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Thiol-benzo-triazolo-quinazolinone covalently modifies Alg44 to inhibit c-di-GMP binding and reduces alginate production by *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that affects a large proportion of cystic fibrosis (CF) patients. CF patients have dehydrated mucus within the airways that leads to the inability of the mucociliary escalator to expel inhaled microbes. Once inhaled, *P. aeruginosa* can persist in the lungs of the CF patients for the remainder of their lives. During this chronic infection, a phenomenon called mucoid conversion can occur in which *P. aeruginosa* can mutate and inactivate their *mucA* gene. As a consequence, transcription of the *alg* operon is highly expressed leading to the copious secretion of the alginate exopolysaccharide, which is associated with decreased lung function and increased CF patient morbidity and mortality. Alginate biosynthesis by *P. aeruginosa* is post-translationally regulated by bis-(3'–5')-cyclic dimeric guanosine monophosphate (c-di-GMP), which binds to the receptor protein Alg44 to activate alginate production. The identification of small molecules that disrupt the binding of c-di-GMP to Alg44 could inhibit the ability of *P. aeruginosa* to produce alginate. In this work, a class of thiol-benzo-triazolo-quinazolinone compounds that inhibited Alg44 binding to c-di-GMP *in vitro* was identified after screening chemical libraries consisting of ~50,000 chemical compounds. Thiol-benzo-triazolo-quinazolinones were shown to specifically inhibit Alg44-c-di-GMP interactions by forming a disulfide bond with the cysteine residue in the PilZ domain of Alg44. The more potent thiol-benzo-triazolo-quinazolinone had the ability to reduce *P. aeruginosa* alginate secretion by up to 30%. These compounds serve as leads in the development of novel inhibitors of alginate production by *P. aeruginosa* after mucoid conversion.

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Supporting Information

Additional methods, figures, and tables as described in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Graphical Abstract

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive genetic disorder in the cystic fibrosis transmembrane conductance regulator (CFTR), a membrane protein that serves as a chloride channel in epithelial cells lining the lungs, respiratory tract, and other organs.¹ Defects in CFTR result in impaired movement of chloride ions down their electrochemical gradient.² As a result, water is transported in the lung epithelial cells leading to a reduction in the airway surface liquid and the resulting collapse of the cilia on the lung epithelium.^{3, 4} In the absence of mucociliary clearance, bacteria and other microbes colonize the airways leading to infections.⁵ As a result, CF patients are at extremely high risk for contracting chronic lung infection from foreign pathogens, including *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia*, and *Pseudomonas aeruginosa*. These infections are acquired early in the life of a CF patient, but over time, *P. aeruginosa* becomes the dominant pathogen causing chronic respiratory infection⁶ that infects over 80% of all CF patients.⁷

P. aeruginosa strains chronically infecting the respiratory tract undergo positive selection for mutations that facilitate long-term survival within the CF patient lung.⁸ Over time, infecting strains incur loss-of-function mutations in the *mucA* gene that lead to a phenomenon known as mucoid conversion. By age 16, over 90% of CF patient have infections with mucoid *P. aeruginosa*.⁷ Mucoid *P. aeruginosa* secretes alginate, a viscous, slime exopolysaccharide which confers bacterial resistance to antibiotics and the host immune system.^{9, 10} The combination of thickened dehydrated host mucus and viscous alginate secretions from the infecting *P. aeruginosa* leads to exacerbation of airway blockage and decreased respiratory function, eventually resulting in CF patient morbidity and mortality. For these reasons, alginate is a major virulence factor for CF patients associated with a decline in lung function and contributes to the average patient life expectancy of 37 years of age.^{11, 12}

Alginate is an exopolysaccharide polymer consisting of mannuronic acid and guluronic acid produced by several bacterial species.¹³ Alginate biosynthesis occurs by a protein complex encoded in the *alg* operon which polymerizes, acetylates, and transports the alginate exopolysaccharide.¹³ The production of alginate in *P. aeruginosa* is regulated at the transcriptional level by the sigma factor known both as AlgT and AlgU.^{14, 15} AlgT/U binds the *alg* promoter to activate transcription of the *alg* operon.¹⁵ Transcription of the *alg* operon is normally inactive in wild-type non-mucoid *P. aeruginosa* due to the actions of MucA and MucB, which together serve as anti-sigma factors that sequester AlgT/U to prevent the sigma factor from activating transcription of the *alg* operon.¹⁶ However, loss-of-function mutations in *mucA* acquired during chronic CF infection¹⁷ cause inactivation of the ability

of MucA to repress AlgT/U.¹⁸ As a result, the *alg* operon is constitutively transcriptionally activated leading to mucoid conversion and the secretion of copious amounts of alginate.

Alginate biosynthesis is further controlled at the post-translational level by a signaling molecule known as bis-(3'-5')-cyclic dimeric guanosine monophosphate (cyclic di-GMP, or c-di-GMP). C-di-GMP is a universal bacterial second messenger that is involved in the regulation of motility, biofilm formation, cell cycle, and virulence.¹⁹ C-di-GMP binds receptor proteins, including proteins that contain a conserved protein domain known as the PilZ domain, to regulate biological output.²⁰ The PilZ domain is a β -barrel fold that contains two conserved motifs, RxxxR and DxSxxG.^{19, 20} Alg44, an inner membrane protein encoded within the *alg* operon, contains a PilZ domain that binds c-di-GMP (PDB: 4RT0).^{21, 22} Alg44 binding to c-di-GMP is critical for alginate biosynthesis since *alg44* alleles that encode Alg44 proteins that could not to bind c-di-GMP also failed to produce alginate.²¹ Together, these results demonstrated that c-di-GMP binding to the PilZ of Alg44 is required for alginate biosynthesis.

P. aeruginosa from CF sputum samples have elevated levels of c-di-GMP.²³ In addition, mutations that lead to constitutive activation of diguanylate cyclases (DGCs), the enzymes that synthesize c-di-GMP, have been identified in *P. aeruginosa* isolated from CF patients.^{8, 24} These observations suggest that there are two independent, sequential steps for *P. aeruginosa* strains to become hypermucoid: constitutive transcription of the *alg* operon and subsequent binding of c-di-GMP to Alg44 to activate the alginate biosynthesis complex. Due to the essential nature of Alg44 binding to c-di-GMP for the activation of alginate secretion by *P. aeruginosa*, we hypothesize that the disruption of Alg44 binding to c-di-GMP by a chemical inhibitor will lead to the reduction of alginate production by *P. aeruginosa* that has already undergone mucoid conversion. Given the adverse effect of alginate on the lung function of CF patients, the use of a small molecule inhibitor of alginate production in a clinical setting could drastically improve the lifestyle and livelihood of CF patients suffering from chronic *P. aeruginosa* infection.

In order to identify small molecule inhibitors of Alg44 binding to c-di-GMP as potential leads for the discovery of inhibitors of alginate biosynthesis *in vivo*, a high-throughput screen of chemical libraries was conducted using the Differential Radial Capillary Action of Ligand Assay (DRaCALA).²⁵ We have previously conducted a high-throughput DRaCALA-based screen to identify ebselen as an inhibitor of diguanylate cyclases.²⁶ In this work, a DRaCALA screen of multiple chemical libraries identified a class of small molecule inhibitors of Alg44 binding to c-di-GMP *in vitro* that had specificity for Alg44 and inhibitory potency in the micromolar range. Structure-activity relationship studies revealed several properties of these compounds important for inhibition, including a critical sulfhydryl functional group. The sulfhydryl group allows for covalent modification of Alg44 through the formation of a disulfide bond to cysteine at position 98. The Cys-98 in Alg44 is conserved in 100% of sequenced *P. aeruginosa* strains indicating that this is a conserved amino acid within the protein and a suitable target for inhibition. Treatment with the identified compounds led to a moderate decrease in alginate production by *P. aeruginosa*. Since the cytoplasm of the bacterium is reducing, the disulfide bond between the inhibitory compounds and Alg44 is likely reversed *in vivo*. This is supported by the ability of the

compounds to decrease alginate production by a similar level of a strain in which the Cys-98 was change to either alanine or serine. Taken together, our results represent the identification of a small molecule inhibitor of Alg44 binding to c-di-GMP that can serve as a lead compound for the development of alginate biosynthesis inhibitors.

RESULTS

DRaCALA screen of chemical libraries identifies inhibitors of Alg44 binding to c-di-GMP.

Previously, a high-throughput assay for Alg44 binding to c-di-GMP was developed using DRaCALA.²⁶ This assay had a Z-factor of 0.626 indicating that it is suitable for high-throughput screening.²⁶ Here we used this assay to screen chemically diverse compound libraries for inhibitors of Alg44 binding to c-di-GMP. In order to have an internal validation for each 384-well plate screened, 16 wells contained only DMSO as a negative control for inhibition (Fig. 1A, blue box) and 8 wells contained 5 μ M or 10 μ M of unlabeled c-di-GMP as a positive control for inhibition (Fig. 1A, green box). The fraction of ³²P-c-di-GMP bound to Alg44 (F_B) for every well was calculated using a previously described equation and averaged for the two plates.²⁵ Small molecules that inhibit Alg44 binding to ³²P-c-di-GMP should cause an increase in ligand mobilization on nitrocellulose and therefore will yield a low fraction of ³²P-c-di-GMP bound to Alg44 similar to the unlabeled c-di-GMP competitor control (Fig. 1B, green circles).

Positive hits were classified as compounds that reduced the F_B of ³²P-c-di-GMP binding to Alg44 by 3 standard deviations below the mean of the all the wells in the 384-well plate, excluding positive controls. In every plate, the F_B values for the positive controls were below the 3 standard deviation cutoff indicating that this is a valid criterion for positive hits. An example of a positive hit is shown in Fig. 1A (red box). After calculating and plotting the F_B of every well in the plate, the F_B of the positive hit (Figure 1B, red star) was below the 3 standard deviations cutoff for hit classification (Figure 1B, dashed line). The chemical identity of the positive hit was a thiol-benzo-triazolo-quinazolinone compound, called AC1LFHI9 on the PubChem Chemistry Database (Figure 2A).

In total, fourteen preliminary positive hits were identified after screening 16 chemical libraries encompassing a total of 49,745 small molecule compounds (Supporting Information (SI), Table S1). To validate the preliminary hits from the DRaCALA screen, an additional sample of each hit compound was obtained from the original chemical library, and DRaCALA was used to re-assess the ability of the compounds to inhibit Alg44 binding to ³²P-c-di-GMP at the same concentration used in the preliminary screen. After re-testing all re-obtained compounds, reproducible inhibition was observed only for AC1LFHI9, which will henceforth be referred to as 'HI9'. The remainder of this manuscript will focus on characterizing the inhibitory properties of HI9.

HI9 and its highly related analog 925 specifically inhibit Alg44 binding to c-di-GMP with IC₅₀ values in the micromolar range.

In order to validate HI9 as an inhibitor of Alg44 binding to c-di-GMP, the compound was obtained independently from the original screening library. ChEMBL561925 (referred to as

‘925’ for the remainder of the manuscript), a highly related analog that differs from HI9 only by an additional methyl group, was also acquired (Figure 2B). To validate inhibition, both HI9 and 925 were tested for their ability to inhibit Alg44 binding to ^{32}P -c-di-GMP by DRaCALA. In order to assess the inhibition potency of these compounds on Alg44 binding to c-di-GMP, DRaCALA was used to measure the fraction of ^{32}P -c-di-GMP bound to Alg44 after incubation with varying concentrations of HI9 and 925. Both compounds were found to inhibit Alg44 ligand binding at similar potencies. The calculated IC_{50} values were $63.5 \pm 1.3 \mu\text{M}$ for HI9 and $58.1 \pm 1.2 \mu\text{M}$ for 925 (Figure 2C). These results suggest that HI9 and 925 are bona fide inhibitors of Alg44 binding to c-di-GMP.

Alg44 belongs to the PilZ domain c-di-GMP binding receptor family. The *P. aeruginosa* genome encodes 7 other PilZ domain proteins (http://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html).^{19, 20, 27} To determine if HI9 and 925 specifically act on Alg44 or broadly inhibit all *P. aeruginosa* PilZ domain proteins, DRaCALA was used to assess the ability of each compound to inhibit ^{32}P -c-di-GMP binding. At a concentration of 200 μM , HI9 inhibited Alg44 binding to c-di-GMP by 94%. In contrast, HI9 only reduced c-di-GMP binding to PA0012, PA2989, PA3353, and PA4324 by 17%, 29%, 25%, and 35%, respectively, compared to DMSO control (Figure 2D). Likewise, 925 inhibited Alg44 binding to c-di-GMP by 98%, but only reduced PA0012, PA2989, PA3353, and PA4324 binding to ^{32}P -c-di-GMP by 30%, 46%, 39%, and 40%, respectively (Figure 2D). Since neither compound was able to inhibit ^{32}P -c-di-GMP binding to the other PilZ domain containing proteins by more than 50%, this suggests that these thiol-benzo-triazolo-quinazolinones preferentially inhibit Alg44 binding to c-di-GMP. Altogether, these results indicate that HI9 and 925 are specific inhibitors of Alg44 binding to c-di-GMP that have IC_{50} values in the micromolar range. Moreover, due to the high similarity between these two compounds, these results suggest the identification of a novel class of chemical compounds that target the ability of Alg44 PilZ domain to bind c-di-GMP.

The inhibitory properties of HI9 and 925 are dependent on the sulfhydryl functional group.

In order to investigate the chemical properties that enable thiol-benzo-triazolo-quinazolinone to inhibit Alg44 binding to c-di-GMP, eight related compounds containing alterations to the ring structure, the alkyl side chain, or the sulfhydryl functional group were ordered from chemical vendors (Figure 3A–H). These compounds were verified by ^1H -NMR (Figure S1) and evaluated for their ability to inhibit Alg44 binding to ^{32}P -c-di-GMP by DRaCALA. At a concentration of 200 μM , the triazole ring itself was not able to inhibit Alg44 ligand binding (Figure 3A and 3I). Moreover, a compound containing the triazole pyrimidinone rings was also unable to inhibit Alg44 (Figure 3B and 3I). Compounds containing only the benzo-quinazolinone rings could not inhibit Alg44 binding to c-di-GMP, (Figure 3I, C and D), suggesting that the triazole ring structure is necessary for inhibition. In order to investigate whether the sulfhydryl group was required for inhibition, another set of compounds containing the benzo-triazolo-quinazolinone rings but lacking the sulfhydryl moiety was tested in this assay. Compounds E and F that lack the sulfhydryl group did not have the ability to inhibit Alg44 binding to ^{32}P -c-di-GMP (Figure 3E and 3F). Moreover, substitution of the sulfhydryl by a thioether group caused a 48% reduction in the inhibition as compared to 925 (Figure 3G and 3I). The inhibitory ability of the thioether compound (Figure 3G) was

abolished by an additional modification in the alkyl side chain (Figure 3H) suggesting that the alkyl side group is also necessary for the inhibition of Alg44 binding to c-di-GMP by these benzo-triazolo-quinazolinone compounds. Altogether, these results reveal that the sulfhydryl group is a key feature of thiol-benzo-triazolo-quinazolinones that facilitates their ability to inhibit Alg44 binding to c-di-GMP. Other structural features such as the alkyl side group also contribute to the inhibitory activity.

HI9 and 925 form a disulfide bond with Alg44.

The sulfhydryl group of thiol-benzo-triazolo-quinazolinones is a key chemical feature required for these compounds to inhibit Alg44 binding to c-di-GMP. Sulfhydryl groups are able to form covalent disulfide bonds with cysteine residues found in proteins. Sequence analysis of *P. aeruginosa* Alg44 has shown that its PilZ domain contains only one cysteine residue²⁸, whereas there are no cysteines in MBP. Therefore, the MBP-Alg44_{PilZ} fusion protein that was used in this work has only one cysteine residue located in the PilZ domain of Alg44. Disulfide bond formation between thiol-benzo-triazolo-quinazolinones and the PilZ domain of Alg44 should remove two protons and increase the molecular weight of MBP-Alg44_{PilZ} by exactly two Daltons less than the molecular weight of the inhibitor. Furthermore, this change should be reversed by the addition of a reducing agent such as dithiothreitol (DTT).

Using mass spectrometry, the molecular weight of the protein was determined after incubation with the compounds or DMSO solvent. Treatment of MBP-Alg44_{PilZ} with DMSO solvent resulted in a species that had the expected molecular weight of 58384 Da (Figure 4A). The molecular weight increased by 296 and 310 Da after a 3-hour incubation with HI9 and 925, respectively (Figure 4B, 4D). These increases in molecular weight corresponded to 2 Da lower than the molecular weight of HI9 (MW = 298 Da) and 925 (MW = 312 Da), supporting the hypothesis of the formation of a disulfide bond. Additionally, incubation of Alg44 with compounds lacking either the tetra heterocyclic structure or the sulfhydryl group (Figure 3, compounds A, B, E and G) did not cause an increase in protein molecular weight, suggesting that the sulfhydryl group and the intact heterocyclic ring system of HI9 and 925 are required for disulfide bond formation to Alg44 (Figure 4F–4I).

Two additional lines of evidence support the covalent linkage of HI9 and 925 to Alg44. First, addition of 20 mM DTT after incubation of Alg44 with HI9 and 925 restored the original molecular weight of DMSO-treated Alg44 indicating the reduction of the disulfide bond (Figure 4C, 4E). Second, site-directed mutagenesis of Cys-98 in Alg44 to alanine (Alg44 C98A) or serine (Alg44 C98S) produced proteins lacking the sulfhydryl group. As expected, mass spectrometry of Alg44 C98A and C98S after the incubation with HI9 and 925 revealed that the compounds had no effect on the protein molecular weight, indicating that a cysteine residue is essential for the compounds to covalently modify the PilZ domain (Figure S2). These results show that the HI9 and 925 form a disulfide bond with the Alg44 protein using the sulfhydryl on the compound and the Cys-98 on Alg44.

To determine consequence of the disulfide bond formation between the compounds and Alg44, the above combinations of protein variants, compound analogs and reducing agent

were tested for their ability to inhibit Alg44 binding to c-di-GMP. Addition of 5 mM DTT to inhibitor-treated Alg44 restored ^{32}P -c-di-GMP binding by over six-fold for HI9 and over four-fold for 925, indicating that the reducing agent rescued the ability of the protein to bind c-di-GMP (Figure 5). The molecular weight of protein variants (C98A and C98S) lacking Cys98 was not altered by treatment with compounds either HI9 or 925 (Supporting Information Figure S2 and see SI for details of molecular weight determination). Both C98A and C98S proteins were not inhibited for c-di-GMP binding by HI9 or 925 at concentrations below 100 μM (SI Figure S3). At higher concentrations of 925, there was a partial inhibition of c-di-GMP binding (SI Figure S3). These findings suggest that the formation of a disulfide bond between the compounds and the PilZ domain of Alg44 inhibits c-di-GMP binding.

The cysteine residue within the PilZ domain of Alg44 is highly conserved.

Inhibition of Alg44 binding to c-di-GMP by thiol-benzo-triazolo-quinazolinones requires the modification of the cysteine residue in the PilZ domain. Resistance to the inhibitory properties of thiol-benzo-triazolo-quinazolinones can arise if the cysteine is not a conserved residue in the Alg44 PilZ domain. In order to determine the level of biological conservation of the Alg44 PilZ domain cysteine residue, a Basic Local Alignment Search Tool (BLAST-P) search was conducted to identify homologous Alg44 proteins in all fully sequenced *P. aeruginosa* genomes available on Pseudomonas.com. All of the *P. aeruginosa* Alg44 protein sequences identified by our search were found to have more than 99.5% identity and have 100% identity in their PilZ domains (SI Figure 4A). Furthermore, a sequence alignment of Alg44 homologs located within the *alg* operon from other Pseudomonal and Azotobacter species showed 100% conservation of RxxxR and DxSxxG sequence motifs in the PilZ domain (SI Figure 4A, blue and green highlights).²⁰ The cysteine residue at position 98 was present in all analyzed *P. aeruginosa* Alg44. When compared to Alg44 from other Pseudomonal and Azotobacter species, all homologs have Cys 98 except *P. putida* (SI Figure 4A, red highlight), which indicates that the cysteine residue is highly conserved among Pseudomonal and Azotobacter species.

HI9 and 925 preferentially inhibit Alg44, but not the other PilZ domain proteins (Fig. 2D). Of the four other PilZ domain proteins tested, PA2989 and PA3353 lack cysteine in the PilZ domain (SI Figure 4B, red highlight). PA0012 has one cysteine that is immediately downstream of the RxxxR motif. PA4324 has three cysteines located in amino acids 64, 74 and 78. HI9 and 925 had low-level inhibitory activity towards the ability of PA0012, PA2989, PA3353 and PA4324 to bind c-di-GMP (Figure 2D), indicating that the location of the cysteine in Alg44 PilZ is critical for inhibition by HI9 and 925.

HI9 and 925 inhibit alginate secretion by *P. aeruginosa*.

Alg44 binding to its ligand c-di-GMP is an essential process for alginate biosynthesis in *P. aeruginosa*.²¹ Treatment of *P. aeruginosa* with HI9 and 925 has the potential to disrupt Alg44 activation of alginate production. The strain used for these studies is *P. aeruginosa* PA14 pMMB-*algU* since PA14 does not produce alginate.²¹ In the absence of induction, PA14 pMMB-*algU* produced a low level of alginate (5.1 ± 0.3 μg of alginate/mL/OD₆₀₀) (Figure 6A). Induction of *algU* with 100 μM IPTG increased alginate secretion to 141 ± 27 μg /mL/OD₆₀₀ (Figure 6A). Treatment with HI9 and 925 reduced alginate secretion to 76

$\pm 30 \mu\text{g/mL/OD}_{600}$ and $75 \pm 20 \mu\text{g/mL/OD}_{600}$, respectively (Figure 6A). As the compounds concentrations are reduced, the level of inhibition is also diminished indicating that these compounds only inhibit alginate production at high concentrations. To test whether or not these compounds are efficiently effluxed out of the cell by the MexA-MexB-OprM multidrug efflux pump, a strain with transposon insertion mutation in the *mexA* gene was tested for inhibition. Expression of *algU* from pMMB plasmid induced the *mexA::tn* strain to produce $78 \pm 11 \mu\text{g/mL/OD}_{600}$ of alginate. Treatment with HI9 and 925 reduced alginate production to 31 ± 6 and $50 \pm 10 \mu\text{g/mL/OD}_{600}$ of alginate, respectively (Figure 6B). Since both compounds were able to inhibit alginate to a similar level in the *mexA::tn* mutant, efflux of the compounds is not the primary reason for the low level of inhibition. To test the requirement for disulfide bond formation on the ability of these compounds to inhibit alginate production, *alg44* alleles were generated with C98A and C98S substitutions. When *algU* is induced from the pMMB plasmid, the strains with *alg44 C98A* or *alg44 C98S* have reduced production of alginate (94 ± 18 and $87 \pm 14 \mu\text{g/mL/OD}_{600}$, respectively) indicating that C98 is required for maximal alginate production (Figure 6C and 6D). Treatment with $300 \mu\text{M}$ of HI9 and 925 inhibited alginate production between 25–45% as compared to the DMSO vehicle control. Furthermore, treatment with Compound 3G, which has a thioether, was able to inhibit alginate production by a similar level (SI Figure 5). Therefore, the disulfide bond formation between the inhibitory molecules and C98 of Alg44 is not required for inhibition of alginate production at these inhibitor concentrations. These results indicate that thiol-benzo-triazolo-quinazolinones are capable of reducing alginate secretion by *P. aeruginosa* independent of their ability to form disulfide bonds.

DISCUSSION

Alginate secreted by *P. aeruginosa* in the CF lung is a major virulence factor that leads to patient morbidity and mortality. There are many strategies for reducing alginate secretion during mucoid *P. aeruginosa* chronic lung infection, including inhibition of transcription of the *alg* operon, inhibition of the glycosyltransferases that polymerize the nucleotide sugars, inhibition of the synthesis of c-di-GMP, inhibition of activation of the Alg44 by c-di-GMP, and degradation of the secreted alginate using exogenously added enzymes. Each approach has its own challenges and drawbacks. Since mucoid conversion often occurs via mutagenesis and inactivation of *mucA*, the result is the constitutive activation of AlgT/U and expression of the *alg* operon. Disruption of this process would require either the restoration of MucA function or inhibition of AlgT/U activity. Inhibitors of glycosyltransferases can be identified, but accessibility of nucleotide sugar analogs to the cytoplasm and selectivity to the target enzyme represent problems. Inhibition of c-di-GMP synthesis is somewhat problematic as there are more than 30 genes in the *P. aeruginosa* genome that have a diguanylate cyclase domain.²⁹ Inhibition of all 30 proteins would require a broad-range inhibitor that can act on the entire set of DGCs present in the cell. Inhibition of Alg44 activation by c-di-GMP is attractive since the redundancy associated with DGCs is not present. However, identifying a specific inhibitor that acts on Alg44 alone is challenging. Lastly, degradation of alginate is another interesting strategy, but there would be issues associated with the delivery of alginase enzymes to the lung and immune recognition of foreign proteins. In this work, a novel class of small molecule derivatives of thiol-benzo-

triazolo-quinazolinone that inhibit Alg44 binding to c-di-GMP was discovered using a high-throughput DRaCALA screen of chemical libraries.

Thiol-benzo-triazolo-quinazolinones inhibit c-di-GMP binding with IC₅₀ values in the micromolar range. These compounds have been previously assayed in other high-throughput screens. Compound 925 showed activity in three different screens. It had antibacterial activity against *Bacillus anthracis* in a broth microdilution assay, antibacterial activity against a *polA*-deficient *B. anthracis* and was able to inhibit *B. anthracis* DnaB helicase activity.³⁰ These findings suggest that compound 925 has the ability to interfere with DnaB helicase activity and DNA replication in *B. anthracis*. 925 was also included in a screen to identify inhibitors of *Mycobacterium tuberculosis* growth, but was not found to be active. HI9 was a part of the chemical library screened for inhibition of DNA replication, RNA synthesis, and siderophore biosynthesis in other bacterial organisms and failed to show any activity in these screens.³⁰ Since HI9 and 925 only differ by a single methyl group, the activity of 925 against DnaB of *B. anthracis* is likely specific for just *B. anthracis*.

Inhibition of Alg44 binding to c-di-GMP by compounds HI9 and 925 requires binding to Cys-98. The Cys-98 residue is located in the interior of the β -barrel of the PilZ domain based on the recently solved structure of the Alg44 PilZ domain.²² An obvious question is how HI9 and 925 gain access to the interior of the β -barrel in order to form a disulfide bond with Cys-98. Several explanations are possible. The first possibility is that there is an opening on the surface of the protein allowing small molecules to diffuse into the center of the β -barrel. This is unlikely since inspection of the structure did not reveal obvious openings on the surface of the PilZ domain. A second possibility is that the protein structure is dynamic such that the opening occurs when the protein is in solution and was not captured in the crystal packing. In this case, any small molecule with a thiol functional group can enter and form a disulfide with Cys98. Compounds that are smaller than HI9 and 925 with thiols were tested. Both triazole-thiol (Figure 3A) and mercapto-triazaindolizine (Figure 3B) were unable to form disulfide bonds with Cys98 (Figure 4F and 4G) indicating that protein flexibility alone does not allow an opening for all small thiol-containing molecules. A third possibility is that these compounds have a unique property to interact with the Alg44 PilZ domain thereby allowing the sulfhydryl to form a disulfide with Cys-98. Two lines of evidence support the idea that thiol-benzo-triazolo-quinazolinone possesses the ability to interact specifically with Alg44. Small compounds, such as triazole-thiol and mercapto-triazaindolizine, cannot interact with Cys98 meaning that the tetra heterocyclic structure of benzo-triazolo-quinazolinone is required for disulfide formation. Furthermore, the thioether form of benzo-triazolo-quinazolinone has low level of activity (Figure 3G). This can be interpreted to mean that, for HI9 and 925, the benzo-triazolo-quinazolinone moiety promotes interaction with the Alg44 protein with low affinity, which allows for disulfide formation and covalent linkage with the protein. Future structural studies should distinguish between these possibilities.

Since HI9 and 925 were demonstrated to inhibit Alg44-c-di-GMP interactions, these two compounds were tested for their ability to inhibit alginate secretion in *P. aeruginosa*. Although these compounds disrupt c-di-GMP binding to Alg44 *in vitro*, it is possible that they could act as either agonists or antagonists of alginate production *in vivo*. Our results

show that both HI9 and 925 caused a modest, but significant reduction in alginate secretion by the *P. aeruginosa* PA14 pMMB-*algU*. The relatively modest ability of HI9 and 925 to inhibit *P. aeruginosa* alginate secretion is likely due to the inability of the compound to form covalent disulfide bond with Alg44 in the cytoplasm. Since the cytoplasm is a reducing environment, the disulfide bond is reduced, which limits the ability of these compounds to inhibit c-di-GMP binding to Alg44. This possibility is supported by the findings that inhibition by HI9 and 925 are similar when the C98 of Alg44 is altered to alanine or serine. Similarly, Compound 3G, the thioether form of benzo-triazolo-quinazolinone, was also able to reduce alginate production by a similar level. This reduced ability is not likely due to efflux of the compounds since mutants in the primary efflux pump (*mexA*) was also inhibited by HI9/925 by a similar level. These results indicate that these compounds enter the cell and inhibit Alg44 sufficiently to reduce alginate production. Although HI9 and 925 inhibition of alginate production was modest, these compounds could serve as leads for the discovery of more potent inhibitors. Two main types of modification include the generation of chemical analogs that increase affinity to Alg44 or increase the stability of covalent linkage between the inhibitor and Alg44. Development of these second generation compounds can be tested for enhanced inhibition of *P. aeruginosa* alginate biosynthesis.

Conclusions.

In summary, we have identified of a class of small molecule compounds, thiol-benzo-triazolo-quinazolinones, that inhibit Alg44-c-di-GMP interactions. These compounds were shown to specifically inhibit Alg44 with a potency in the micromolar range through covalent modification of Cys-98 on the Alg44 protein. One compound derivative exhibited a small but significant reduction in alginate biosynthesis by *P. aeruginosa*. This work shows that thiol-benzo-triazolo-quinazolinone represents a lead compound for the future development of chemical inhibitors of alginate secretion by *P. aeruginosa*, which could reduce the adverse effects of alginate production on CF patients.

METHODS

Buffers and Compounds

C-di-GMP binding buffer (10 mM Tris pH 8.0, 100 mM NaCl, 5 mM MgCl₂) was used for all DRaCALA binding assays. His Buffer A (10 mM Tris pH 8.0, 300 mM NaCl, 25 mM imidazole) and His Buffer B (10 mM Tris pH 8.0, 300 mM NaCl, 250 mM imidazole) were used for protein purification. Compounds HI9, 925, 3C, 3D, 3F and 3H were purchased from [Hit2Lead.com](https://www.hit2lead.com) (Catalog numbers: 7354238, 7931247, 5228972, 5228974, 6951950, and 5228974 respectively). Compounds 3A and 3B were purchased from LabNetwork (Catalog numbers: 037233–25g-OAKW and STOCK1S-73186, respectively). Compounds 3E and 3G were purchased from Vitas-M Laboratories (Catalog numbers: STL038216 and STK559651, respectively).

Bacterial strains, plasmids and growth conditions

E. coli strains BL21 (DE3) and T7Iq were used for protein purification. Strains for protein purification were grown at 30°C in LB-M9 media (5 g/L yeast extract, 10 g/L tryptone, 2

g/L glucose, 1 g/L sodium succinate hexahydrate, 1 g/L NH_4SO_4 , 0.5 g/L NaCl, 2 g/L KH_2PO_4 , 7 g/L anhydrous Na_2HPO_4 , 3 mM MgSO_4) supplemented with 50 $\mu\text{g/mL}$ carbenicillin antibiotic. *E. coli* DH5 α was grown at 37°C in LB media supplemented with carbenicillin (50 $\mu\text{g/mL}$). *P. aeruginosa* PA14 containing pMM6580 (pMMB-AlgU) harboring AlgU²¹ was grown at 37°C in LB media and induced with 100 μM IPTG and assayed for alginate biosynthesis. The PA14 *mexA::tn* strain was obtained from the Ausubel lab.³¹ PA14 alg44 C98A and C98S were generated by cloning the *alg44* gene and 1 kb of flanking sequence around C98 using primers (Table S3). The 2 kb fragment was cloned into pEX-Gn.³² The C98A and C98S site-directed mutagenesis was performed using primers (Table S3) and sequence verified. The C98A and C98S mutations were introduced into the chromosome by allelic exchange as previously described.³²

Site-Directed Mutagenesis of Alg44

pVL877 (pVL847-His-MBP-Alg44_{PilZ}) was mutated using primers (Table S3) to create *alg44* genes containing C98S and C98A mutations using the Q5 Site-Directed Mutagenesis Kit from New England Biolabs. The presence of the desired mutation within each plasmid was confirmed by restriction digest and DNA sequencing.

Purification of His-MBP-Alg44_{PilZ} (Alg44)

E. coli BL21 (DE3) or T7Iq expressing the His-MBP-Alg44_{PilZ} (Alg44) fusion protein (pVL877)²¹, His-MBP-Alg44_{PilZ} C98A, and His-MBP-Alg44_{PilZ} C98S were grown in LB-M9 media supplemented with carbenicillin (50 $\mu\text{g/mL}$) and induced with 500 μM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were pelleted by centrifugation and resuspended in His Buffer A. Cells were lysed by sonication after addition of DNase I (10 $\mu\text{g/mL}$), lysozyme (25 $\mu\text{g/mL}$), and phenylmethanesulfonyl fluoride (1 mM). Overexpression of Alg44 in lysates was confirmed by SDS-PAGE. Alg44 was purified from the whole-cell lysates over a nickel-nitrilotriacetic acid (Ni^{2+} -NTA) column using a fast protein liquid chromatography (FPLC) instrument. After elution, the purified protein fractions were pooled, dialyzed against 10 mM Tris pH 8.0, 100 mM NaCl, 25% glycerol, flash-frozen with liquid nitrogen, and stored at -80°C. Purification was confirmed by SDS-PAGE, and the concentration of purified Alg44 was determined using a NanoDrop UV-Vis spectrophotometer.

Synthesis of radiolabeled c-di-GMP (³²P-c-di-GMP)

Radiolabeled GTP (α -³²P-GTP) was mixed with the diguanylate cyclase WspR D70E in c-di-GMP binding buffer and incubated for 18 hours at 37°C. Residual WspR was separated from ³²P-c-di-GMP by centrifugation over a 3 kDa molecular weight cut-off filter. The reaction efficiency was assessed by thin-layer chromatography.

DRaCALA

Purified proteins were diluted to target concentrations in c-di-GMP binding buffer and kept on ice. Chemical inhibitory compounds, if necessary for the experiment, were diluted in c-di-GMP binding buffer to target concentrations and transferred to wells on a 96-well plate. Diluted proteins were mixed with the compounds in the wells and were incubated on a plate

shaker for 5 minutes to allow binding to take place. ^{32}P -c-di-GMP was added to the wells, and mixtures were incubated again for five minutes on a plate shaker to allow the ligand to bind to the protein. Approximately 2 μL from each well were spotted on dry nitrocellulose membrane using a pin-tool. Spots were allowed to air-dry and were imaged for radioactivity using a Fujifilm phosphorimager. The radioactive signal of each spot was quantified and used to calculate the fraction of ligand bound to protein (F_B) using the equation described previously.²⁵

DRaCALA-based Screen for Alg44 Inhibitor

Chemical libraries were obtained from the ICCB-Longwood Screening Facility at Harvard Medical School. The compounds were dissolved in DMSO solvent and transferred to wells in 384-well plates and further diluted in 20 μL of c-di-GMP binding buffer. 10 μL of 1 μM purified Alg44 were dispensed to wells using a Biotek MultiFlo liquid dispensing machine and allowed to incubate for five minutes. 10 μL of 0.2 nM ^{32}P -c-di-GMP were then dispensed to the wells, and mixtures were incubated again for five minutes. The final concentrations of the compounds in the wells were 12.5 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, or 25 μM depending on the origin of the compound library. 1 μL from each well was spotted on dry nitrocellulose membrane using a 384-well pin-tool, and spots were allowed to air dry for several minutes. The F_B of spots was determined by quantifying radioactive signal using a Fujifilm phosphorimager.

Determination of Molecular Weight of Alg44

Purified Alg44, Alg44 C98A and Alg44 C98S (50 μM) diluted in c-di-GMP buffer were incubated at room temperature with compounds (200 μM) for 3 hours. Afterwards, if appropriate, the mixtures were further incubated with 20 mM dithiothreitol (DTT) for 20 minutes. After incubation, protein samples were acidified with 1 μL 10% TFA and analyzed by LCMS. Protein was eluted with a linear gradient of 15–90% solvent B in 20 min, followed by 5 min wash at 90% B and equilibration at 15% B for 10 min. Solvent A is 2.5% ACN in water with 0.1% formic acid, and solvent B is 75% ACN in water with 0.1% formic acid. Spectra of m/z 500–1700 were acquired with an Orbitrap Fusion Lumos mass spectrometer with resolution of 15,000 at m/z 200. Source fragmentation of 30% was applied to disrupt potential clusters. 5 spectra (micro scans) were averaged for each spectrum recorded. After acquisition, mass spectra over the chromatography peak were averaged. Averaged spectrum was deconvoluted using MagTran program³³ (A universal algorithm for fast and automated charge state deconvolution of electrospray mass-to-charge ratio spectra). Molecular weight of the protein was calculated as described.

Quantification of alginate biosynthesis by *P. aeruginosa*

P. aeruginosa strains harboring pMMB-AlgU was grown to $\text{OD}_{600} \sim 0.5$ and induced for 3 additional hours with 100 μM IPTG. After centrifugation, the alginate-containing supernatant was precipitated by adding one volume of isopropanol at -20°C followed by centrifugation for 15 minutes at 16,000 g . The alginate pellet was resuspended in double distilled water and quantified by the uronic acid assay as described previously.³⁴ Briefly, 117 μL of alginate resuspensions were added to 33 μL of carbazole reagent (0.1% carbazole in absolute ethanol) and 1 mL of borate-sulfuric acid reagent (0.1 M K_3BO_3 in concentrated

H₂SO₄). The reaction was incubated at 55°C for 30 minutes, and the absorbance at 530 nm was measured using a spectrophotometer. Absorbance values were converted to units of alginate concentration by using an alginate standard curve. Alginate concentrations were reported in µg alginate/mL/OD₆₀₀ of bacterial culture. Statistical significance was determined on Prism software using T-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Identification of a chemical inhibitor of Alg44 binding to c-di-GMP. (A) Image of a subsection of DRaCALA spots from one plate screen. Spot boxed in red corresponds to a well that contained a compound that disrupted Alg44 binding to ^{32}P -c-di-GMP. Spots boxed in green and blue correspond to wells containing 5 μM unlabeled c-di-GMP and DMSO, respectively. (B) Quantification of fraction bound from the entire 384-well plate. The fraction of ^{32}P -c-di-GMP bound to Alg44 of each spot was quantified and plotted against its well position on the plate. Red star corresponds to the red-boxed well in panel A. Blue circles correspond to wells containing DMSO solvent only. Green diamonds correspond to

wells containing 5 μ M and 10 μ M unlabeled c-di-GMP, respectively. Dashed line represents the 3 standard deviations cut-off for classification of hit compounds. The compound identified from screening this plate was AC1LFH19 (HI9), a derivative of thiol-benzotriazolo-quinazolinone.

Figure 2.

HI9 and 925 specifically inhibit Alg44 binding to c-di-GMP. Chemical structure, PubChem designation, and molecular weight of (A) HI9 and (B) 925. (C) Fraction of ^{32}P -c-di-GMP bound to Alg44 in the presence of varying concentration of HI9 (solid line, black circles) and 925 (dashed line, white circles). The calculated IC_{50} for HI9 and 925 were $63.5 \pm 1.3 \mu\text{M}$ and $58.1 \pm 1.2 \mu\text{M}$, respectively. (D) Fraction of ^{32}P -c-di-GMP bound to *P. aeruginosa* PilZ domain proteins in the presence of 200 μM compounds or DMSO solvent. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$. Data represents at least 3 experimental replications.

Figure 3. Inhibition of Alg44 binding to ^{32}P -c-di-GMP by structural analogs of HI9 and 925. (A-H) Chemical structures of analogs. (I) Fraction ^{32}P -c-di-GMP bound to Alg44 after treatment with DMSO, HI9, 925, and Compounds A-H at a final concentration of 200 μM . *** $p < 0.001$; ns $p > 0.05$. Data represents at least 3 experimental replications.

Figure 4.

HI9 and 925 form a disulfide bond with the PilZ domain of Alg44 to inhibit c-di-GMP binding. Mass spectra of MBP-Alg44_{PilZ} after incubation with (A) DMSO, (B) HI9, (C) HI9+DTT, (D) 925, (E) 925+DTT, (F) Analog 3A, (G) Analog 3B, (H) Analog 3E, and (I) Analog 3G (See Figure 3 for chemical structure of compounds). Compounds were tested against MBP-Alg44_{PilZ} at a final compound concentration of 200 μ M and 20 mM DTT where indicated.

Figure 5.

Reducing agent can reverse inhibition of c-di-GMP binding to Alg44 by HI9 and 925.

Fraction of ^{32}P -c-di-GMP bound to Alg44 after treatment with 200 μM thiol-benzo-triazolo-quinazolinones and 5 mM DTT, where indicated. ns $p > 0.05$; *** $p < 0.001$. Data represents at least 3 experimental replications.

Figure 6.

HI9 and 925 reduce alginate secretion by *P. aeruginosa* PA14. Quantification of alginate secretion by (A) PA14 pMMB-*algU*, (B) PA14 *mexA::tn*, (C) PA14 *alg44(C98A)* pMMB-*algU* or (D) PA14 *alg44(C98S)* pMMB-*algU* after treatment with 300 μM or the indicated concentration (in μM) of thiol-benzo-triazolo-quinazolinone or DMSO solvent control. Alginate biosynthesis was induced by the addition of 100 μM IPTG. Alginate concentrations were normalized to the culture optical density and reported in units of μg/mL/OD₆₀₀ of bacterial culture. Bars on the graphs represent the mean value of six independent experiments, with error bars representing the SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.