Title: Auranofin inhibits virulence in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is widely attributed as the leading cause of hospital-acquired infections. Due to intrinsic antibiotic resistance mechanisms, as well as the ability to form biofilms, *P. aeruginosa* infections are notoriously difficult to treat. *P. aeruginosa* employs multiple virulence mechanisms to establish infections, and the global virulence regulator, Vfr, plays an important role in modulating these mechanisms. An attractive strategy to combat *P. aeruginosa* infections is the use of anti-virulence compounds. Here, we report the identification of auranofin, an FDA-approved drug, that attenuates virulence in *P. aeruginosa*. We present evidence that auranofin exerts its action by targeting Vfr, which in turn leads to inhibition of quorum sensing (QS), Type III secretion (T3SS) and Type IV pili (TFP). Consistent with potent inhibition of QS and TFP expression, we show that auranofin attenuates biofilm maturation, and when used in combination with colistin, displays strong synergy in eradicating *P. aeruginosa* biofilms both in flow chambers and in a murine implant model. Auranofin may have immediate applications as an anti-virulence drug against *P. aeruginosa* infections.

Introduction

Pseudomonas aeruginosa is a prevalent opportunistic pathogen known for its potent virulence, environmental versatility and multi-drug resistance. Its role as the leading cause of hospital-acquired infections (HAIs) among immuno-compromised patients is well documented¹. Notably, it is one of the main causes of pneumonia in cystic fibrosis (CF) patients², and wound infections in surgical patients or burn victims³. Furthermore, *P. aeruginosa* has been known to thrive in a variety of ecological niches⁴, allowing colonization on abiotic surfaces like catheters and other implanted medical devices^{5,6}. Increasing incidences of multi-drug resistant (MDR) *P. aeruginosa* infections^{7,8} in the clinic pose a serious threat to global health, underscoring the need to develop new strategies to combat these pathogens.

P. aeruginosa carries several intrinsic antibiotic resistance mechanisms involving the collaboration of restricted uptake through the outer membrane⁹, the use of energy-dependent efflux pumps^{10,11}, and expression of β -lactamases^{12,13}. In addition, *P. aeruginosa*, like many other bacteria, exhibits a form of social behavior known as biofilm formation¹⁴, which further increases its tolerance to antibiotics¹⁵. A biofilm is a community of bacteria encased in a polymeric mesh consisting of extracellular DNA (eDNA), proteins and polysaccharides. In the clinic, significantly higher patient mortality has been associated with the formation of biofilms in the lungs of CF patients¹⁶. Specifically, the presence of biofilms increases the complexity of treatment due to the function of the extracellular matrix as a physical barrier against, and sponge for, antimicrobial drugs¹⁷. The small pore size of the biofilm mesh effectively reduces penetration of lysozymes and macromolecular drugs, presumably decreasing the immediate concentrations of these agents surrounding the cells. Additionally, the biofilm matrix is also highly negatively charged due to the presence of eDNA and polysaccharides, which protect the colony from positively-charged aminoglycosides¹⁸. Consequently, the inhibition of biofilm

formation, maturation and/or function remains a principal consideration in the search for new treatment options against *P. aeruginosa*.

An attractive alternative strategy to control bacterial infections has been the use of small molecules that block virulence. Such anti-virulence compounds may reduce the ability of bacteria to infect the host^{19,20}, and/or attenuate bacterial defenses against host immune cells²¹, thereby facilitating clearance of the infection site²². The virulence of *P. aeruginosa* is multifaceted but largely controlled by the global virulence factor regulator, Vfr²³. Vfr is a cyclic adenosine monophosphate (cAMP)-dependent transcription factor that coordinates expression of roughly 200 genes^{24,25}. It regulates virulence directly by activating expression of secretion systems²⁶ (e.g. Type III) and the pilus formation machinery²⁷ (Type IV). The Type III secretion system (T3SS) in *P. aeruginosa* mediates cytotoxicity against host cells through the delivery of molecular effectors that can disrupt host processes²⁸. The Type IV Pilus (TFP) is important for twitching motility²⁹, and often associated with the establishment and maturation of *P. aeruginosa* biofilms. Vfr also controls quorum sensing (QS)³⁰, a cell-cell communication network involving the *las*-, *rhl*- and *pqs*-encoded systems, which plays a role in the production of several virulence factors including extracellular proteases, biosurfactants and toxins²¹, and is important for biofilm maturation³¹ and immune evasion. While many small molecules that inhibit QS and T3SS have been reported³²⁻³⁴, no inhibitors of Vfr and TFP have been discovered to date.

In this work, we identified auranofin, an FDA-approved, gold(I)-based anti-inflammatory drug, as a potent inhibitor of virulence in *P. aeruginosa*. We show that auranofin inhibits QS, T3SS and TFP formation at low micromolar concentrations. It does this in part by targeting cysteines in Vfr and abolishing the ability of Vfr to bind DNA. Using flow cell biofilm

87 assays, we demonstrate that auranofin is able to attenuate biofilm maturation. Furthermore,
88 auranofin displays synergistic effects in eradicating *P. aeruginosa* biofilms *in vitro*, when used
89 in combination with colistin. We propose that auranofin can potentially be re-purposed as an
90 anti-virulent drug to treat *P. aeruginosa* infections.

Results

Auranofin inhibits QS in *Pseudomonas aeruginosa*

We initially discovered auranofin in a screen to identify small molecules that inhibit QS in *P. aeruginosa*. We screened ~6,000 compounds from various libraries using a *lasB*-*gfp*(ASV) strain³⁵, where expression of a fusion construct between LasB N-terminus and an unstable GFP variant (GFP(ASV)) is controlled by constitutively expressed LasR, the cognate transcriptional regulator of the *las* pathway. Inhibitors of this pathway should reduce GFP fluorescence in this assay. In addition, since the *las* pathway controls both the *rhl* and *pqs* pathways, compounds identified in this screen could potentially shut down the entire QS network. We found that auranofin exhibits sub-micromolar (IC₅₀) inhibition of LasB-GFP expression with minimal effects on cell growth (Fig. 1a, S1). Auranofin also strongly inhibits fluorescence in two other reporter strains, *rhlA*-*gfp*(ASV) and *pqsA*-*gfp*(ASV)³⁶, both under the control of their respective transcriptional activators, RhlR and PqsR (Fig. 1b&c). To validate our findings, we examined the effects of auranofin on the levels of RNA transcripts for key QS-regulated genes, as well as on the production of various QS-controlled virulence factors. Auranofin results in the down-regulation of *lasA* and *rhlA* transcripts (Fig. S2), and significantly reduces the levels of elastase (LasB), rhamnolipids and pyocyanin (Fig. 2). Taken together, these results establish that auranofin is a potent QS inhibitor.

Auranofin inhibits expression of Type III secretion system (T3SS) and Type IV pili (TFP) in *P. aeruginosa*

To gain insights into the mechanism(s)-of-action of auranofin, we compared the protein profiles of *P. aeruginosa* in the presence and absence of auranofin. Consistent with it being a QS inhibitor, treatment with auranofin caused reduction in the levels of proteins involved in or regulated by QS (Table S1). Interestingly, we also observed down-regulation of proteins

involved in T3SS and TFP formation. We confirmed that T3SS was down-regulated using immunoblot analyses (Fig. S4). We also showed that auranofin is able to inhibit TFP-dependent motility, including swarming and twitching^{37,38} (Fig. 3). Our observations here are, in part, due to transcriptional down-regulation of these systems (Fig. S2). These results imply that auranofin either has multiple targets, and/or it targets a transcriptional regulator upstream of QS, T3SS and TFP.

Auranofin inhibits Vfr *in vitro* but has other targets in cells

QS, T3SS and TFP formation are positively regulated by Vfr²⁵. We asked if auranofin directly targets Vfr function. To test this, we examined the ability of auranofin to inhibit the function of Vfr in binding to promoter sequences *in vitro* using electrophoretic mobility shift assays (EMSA). We first showed that purified Vfr is able to bind DNA fragments containing the promoter regions of representative QS (*lasR*) and virulence (*toxA*, *regA*) genes (Fig. S3), as previously reported³⁹. We further demonstrated that auranofin effectively prevents Vfr binding to these promoters in a dose-dependent manner, indicating that it inhibits Vfr function *in vitro* (Fig. 4a, c & d). Gold(I)-based compounds are thiophilic, and auranofin has previously been shown to react covalently with cysteine residues within target proteins^{40,41}. To ascertain if its inhibition of Vfr proceeded via covalent modification of cysteines, we generated a cysteine-less Vfr variant (VfrΔ5) in which all five cysteines were replaced by serine residues. This variant is functional as judged by its ability to bind the *lasR*, *toxA* and *regA* promoters *in vitro* (Fig. S3). Remarkably, auranofin is no longer able to prevent binding of VfrΔ5 to these same promoters (Fig. 4b, c & d). These results establish that auranofin inhibits Vfr *in vitro* via its cysteine residues.

To determine if Vfr is the main target of auranofin in cells, we complemented the Δ*vfr* strain with wild-type Vfr and the VfrΔ5 variant, and examined the levels of elastase, T3SS

expression and twitching motility in the presence and absence of auranofin. Both wild-type Vfr and the Vfr Δ 5 variant were able to complement the Δ yfr strain in these assays (Fig. S4). We showed that auranofin inhibits all three phenotypes in the wild-type Vfr complemented strain, as expected. Surprisingly, auranofin was still able to inhibit these phenotypes in the Vfr Δ 5 complemented strain, unlike what we have observed *in vitro*. While we have demonstrated that auranofin does inhibit Vfr via its cysteine residues *in vitro*, our data suggests auranofin may have other targets, in addition to Vfr.

Auranofin impedes the development of *P. aeruginosa* biofilms

Both TFP and QS are required during biofilm development^{42,43}. TFP are important for initial surface attachment of cells, as well as for the organization of cells into the typical “mushroom” architecture of mature *P. aeruginosa* biofilms. QS is also critical for biofilm maturation, although the mechanisms here are less understood. Since auranofin inhibits both TFP formation and QS networks, we expect it to also have an impact on the development of *P. aeruginosa* biofilms. To test this, we monitored biofilm formation and maturation in the presence and absence of auranofin over a period of 72 h in continuous flow chambers. The formation of fully matured, canonical mushroom-shaped biofilm structures for wild-type *P. aeruginosa* was readily observed within 72 h (Fig. 5). Interestingly, while auranofin did not have obvious effects on the initial surface attachment of cells, the formation of mushroom-shaped structures was less pronounced; biofilms formed in the presence of auranofin appeared more flat, similar to biofilms of QS or TFP-null strains⁴⁴. Our data clearly indicate that despite permitting biofilm attachment and growth, auranofin attenuates the progression of biofilm maturation, possibly by inhibiting QS and TFP.

Auranofin exhibits synergistic effects with colistin in killing *P. aeruginosa* biofilms

Since auranofin affects the maturation of the biofilm, we asked whether it may display synergy with colistin, a last resort drug commonly used to treat *P. aeruginosa* infections. We evaluated the combined effects of auranofin and colistin by monitoring the ratio of live versus dead cells within *P. aeruginosa* flow cell biofilms after treatment. As previously reported, colistin treatment alone results in a sharp decline in cell viability across a 2-day old biofilm, with the exception of several surviving colistin-tolerant subpopulations⁴⁴ (Fig. 6a). Remarkably, adding auranofin prior to biofilm formation allows subsequent colistin additions to completely eradicate the *P. aeruginosa* biofilm. In fact, co-treatment of a 2-day biofilm with auranofin and colistin also results in complete killing. These results demonstrate synergy between auranofin and colistin in a flow cell biofilm.

To test whether auranofin and colistin can work together *in vivo*, we surgically inserted silicone implants coated with 1-day old *P. aeruginosa* biofilms into the peritoneal cavity of mice, and subsequently treated them locally with colistin, auranofin, or a combination of both drugs. Remarkably, combination therapy of auranofin and colistin resulted in significantly lower bacterial titer counts from the implant than either auranofin or colistin alone (Fig. 6b). This confirms the synergy between auranofin and colistin in a murine model.

Discussion

Auranofin is approved by the FDA for the treatment of rheumatoid arthritis. It has been shown to reduce inflammation of joints in rheumatic patients⁴⁵; however, the precise mechanism of how this occurs is not clear. Auranofin also exhibits anti-cancer and anti-parasitic properties^{40,46,47}. Furthermore, auranofin is able to kill a broad spectrum of Gram-positive bacteria, and also mycobacteria⁴¹. All of these activities may be attributed to auranofin's ability to disrupt the thiol-redox balance in cells, in part by targeting the thioredoxin reductases in each of these systems⁴⁸. Interestingly, auranofin is largely ineffective against most Gram-negative bacteria, presumably because of lack of penetration of the bacterial outer membrane⁴⁹. Here, we have demonstrated that auranofin is additionally able to inhibit virulence in *P. aeruginosa*. Despite not being able to accumulate at high concentrations in cells, auranofin causes downregulation of QS, T3SS and TFP formation. One possible target is Vfr, which controls all of these phenotypes. Consistently, we have shown that auranofin inhibits the ability of Vfr to bind to target promoter sequences in vitro. The fact that a cysteine-less Vfr variant is not inhibited in vitro indicates that auranofin also targets cysteines within this protein. Interestingly, *P. aeruginosa* does not gain resistance against auranofin even when expressing cysteine-less Vfr, suggesting that it has multiple targets in cells. This is probable, given that auranofin is a general thiol-targeting reagent⁴⁰. Understanding the mechanism of action of auranofin would require identifying these unknown targets.

Current clinical treatment options of *P. aeruginosa* infections involve the usage of carbapenems, aminoglycosides and ciprofloxacin, to name a few¹⁶. These drugs are often used in combination or singularly in treatment. Unfortunately, many strains of *P. aeruginosa* have developed resistance to such compounds and drastically reduce the pool of available treatment options for clinicians⁷. Colistin remains one of the few drugs effective against most strains of *P. aeruginosa*. Its usage, however, remains limited and is confined to last resort situations due

to its toxicity⁵⁰. We have shown that auranofin and colistin combine synergistically to completely eradicate a *P. aeruginosa* biofilm, something not observed with colistin treatment alone. This synergy is also observed in a murine implant model. We believe that the synergy between auranofin and colistin works in both directions. It has been shown that disrupting outer membrane permeability (here with the use of colistin) allows auranofin to penetrate the cell envelope and kill Gram-negative bacteria, through its potential action on thioredoxin reductase. At the same time, the inhibition of TFP and QS (by auranofin) during biofilm development may prevent the formation of colistin-tolerant subpopulations within the biofilm, thereby allowing complete eradication. The unique synergistic effects of auranofin and colistin may be exploited as a new treatment regimen for *P. aeruginosa* infections. Given that auranofin is already an FDA-approved drug, it may not be in the very far future that this approach can be tested.

Materials and Methods

Detailed methods can be found in SI Appendix, Materials and Methods. All bacterial strains and plasmids are summarized in Table S2 and S3. Oligonucleotides for PCR are listed in Table S4.

Acknowledgements

We thank Young-Tae Chang (POSTECH) for allowing access to small molecule libraries used in this study. We acknowledge Song Lin Chua (SCELSE) for his help in processing and submitting our samples for proteomics analysis. We thank Yingying Li (SCELSE) for her guidance in RT-PCR experiments. We are grateful to Arne Rietsch (Case Western Reserve University) and Katrina Forest (University of Wisconsin-Madison) for their generous gifts of the α -PcrV and α -Vfr antibodies, respectively. This research was supported by the National Research Foundation and the Ministry of Education of Singapore under its Research Centre of Excellence Programme.

Contributions

L.Y., M.G., and S.-S.C. conceived the project and directed the study. Z.H. conducted the QS inhibitor screening assay. L.Z.W.T. conducted all biochemical experiments. J.K.H.Y. and M.M.S.S. performed the mouse model and the flow cell biofilm experiments. B.Y.W. conducted the rhamnolipid analysis with supervision from S.F.Y.L.. L.Z.W.T. and S.-S.C. wrote the manuscript with input from all authors.

Competing financial interests

M.G., S.-S.C., L.Y., L.Z.W.T., and Z.H. have filed an international patent application (PCT/SG2016/050436, filed 6th September 2016) on the work described in this manuscript.

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Figure Legends

Figure 1. Auranofin inhibits all levels of QS with minimal effects on cell growth. Representative fluorescence induction profiles (relative fluorescence units normalized to cell density, over time) for (a) LasR-mediated expression of LasB-GFP, (b) RhIR-mediated expression of *P_{rhIA}-gfp*, and (c) PqsR-mediated expression of *P_{pqsA}-gfp*, in the presence of indicated concentrations of auranofin, whose chemical structure is shown on the top. Dose-dependent inhibition curves for each assay are plotted on the right. Error bars represent standard deviation from technical triplicates.

Figure 2. The production of key QS-controlled virulence factors is reduced in the presence of auranofin. (a) Elastase activity in the supernatants of PAO1 and Δ *lasIrhII* cultures treated with indicated concentrations of auranofin. (b) Relative abundance of rhamnolipid fragments isolated from PAO1 cells treated with 20 μ M auranofin, as quantified using multiple reaction monitoring mass spectrometry. The chemical structure of rhamnolipid and its measured fragments are shown on the right. (c) Relative levels of pyocyanin in supernatants of PAO1 and Δ *lasIrhII* cultures treated with indicated concentrations of auranofin, as measured at absorbance at 691 nm. Error bars represent standard deviation from biological triplicates.

Figure 3. Motility phenotypes that depend on TFP (i.e. swarming and twitching) are reduced in the presence of auranofin. (A) Swarming (0.5% LB agar), (b) swimming (0.3% LB agar), and twitching (1% LB agar) motilities of PAO1 and PAO1 Δ *yfr* strains in the presence or absence of 20 μ M auranofin. For twitching, cells at the base of the plates were stained with Coomassie blue.

Figure 4. Auranofin inhibits Vfr binding to target promoter sequences in vitro by reacting with cysteine residues. Polyacrylamide gels showing specific binding of (a) Vfr, (b) VfrΔ5 to P_{lasR} , or both proteins to (c) P_{toxA} and (d) P_{regA} , in the presence of indicated concentrations of auranofin. The coding region of the gene *rsaL* is used as a probe to control for non-specific binding.

Figure 5. Auranofin attenuates the progression of *P. aeruginosa* biofilms. Confocal fluorescence microscopy images illustrating the formation and maturation of PAO1-*gfp* biofilms over three days in the presence or absence of 1 μM under continuous flow conditions. Scale bar = 20 μm

Figure 6. Auranofin displays strong synergy in eradicating *P. aeruginosa* biofilms when used in combination with colistin. (a) Confocal fluorescence microscopy images of two-day old PAO1-*gfp* biofilms before and after treatment with 10 μg/ml colistin. Auranofin was not added (top panels), added prior to biofilm formation (middle panels), or added together with colistin (bottom panels). Dead cells were revealed by staining with propidium iodide. Each panel represents a square surface area of 212 x 212 μm. (b) Mean c.f.u per ml of *in vivo* PAO1 biofilms obtained from silicone implants in the peritoneal space of mice, treated with auranofin, colistin or a combination of both drugs. * $P < 0.01$, Student's *t*-test.

412 **Figure 1**

414 **Figure 2**

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Figure 3

418 Figure 4

419 **Figure 5**

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421 **Figure 6**