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Virulence attenuating combination therapy: a potential multi-target synergy approach to treat *Pseudomonas aeruginosa* infections in cystic fibrosis patients

Elana Shaw [a] and William M. Wuest *[ab]

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The World Health Organization considers the discovery of new treatments for *P. aeruginosa* a top priority. Virulence attenuating combination therapy (VACT) is a pragmatic strategy to improve bacterial clearance, repurpose outmoded antibiotics, improve drug efficacy at lower doses, and reduce the evolution of resistance. *In vitro* and *in vivo* studies have shown that adding a quorum sensing inhibitor or an extracellular polymeric substance repressor to classical antibiotics synergistically improves antipseudomonal activity. This review highlights why VACT could specifically benefit cystic fibrosis patients harboring chronic *P. aeruginosa* infections, outlines the current landscape of synergistic combinations between virulence-targeting small-molecules and anti-pseudomonal drugs, and suggests future directions for VACT research.

Introduction

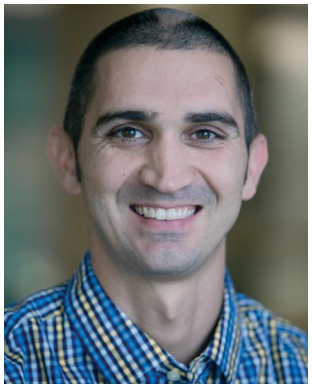
Cystic fibrosis and *P. aeruginosa*: a deadly combination

Individuals with cystic fibrosis (CF) carry a genetic mutation that causes defects in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The clinical result is an overproduction of thick, viscous mucus in the organ systems, notably the lungs.[1–10] Airway mucus promotes chronic respiratory infections by physically shielding bacteria from antibiotics and impeding mucociliary clearance (MCC) of inhaled pathogens.[8,11] To make matters worse, CFTR-dependent disruptions to complement-mediated immunity impair the ability of monocytes to phagocytose and kill bacteria.[12,13] Consequently, bacteria thrive in CF lungs and decimate lung function (Fig. 1).[14] A CFTR-mediated metabolic defect that results in excessive succinate release

[a] *Department of Chemistry, Emory University, 1515 Dickey Drive, Atlanta, Georgia 30322, USA. E-mail: william.wuest@emory.edu*
[b] *Emory Antibiotic Resistance Center, Emory University School of Medicine, 201 Dowman Drive, Atlanta, Georgia 30322, USA*

Elana Shaw

Elana Shaw earned her BS in Chemistry at Emory University in Dec 2019 where she worked on the synthesis of novel precision antibiotic candidates in the Wuest Lab. She is currently a post-baccalaureate fellow at the National Institutes of Health studying the biomolecular underpinnings of rare immunological diseases.



William M. Wuest

Bill Wuest received his B.S. in Chemistry/Business (Notre Dame), his Ph.D. in organic chemistry (UPENN) and was a NIH Postdoctoral Fellow at Harvard Medical School before beginning his independent career at Temple University in 2011. In 2017, he moved to Emory University where he is currently a Georgia Research Alliance Distinguished Investigator and Associate Professor of Chemistry. His research focuses on the diverted total synthesis of pathogen-specific antibiotics. Bill is the recipient of a number of awards including the NIH ESI MIRA, NSF CAREER Award, ACS Medicinal Chemistry David W. Robertson Award, and the ACS Infectious Diseases Young Investigator Award.

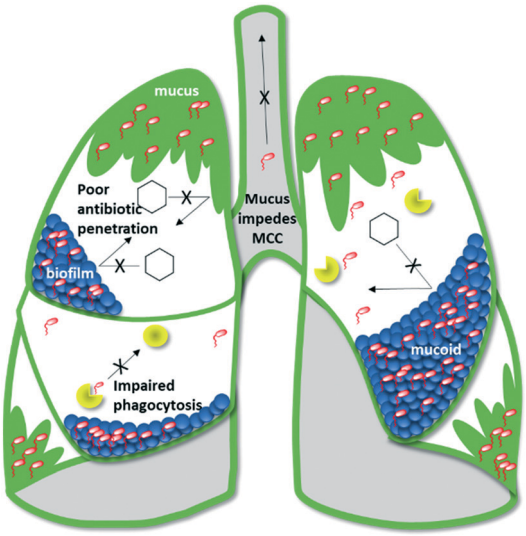


Fig. 1 Depiction of a CF lung.

particularly favors colonization by succinate-metabolizing *Pseudomonas aeruginosa*.[15] Chronic infection by this opportunistic Gram-negative rod is the leading cause of morbidity and mortality for CF patients.[6,7,15–18]

Treating *P. aeruginosa* with antibiotics is exceptionally difficult:[19–24] the bacteria's outer membrane under expresses OprD porins by which antibiotics can enter the cell and over expresses RND (resistance-nodulation-division) efflux pumps which expel antibiotics. In addition, *P. aeruginosa* has an inducible gene that codes for AmpC cephalosporinase, a potent beta-lactamase.[24,25] The bacteria can also mutate to overproduce a protective, charged, alginate-filled biofilm.[9,26–30] Bacteria in biofilms are up to 1000 times more resistant to antibiotic treatment than their planktonic counterparts, making this mucoidal phenotype infamously difficult to treat.[31–37] To make matters worse, CF patients require frequent antibiotics, placing near-constant pressure on *P. aeruginosa* to evolve new resistance traits.[38]

On average, CF patients initially contract *P. aeruginosa* at age 2.6. By adolescence, 85% of CF patients are actively harboring the bacteria in their lungs.[5,14,31,33] To treat the infection, CF patients cycle through bactericidal monotherapies (including colistin (COL), meropenem (MER), tobramycin (TOB), ceftazidime (CFT), gentamycin (GEN), and azithromycin (AZM)).[23–25,39–42] Though these monotherapies may dampen *P. aeruginosa* flare-ups, they often fail to achieve full bacterial clearance due to the pathophysiology of CF (Fig. 1). Bacteria that persist in mucus or biofilms may select for resistance, repopulate the lung, and evolve mucoidy. Each failed treatment attempt increases the likelihood of eventually colonizing a mucoidal multidrug resistant (MDR) or extensively drug-resistant (XDR) strain.[43–50] As such, this current treatment paradigm promotes heinous chronic infections and the World Health Organization (WHO) has assigned top priority to discovering novel therapies for treating *P. aeruginosa*.[17]

Virulence attenuating combination therapy (VACT)

Modern insights into how bacteria specifically interact with the body to cause disease have shed light on new potential drug targets: virulence factors.[44,51] Virulence inhibitors specifically interfere with the disease process, thereby preserving the host microbiome and minimizing the risk of selecting for resistance.[44,52] VACT couples virulence-targeting small-molecules (to disarm pathogens) with a bactericidal antibiotic (to kill pathogens).

CF patients suffering from intractable mucoid *P. aeruginosa* are a strong example of a population that could benefit from VACT. In CF lungs, VACT may improve antibiotic efficacy by attenuating biofilm and reducing *P. aeruginosa* virulence factor production to improve the antipseudomonal activity of antibiotics. Substantial *in vitro* and *in vivo P. aeruginosa* VACT studies support this theory. This review summarizes these synergistic combinations in accordance with their virulence target, including 1) quorum sensing (QS) systems and 2) biofilm extracellular polymeric substance (EPS) as well as advocates for future VACT studies that target the type 3 secretion system (T3SS) (Fig. 2).

Discussion

Quorum sensing

QS describes the process by which bacteria communicate with one another by synthesizing, releasing, and responding to the population-dependent concentration of small molecules known as autoinducers (Fig. 3).[53–55] *P. aeruginosa* secretes two main classes of autoinducer: acyl-homoserine lactones (HSLs) and 2-heptyl-3-hydroxy-4-quinolone (PQS) (Fig. 3).[56] When the environmental concentration of autoinducers reaches a threshold, transcriptional regulators alter gene expression to promote survival.[55] *P. aeruginosa*'s three QS systems, (*Las*, *Rhl*, and PQS), work together to control over 300 genes.[57–60] Many of those genes code for potent virulence factors such as LasB elastase, LasA protease, the T3SS, exotoxin A, and pyocyanin.[60–62]

Quorum signaling also allows individual planktonic bacteria to make group-behavioral decisions, notably the choice to form a biofilm (Fig. 3).[63] During biofilm formation, bacterial cells aggregate together within a self-produced matrix of EPS.[64–66] Inside of the EPS, *P. aeruginosa* can persist, shielded from the host immune system, environmental stresses, and many antibiotics.[37,63] Additionally, biofilms facilitate horizontal gene transfer, which can lead to the development of resistance.[67]

Functional QS systems are vital for *P. aeruginosa* pathogenesis.[56,68] In mouse and rat models, *P. aeruginosa* mutants that lacked QS genes caused less lung pathology, suggesting that cell–cell signaling plays a key role in acute virulence.[69,70] In addition, sputum cultures from CF patients infected with chronic *P. aeruginosa* were discovered to contain significant amounts of HSLs and PQS, indicating that all three QS systems are deeply involved in human infection.[56,71,72] Thus, selectively perturbing *P. aeruginosa*'s

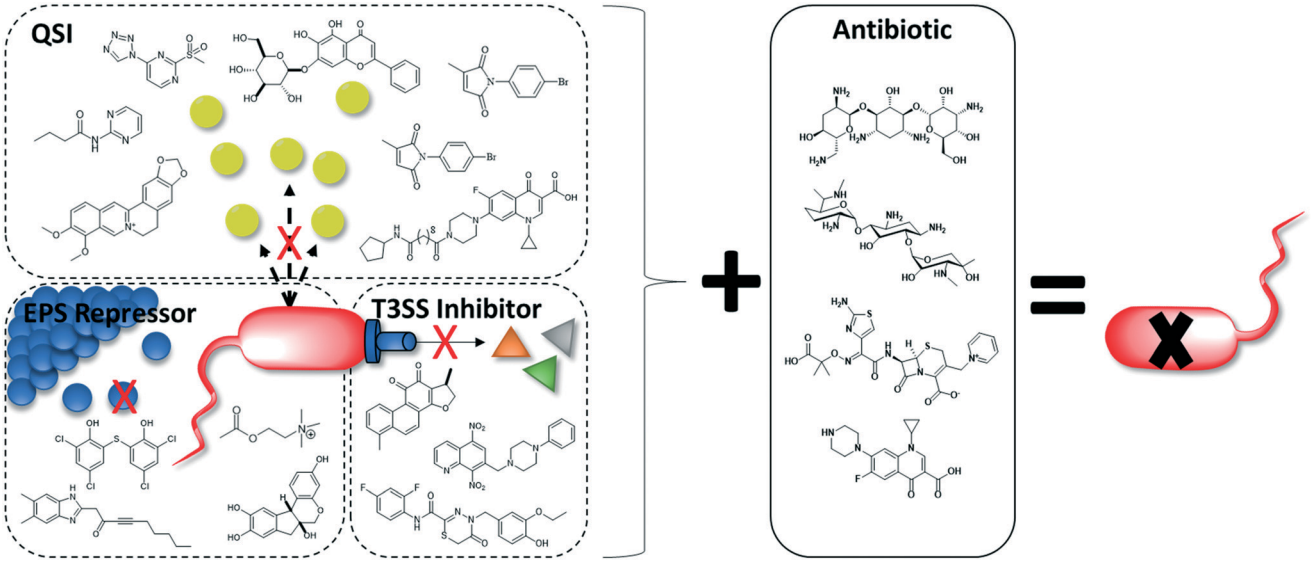


Fig. 2 Adding a virulence inhibitor to antibiotic therapy improves *P. aeruginosa* killing.

QS systems with small molecules is an extremely promising strategy for curtailing pathogen virulence in both acute and chronic infections.

Quorum sensing inhibitors and VACT

Small molecule quorum sensing inhibitors (QSIs) have demonstrated therapeutic anti-pseudomonal potential.[73–77] Alone, they have been found to reduce virulence factor production, biofilm formation, bacterial motility, and pathogen virulence.[78–81] In mouse and rat infection models, QSIs alone induced immunogenicity, improved bacterial clearance, decreased lung pathology, and improved survival.[69,70,78,82]

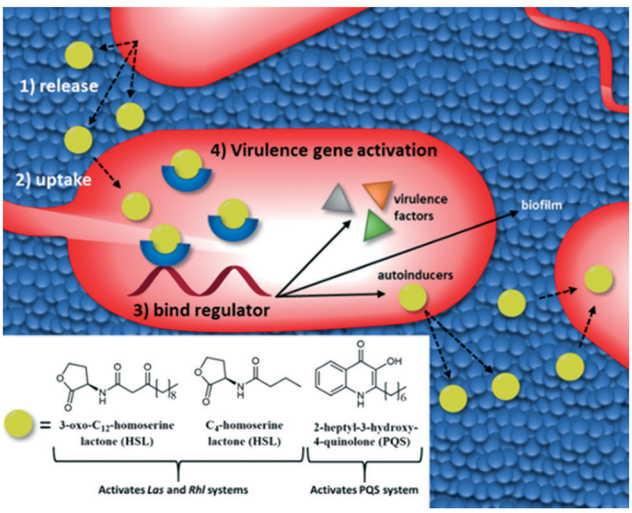


Fig. 3 The QS systems of *P. aeruginosa* are activated when autoinducers bind to transcriptional regulators upregulating autoinducers, virulence factor production, and biofilm formation.

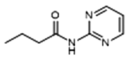
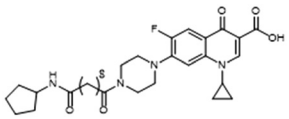
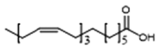
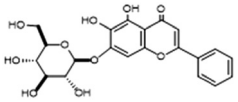
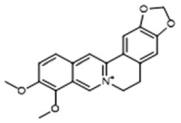
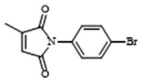
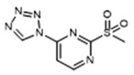
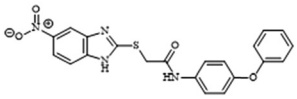
The addition of a QSI to antibiotic therapy generally attenuates biofilm to improve antibiotic penetration into the cell while also reducing virulence factor production. Strong QSI candidates for VACT must be non-cytotoxic to human cells and synthetically accessible (<5 steps) or commercially available (for feasible large-scale testing). Laboratories have been exploring VACT *in vitro* and *in vivo* with promising results as discussed herein.

Furiga and colleagues took inspiration from the structure of C_4-HSL (Fig. 3), a key signaling molecule in CF lung infections, to develop *N*-(2-pyrimidyl)butanamide (**C11**) (Table 1, entry A). **C11** downregulates *Las* and *Rhl* QS systems, decreasing virulence factors LasB and RhlA. **C11** also notably attenuates both aerobic biofilms and the more robust anaerobic biofilms that predominate in CF lung infection. When combined with CIP, TOB, and COL, **C11** inhibited biofilm in a dose-dependent manner to improve antibiotic efficacy. They hypothesize that **C11** tampers with QS, causing the bacteria to transition from a biofilm to a planktonic state where antibiotics have easier access for killing. **C11** is a strong target for *in vivo* studies because it is stable, not cytotoxic to human cells, and synthetically accessible.[83]

Similarly inspired by the structure of HSLs, Bortolotti and colleagues conjugated an antagonistic C_{12}-HSL analog to CIP. They observed that their hybrid molecule, **ET37**, reduced biofilm formation and the development of CIP tolerance in clinical strains of *P. aeruginosa* (Table 1, entry B).[84]

Aware that linolenic acid (LNA), an essential fatty acid, has antimicrobial properties, Chanda *et al.* added LNA to TOB therapy and found that the combination synergistically attenuated biofilm and virulence factor production by interfering with all three QS systems (Table 1, entry C).[85] The LNA and TOB combination is promising for future *in vivo* exploration and eventual studies in CF patients for two reasons: 1) the regiment is less toxic than TOB alone because

Table 1 QSI chemical structures and VACT testing summary

Entry	Structure	Synergy with	Tested *in vitro*?	Tested *in vivo*?
A		CIP, TOB, COL	Yes	No
B		CIP	Yes	No
C		TOB	Yes	No
D		TOB	Yes	Yes
E		AZM	Yes	Yes
F		TOB	Yes	No
G		CIP	Yes	Yes
H		TOB, MER	Yes	No

the addition of LNA allowed TOB efficacy at lower doses and 2) the VACT has been found to disrupt the production of alginate, a key contributor to chronicity in *P. aeruginosa* lung infections.

Work by Brackman and colleagues showed the addition of baicalin hydrate (BH) to TOB significantly increased biofilm killing *in vitro* (Table 1, entry D). They also found that a VACT regiment of BH and TOB improved survival in a *C. elegans* infection model.[86] BH is commercially available, making larger-scale VACT studies (for example, in conditions that mimic the CF lung environment) very feasible.

Berberine (BER) is an isoquinolone alkaloid that has been approved for over-the-counter use in China to treat gastrointestinal infections and is being studied as an anti-diabetic and antimicrobial.[87,88] Li and coworkers found that sub-MIC regimens of BER and AZM interfered with the *Las* and *Rhl* QS systems to inhibit biofilm and virulence factor production (notably alginate) (Table 1, entry E). The VACT also proved effective against 10 clinical *P. aeruginosa* isolates cultured from the sputum of CF patients. Excitingly, infected mice treated with a regimen of 0.8 mg kg^{-1} of AZM combined with 3.2 mg kg^{-1} of BER showed markedly increased survival and decreased lung inflammation.[89]

Intrigued by the antibacterial properties of itaconimides, Fong and colleagues synthesized an analog library based on structure–activity relationships (SAR) and found that 10 μM (non-cytotoxic up to 40 μM) of itaconimide **12a** in combination with TOB completely eradicated an entire population of 72 hour old *P. aeruginosa* biofilm (Table 1, entry F). They hypothesize that **12a** works by inhibiting the PQS system via the *Las* system.[90] The VACT's ability to entirely remove preformed biofilms may suggest exciting utility against chronic mucoid infection. However, before initiating further clinically oriented studies, it is necessary to test **12a** for reactivity with thiol containing compounds such as glutathione. Such reactivity has been linked to cell-death.[91]

Using bioisosteric modification of known, single-target inhibitors, Thomann *et al.* developed compound **6**, which is a multi-target drug that simultaneously inhibits the transcriptional regulator, *PqsR*, and a key enzyme for PQS production, *PqsD* (Table 1, entry G).[92] Alone, **6** interfered with iron metabolism, decreased virulence factor production, and inhibited biofilm formation (IC_{50} = 100 μM) and eDNA production. It was also efficacious *in vivo*, increasing the survival of *Galleria mellonella* (low toxicity observed). The VACT combination of 1 μM CIP + 50 μM **6** restored CIP activity against *P. aeruginosa* biofilm. They hypothesize that this synergy is due to the ability of **6** to inhibit eDNA, which hinders CIP.

Excitingly, Maura and colleagues found that another *PqsR* inhibitor, **M64**, is one hundred times more potent at inhibiting biofilm than compound **6** (Table 1, entry H).[93] Pre-treatment with **M64** restored TOB and MER efficacy against antibiotic-tolerant biofilms. Concurrent treatment with **M64** and MER or TOB decreased biofilm CFU by 178 and 17 times respectively.

In recent years, a plethora of auspicious QSIs have been discovered. Many of these inhibitors are commercially available, have low toxicity, or inhibit alginate (the main component of mucoid *P. aeruginosa* biofilm). For these reasons, such compounds and their synthetic analogs merit VACT studies in the context of CF. A summary of promising QSIs for VACT studies can be found in Table 2.[78,79,81,94–97]

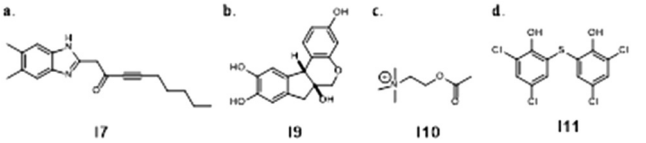


Fig. 4 Chemical structures of EPS inhibitors by the Lewenza Lab used in VACT.

Extracellular polymeric substance repressors

During the early stages of biofilm formation, bacteria generate a matrix made up of extracellular polymeric substances (EPSs).[98] *P. aeruginosa* produces three EPSs: *Pel*, *Psl*, and alginate. *Pel* is believed to function as an eDNA cross-linker and has been implicated in the development of antimicrobial resistance.[99,100] *Psl* may act as a signal to initiate biofilm formation and is a critical structural element of biofilms during the microcolony formation stage.[101,102]

Pel and *Psl* are exciting targets for VACT because inhibiting EPSs reduces or eliminates biofilm, making bacteria more susceptible to antimicrobials.[100] The Lewenza Lab used a high-throughput gene expression screen to find compounds that repress *Pel*.[103] When studied in combination with COL, polymyxin B (PB), TOB, GEN, and CIP, EPS inhibitors **I7-I11** attenuated mucoidal biofilms to improve antibiotic penetration and efficacy (Fig. 4). Notably, compound **I7** also demonstrated ability to attenuate biofilm in mucoidal *P. aeruginosa*, making it a prime candidate for *in vivo* exploration in the context of CF.

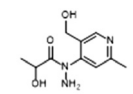
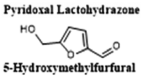
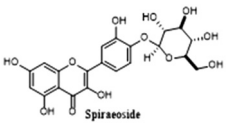
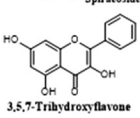
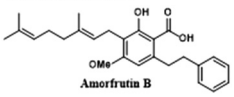
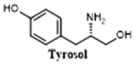
Type 3 secretion system inhibitors and VACT

Type 3 secretion systems (T3SSs) play leading roles in *P. aeruginosa* virulence and are a strong yet unexplored candidate for VACT.[104,105] The T3SS is broadly responsible for transferring proteins out of the bacterial cell and highly conserved amongst Gram-negative pathogens.[105,106] *P. aeruginosa* expresses two distinct T3SSs: the fT3SS and the iT3SS.[107] The fT3SS expels flagellar proteins, enabling chemotaxis and biofilm formation and secreting effector toxins.[107–109] The iT3SS, or injectosome, ejaculates cytotoxic effector toxins (ETA, ExoS, ExoT, ExoU, ExoY) directly into the host cell's cytoplasm (Fig. 5).[80,110,111]

The most potent effector toxin is ExoU.[18,22] ExoU has been found to lyse lung cells and destroy the alveolar epithelia, leading to septic shock and severe acute lung damage.[112,113] ExoS prevents human neutrophils from producing reactive oxygen species (ROS), which are vital for killing phagocytosed bacteria.[114] ExoT inhibits mammalian cytokinesis, causing slowed wound healing.[9,115] ExoY causes apoptosis.[113,116] In addition to their individual functions, the four major effector toxins collude to kill and impair alveolar neutrophils and macrophages, severely handicapping the phagocytotic immune response to bacteria.[117]

Inhibiting the T3SS with small-molecules is a highly appealing anti virulence strategy: the T3SS is specific to pathogenic bacteria, limiting the chance of off-target effects.[18,80,118–121] In addition, because the T3SS is not vital for the bacteria's survival, disrupting it should not place evolutionary pressure on *P. aeruginosa* to evolve new resistance mechanisms.[122] Ideally, a selective T3SS inhibitor

Table 2 Summary and evaluation of promising QSIs not tested for synergy

Compound	Reduces virulence factors	Inhibits biofilm?	Commercially available?
 Pyridoxal Lactohydrazone	Alginate, pyocyanin	Yes	No (2 synthetic steps)
 5-Hydroxymethylfurfural	Alginate, rhamnolipid (sub-MIC)	Yes	Yes
 Spiraeoside	Elastase, pyocyanin, violacetin	Yes	Yes
 3,5,7-Trihydroxyflavone	Pyocyanin	Yes (IC_{85} = 100 μM)	Yes
 Amorfrutin B	Elastase, pyocyanin (IC_{50} = 10 μM)	Yes (IC_{50} = 50 μM)	No
 Tyrosol	Elastase, pyocyanin	Yes	Yes

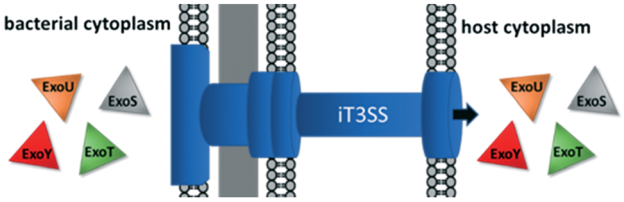


Fig. 5 The T3SS secretes effector toxins into the host cytoplasm.

would stop *P. aeruginosa* from impairing phagocytosis, empowering the host immune system to kill the bacteria.[118] However, T3SS inhibitors have never been studied for synergy with bactericidal antibiotics. Several promising T3SS inhibitors and VACT candidates are featured in Fig. 6 and discussed herein. However, for a thorough review of targeting the T3SS to fight *P. aeruginosa*, see Anantharajah *et al.*[123] and Duncan *et al.*[124]

Promising T3SS inhibitors for future VACT studies

Sheremet and colleagues studied the activity of flourothiazinon (FT), a 2,4-disubstituted-4*H*-[1,3,4]-thiadiazine-5-one, against *P. aeruginosa* infections in a mouse model.[122,125] They found that mice dosed with 50 mg kg^{-1} of FT twice daily for 4 days after being infected with various lethal doses of multi-drug resistant clinical isolates of *P. aeruginosa* often survived the infection and displayed less lung pathology, lower levels of systemic inflammation, increased clearance of bacteria in their lungs and spleen, and no bacteremia. This suggests that at both a systemic level and in the lungs, FT intercepts *P. aeruginosa*'s characteristic attempt to suppress host immunity, improving phagocytotic clearance of pathogen from the cells to allow survival. *In vitro* experiments showed that FT specifically inhibited secretion of ExoT and ExoY and significantly decreased bacterial cytotoxicity. They also found that FT restored the ability HeLa cells to phagocytose bacteria in a dose dependent manner. When plated with FT, *P. aeruginosa* growth was not affected. The efficacy, specificity, and documented low toxicity[104] of FT make it a highly promising candidate for VACT.

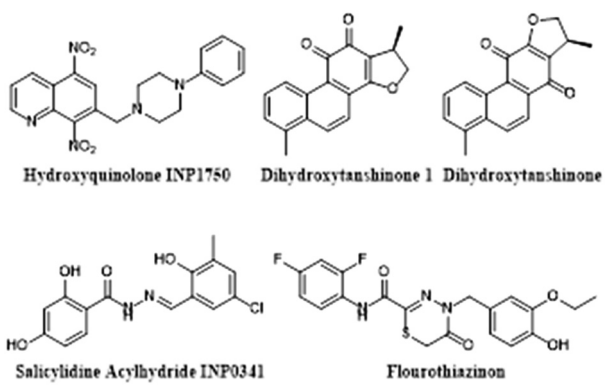


Fig. 6 Promising T3SS inhibitors for future VACT study.

Anantharajah and colleagues explored two classes of T3SS inhibitors for antibiotic potential: salicylidine acylhydrides and hydroxyquinolones.[107] These molecular classes were promising as they had previously been found to inhibit transcription of the iT3SS in other gram negative pathogens including *Y. pseudotuberculosis*, *Shigella*, *Salmonella*, and *Chlamydia*.[119–121] The team compared the anti-pseudomonal activity of the two chemical classes by selecting a non-cytotoxic model molecule from each and testing it in a series of *in vitro* experiments. From the salicylidine acylhydrides, they selected INP0341 (SA INP0341), and from the hydroxyquinolines, they selected INP1750 (HINP1750). They found that both INPs reduced *P. aeruginosa*'s cytotoxicity in phagocytotic and epithelial eukaryotic cells. Further experimentation revealed that SA INP0341 interferes with iT3SS gene transcription while HINP1750 mediates cytotoxicity by inhibiting a key ATPase homologous to the iT3SS and the fT3SS to decrease ExoS secretion and flagellar motility. HINP1750 had a more robust effect on T3SS inhibition as it was active against both T3SS systems, making it a stronger candidate for future study in acute animal infection models and VACT. However, before such studies take place, it is necessary to test the mutagenicity of HINP1750 with an Ames test or its equivalent—nitroaromatic groups have been linked to mutagenicity and HINP1750 contains 2 nitro groups attached to an aromatic ring.[126]

The iT3SS injectosome needle is composed of repeated subunits of a single protein termed PscF. Before PscF is secreted to form the needle, it is protected by two chaperonins, PscE, and PscG. Without protection from the chaperonins, PscF will degrade in the cytosol, and the injectosome needle will not form. Thus, inhibiting the interactions between PscF and the chaperonins will prevent biogenesis of the iT3SS and its resulting cytotoxicity.[80,111] This effect is confirmed by PscE/PscG knockout studies.[111] In July 2019, Feng and colleagues made a serendipitous discovery that non-bactericidal tanshinones compete with PscF for PscE-PscG binding.[80] They found that dihydrotanshinone 1 (dHTSN1) demonstrated an IC_{50} of 0.68 μM, and dihydrotanshinone (dHTSN) demonstrated an IC_{50} of 1.5 μM in a fluorescence polarization (FP) assay. In addition, the compounds were found by Western Blot to decrease ExoS secretion at concentrations of 100 μM in Ca^{2+} depleted (T3SS stimulating) conditions. Western blot also revealed that the tanshinones repressed *P. aeruginosa*-induced macrophage lysis by reducing bacterial caspase-1 release. Caspase-1 is normally released by the T3SS, further suggesting that the tanshinones inhibit T3SS biogenesis.[127] In mouse models, 90% of mice challenged with LD_{70} of PA01 survived when dosed with either dHTSN or dHTSN1. These mice showed less bacteria in their lungs, lower levels of inflammation, and less alveolar damage. The impressive anti virulence properties of tanshinones demonstrated *in vitro* and *in vivo* make them prime candidates for VACT exploration.

Conclusions

Despite a cornucopia of promising academic work in the development of virulence inhibitors such as QSIs, EPS repressors, and T3SS inhibitors, these compounds have yet to progress to clinical trials. One potential explanation for this disconnect is that large pharmaceutical companies are likely deterred by the financial risk of such an endeavor for two reasons. 1) The path by which such a compound would pass phase 3 is unclear and has yet to be evaluated and 2) testing molecules that specifically inhibit virulence and not bacterial growth in humans might raise some ethical questions. However, with antibiotic resistance on the rise, failing to pursue non-bactericidal options, even in combination with approved drugs, is potentially both dangerous and a missed opportunity to revolutionize our antiquated approach to treating infectious disease.

Virulence inhibitors (notably QSIs and EPS repressors) have demonstrated a strong ability to potentiate antipseudomonal antibiotics in both *in vitro* and animal models. There is a long road from *in vitro* and murine model experimentation to clinical testing. However, the early promising results of VACT merit further exploration: first, synergy testing of known and novel QSIs and EPS inhibitors is needed to identify new and potent VACT combinations. Computational screening methods can be used to identify highly specific, multi-target virulence inhibitors for such testing. Second, labs must begin testing VACT regimens in conditions that replicate the CF lung. For example, in the presence of alginate, CF sputum,[128] or in genetically engineered mouse models (GEMM) with CFTR defects. Third, VACT studies with T3SS (and other virulence) inhibitors demand exploration. It is our hope that the next generation of chemists will use virulence-inhibiting small-molecules to resuscitate antibiotics and annihilate resistant bacteria.

Conflicts of interest

There are no conflicts to declare.

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