsD inhibitor resulting in a global and complete shutdown of quinolone biosynthesis in *P. aeruginosa*.

The active site of PqsD comprises a nucleophilic cysteine residue, which is involved in the transfer of the anthraniloyl moiety.[23] We have demonstrated with purified enzyme that simple electrophilic α-chloroacetamide probes have a remarkable selectivity for this cysteine residue (Cys112), and its mutation to alanine prevented probe labeling.[15]

We now aimed for a live-cell labeling strategy using the α-chloroacetamide probe CA (Figure 1b, Figure S1). Hereby, we incubated intact cells with the probe, removed excess probe by washing steps and after cell lysis employed click chemistry to append a fluorescent tetramethylrhodamine tag to the terminal alkyne group for visualization by SDS-polyacrylamide gel electrophoresis (PAGE). However, when we applied the probe at 100 μM to live cells of a stationary phase culture of *P. aeruginosa*, no fluorescent band in the size range of PqsD was detected. Coomassie staining indicated, that PqsD was of low abundance in the native proteome of *P. aeruginosa*, which was likely too low for *in situ* labeling by the CA probe (Figure 1b). We thus speculated that *Escherichia coli* cells overexpressing PqsD may provide a useful *in situ* model for in-cell labeling. Indeed, incubation of cells with 100 μM of CA probe revealed a band only for *E. coli* cells expressing PqsD but not for the parent *E. coli* strain (Figure 1c). The labeling hereby achieved remarkable sensitivity down to submicromolar probe concentrations (Figure 1d). The band of the corresponding protein size was cut out and after tryptic digest subjected to proteomic analysis by mass spectrometry. The results confirmed the sequence of PqsD and in addition the active site cysteine as the site of covalent attachment of the probe (Figure S2). Since our probe selectively and specifically labeled the active site of PqsD in the background of the native proteome of live *E. coli* cells, we now aimed to apply this probe as tool for live-cell compatible

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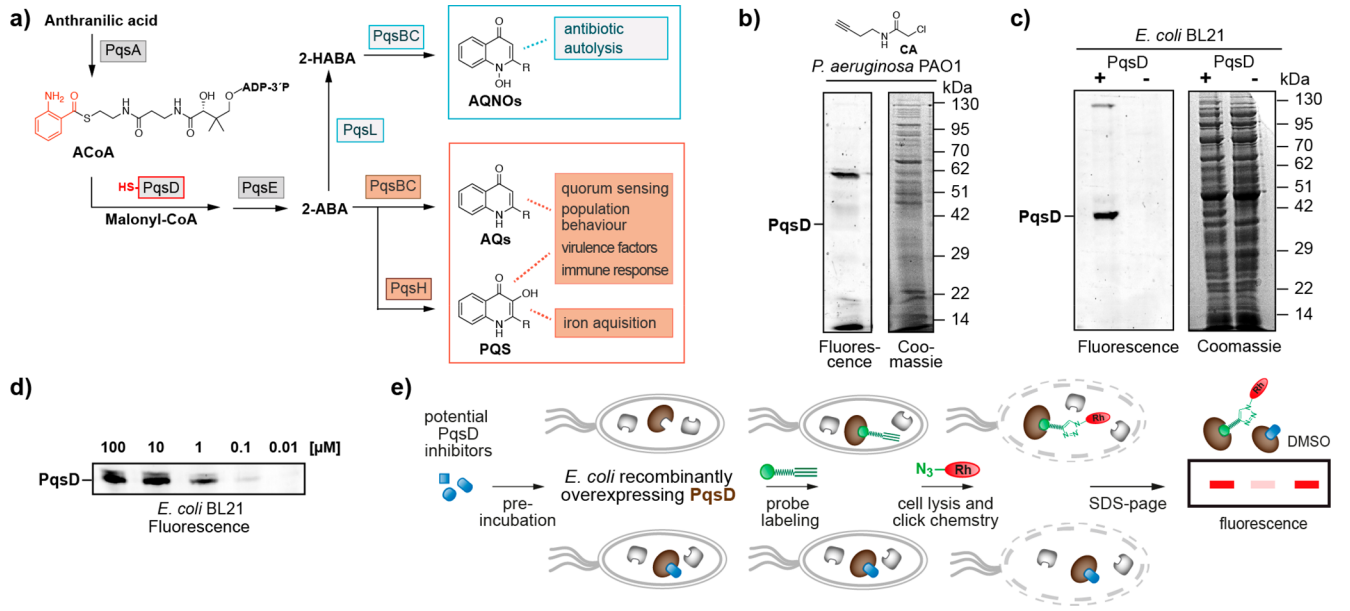

Figure 1. *In situ* labeling of PqsD. (a) Biosynthesis of the different quinolone classes of *Pseudomonas aeruginosa* and their biological effects. (b) *In situ* labeling of *P. aeruginosa* PAO1 by probe CA showed no fluorescent band for PqsD. (c) Live *E. coli* BL21 cells overexpressing PqsD labeled by probe CA (100 μM) resulted in a strong band for PqsD compared to the parent BL21 strain. (d) Dose-down of probe CA with live *E. coli* overexpressing PqsD. (e) Scheme of the competitive labeling platform. 2-HABA: 2′-hydoxyaminobenzoylacetate.

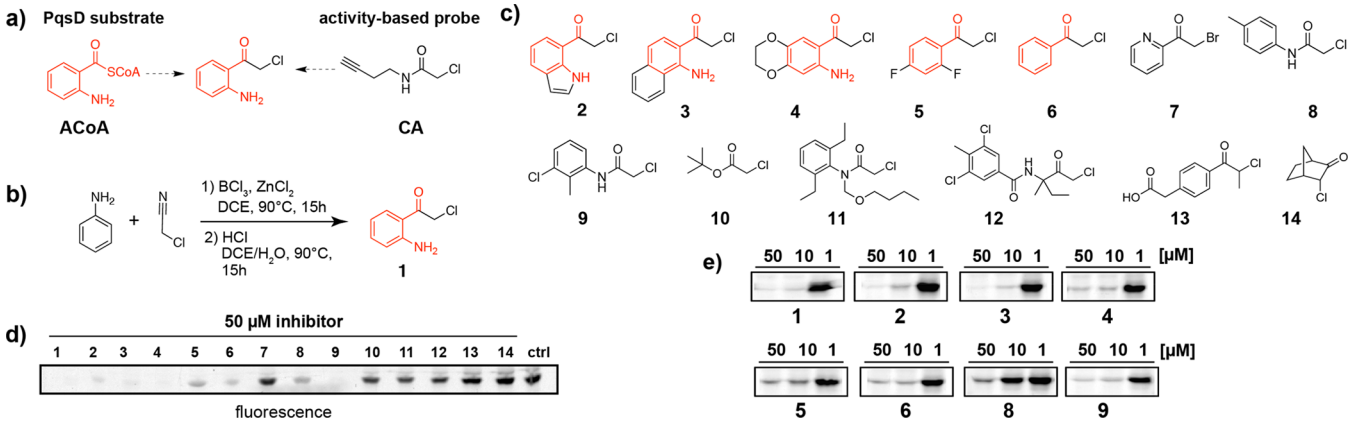

Figure 2. *In situ* screening of potential PqsD inhibitors. (a) Inhibitor design based on the anthraniloyl-CoA core. (b) Synthesis of α-chloroacetophenone **1**. (c) Focused compound library of PqsD substrate analogs. (d) Initial screening of the compound library in PqsD overexpressing *E. coli* cells. (e) Concentration-dependent labeling experiments with active inhibitors.

inhibitor discovery. Effective PqsD inhibitors would block the active site and thereby inhibit intracellular labeling by the probe (Figure 1e). To this aim, we compiled a small focused library of 14 compounds comprising an α-halocarbonyl-moiety, some of which mimicked the core of the native PqsD substrate anthraniloyl-CoA (Figure 2a). We hypothesized that fusing of the core structure of anthranilate and the electrophilic α-chloroacetyl group of the probe CA could lead to covalently binding inhibitors exhibiting high affinity toward PqsD. 2′-Amino-α-chloroacetophenone **1** was synthesized using Houben–Hoesch acylation (Figure 2b) and other compounds (**2**–**14**) were obtained commercially. For an initial screening, PqsD expressing *E. coli* cells were preincubated with the library compounds at 50 μM for 15 min followed by incubation with 100 μM probe CA. Successful inhibitors that bind to the active site of PqsD would be detected by reduction in fluorescence signal intensity in the gel. All compounds with an anthranilic acid-derived core were active and completely abolished labeling. Compounds that did not considerably reduce probe labeling (**10**–**14**) had a rather bulky nonflat architecture. For compound **7**, we considered electronic reasons of the pyridine core being responsible for its poor activity. The remaining compounds were tested in concentration series.

Quantifying the intensity of competitive probe labeling over a broad range of concentrations ranging from 0.05 to 100 μM provided the unique opportunity to determine IC_{50} values for PqsD inhibition in living cells (Figure 3a, Figures S3–S5). Compounds resembling the anthranilic acid core of the native substrate were among the most potent inhibitors, such as **1**–**6** with an α-chloroketone motif exhibiting IC_{50} values of 1–3 μM (Figure 3a). Also compound **9** with an α-chloroacetamide motif was highly active, while closely related compound **8** was significantly less active, indicating that already small changes in substitution pattern had major impacts. Using proteomic analysis of PqsD inhibited by compound **1** in live cells, confirmed that the compound only bound to the active site cysteine

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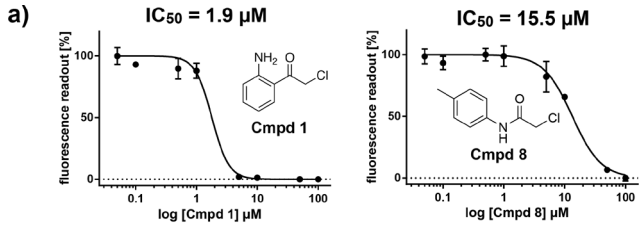
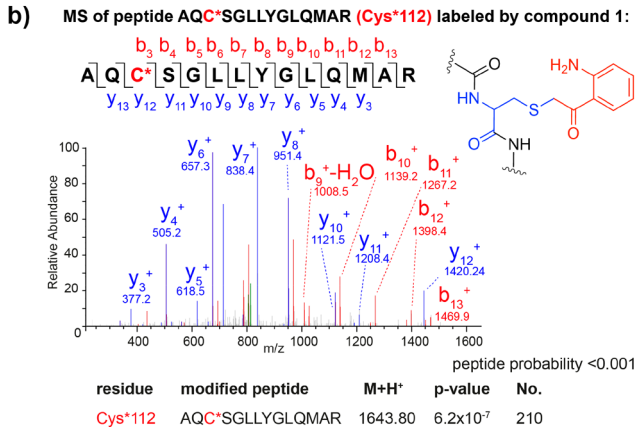


Cmpd	IC_{50} (μM)	Cmpd	IC_{50} (μM)
1	1.9 ± 0.3	5	1.3 ± 0.2
2	2.5 ± 0.5	6	1.8 ± 0.2
3	1.2 ± 0.1	8	15.5 ± 2.6
4	2.2 ± 0.2	9	2.1 ± 0.3



residue	modified peptide	M+H+	p-value	No.
Cys*112	AQC*SGLLYGLQMAR	1643.80	6.2×10^{-7}	210

Figure 3. Inhibition of PqsD *in situ*. (a) Dose−response curves of compounds **1** and **8** and IC_{50} values of all active compounds. (b) Tryptic peptide fragment with compound **1** covalently bound to the active site cysteine of PqsD after *in situ* inhibition.

(Cys112) although the protein structure comprises in total six cysteines, five of which are not involved in catalysis (Figure 3b). This indicates a highly selective mechanism-based mode of action. Off-target protein bands labeled by simple electrophilic probes in *P. aeruginosa* were not competed by compound **1**, suggesting the inhibitor was more specific than these probes (Figure S6). Consequently, we were interested to investigate if also quinolone production of *P. aeruginosa* could be inhibited. Since primary amines facilitate the accumulation of compounds in Gram-negative bacteria[25] and due to the close structural resemblance with the anthranilic substrate, we selected compound **1** for *in situ* assays with *P. aeruginosa*. Growth remained largely unaffected with compound **1** and **2** at concentrations up to 100 μM, ruling out that potential effects on virulence would be an artifact of growth inhibition (Figure S7). We thus could investigate the effects on the biosynthesis of quinolones. PqsD is responsible for the biosynthesis of more than 50 quinolones, including AQs such as HHQ and its 3-hydroxy-derivative PQS, as well as different AQNOs.[16] We established a LC-MS/MS method using characteristic mass transitions for quantitative analysis of metabolites in the supernatants of *P. aeruginosa* (Table S1, Figures S8−S10). A library of phenazines and synthetic AQs and AQNOs that we reported recently served as standards.[26] We also analyzed phenazines, including phenazine-1-carboxamide (PHZ-CA), 1-hydroxyphenazine (1-OH-PHZ), and pyocyanin (PYO), which are produced as virulence factors partially under control of the PQS quorum sensing system (Figure 4a).

Quantifying quinolone production over time revealed that AQs and AQNOs are produced between 5 and 10 h and then remain constant to 24 h (Figure S11). We thus incubated cultures of *P. aeruginosa* strains PAO1 and PA14 with different concentrations of **1** for 24 h and used extracted culture

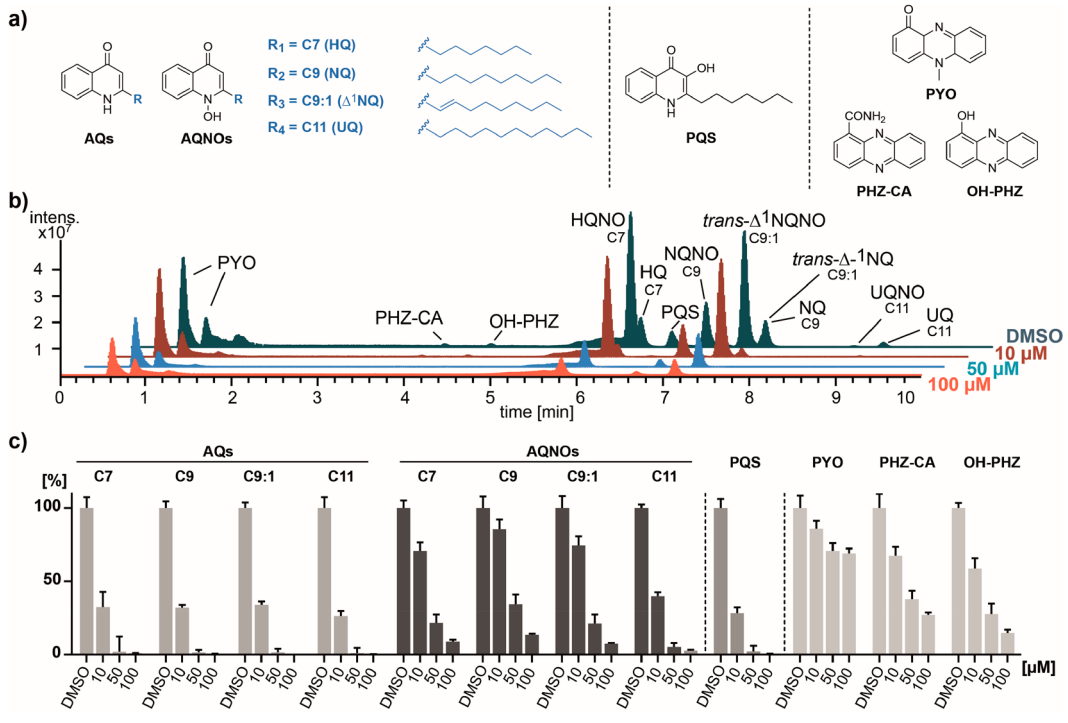


Figure 4. Quinolone and phenazine inhibition in *P. aeruginosa* PAO1 cultures treated with compound **1**. (a) Structures of quinolones and phenazines analyzed by LC-MS/MS. (b) Chromatograms of mass transitions from extracted supernatants after incubation with **1** at different concentrations for 24 h. (c) Percentage of inhibition of extracellular quinolones and phenazines.

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supernatants for LC-MS/MS analysis. Increasing concentrations of the PqsD inhibitor considerably decreased the intensity of quinolones and phenazines in both strains (Figure 4b, Figure S12a). Quantification of individual quinolones revealed a global down-regulation of AQs and AQNOs. The production of all major quorum sensing signals, PQS and the chain length congeners of HHQ was completely inhibited in *P. aeruginosa* PAO1 between 50 and 100 μM with an average IC_{50} < 10 μM and to a slightly lesser degree in *P. aeruginosa* PA14 (Figure 4c, Figures S12b and S13). Inhibition of quinolone signal production consequently caused the down-regulation of phenazines, which was strongest for 1-OH-PHZ and weakest for pyocyanin. Finally, also production of the different quinolone *N*-oxide congeners was inhibited by compound **1** in dose response manner including the three major *N*-oxides HQNO, NQNO and *trans*-Δ^1-NQNO. In comparison, compound **2** inhibited quinolone production less effectively (Figure S14). External addition of synthetic 2-ABA[27] to a culture of *P. aeruginosa* PAO1 grown with 50 μM of compound **1** partially restored HHQ production (Figure S15). Our results with *P. aeruginosa* confirm compound **1** as highly active inhibitor with unprecedented efficacy in the global inhibition of quinolone biosynthesis and down-regulation of phenazine production.

P. aeruginosa is listed by the World Health Organization on the highest priority level requiring urgently new treatment options.[28] Quinolone-based quorum sensing has attracted much attention due to its decisive impact on the virulence of *P. aeruginosa*. Over the past years, the biosynthesis of quinolones in *P. aeruginosa* has been investigated in detail and revealed possible intervention points.[22,27,29] For instance, inhibitors of PqsA, PqsBC as well as PqsE have been developed by rational design and fragment-based screening, respectively.[27,30,31] PqsD has been in the focus of inhibitor development with pioneering work led by the Hartmann group.[32−36] Although potent *in vitro* inhibitors have been reported, *in situ* efficacy remained rather moderate.[37] More potent effects on virulence could only be achieved by dual inhibition of PqsD and the transcriptional regulator PqsR.[38] To the best of our knowledge, our anthranilic acid-derived covalent inhibitor **1** is the most potent *in situ* PqsD inhibitor reported so far that causes global inhibition of quinolone biosynthesis. While compound **1** exhibits moderate toxicity to eukaryotic cells (Figure S16), substituted α-chloroacetylindoles such as **2** were well tolerated in a mouse model.[39] Thus, our inhibitors may be promising candidates for the development of an anti-infective drug against *P. aeruginosa*. While many quinolones are signals controlling virulence, quinolone *N*-oxides serve as weapons against competing bacterial species and likely support the colonization of various niches.[20] Our group recently demonstrated major activity of unsaturated *trans*-Δ^1-NQNO against *Staphylococcus aureus* while the saturated congeners are much less potent[26] and we speculated about functional specialization of different AQNOs.[40] Our potent PqsD inhibitors may hereby serve as valuable tools to dissect the roles of the diverse quinolones produced by *P. aeruginosa*.

In conclusion, we could demonstrate the power of a competitive *in situ* labeling strategy using an electrophilic activity-based probe and target protein expressing *E. coli* cells. This strategy allowed to screen for customized enzyme inhibitors in a live-cell model system. We established this strategy for the quinolone biosynthesis enzyme PqsD as an example and were able to identify potent inhibitors with unprecedented efficacy in the inhibition of quinolone biosynthesis in *P. aeruginosa*.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b07629.

Additional data and methods (PDF)

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Notes

The authors declare no competing financial interest.

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