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Inhibition of *Pseudomonas aeruginosa* Alginate Synthesis by Ebselen Oxide and Its Analogues

Soo-Kyoung Kim, Huy X. Ngo, Emily K. Dennis, Nishad Thamban Chandrika, Philip DeShong, Sylvie Garneau-Tsodikova,* and Vincent T. Lee*

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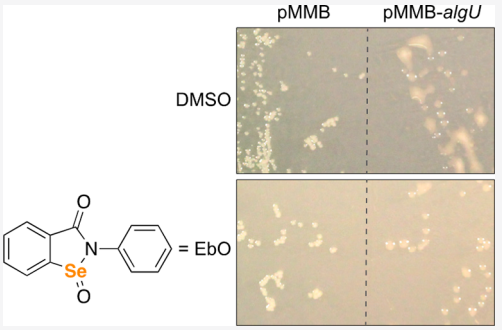
ABSTRACT: *Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that is frequently found in the airways of cystic fibrosis (CF) patients due to the dehydrated mucus that collapses the underlying cilia and prevents mucociliary clearance. During this life-long chronic infection, *P. aeruginosa* cell accumulates mutations that lead to inactivation of the *mucA* gene that results in the constitutive expression of *algD-algA* operon and the production of alginate exopolysaccharide. The viscous alginate polysaccharide further occludes the airways of CF patients and serves as a protective matrix to shield *P. aeruginosa* from host immune cells and antibiotic therapy. Development of inhibitors of alginate production by *P. aeruginosa* would reduce the negative impact from this viscous polysaccharide. In addition to transcriptional regulation, alginate biosynthesis requires allosteric activation by bis (3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) binding to an Alg44 protein. Previously, we found that ebselen (Eb) and ebselen oxide (EbO) inhibited diguanylate cyclase from synthesizing c-di-GMP. In this study, we show that EbO, Eb, ebsulfur (EbS), and their analogues inhibit alginate production. Eb and EbS can covalently modify the cysteine 98 (C98) residue of Alg44 and prevent its ability to bind c-di-GMP. However, *P. aeruginosa* with Alg44 C98 substituted with alanine or serine was still inhibited for alginate production by Eb and EbS. Our results indicate that EbO, Eb, and EbS are lead compounds for reducing alginate production by *P. aeruginosa*. Future development of these inhibitors could provide a potential treatment for CF patients infected with mucoid *P. aeruginosa*.



KEYWORDS: *Alg44, alginate, cystic fibrosis, inhibitors, Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic pathogen that capitalizes on deficiencies in the host immune response to cause acute and chronic infections.[1] Despite not being one of the abundant commensal bacteria in the normal microflora of typical healthy humans,[2,3] *P. aeruginosa* has a tremendous ability to cause opportunistic infections as demonstrated by the prevalence of this bacteria in healthcare-associated infections (HAIs).[4] In addition to these infections, *P. aeruginosa* is often found in the airways of cystic fibrosis (CF) patients.[5] CF patients have a defect in cystic fibrosis transmembrane regulator (CFTR)[6] that is a symporter for water and chloride ions[7] to maintain the appropriate hydration of the airway surface liquid.[8,9] In CF patients, the dehydrated mucus collapses on the cilia leading to decreased mucociliary escalatory clearance of mucus and trapped microbes.[8,9] As a consequence, CF airways have a suboptimal immune response and an increased microbial load.[10] Airway infections have decreased the lifespan of CF patients and led to aggressive antibiotic therapies that have greatly extended life expectancies. However, *P. aeruginosa* is one bacterium that is not easily eliminated by antibiotics. Despite these treatments, *P. aeruginosa* has formed lifelong associations with patients as determined by sequencing of genomes of longitudinal isolates from individual CF patients.[11−14] Even more problematic is that, over time in the patient, *P. aeruginosa* undergoes mutagenesis through mutation in the DNA repair gene *mutS*,[11,13] resulting in accumulation of additional mutations in the *P. aeruginosa* genome. One of the genes often mutated is *mucA*,[15] which leads to mucoid conversion. *P. aeruginosa* strains with *mucA* mutations lack the MucA protein and release AlgT/U sigma factor.[15−17] The AlgT/U activates the constitutive expression of the *algD-algA* operon that encodes the alginate biosynthesis proteins.[15,18] The overexpression of the alginate biosynthesis proteins leads to the constitutive production of the mucoid alginate polysaccharide. The combination of dehydrated mucus and the mucoid alginate polysaccharide leads to further occlusion of the airway and loss of lung function. Since *P. aeruginosa* producing alginate has enhanced resistance to antibiotics, inhibition of alginate

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production may reduce airway blockage and antibiotic resistance.

In addition to transcriptional regulation, alginate production also requires allosteric activation by bis (3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) through binding to the PilZ domain of the Alg44 protein ($Alg44_{PilZ}$), a component of the biosynthesis complex in the cytoplasmic membrane.[19−21] c-di-GMP is a signaling nucleotide widely used by bacteria.[22] The molecule is synthesized from two guanosine triphosphate (GTP) molecules by diguanylate cyclases, which are regulated by the product through binding to the allosteric inhibitory site (I-site).[23,24] Similar to other secondary signaling molecules, c-di-GMP binds receptors to allosterically alter protein function.[25] In the case of Alg44, ablation of the c-di-GMP binding residues in the PilZ domain results in strains that fail to produce alginate polysaccharide.[20,21] On the basis of analogy to the bacterial cellulose synthase (Bcs) system, c-di-GMP binding to the PilZ domain is thought to shift the PilZ domain that occludes the catalytic site in the glycosyltransferase.[26] As a result, nucleotide activated monosaccharides can enter the active site, polymerize, and be exported out of the bacterial cell envelope.[27] So, c-di-GMP synthesis and its binding to Alg44 serve as potential targets of inhibition to prevent alginate biosynthesis.

Different strategies have been taken to ameliorate the negative effect of alginate production. One is using enzymes to degrade alginate polymers.[28,29] Another is to identify small molecule inhibitors that either prevent expression of the *algD-algA* operon, synthesis of c-di-GMP signaling, or c-di-GMP binding to Alg44. Previous studies have identified two classes of inhibitors that interfere with c-di-GMP activation of alginate. One class is thiol-benzo-triazolo-quinazolinone that interacts with the PilZ domain of Alg44 and prevents this domain from binding c-di-GMP.[30] A second class of inhibitor is ebselen (Eb), which occupies the I-site of diguanylate cyclases and inhibits the biosynthesis of c-di-GMP.[31] Since Eb is a compound that has undergone phase III clinical trials for ischemic stroke[32] and acute hearing loss,[33] it was anticipated that Eb analogues would have a fast-track to application as a chemotherapeutic for treatment of CF. Because of its safety profile and its effectiveness at inhibiting c-di-GMP, we tested whether Eb can inhibit alginate production, which is regulated by c-di-GMP. Here we show that Eb, ebselen oxide (EbO), ebsulfur (EbS), and their analogues (Figure 1) can inhibit alginate production by *P. aeruginosa*. From this small structure−activity relationship (SAR) study, we show that specific positions of Eb can be modified without affecting inhibitory activity toward alginate. One particular modification, EbO, improved the selectivity index for inhibition of alginate production by about 20-fold. We show that Eb can covalently modify PilZ domain of Alg44 using cysteine 98 (C98) and that this modification prevents c-di-GMP binding. However, in *P. aeruginosa* cells, modification of Alg44 at C98 is not required for alginate inhibition. Together, these results indicate that EbO, Eb, EbS, and their analogues can inhibit alginate production by *P. aeruginosa* and may provide a strategy to reduce disease burden by mucoid bacteria in the CF airway.

RESULTS

Preparation of EbO, Eb, EbS, and Their Analogues. We have previously reported the synthesis and characterization of EbS and its analogues **1a**−**h**, and **2a**,**b**.[34,35] Herein, the synthesis of Eb and its analogues **3b**, **3g**, and **3h** was carried

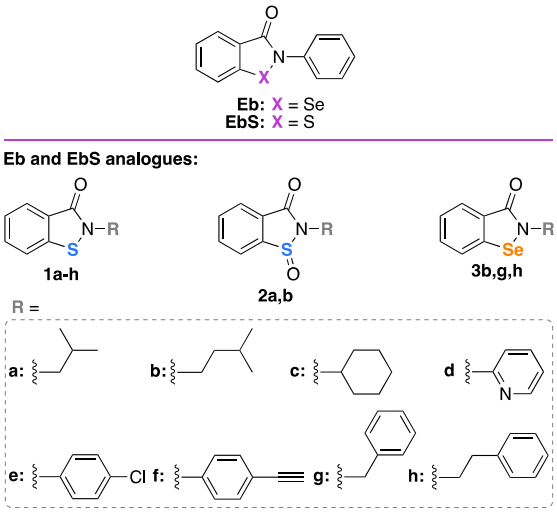

Figure 1. Chemical structures of compounds used in this study.

out in two steps as previously reported[36] for analogous compounds (Scheme S1). The first step involved the coupling of 2-iodobenzoyl chloride with various amines in the presence of a base to afford intermediates **4**−**8** in 25%-quantitative yields. The resulting 2-iodobenzamides, when subjected to a combination of CuI, 1,10-phenanthroline, and KSeCN in the presence of a base (Cs_2CO_3) underwent cyclization to provide Eb, **3b**, **3g**, and **3h** in 4−13% yields.[36]

EbO, Eb, and Their Analogues Inhibit Alginate Secretion by *P. aeruginosa*. Prior studies have shown that Eb and its oxidized analogue, EbO, interfere with the diguanylate cyclase WspR by covalent modification of cysteine and by occupying the I-site to inhibit its activity.[31] Since c-di-GMP binding to Alg44 is required for alginate production, we tested whether Eb and EbO can reduce alginate secretion by *P. aeruginosa*. To assess inhibition of alginate secretion, we employed the *P. aeruginosa* PA14 strain harboring pMMB-*algU*.[20,30] The parental PA14 strain with pMMB vector control produced little alginate (11.96 ± 1.12 μg/mL/OD_{600}) (Figure 2).[30] Induction of *algU* expression with 200 μM IPTG led to copious production and export of alginate polysaccharide (304.2 ± 10.8 μg/mL/OD_{600}) (Figure 2A; DMSO control). When treated with 100 μM of Eb or EbO, alginate production by *P. aeruginosa* PA14 pMMB-*algU* was reduced by ∼50% and ∼70%, respectively (Figure 2A). To confirm the potency of Eb and EbO for alginate inhibition, *P. aeruginosa* PA14 was treated with varying concentrations of Eb or EbO (0, 25, 50, and 100 μM). Eb inhibition only occurred at 100 μM concentration (Figure 2B; green circles). In contrast, 25 μM of EbO was sufficient to inhibit alginate secretion (Figure 2B; yellow circles). From these data, the IC_{50} values were calculated to be 80 μM for Eb and 14 μM for EbO (Table 1). Addition of compounds to extracts of alginate did not interfere with the assay (Figure S17), suggesting that the observed inhibition occurs during the growth of the bacterial cultures. Treatment with 100 μM of Eb derivatives **3b**, **3g**, and **3h** resulted in inhibition of alginate production by *P. aeruginosa*, albeit to different degrees (Figure 2C). The greater inhibition by **3g** and **3h** with planar aromatic groups as compared to **3b** with the isobutyl group suggest that properties such as size and geometry of this side chain impacts interaction with the cellular target. Taken together, these data indicated that Eb

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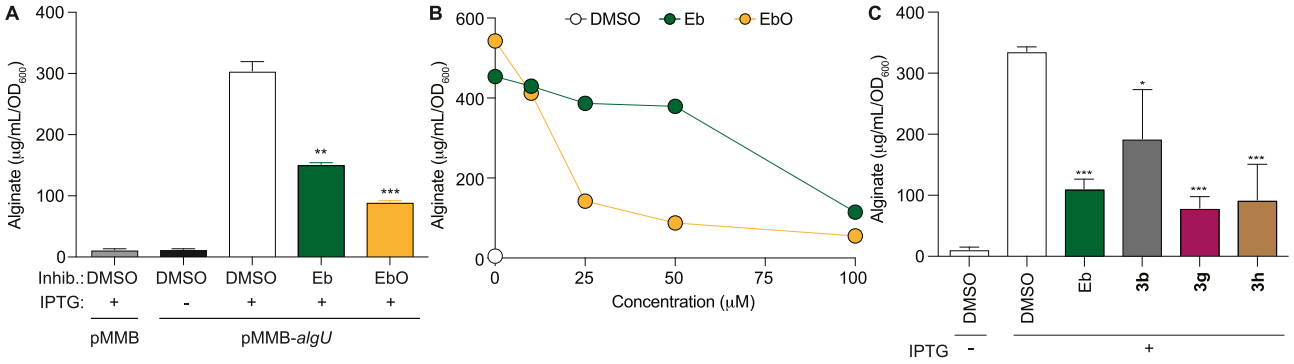

Figure 2. Ebselen (Eb) and ebselen oxide (EbO) inhibit alginate production by *P. aeruginosa*. *P. aeruginosa* PA14 pMMB-*algU* for alginate production by the addition of 200 μM IPTG was quantified in the presence of (A) 100 μM Eb (green), EbO (yellow), DMSO (white); or (B) varying concentration of Eb (green circles) or EbO (yellow circles); or (C) 100 μM of Eb analogues **3b** (gray), **3g** (crimson), and **3h** (brown). The graph indicates the average alginate production per OD_{600} for six independent experiments. *, **, and *** indicate p values <0.05, 0.005, and 0.0005, respectively, by t-test.

Table 1. Selectivity Index (SI) for Alginate Inhibition

Cpd	IC_{50} (μM)[a]	CC_{50} (μM)[b]	SI (CC_{50}/IC_{50})[c]
Eb	80	109.1	1.36
EbO	14	413.5	29.5
EbS	11	79	7.18
1a	10	29.8	2.98
1b	14	18.4	1.31
1c	11	26.3	2.39
1d	10.5	94.8	9.03
1e	18	55.5	3.08
1f	32	127.3	3.98
1g	7	32.7	4.67
1h	8	41.4	5.18

[a]The half-maximal inhibitory concentration (IC_{50}) values were calculated using data from Figures 2 and 4 for 50% reduction in alginate production. [b]Cytotoxicity concentration 50% (CC_{50}) values were calculated using data from Figure 6 for 50% cell death. [c]Selectivity index (SI) CC_{50}/IC_{50}.

and EbO and their analogues are able to inhibit alginate production by *P. aeruginosa*.

Eb, EbO, and EbS Do Not Inhibit Growth of *P. aeruginosa*. One possibility for the observed inhibition of alginate production is that Eb, EbO, and related compounds are inhibiting growth of *P. aeruginosa*. If this is the case, then addition of these compounds to cultures of parental PA14 should alter the growth curve. When Eb, EbO, and EbS were tested at 100 μM, the PA14 growth curve were mostly unaffected (Figures 3A and S18). When the colony forming unit (CFU) of these cultures were enumerated by serial dilution and plating, the number of CFUs were all between 0.4 $\times 10^9$ and 1.1 $\times 10^9$ per optical density at 600 nm (OD_{600}) (Figure 3B). These results indicate that Eb, EbO, and EbS are not causing toxicity to *P. aeruginosa*. For the *P. aeruginosa* PA14 pMMB-*algU* strain, induction of alginate production by the addition of IPTG shifts the metabolism to alginate production. As a consequence, the OD_{600} of the bacteria cultures drops noticeably. When Eb, EbO, and EbS are added at the same time as the IPTG inducer, the growth of the bacteria is partially restored (Figure 3C). At the end of the growth curve, the CFU per OD_{600} of bacteria treated with Eb, EbO, and EbS is indeed higher than the DMSO control (Figure 3D). Together, these results indicate that Eb, EbO, and EbS are acting specifically to inhibit alginate production by *P. aeruginosa*.

EbS and Its Analogues Potentiate Alginate Inhibition in *P. aeruginosa*. Eb is a small molecule with a selenium atom that serves as the active moiety for inhibition.[37] Previous studies have shown that EbS, the sulfur analogue of Eb, act to inhibit growth of eukaryotic parasites,[38] yeast,[34] and broad spectrum antibacterial activity[35,39] and has IC_{50} similar or better than Eb. To identify Eb analogues that have greater potency for inhibition of alginate production, a SAR was established for compounds that include the replacement of the Se atom with sulfur, alteration of the phenyl ring, or a combination of both types of modifications (Figure 1). Induction of pMMB-*algU* by 200 μM IPTG in the presence of the DMSO solvent carrier led to the production of alginate (Figure 4). When treated with 100 μM EbS or EbS analogues **1a**−**1h**, alginate production was reduced by approximately 3- to 25-fold (Figure 4A). When treated with oxidized versions of EbS (EbSO) analogues **2a** and **2b**, alginate production was either not affected (**2b**) or, interestingly, increased by approximately 1.4-fold (**2a**) (Figure 4B). The potency of EbS and EbS analogues **1a**−**1h** was determined by treatment with varying concentrations (25, 50, or 100 μM) of EbS or EbS analogues (Figure 4C). Treatment with EbS reduced alginate secretion (102, 80, or 58 μg/mL/OD_{600}) at 25, 50, or 100 μM of concentration, respectively, compared to 533 μg/mL/OD_{600} of DMSO control (Figure 4C). Alginate production by *P. aeruginosa* PA14 pMMB-*algU* was inhibited by EbS analogues **1a**−**1h** by 70% to 90%. The maximal inhibition of alginate production was greater than 90% for EbS analogues, **1g** and **1h**, even at 25 μM concentration (Figure 4C). The IC_{50} value was from 22 to 24 μM for EbS and EbS analogues (Table 1). These results indicated that EbS and EbS analogues are also potent inhibitors of alginate production by *P. aeruginosa* compared to Eb. Prior studies showed that an oxidized version of EbS (EbSO), the sulfoxide form of EbS, is inactive against other bacteria.[35] At 100 μM, the two EbSO analogues, **2a** and **2b**, failed to inhibit alginate production indicating that the reduced sulfur atom is required for alginate inhibition (Figure 4B).

Eb, EbS, and Their Analogues Promote Formation of Disulfide/Selenylsulfide Bond with PilZ Domain of Alg44. Eb and EbS perform their inhibitory activity through the formation of Se−S or S−S adducts, respectively, on

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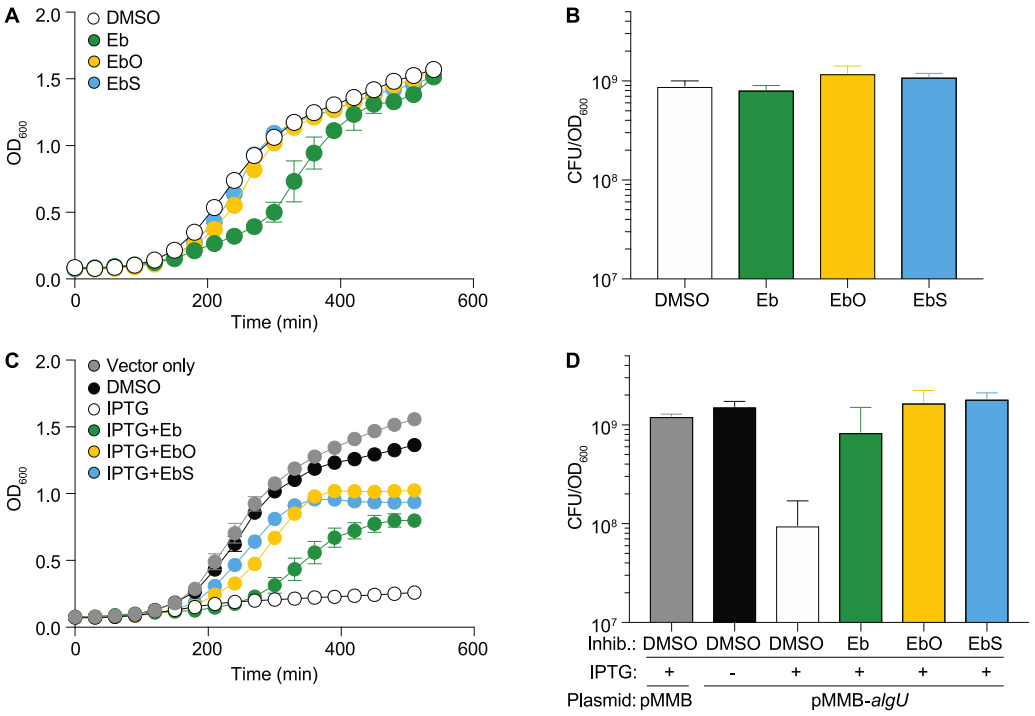

Figure 3. Effect of compounds on bacterial growth. The cell growth of *P. aeruginosa* (A) PA14 wild-type or (C) PA14 pMMB-*algU* in the presence of indicated compounds at 100 μM was measured by OD at 600 nm. In a separate experiment, the colony forming unit (CFU) per OD_{600} was determined by measuring OD_{600} and CFU by serial dilution for (B) PA14 wild-type or (D) PA14 pMMB-*algU* treated with 100 μM of indicated compounds for 6 h. All experiments were performed in triplicate. Error bars indicate standard deviation from the mean.

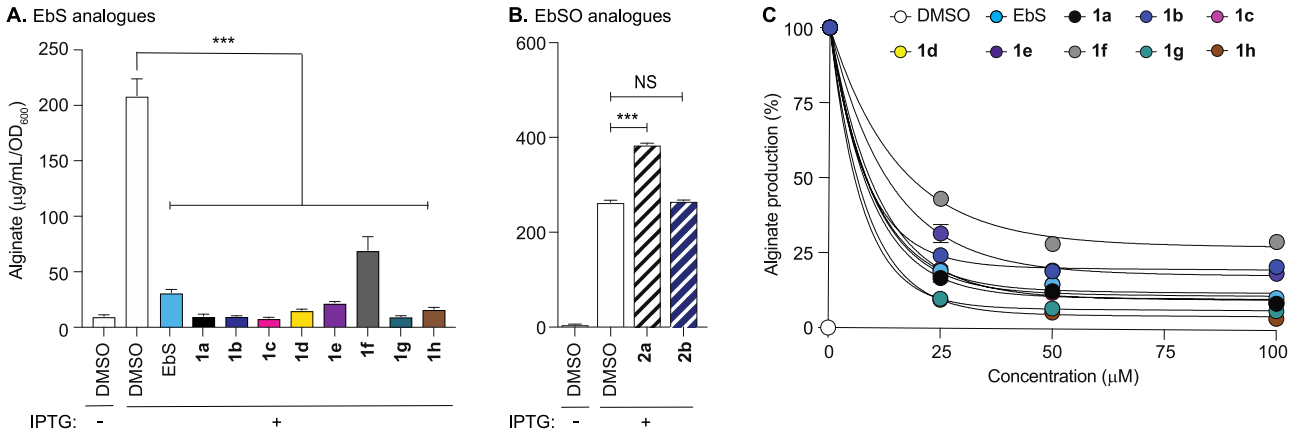

Figure 4. Inhibition of alginate production by EbS and EbS analogues **1a**−**1h**. Quantification of alginate secretion by *P. aeruginosa* PA14 pMMB-*algU* treated with 100 μM of (A) EbS, EbS analogues **1a**−**1h**, or DMSO; or (B) EbSO analogues **2a** and **2b**. (C) Alginate production in the presence of varying concentration of EbS or EbS analogues **1a**−**1h**. *P. aeruginosa* PA14 pMMB-*algU* was induced by the addition of 200 μM IPTG for alginate production and normalized by the optical density in the unit μg/mL/OD_{600} of bacterial culture. The graph average and SD show six independent experiments. *** indicates p value <0.0005, by one-way ANOVA.

cysteines of target proteins.[35,40] Previous studies in *P. aeruginosa* have shown that Eb inhibits WspR activity by modifying the N-terminal cysteine.[31] This cysteine modification by Eb occupies the inhibitory site (I-site) on WspR[31] that normally allows feedback inhibition of diguanylate cyclase by the c-di-GMP product.[23] The alginate biosynthesis pathway encodes *alg44*, a PilZ domain c-di-GMP binding protein, that is required for alginate production. Furthermore, Alg44 protein encodes one cysteine residue (C98) that has previously been shown to be modified by thiol-benzo-triazolo-quinazolinone.[30] We hypothesized that Eb, EbO, and/or EbS could form a covalent bond between selenium or sulfur and the C98 of Alg44 to prevent c-di-GMP binding, thereby reducing alginate production. To test if the covalent modification of Alg44 occurred, the molecular weights of the PilZ domain of Alg44 ($Alg44_{PilZ}$) treated with compounds or DMSO were determined by mass spectrometry. Purified MBP-$Alg44_{PilZ}$ protein treated with DMSO showed the expected molecular weight of 58 384 Da (Figure 5A). When $Alg44_{PilZ}$ was incubated with Eb, EbO, or EbS for 1 h, the molecular weight was increased by 274, 273, and 228 Da, respectively, as compared to DMSO control (Figure 5A). The increase in molecular weights for Eb and EbS matched the expected mass of these two compounds, indicating that the protein is covalently modified by these compounds. However, when treated with EbO the molecular weight of MBP-$Alg44_{PilZ}$ was

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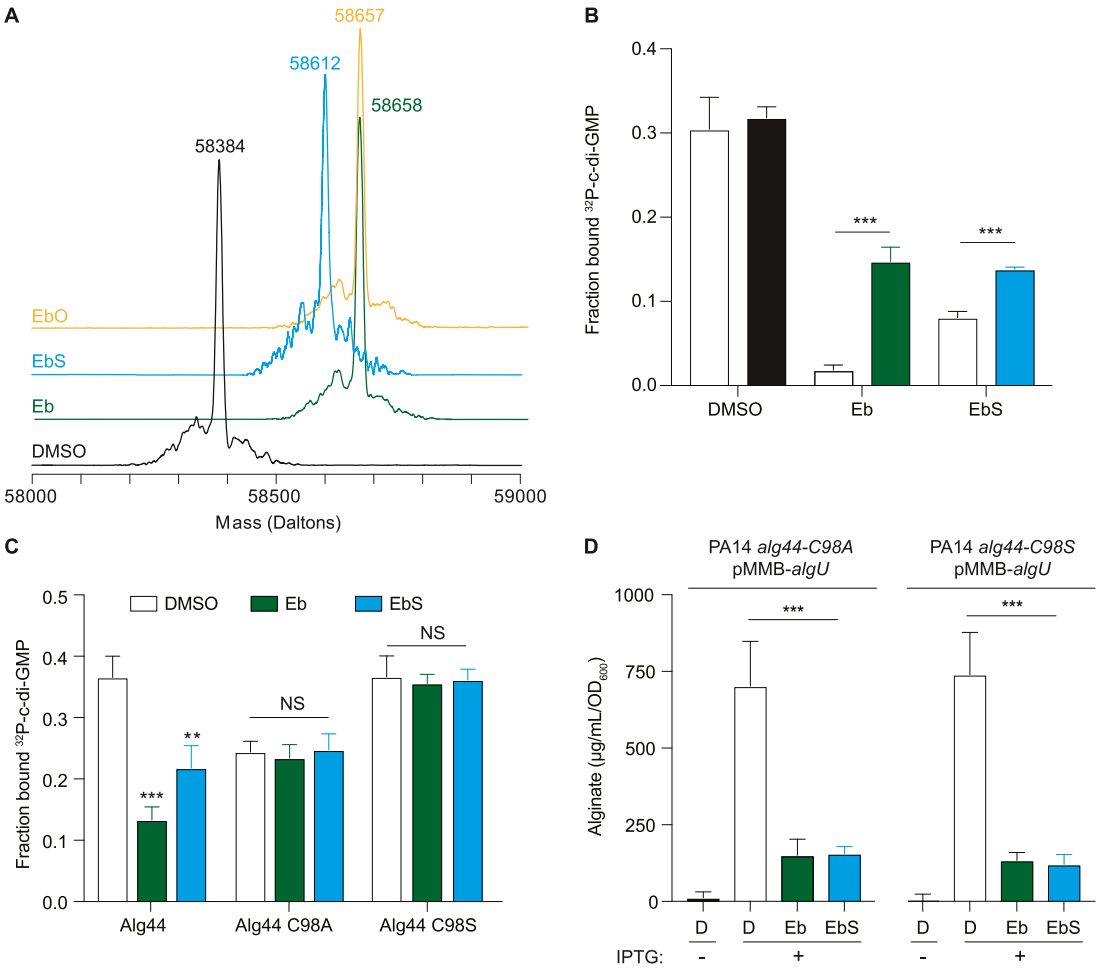

Figure 5. Eb, EbO, and EbS can covalently modify Alg44$_{PilZ}$ on cysteine at position 98 to prevent binding to c-di-GMP, but inhibition of alginate production is independent of this covalent modification. (A) Mass spectra of His-MBP-Alg44$_{PilZ}$ after incubation in the presence of Eb, EbS, EbO, or DMSO. (B) Fraction bound of ^{32}P-c-di-GMP to His-MBP-Alg44$_{PilZ}$ after treatment with DMSO, 100 μM Eb or EbS followed by additional incubation without DTT (white bars) or with 1 mM DTT (colored bars). (C) Alginate production by *P. aeruginosa* PA14 *alg44* (*C98A* and *C98S*) pMMB-*algU* was induced with 0.2 mM IPTG treated with 100 μM Eb, EbS, or DMSO. (D) Fraction bound of ^{32}P-c-di-GMP to His-MBP-Alg44$_{PilZ}$, Alg44$_{PilZ}$ (C98A) or Alg44$_{PilZ}$ (C98S) in the presence of 100 μM Eb, EbS, or DMSO (indicated as D). Graphs show average of alginate produced from three independent experiments. $^{**}p < 0.005$; $^{***}p < 0.0005$ by one-way ANOVA.

increased by the same amount as Eb (Figure 5A), suggesting that EbO is first reduced to Eb prior to modification of the protein.

Given that the molecular weight of MBP-Alg44$_{PilZ}$ increased by molecular weight of the compounds, we postulated that the modification of Alg44$_{PilZ}$ by Eb, EbO, or EbS inhibits c-di-GMP that is required for alginate production. To investigate this possibility, we tested the ability of compounds to inhibit c-di-GMP binding to Alg44$_{PilZ}$. Addition of 100 μM of Eb and EbS reduced ^{32}P-c-di-GMP binding to Alg44$_{PilZ}$ by 90 and 75%, respectively, compared to DMSO control (Figure 5B). The covalent selenylsulfide (Se−S) and disulfide bonds between the protein and Eb/EbS are reversed by a reducing agent such as dithiothreitol (DTT) and should restore Alg44$_{PilZ}$ binding to c-di-GMP. After incubation of Alg44$_{PilZ}$ with compounds for 1 h, the addition of 1 mM DTT restored the ability of Alg44$_{PilZ}$ to bind c-di-GMP by 8-fold for Eb and 1.7-fold for EbS (Figure 5B). To determine the residue in Alg44$_{PilZ}$ that is modified by Eb or EbS, the one cysteine (C98) in the PilZ domain of Alg44 was tested since previous studies have shown that it is modified by thiol-benzo-triazolo-quinazolinone through a disulfide bond.[30] The Alg44$_{PilZ}$ (C98A) or (C98S) proteins lacking a sulfhydryl group were tested for their binding to ^{32}P-c-di-GMP in the presence of Eb or EbS. Both Alg44$_{PilZ}$ (C98A) and (C98S) proteins were able to bind ^{32}P-c-di-GMP (Figure 5C). As expected, both proteins lacking C98 were not inhibited by Eb and EbS (Figure 5C), suggesting that C98 of Alg44 is the target of Eb and EbS. Together these studies show that, in vitro, the Alg44 protein can be modified at C98 by Eb, EbS, and their analogues, which in turn inhibit the ability of Alg44 to bind c-di-GMP.

Eb and EbS Modification of Alg44 Is Not Required for Alginate Inhibition. In order to test whether the only mechanism of action by which Eb and EbS inhibit alginate production is by modifying C98 of Alg44, the *P. aeruginosa* PA14 strains with chromosome replacements of *alg44* with *alg44* (*C98A*) or (*C98S*) were used in the alginate inhibition assay.[30] Induction of pMMB-*algU* with IPTG allowed for increased alginate production (Figure 5D) indicating that C98 is not required for alginate production.[30] When treated with 100 μM Eb or EbS, the PA14 strains with *alg44* (*C98A*) and *alg44* (*C98S*) were also inhibited by Eb or EbS at a level similar to wild-type *P. aeruginosa* (Figures 2, 4, and 5D). These results indicate that Eb and EbS were able to inhibit alginate

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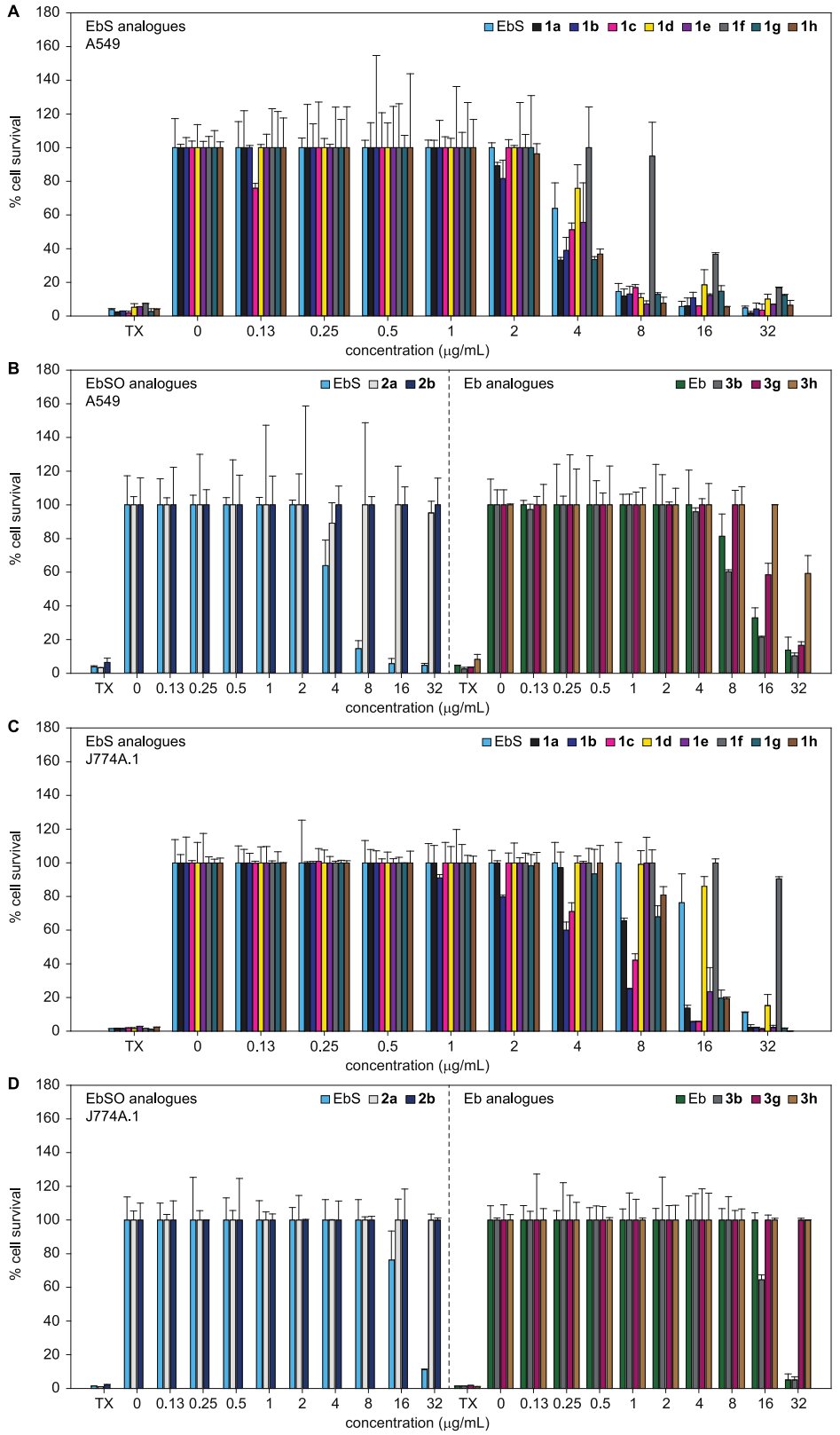

Figure 6. Evaluation of cytotoxicity for (A) EbS and EbS analogues **1a**−**1h** against A549 cell line; (B) EbS (same data as in panel A for ease of comparison), oxidized version of EbS (EbSO) analogues **2a** and **2b**, Eb, and Eb analogues **3b**, **3g**, and **3h** against A549 cell line; (C) EbS and EbS analogues **1a**−**1h** against J774A.1 cell line; and (D) EbS (same data as in panel B for ease of comparison), EbSO analogues **2a** and **2b**, Eb, and Eb analogues **3b**, **3g**, and **3h** against J774A.1 cell line. Controls include treatment with Triton-X (TX, 1% *v*/*v*, positive control) and 0.5% DMSO (negative control). In instances where >100% cell survival was observed, we displayed the data as 100% cell survival (normalized data). Experiments were performed in two independent samples. The average and standard deviation are shown. Note: The corresponding non-normalized data are presented in Figure S19.

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secretion by *P. aeruginosa* through a cellular target that is independent of C98 in Alg44.

Cytotoxicity of Eb and EbS Analogues on Mammalian Cells. For Eb and analogues to be utilized as alginate inhibitors in CF patients, the compounds have to be tested for safety toward mammalian cells. All Eb and EbS analogues were tested against A549 human adenocarcinoma cells and J774A.1 mouse macrophage cells to determine the cytotoxic effect in the range of 0.13−32 μg/mL (Figures 6 and S19; Table S1). Both lung and macrophage cell lines were chosen as *Pseudomonas* causes chronic lung infections in CF patients and as macrophages are the first responders to the infection, a chemotherapeutic should not be toxic to these cells. Against the A549 cells, we observed that EbS had an CC_{50} of ∼4 μg/mL and EbS analogues **1a**−**1h** also displayed similar CC_{50} values. Only one EbS analogue, **1f**, was less cytotoxic with more than 90% cell survival at 8 μg/mL. The EbSO analogues exhibited greatly improved profiles with both **2a** and **2b** having full cell survival at 32 μg/mL. Eb had an approximately 4-fold better CC_{50} value than EbS, which matched reports of cytotoxicity from HEK-293 cell studies.[34,35] For the Eb analogues, CC_{50} values only varied by 2-fold from Eb with CC_{50} values of about 8, 16, and 32 μg/mL for **3b**, **3g**, and **3h**, respectively. Overall, EbSO analogues displayed the best CC_{50} values followed by the Eb analogues and then the EbS analogues and this pattern was observed when comparing analogues with the same R groups. The EbSO analogues, **2a** and **2b** (CC_{50} >32 μg/mL), had at least an 8-fold improvement in CC_{50} value compared to the EbS analogues, **1a** and **1b** (CC_{50} ∼4 μg/mL). In addition, the corresponding Eb analogue, **3b** (CC_{50} ∼8 μg/mL), exhibited 2-fold improvement compared to **1b**. Similarly, Eb analogues **3g** (CC_{50} ∼16 μg/mL) and **3h** (CC_{50} >32 μg/mL) displayed 4-fold and at least 8-fold improvement over the corresponding EbS analogues, respectively. The trends observed with the A549 cells remained consistent with the J774A.1 macrophages, but with somewhat greater cell survival. EbS displayed 76% cell survival at 16 μg/mL (∼4-fold greater CC_{50} than with A549). EbS analogues **1d** and **1f** exhibited the least cytotoxicity of the EbS analogues, **1a**−**1h**, with greater than 80% cell survival at 16 μg/mL. All other Eb analogues displayed ≥60% cell survival at 4 μg/mL and less than 40% survival at 16 μg/mL. The EbSO analogues, **2a** and **2b**, again displayed total cell survival at the highest concentration, 32 μg/mL. Full cell survival was also observed for Eb at 16 μg/mL and Eb derivatives **3g** and **3h** at 32 μg/mL.

Finally, we tested EbO in a concentration range of 1 to 256 μg/mL against HEK-293, A549, and J774A.1 cell lines to establish its toxicity (Figures 7 and S20; Table S1). EbO displayed 100% cell survival at 64 μg/mL against the J774A.1 cell line. At the same concentration (64 μg/mL) EbO exhibited 60% and >30% cell survival against the HEK-293 and A549 cell lines. Compared to other Eb derivatives, EbO displayed even better safety profile against mammalian cell lines.

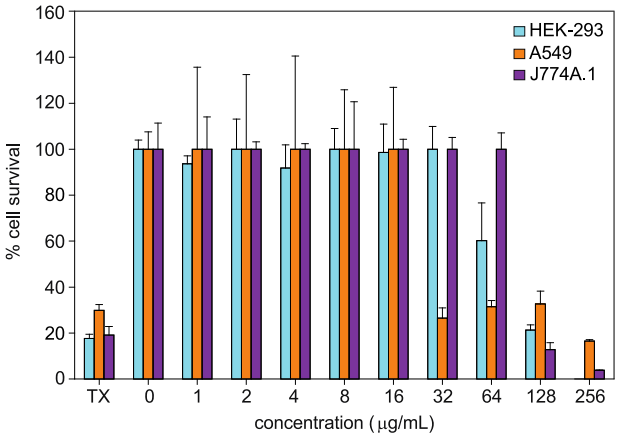

Figure 7. Evaluation of cytotoxicity for EbO against HEK-293, A549, and J774A.1 cell lines. The positive control used was treatment with Triton-X (TX, 1% *v*/*v*). In instances where >100% cell survival was observed, we displayed the data as 100% cell survival (normalized data). Experiments were performed in four independent samples. The average and standard deviation are shown. Note: The corresponding non-normalized data are presented in Figure S20.

DISCUSSION

P. aeruginosa is a leading cause of infection of CF patients. Mucoid conversion of *P. aeruginosa* through mutational inactivation of the *mucA* gene is correlated with increased production of alginate and the concomitant decline in lung function, leading to morbidity and mortality. One possible approach to dealing with the mutant bacteria is to inhibit the synthesis of alginate. The results from this current study indicate that Eb, EbO, EbS, and their analogues are effective in reducing the production of alginate by *P. aeruginosa*. On the basis of the compounds tested, the Se/S atom in the molecule is the active moiety. In the case of EbS, the sulfur atom must be in the reduced state since EbSO are inactive. In contrast, EbO appears to be active, but is reduced to Eb prior to activity. Whether EbO can serve as a pro-drug requires further studies. The Eb, EbO, and EbS scaffolds appear to tolerate a wide variety of modifications and may allow future development of compounds with greater potency or chemical probes. The results presented in this limited SAR indicate that the phenyl ring enhances activity in comparison to the isobutyl group.

Eb is a compound that has undergone phase III clinical trials for ischemic stroke and acute hearing loss as well as a phase II clinical trial for bipolar/manic disorder. Eb was proven to be safe through oral administration during clinical trials, suggesting it is well tolerated. We calculated the selectivity index (SI) values of these compounds for alginate inhibition (Table 1). The SI for a compound is defined as the ratio of its cytotoxicity concentration 50% (CC_{50}) value against half-maximal inhibitory concentration (IC_{50}) value. It is important to note that differences in inhibitors binding to proteins present in the different assays were not accounted for in the selectivity discussion to follow. The analysis shown here suggests that Eb, while able to inhibit alginate production, has a narrow therapeutic window (SI value of 1.36). EbS has a similar toxicity profile to mammalian cells, but has improved inhibitory activity toward alginate production and has an improved therapeutic index with SI values ranging from 1.31 to 9.03. EbO, which is typically considered inactive, is indeed less toxic to A549, HEK-293, and J774A.1 cells, but yet has similar inhibitory activity as EbS. Thus, EbO represents a lead compound for further development for compounds with enhanced therapeutic index with an SI value of 29.5.

The alginate assay is based on induction of a plasmid encoded *algU*/*T*. The observed inhibition of alginate production by Eb, EbS, and their analogues likely occurs downstream of transcription of the *algD-algA* alginate biosynthesis operon. Targets upstream of *algD-algA* transcription are

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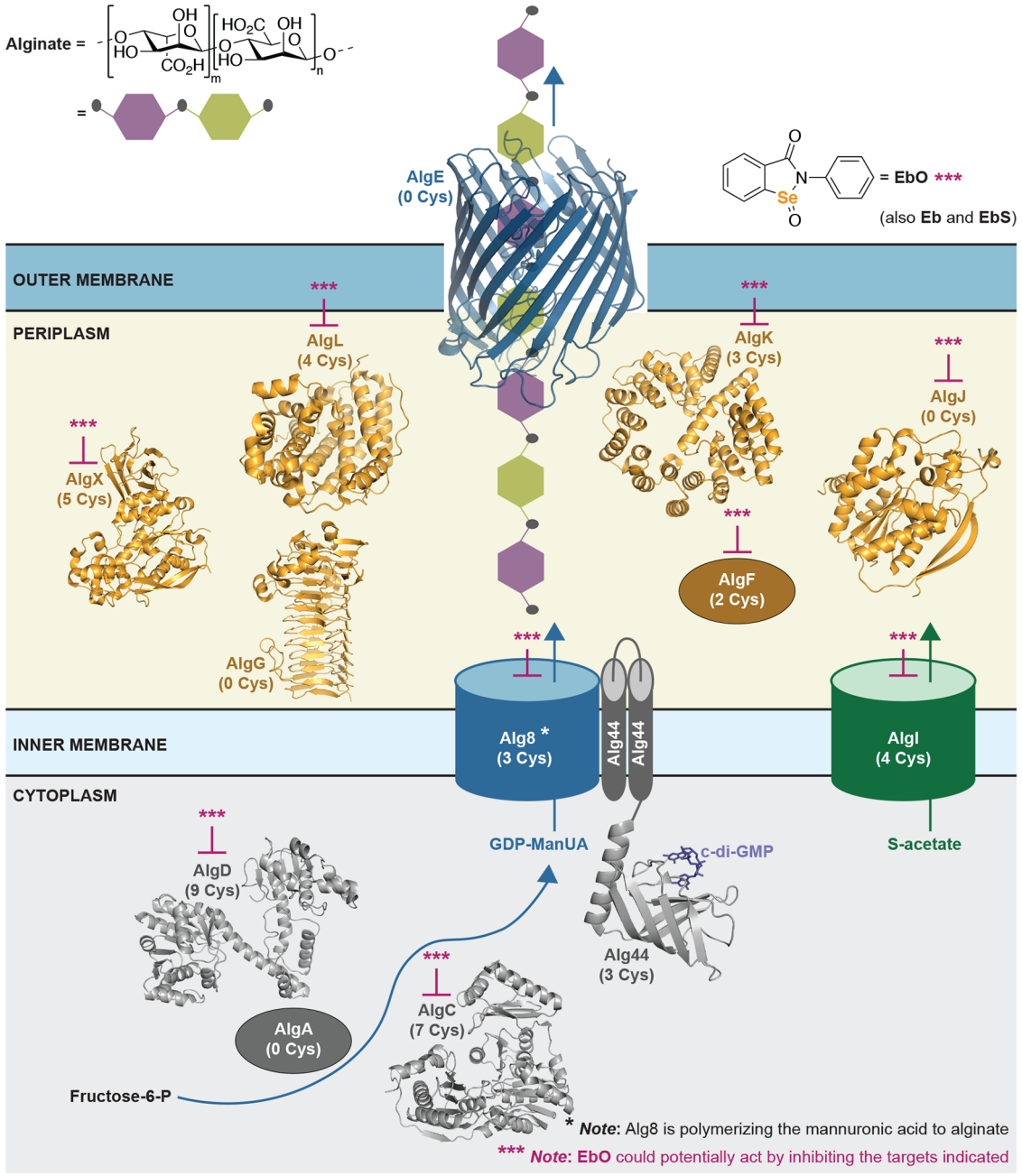

Figure 8. Model for inhibition of *P. aeruginosa* alginate production by EbO and analogues. Schematic diagram for the alginate biosynthetic proteins encoded in the *algD-algA* operon and the *algC* gene. The protein structures are placed in the cellular location (outer membrane, periplasm, and inner membrane) based on previous literature including AlgD (PDB ID: 1MUU),[41] Alg44 (PDB ID: 4RT0),[21] AlgK (PDB ID: 3E4B),[42] AlgE (PDB ID: 3RBH),[43] AlgG (PDB ID: 4NK6),[44] AlgX (PDB ID: 4KNC),[45] AlgL (PDB ID: 4OZV; unpublished), and AlgJ (PDB ID: 4O8V).[46] Structures for Alg8, AlgI, AlgF, and AlgA are not yet available. The number of conserved cysteine residues is indicated in parentheses.

unlikely since these compounds did not cause a general growth defect in *P. aeruginosa*. Furthermore, the alginate assay corrected for cell number through OD_{600} measurements. The obvious target for inhibition is Alg44 since c-di-GMP is required for alginate production.[20,31] When tested in vitro, Eb, EbO, and EbS can interfere with c-di-GMP binding to Alg44 supporting the idea that Alg44 is the target (Figure 5A−C). However, *P. aeruginosa* with *alg44* (*C98A*) or *alg44* (*C98S*) are still inhibited by Eb and EbS despite not having a cysteine required for cross-linking (Figure 5D), suggesting that inhibition by Eb and EbS must target another aspect of alginate production. Analyzing the genes in the *algD-algA* operon and the *algC* gene, that is required for generating the mannuronic acid precursor, only a subset of genes have cysteines including AlgD, Alg8, Alg44, AlgK, AlgX, AlgL, AlgI, and AlgG (Figure 8). In this manuscript, we have demonstrated that Alg44 is not the sole target for EbO and analogues. In contrast, AlgE, AlgG, AlgJ, and AlgA do not have cysteine residues and are likely not the target of inhibition. Future studies will reveal the target of inhibition by EbO (Eb and EbS), perhaps using EbO probes with modifications that target proteins through covalent bonds. In addition, studies using clinical isolates of *P. aeruginosa* from CF patients will

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enable determination whether the observed inhibition can act to broadly inhibit alginate production.

If these efforts are successful in developing alginate inhibitors, how does this fit into current treatment for CF patients? The most direct approach is to restore the function of CFTR through correcting the mutation in the gene. While gene therapy has yet to be effective for CF patients,[47] a different approach has been taken to restore CFTR protein function.[48] Many classes of the CFTR mutations produce the CFTR protein; however, the protein fails to reach the plasma membrane or has a defect in channel function.[49] In the past decades, CFTR correctors were developed through screening for small molecules that restored activity of G551D-CFTR alleles in cell-based assays.[50,51] In 2012, Vertex Pharmaceuticals received FDA approval for ivacaftor, which was shown to improve lung function in patients with G551D allele.[52−54] However, the majority of the CF patients (>90%) have the Δ508 allele that does not respond to ivacaftor.[55] Subsequent screening identified compounds that corrected the Δ508-CFTR[56,57] and when used in combination with modulators are highly effective for improving lung function and reducing microbial load in CF patients with Δ508-CFTR.[58,59] Orkambi, a combination of ivacaftor/lumacaftor, and trikafta, comprised of elexacaftor/tezacaftor/ivacaftor, were approved by the FDA in 2015 and 2019, respectively. For those CF alleles that do not respond to the treatment or for patients that have serious complications as a result of the above treatment, infection by *P. aeruginosa* and mucoid conversion still represent a serious condition. Reduction of alginate produced by *P. aeruginosa*, while not curative, would reduce the impact of mucoid conversion. The results described here represent one approach to reduce the alginate in the airway and prevent the negative impact of mucoid *P. aeruginosa* on lung function. Future development of EbO analogues will lead to the development of compounds with improved therapeutic index.

CONCLUSIONS

In summary, we have identified novel EbO, Eb, and EbS analogues that can inhibit alginate production by *P. aeruginosa*. From the limited SAR performed we could emphasize that specific positions of Eb and EbS can be modified without affecting inhibitory activity toward alginate. Both Eb and EbS analogues were able to covalently modify PilZ domain of Alg44 using cysteine 98 (C98) and this modification prevented c-di-GMP from binding to Alg44. The Eb and its oxidized version (EbO) were shown to inhibit alginate production by *P. aeruginosa*. EbO exhibited better potency at inhibiting Alg44 with lower micromolar value when compared to Eb. The Eb analogues **3g** and **3h** performed better than Eb in alginate inhibition assays, indicating the possibilities for more vigorous scaffold expansion. In the case of EbS, the parent compound and its analogues displayed better inhibition of alginate production compared to Eb. However, contrary to the result observed for EbO, the oxidized version of EbS (EbSO) failed to inhibit alginate production. The results from mass spectrometry studies revealed that Eb, EbS, and their analogues form selenylsulfide and disulfide bonds with the PilZ domain of Alg44. Even though an obvious target for inhibition is Alg44, the inhibition of alginate production in *P. aeruginosa* PA14 strains with *alg44* (*C98A*) or *alg44* (*C98S*) indicate another cellular target that is independent of C98 of Alg44. Finally, when tested against A549 and J774A.1 cell lines for their toxicity, EbO analogues displayed better safety profiles and have a greater selectivity index for inhibiting alginate production by *P. aeruginosa*. From this study, it could be concluded that EbO represents an interesting avenue for the future development of chemical inhibitors for alginate secretion by *P. aeruginosa*, which could in turn reduce the adverse side effects of alginate production on CF patients.

METHODS

Materials and Instrumentation. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), and AK scientific (Union City, CA), and used without further purification. Chemical reactions were monitored by thin layer chromatography (TLC) using Merck Silica gel 60 F_{254} plates and visualization was achieved using UV light. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz (or 125 MHz), respectively, on a Varian 400 MHz spectrometer (MR400) (or a Varian 500 MHz spectrometer; VNMRS 500), using the indicated deuterated solvents. Chemical shifts (δ) are given in parts per million (ppm). Coupling constants (J) are given in Hertz (Hz), and conventional abbreviations used for signal shape are as follows: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; tt = triplet of triplets.

Synthesis and Characterization of Compounds in This Study. We previously synthesized and characterized EbS as well as compounds **1a−h** and **2a**,**b**.[34,35] The previously reported synthetic scheme[36] was used to prepare Eb and its analogues **3b**, **3g**, and **3h** (Scheme S1). All the compounds synthesized are presented in Figure 1.

Preparation of Compound 5. To a solution of aniline (0.40 mL, 4.50 mmol) and Et_3N (0.90 mL, 9 mmol) in anhydrous CH_2Cl_2(20 mL), 2-iodobenzoyl chloride (0.5 mL, 3.75 mmol) was added dropwise. The product was washed with 1 N HCl (50 mL) and a saturated solution of sodium bicarbonate (50 mL) and dried with $MgSO_4$. The organic layer was removed in vacuo and the crude product was purified via recrystallization in *i*-PrOH to afford the known compound **5**[36] (299 mg, 25%) as a pink solid: ^{1}H NMR (400 MHz, $CDCl_3$, Figure S1) δ 7.89 (d, J = 8.4 Hz, 1H), 7.62 (d, J = 8.0 Hz, 2H), 7.51 (d, J = 6.8 Hz, 1H), 7.48−7.30 (m, 4H), 7.20−7.10 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$, Figure S2) δ 167.4, 142.3, 140.2, 137.7, 131.6, 129.3, 128.7, 128.5, 125.1, 120.3, 92.6.

Preparation of Compound 6. To a solution of isoamylamine (1.0 mL, 9.0 mmol) and Et_3N (2.0 mL, 15.0 mmol) in anhydrous CH_2Cl_2(20 mL), 2-iodobenzoyl chloride (1.0 mL, 3.75 mmol) was added dropwise. The product was washed with 1 N HCl (50 mL) and a saturated solution of sodium bicarbonate (50 mL) and dried with $MgSO_4$. The organic layer was removed in vacuo to afford the known compound **6**[60] (2.4 g, quantitative yield) as a yellow solid: ^{1}H NMR (400 MHz, $CDCl_3$, Figure S3) δ 7.81 (d, J = 8.0 Hz, 1H), 7.33 (d, J = 3.6 Hz, 2H), 7.10−7.00 (m, 1H), 5.78 (s, 1H), 3.43 (q, J = 7.6 Hz, 2H), 1.69 (nonet, J = 6.4 Hz, 1H), 1.49 (q, J = 7.6 Hz, 2H), 0.93 (d, J = 6.4 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$, Figure S4) δ 169.5, 142.7, 139.9, 131.1, 128.4, 128.3, 92.6, 38.6, 38.4, 26.1, 22.6.

Preparation of Compound 7. To a solution of benzylamine hydrochloride (0.65 g, 4.50 mmol) and Et_3N (0.90 mL, 9 mmol) in anhydrous CH_2Cl_2(20 mL), 2-iodobenzoyl chloride (0.5 mL, 3.75 mmol) was added dropwise. The product was washed with 1 N HCl (50 mL) and a saturated solution of sodium bicarbonate (50 mL) and dried with $MgSO_4$. The organic layer was removed in vacuo and the crude

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product was purified via recrystallization in *i*-PrOH to afford the known compound **7**[36] (639 mg, 51%) as a white solid: ^{1}H NMR (400 MHz, CD_3OD, Figure S5) δ 7.88 (d, J = 8.0 Hz, 1H), 7.46−7.40 (m, 3H), 7.30−7.37 (m, 3H), 7.24−7.28 (m, 1H), 7.14 (app. tt, J = 7.6, 2.0 Hz, 1H), 4.53 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$, Figure S6) δ 169.4, 142.2, 140.1, 137.7, 131.4, 129.0, 128.5, 128.4 (2 carbons), 127.9, 92.6, 44.4.

Preparation of Compound 8. To a solution of phenethylamine (1.1 mL, 9.0 mmol) and Et_3N (2.0 mL, 15.0 mmol) in anhydrous CH_2Cl_2(20 mL), 2-iodobenzoyl chloride (1.0 mL, 3.75 mmol) was added dropwise. The product was washed with 1 N HCl (50 mL) and a saturated solution of sodium bicarbonate (50 mL) and dried with $MgSO_4$. The organic layer was removed in vacuo to afford the known compound **8**[61] (2.6 g, quantitative yield) as a yellow solid: ^{1}H NMR (400 MHz, CD_3OD, Figure S7) δ 7.86 (d, J = 8.0 Hz, 1H), 7.38 (app. tt, J = 8.0, 1.2 Hz, 1H), 7.32−7.24 (m, 4H), 7.24−7.16 (m, 2H), 7.20−6.80 (m, 1H), 3.56 (t, J = 8.0 Hz, 2H), 2.92 (t, J = 7.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$, Figure S8) δ 169.6, 142.4, 140.0, 138.8, 131.2, 129.0, 128.9, 128.31, 128.30, 126.8, 92.6, 41.3, 35.6.

Preparation of Compound Eb. The known compound **Eb** was prepared using a previously published protocol.[36] Compound **5** (0.299 g, 0.93 mmol), copper(I) iodide (0.18 g, 0.93 mmol), 1,10-phenanthroline (0.17 g, 0.93 mmol), cesium carbonate (0.81 g, 2.5 mmol), and potassium selenocyanate (0.16 g, 1.2 mmol) were suspended in DMF (5 mL). The mixture turned red and was heated to 100 °C for 12 h. The reaction mixture was cooled to room temperature, diluted with EtOAc (40 mL), and filtered through Celite. The filtrate was washed with cold H_2O (2 × 20 mL) and brine (2 × 20 mL), and dried with $MgSO_4$. The organic layer was removed in vacuo and the crude product was purified via recrystallization from EtOH and flash column chromatography (SiO_2, 49:1/CH_2Cl_2:MeOH) to afford the known compound **Eb**[36] (14 mg, 6%) as colorless needles: ^{1}H NMR (400 MHz, $CDCl_3$, Figure S9) δ 8.10 (d, J = 7.6 Hz, 1H), 7.68−7.56 (m, 4H), 7.48−7.43 (m, 1H), 7.41 (t, J = 8.0 Hz, 2H), 7.26 (t, J = 7.6 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, Figure S10) δ 169.6, 139.3, 137.8, 132.7, 129.6, 129.5, 127.0, 126.8, 125.6, 123.9, 121.4.

Preparation of Compound 3b. Compound **6** (0.32 g, 1 mmol), copper(I) iodide (0.19 g, 1 mmol), 1,10-phenanthroline (0.18 g, 1 mmol), cesium carbonate (0.70 g, 2 mmol), and potassium selenocyanate (0.16 g, 1.2 mmol) were suspended in DMF (5 mL). The mixture turned red and was heated to 100 °C for 3 h. The reaction mixture was cooled to room temperature, diluted with EtOAc (40 mL), and filtered through Celite. The filtrate was washed with cold H_2O (2 × 20 mL) and brine (2 × 20 mL), and dried with $MgSO_4$. The organic layer was removed in vacuo and the crude product was purified via recrystallization from EtOAc and twice via flash column chromatography (SiO_2, 49:1/CH_2Cl_2:MeOH then 19:1/CH_2Cl_2:MeOH) to afford the known compound **3b**[60] (26 mg, 10%) as white solid: ^{1}H NMR (400 MHz, $CDCl_3$, which matches the literature,[60] Figure S11) δ 8.01 (d, J = 7.6 Hz, 1H), 7.61 (d, J = 8.0 Hz, 1H), 7.55 (t, J = 7.6 Hz, 1H), 7.39 (t, J = 7.6 Hz, 1H), 3.86 (t, J = 6.8 Hz, 2H), 1.67 (nonet, J = 6.4 Hz, 1H), 1.59 (q, J = 6.4 Hz, 2H), 0.95 (d, J = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$, which matches the literature,[60] Figure S12) δ 167.3, 137.8, 132.0, 129.0, 127.9, 126.4, 124.1, 43.4, 39.6, 25.9, 22.7.

Preparation of Compound 3g. The known compound **3g** was prepared using a previously published protocol.[36] Compound **7** (0.34 g, 1 mmol), copper(I) iodide (0.19 g, 1 mmol), 1,10-phenanthroline (0.18 g, 1 mmol), cesium carbonate (0.70 g, 2 mmol), and potassium selenocyanate (0.16 g, 1.2 mmol) were suspended in DMF (5 mL). The mixture turned red and was heated to 100 °C for 12 h. The reaction mixture was cooled to room temperature, diluted with EtOAc (40 mL), and filtered through Celite. The filtrate was washed with cold H_2O (2 × 20 mL) and brine (2 × 20 mL), and dried with $MgSO_4$. The organic layer was removed in vacuo and the crude product was purified via recrystallization from *i*-PrOH and flash column chromatography (SiO_2, 49:1/CH_2Cl_2:MeOH then 19:1/CH_2Cl_2:MeOH) to afford the known compound **3g**[36] (38 mg, 13%) as white solid: ^{1}H NMR (400 MHz, $CDCl_3$, Figure S13) δ 8.04 (d, J = 7.6 Hz, 1H), 7.91 (d, J = 6.4 Hz, 1H), 7.76−7.64 (m, 2H), 7.39 (d, J = 7.2 Hz, 2H), 7.36−7.24 (m, 3H), 5.36 (d, J = 14.8 Hz, 1H), 4.60 (d, J = 14.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, which matches the literature,[36] Figure S14) δ 168.2, 145.1, 136.7, 134.4, 133.3, 130.8, 129.3, 129.0, 128.7, 128.6, 126.6, 45.4.

Preparation of Compound 3h. Compound **8** (0.35 g, 1 mmol), copper(I) iodide (0.19 g, 1 mmol), 1,10-phenanthroline (0.18 g, 1 mmol), cesium carbonate (0.70 g, 2 mmol), and potassium selenocyanate (0.16 g, 1.2 mmol) were suspended in DMF (5 mL). The mixture turned red and was heated to 100 °C for 3 h. The reaction mixture was cooled to room temperature, diluted with EtOAc (40 mL), and filtered through Celite. The filtrate was washed with cold H_2O (2 × 20 mL) and brine (2 × 20 mL), and dried with $MgSO_4$. The organic layer was removed in vacuo and the crude product was purified via recrystallization from *i*-PrOH and twice via flash column chromatography (SiO_2, 49:1/CH_2Cl_2:MeOH then 19:1/CH_2Cl_2:MeOH) to afford the known compound **3h**[62] (12 mg, 4%) as white solid: ^{1}H NMR (400 MHz, $CDCl_3$, which matches the literature,[62] Figure S15) δ 8.03 (d, J = 8.0 Hz, 1H), 7.56 (d, J = 3.6 Hz, 2H), 7.42−7.36 (m, 1H), 7.32−7.20 (m, 6H), 4.09 (t, J = 6.8 Hz, 2H), 3.02 (t, J = 7.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$, Figure S16) δ 167.2, 148.3, 138.0, 137.9, 131.9, 129.02, 128.97, 128.8, 128.6, 127.2, 126.7, 126.1, 123.9, 46.2, 36.4.

Bacterial Strains, Plasmids, Cells, and Growth Conditions. For the alginate production assay, *P. aeruginosa* strains PA14, PA14 *algU* (*C98A*), or *algU* (*C98S*) harboring pMMB-*algU* or pMMB were grown in Luria−Bertani (LB) broth at 37 °C supplemented with 50 μg/mL carbenicillin and 0.2 mM IPTG. For protein purification, *E. coli* strains harboring pVL847 were grown in LB M9 medium (5 g/L yeast extract, 10 g/L tryptophan, 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 $MgSO_4$) at 30 °C supplemented with 15 μg/mL gentamicin and 1 mM IPTG. Growth was determined by measuring optical density at 600 nm (OD_{600}) using a SpectraMax M5 plate reader. For the cytotoxicity assays, the human lung carcinoma cell line A549 and the mouse macrophage cell line J774A.1 that were utilized were a kind gift from Prof. David K. Orren (University of Kentucky, Lexington, KY) and Prof. David J. Feola (University of Kentucky, Lexington, KY), respectively. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; catalog # VWRL0100, VWR, Chicago, IL) supplemented with 10% fetal bovine serum (FBS; from ATCC) and 1% penicillin/streptomycin (from ATCC) at 37 °C with 5% CO_2.

Quantification of Alginate Secretion by *P. aeruginosa*. *P. aeruginosa* PA14 harboring pMMB-*algU* were grown

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overnight in LB broth, subcultured with 50 μg/mL carbenicillin at 37 °C and 0.2 mM IPTG for 6 h. Induced bacteria were collected by centrifuge; the supernatant containing alginate was precipitated by the addition of equal volume of isopropanol at −20 °C. After precipitation by centrifuge for 20 min, the alginate pellet is resuspended in distilled water and assay by the addition of 0.1% carbazole in absolute ethanol and borate-sulfuric acid reagent.[63] The reaction was incubated at 55 °C for 30 min. These samples were measured at 530 nm using the SpectraMax M5 plate reader. This is a spectrometric assay in which the uronic acid sugars were modified by carbazole to produce a compound that absorbs at 530 nm. The values were calculated and converted to unit of alginate production using commercially available alginate to generate a standard curve. Alginate concentration was normalized by alginate/mL/OD_{600} of bacterial culture (Figures 2 and 4). Addition of compounds to extracts of alginate did not interfere with the assay (Figure S17).

Effect of Compounds on Bacterial Growth. *P. aeruginosa* PA14 was subcultured 1:50 dilution in LB broth containing 0, 25, 50, and 100 μM concentration of compounds and monitored by measuring OD_{600} values for 15 h at 30 or 37 °C, as indicated (Figures 3 and S18). In addition, the colony forming units (CFU), which reflects the number of viable bacteria, was enumerated by counting colonies formed after serial dilution and plating on LB agar plates. The experiments were performed in triplicate.

Purification of His-MBP-Alg44$_{PilZ}$ (C98A) and (C98S). *E. coli* T7Iq strains harboring pVL847 His-MBP-Alg44$_{PilZ}$ (C98A) and (C98S) were grown overnight, subcultured in LB M9 medium supplemented with 15 μg/mL gentamicin for 4 h at 30 °C, then the addition of 1 mM IPTG for 4 h. Induced cells were collected by centrifuge and resuspended in 10 mM Tris, pH 8, 100 mM NaCl, and 25 mM imidazole. Bacteria were lysed by the addition of 10 μg/mL DNase, 25 μg/mL lysozyme, and 1 mM PMSF. Insoluble material was removed by centrifuge. His-MBP-Alg44$_{PilZ}$ (C98A) and (C98S) in lysates were purified by a Ni^{II}−NTA column using an AKTA fast protein liquid chromatography (FPLC) in 10 mM Tris, pH 8, and 100 mM NaCl using an imidazole gradient from 25 mM to 250 mM. The purification of protein was dialyzed for 1 h and overnight against 10 mM Tris, pH 8, 100 mM NaCl. After then, protein was dialyzed for 4 h against 10 mM Tris, pH 8, 100 mM NaCl, and 50% glycerol. The purification of protein was aliquoted, frozen with liquid nitrogen, and stored at −80 °C.

Synthesis of ^{32}P-c-di-GMP. Radiolabeled GTP (α-^{32}P-GTP) was mixed with WspR D70E encoding diguanylate cyclase in c-di-GMP binding buffer (10 mM Tris, pH 8, 100 mM NaCl, and 5 mM $MgSO_4$) and incubated for 2 h at 37 °C. After incubation, the mixture was performed for WspR separation from ^{32}P-c-di-GMP using 3 kDa molecular weight cutoff filter.

DRaCALA. To confirm binding of purified His-MBP-Alg44$_{PilZ}$, Alg44$_{PilZ}$ (C98A) and (C98S) to ^{32}P-c-di-GMP, 10 μM purified protein was incubated with Eb, EbS (100 μM), or DMSO for 1 h. The mixture was incubated again in the presence of 1 mM DTT for 1 h. The total 10 μL of each sample was incubated with 10 μL of 0.8 nM ^{32}P-c-di-GMP in c-di-GMP binding buffer (10 mM Tris, pH 8, 100 mM NaCl, and 5 mM $MgSO_4$). The mixture was shaken for 1 min at room temperature, spotted on a nitrocellulose sheets, dried, and imaged using Fujifilm phosphorimager.[64] The intensity of radiolabeled signal was quantified by Fujifilm Multi Gauge software v3.0. These data are presented in Figure 5.

Determination of Molecular Weight of Alg44$_{PilZ}$ by Liquid Chromatography Mass Spectrometry (LC-MS). Ten μM Alg44$_{PilZ}$ were incubated at room temperature in the presence of 100 μM Eb, EbO, or EbS for 1 h. After incubation, protein samples were acidified with 1 μL 10% TFA and analyzed by LC-MS. 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% MeCN in water with 0.1% formic acid, and solvent B is 75% MeCN 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. Five spectra (microscans) were averaged for each spectrum recorded. After acquisition, mass spectra over the chromatography peak were averaged. Averaged spectrum was deconvoluted using MagTran program[65] (a universal algorithm for fast and automated charge state deconvolution of electrospray mass-to-charge (m/z) ratio spectra). These data are presented in Figure 5.

Mammalian Cytotoxicity Assays for Eb, EbS, and Their Analogues. Ebselen (Eb), ebsulfur (EbS), and all analogues were tested against the A549 and J774A.1 cell lines to evaluate mammalian cytotoxicity. A resazurin cell viability assay was used as previously described with minor modifications.[66] A549 and J774A.1 cells were counted using a hemacytometer and plated into 96-well plates at a concentration of 4×10^3 cells/mL and 1×10^4 cells/mL, respectively. After 24 h incubation, cells were treated with compound. The compounds were tested in concentrations ranging from 0.13 to 32 μg/mL with final concentration of DMSO at 0.5%. 1% Triton-X was used as a positive control. After 24 h treatment, 10 μL of 2 mM resazurin was added to the plates and incubated at 37 °C in the dark for 5 h. Plates were read with an excitation of 360 nm and emission of 390 nm and normalized to no drug (DMSO only) control (Figure 6). It is important to note that testing xenobiotics at sub-IC_{50} concentrations can result in an increase in cell growth, resulting in >100% cell survival in the treatment groups.[67] In instances where >100% cell survival was observed, we displayed the data as 100% cell survival in Figure 6. We are providing the data with the non-normalized observed % in Figure S19. All compounds were tested in duplicate. The corresponding values of the concentrations of Eb and EbS analogues **1a**−**1h**, **2a**, **2b**, **3b**, **3g**, **3h**, **Eb**, and **EbS** in micromolar used for cytotoxicity experiments are reported in Table S1. In addition, EbO was tested against the A549, HEK-293, and J774A.1 cell lines in a concentration range of 1 to 256 μg/mL. Experiments were done in quadruplicate and the 100% normalized data are reported in Figure 7. We are providing the data with the non-normalized observed % in Figure S20.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00045.

A synthetic scheme for the synthesis of the compounds presented (Scheme S1); a table of the data used to prepare Figures 6 and 7 (Table S1); figures presenting

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^{1}H and ^{13}C spectra for molecules synthesized (Figures S1−16); a figure demonstrating that addition of compounds does not interfere with detection by the alginate assay (Figure S17); a figure showing that compounds have no effect on the growth of *P. aeruginosa* PA14 when incubated in LB containing varying concentrations of compounds for 15 h at 30 °C (Figure S18); as well as non-normalized cytotoxicity bar graphs (Figure S19 and S20) (PDF)

AUTHOR INFORMATION

Corresponding Authors

Sylvie Garneau-Tsodikova − *Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536, United States;* orcid.org/0000-0002-7961-5555; Email: sylviegtsodikova@uky.edu

Vincent T. Lee − *Department of Cell Biology and Molecular Genetics, University of Maryland at College Park, College Park, Maryland 20742, United States;* orcid.org/0000-0002-3593-0318; Email: vtlee@umd.edu

Authors

Soo-Kyoung Kim − *Department of Cell Biology and Molecular Genetics, University of Maryland at College Park, College Park, Maryland 20742, United States*

Huy X. Ngo − *Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536, United States*

Emily K. Dennis − *Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536, United States*

Nishad Thamban Chandrika − *Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536, United States;* orcid.org/0000-0002-3605-169X

Philip DeShong − *Department of Chemistry and Biochemistry, University of Maryland at College Park, College Park, Maryland 20742, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsinfecdis.1c00045

Notes

The authors declare no competing financial interest.

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