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High-throughput screening using the differential radial capillary action of ligand assay identifies ebselen as an inhibitor of diguanylate cyclases

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Abstract

The rise of bacterial resistance to traditional antibiotics has motivated recent efforts to identify new drug candidates that target virulence factors or their regulatory pathways. One such antivirulence target is the cyclic-di-GMP (cdiGMP) signaling pathway, which regulates biofilm formation, motility, and pathogenesis. *Pseudomonas aeruginosa* is an important opportunistic pathogen that utilizes cdiGMP-regulated polysaccharides, including alginate and pellicle polysaccharide (PEL), to mediate virulence and antibiotic resistance. CdiGMP activates PEL and alginate biosynthesis by binding to specific receptors including PelD and Alg44. Mutations that abrogate cdiGMP binding to these receptors prevent polysaccharide production. Identification of small molecules that can inhibit cdiGMP binding to the allosteric sites on these proteins could mimic binding defective mutants and potentially reduce biofilm formation or alginate secretion. Here, we report the development of a rapid and quantitative high-throughput screen for inhibitors of protein-cdiGMP interactions based on the differential radial capillary action of ligand assay (DRaCALA). Using this approach, we identified ebselen as an inhibitor of cdiGMP binding to receptors containing an RxxD domain including PelD and diguanylate cyclases (DGC). Ebselen reduces diguanylate cyclase activity by covalently modifying cysteine residues. Ebselen oxide, the selenone analogue of ebselen, also inhibits cdiGMP binding through the same covalent mechanism. Ebselen and ebselen oxide inhibit cdiGMP regulation of biofilm formation and flagella-mediated motility in *P. aeruginosa* through inhibition of diguanylate cyclases. The identification of ebselen provides a proof-of-principle that a DRaCALA high-throughput screening approach can be used to identify bioactive agents that reverse regulation of cdiGMP signaling by targeting cdiGMP-binding domains.

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Supporting Information

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INTRODUCTION

The rise of bacterial resistance to traditional antibiotics is an increasingly important medical problem that has motivated the search for molecules that target bacterial processes involved in pathogenesis.¹ Virulence factors and the genetic networks that regulate their expression are two classes of targets for small molecule interference (reviewed in ^{2, 3}). One such regulatory pathway is based on the intracellular cyclic-di-GMP (cdiGMP) signaling molecule.⁴ CdiGMP is a nucleotide second messenger that regulates cellular responses to stimuli by binding to receptor proteins and allosterically altering their activity.^{4, 5} When cdiGMP is made at high levels by diguanylate cyclases (DGCs) in *P. aeruginosa*,^{6, 7} cdiGMP binds to several receptors to alter phenotypes.^{8–10} CdiGMP binds to FleQ to activate transcription of the *pel* operon, which is responsible for PEL polysaccharide production, and other genes.⁸ CdiGMP binding to the inhibitory site (I-site) of PelD is required for PEL polysaccharide biosynthesis.¹⁰ Similarly, cdiGMP binding to Alg44 is required for synthesis of alginate. These results demonstrate that cdiGMP can act as an allosteric activator of polysaccharide biosynthesis systems that participate in pathogenesis and antibiotic resistance.¹¹ Furthermore, cdiGMP regulates its own synthesis by binding to an inhibitory site (I-site) on DGCs.¹² Small molecule inhibitors that prevent cdiGMP binding to these receptor proteins may abrogate polysaccharide secretion and pathogenesis in *P. aeruginosa*.

Allosteric site inhibitors can be identified through rationally designed compounds based on the native second messenger^{13–16} or identified using an unbiased high-throughput screen based on the scintillation proximity assay (SPA)¹⁷ or fluorescence anisotropy.¹⁸ Medicinal chemistry to synthesize analogs is advantageous because the backbone scaffold has inherent affinity for the binding site. Chemical analogs of cdiGMP have been developed which are able to inhibit receptor binding to cdiGMP and alter the activity of these proteins.^{13–16} However, many such cdiGMP analogues are not membrane permeable and therefore have limited bioactivity. High-throughput screens for allosteric inhibitors often utilize luminescent, fluorescent, or colorimetric readouts for protein binding of small molecules.^{19, 20} Subsets of compounds in the chemical libraries interfere with these spectroscopic readouts. This introduces false positives or false negatives that can lengthen the hit validation process.¹⁹ Recently, an *in vivo* enzyme reporter assay has been used to screen for compounds that inhibit DGC activity and successfully identified several lead compounds that reduced biofilm formation through modulation of intracellular cdiGMP levels.²¹ A high-throughput platform to identify inhibitors of protein-ligand interactions that avoids spectroscopic properties of compounds in chemical libraries would be a useful complement to these approaches.

We sought to identify small molecular weight compounds that inhibit allosteric interactions between cdiGMP and receptor proteins using the differential radial capillary action of ligand assay (DRaCALA) that we have recently developed.^{22, 23} DRaCALA is based on the differential mobility of free and protein-bound radiolabeled ligand after spotting on nitrocellulose. The distribution of radioactivity is imaged directly by a phosphorimager and the fraction bound of each reaction can be quantified by dividing the corrected intensity of the bound ligand by the total intensity of the spot.²² Direct measurement of radioactivity

eliminates interference based on spectroscopic properties of molecules in chemical libraries. Here we present a high-throughput adaptation of DRaCALA to screen a compound library for inhibition of binding between PelD and cdiGMP.²² Using this platform, we identify ebselen (Eb) and ebselen oxide (EbO) as covalent inhibitors of cdiGMP allosteric binding to I-site containing proteins, DGC activity *in vitro* and show these compounds interfere with cdiGMP-regulated phenotypes *in vivo*.

RESULTS AND DISCUSSION

DRaCALA high throughput screening (HTS) platform for identifying inhibitors of protein-small molecule interactions

Although previous work has shown that DRaCALA can measure protein-small molecule interactions in a single reaction, we adapted DRaCALA as a HTS platform for protein small molecule interactions. We evaluated the utility of DRaCALA-HTS by determining three measures of the power of a HTS assay, including the Z-factor, coefficient of variance (CV) and signal to noise (S/N) ratio.^{24, 25} Using the workflow shown in Figure 1, the Z-factor, CV and S/N ratio for DRaCALA were determined by measuring Alg44 binding to ³²P-cdiGMP in a 384-well plate containing alternating columns of either 25 μ M unlabeled cdiGMP or buffer alone (Figure 2). When this plate was screened by HTS-DRaCALA, two distinct populations of binding interactions were present (Figure 2A). These values were then plotted by the well position in the plate (Figure 2B). The average fraction bound of the reactions without competitor was 0.165 ± 0.01 and the average of the reactions with cdiGMP competitor was 0.007 ± 0.005 . This allowed us to obtain a Z-factor of 0.626, a CV of 8.9% and S/N of 24.9. These parameters are comparable to SPA and are indicators of a powerful screening method.²⁶

Measuring binding constants such as the dissociation constant (K_d) is another use for a high-throughput binding assay. The ability to calculate binding constants using DRaCALA demonstrates the sensitivity of this assay and provides a method for rapid validation of positive hits. We assessed the dissociation constant (K_d) of Alg44 binding to ³²P-cdiGMP by serially diluting the protein and performing HTS-DRaCALA. When measured in the 384 well format, we obtained a K_d of 239 ± 7 nM (Figure 2C and 2D). The K_d of Alg44 binding to ³²P-cdiGMP calculated in the 384-well format was similar to the K_d determined by measuring fraction bound in individual reaction mixtures of 380 ± 20 nM (Figure S1). These results show that DRaCALA is amenable to high-throughput analysis of inhibitors of protein-ligand interactions. Although the following data uses PelD as a model cdiGMP binding protein, the principle shown here with Alg44 applies to other protein interaction with low molecular weight ligands.

DRaCALA-based HTS identifies ebselen as an inhibitor of the allosteric binding of cdiGMP to PelD

CdiGMP binding to PelD, an inner membrane protein, is required for the synthesis of PEL polysaccharide and biofilm formation by *P. aeruginosa*.¹⁰ With an ultimate goal of developing compounds that interfere with cdiGMP signaling, we first sought to identify small molecules that inhibit cdiGMP binding to allosteric sites on proteins. We used

DRaCALA-HTS to screen the NIH Clinical Collection 1 (NCC1) library for compounds that inhibited ^{32}P -cdiGMP binding to PelD as described in Figure 1. The NCC1 is a library that includes compounds with favorable pharmacological properties and toxicity profiles that have progressed through clinical trials.²⁷ The distribution of radiolabeled cdiGMP was imaged by a phosphorimager and used to calculate fraction bound (Figure 3). As expected, no binding was observed when excess unlabeled cdiGMP was added in addition to ^{32}P -cdiGMP (Figure 3; green box and arrow). Another well had a fraction bound below the positive cutoff of 3 standard deviations below the average (Figure 3; red box and arrow). This well contained ebselen (Eb), a selenium-containing compound (Figure 4A). Eb was repurchased and its identity was verified using UV absorbance spectroscopy and mass spectrometry (Figure S2).²⁸ Eb has been used in phase III clinical trials to treat ischemic brain injury and has favorable drug-like properties.^{29, 30} We chose to follow-up this finding by characterizing the specificity and efficacy of Eb as an inhibitor of cdiGMP protein interactions *in vitro* and *in vivo*.

DRaCALA screens identify compounds that belong to three general categories: 1. specific inhibitors that alter the ligand-binding site, 2. compounds that alter chemical properties of the ligand, and 3. compounds that non-specifically denature proteins. Compounds in category 1 are of interest and must be differentiated from those in categories 2 and 3. Compounds that are general protein denaturants can be eliminated by testing for their ability to inhibit a completely different protein-ligand pair such as cAMP binding to cAMP receptor protein (CRP). Compounds that act to alter the ligand can be removed by testing for their ability to inhibit binding of the same ligand to structurally distinct binding proteins.¹⁹ For cdiGMP, this is a particular concern since a number of planar compounds have been shown to facilitate the formation of cdiGMP into G-quadruplex-based conformations with unique protein binding properties.^{31, 32} We utilized these secondary assays to narrow the mechanism of Eb activity.

Eb and its oxidized analogue, ebselen oxide (EbO), covalently modify DGC to inhibit cdiGMP-receptor interactions and reduce DGC activity

The specificity of Eb was determined by testing its ability to inhibit cdiGMP binding to other known binding domains. Three classes of cdiGMP-binding proteins were tested, including: WspR, a DGC that has an I-site (RxxD) domain similar to the binding site found in PelD^{7, 33}; RocR, PvrR and FimX, phosphodiesterases (PDEs) that bind cdiGMP at their active site^{34, 35}; and Alg44 and PA3353, PilZ domain-containing proteins.^{6, 9} Inhibition of ^{32}P -cdiGMP binding to each protein was assessed by DRaCALA. Eb (50 μM) reduced cdiGMP binding to PelD by 80% and WspR by 90% compared to the DMSO control (Figure 4B; blue bars). Ebselen did not reduce ^{32}P -cdiGMP binding to the PDE or PilZ proteins from *P. aeruginosa* tested here by more than 25% (Figure 4B; blue bars). Further experiments will determine whether PilZ or PDE proteins from other organisms are similarly resistant to Eb. Under these conditions, the half maximal inhibitory concentration (IC_{50}) of ebselen for PelD was $5 \pm 2 \mu\text{M}$ and that of WspR was $13.6 \pm 0.5 \mu\text{M}$ (Figure 4C blue curve and Fig. S3). These data indicate that Eb is a specific inhibitor of cdiGMP binding to PelD and WspR with affinity in the micromolar range.

Eb has previously been shown to inhibit protein activity by the formation of a covalent bond between its selenium and the thiols in cysteine residues.^{28, 30, 36, 37} Alternatively, Eb can also alter protein activity independently of selenium through the remainder of its chemical scaffold.^{30, 38} To assess the importance of the oxidation state of Eb for its ability to inhibit cdiGMP-receptor interactions, we determined the ability of ebselen oxide (EbO) to inhibit cdiGMP binding to the same panel of proteins described above (Figure 4A–B). Addition of EbO slightly inhibited cdiGMP binding to PelD and WspR by ~60% and ~30%, respectively, but did not reduce cdiGMP binding to PDE or PilZ-domain containing proteins (Figure 4B). To assess the relative inhibition of Eb compared to EbO, we measured the apparent IC₅₀ for PelD and WspR. EbO inhibited cdiGMP binding to PelD with an IC₅₀ of 5±2 μM, which is similar to the IC₅₀ for Eb (Figure 4C). The IC₅₀ of EbO for WspR could not be determined because the curve did not show inhibition of cdiGMP binding (Figure S3). Although Eb is able to rapidly inhibit cdiGMP binding to WspR, we noticed that preincubation of WspR with EbO for 30 minutes before the addition of ³²P-cdiGMP reduced binding by 86% (Figure 4D), indicating that the kinetics of inhibition by EbO are slower than those of Eb. Since cdiGMP allosterically inhibits DGC enzymatic activity by binding to the I-site and Eb and EbO bind WspR to inhibit cdiGMP access to this site,¹² we asked if Eb and EbO also antagonized WspR activity. The velocity of WspR DGC activity was determined in the presence of DMSO, cdiGMP, Eb or EbO (Figure 4E). Eb and EbO reduced WspR activity by 87% (Figure 4E). CdiGMP reduced WspR activity in a similar manner (Figure 4E). We analyzed the effect of Eb on the kinetics of WspR DGC activity and found that Eb reduced WspR velocity in a concentration dependent manner (Figure 4F).¹⁵ These results demonstrate that Eb and EbO not only block cdiGMP binding to the I-site of WspR but also inhibit DGC activity.

Ebselen has previously been shown to inhibit activities of some enzymes by modifying cysteine residues either in the active site or at positions on the protein that cause inhibitory changes in protein conformation.^{30, 39, 40} The effect of linkage to cysteine residues in WspR was determined by measuring the activity of WspR in the presence of Eb or EbO with or without DTT. DTT treatment of WspR that had been preincubated with Eb or EbO returned WspR activity to the level of the DMSO control (Figure 5A). This suggests that reduction of selenocysteine bonds can reverse Eb and EbO inhibition of WspR activity. To determine whether Eb and EbO were covalently linked to WspR, we used liquid chromatography mass spectrometry (LCMS) to analyze WspR incubated with no ligand, with Eb or with EbO (Figure 5B–D). When WspR was incubated with Eb, its mass increased by two times the mass of Eb, suggesting that two ebselen molecules have covalently bonded to WspR (Figure 5C). When the WspR-Eb complex was treated with DTT, which reduces sulfur-selenium bonds, the mass matched that of the unmodified WspR protein (Figure 5C). Since WspR has two cysteines,³³ these results show that both cysteines are covalently modified by Eb. The same procedure was followed to determine whether EbO inhibits WspR through a covalent mechanism. Remarkably, when EbO was incubated with WspR for 30 minutes, the molecular weight was the same as WspR treated with Eb (Figure 5D). This suggests that EbO was being reduced either by the protein or the assay buffer to Eb and covalently linked to WspR.⁴¹ We tested whether WspR enzymatic activity is required to convert EbO to Eb and found the ratio of EbO to Eb to be the same in the heat-killed WspR compared with the

untreated protein. These results indicate that the reduction of EbO is independent of WspR activity (Figure S4). Further, exposure of EbO to the protein containing buffer reduced EbO to Eb, indicating that there is residual reducing agent in the buffer since the purchased, untreated EbO contained no Eb (Figure S2). The covalent modification of WspR by EbO treatment can also be reversed by DTT (Figure 5D). Together these results suggest that EbO inhibits WspR activity through its reduced form, Eb, which covalently modifies both cysteines in WspR to inhibit DGC activity. This two-step mechanism also explains the time dependence of EbO inhibition of WspR.

The two cysteines in WspR modified by ebselen are located in the N- and C-terminal domains. To determine which residue is critical for inhibition by Eb, we performed site-directed cysteine to alanine mutagenesis to generate C49A and C240A alleles. These alleles were cloned into expression vectors and purified for mass spectrometric and enzymatic inhibition analyses. Both C49A and C240A purified proteins had the predicted molecular weight of 42,352 D (Supplemental Table 3). Treatment with Eb and EbO increased the molecular weight of the C49A and C240A proteins to 42,625, which matches the predicted molecular weight of WspR with one covalently modified Eb (Supplemental Table 3). The molecular weight of the protein with two Eb modifications (42,900 D) was not observed for either protein indicating that Eb can modify the remaining cysteine in each of the protein with a single alanine substitution. The effect of Eb modification was assessed by determining the DGC enzymatic activity of the C49A and C240A proteins in the presence of Eb, EbO, cdiGMP or the DMSO solvent carrier. Wild-type WspR is inhibited by cdiGMP, Eb and EbO (Figure 5E). WspR with C49A substitution is still susceptible to inhibition by cdiGMP, Eb and EbO (Figure 5F). WspR with C240A substitution is inhibited by cdiGMP, but completely refractory to Eb and EbO (Figure 5G). These results indicate that the C-terminal cysteine at position 240 must be modified by Eb to inhibit DGC activity. We asked whether the I-site is required for inhibition by generating an AxxA substitution that abolishes the RxxD I-site in WspR. The AxxA protein was no longer inhibited by cdiGMP, Eb or EbO (Figure 5H), indicating that the Eb modified C240 must interact with the I-site to mediate inhibition of WspR DGC activity.

Eb and EbO alter cdiGMP-mediated phenotypes in *Pseudomonas aeruginosa*

In *P. aeruginosa*, increased cdiGMP levels have been shown to repress swimming through an unknown molecular mechanism and to promote biofilm formation by increasing transcription of the *pel* operon and allosterically activating PelD.^{8, 10} We were interested in whether Eb and EbO would alter these cdiGMP-controlled phenotypes by inhibiting DGC activity *in vivo*. We first determined whether Eb and EbO would cause a growth defect in *P. aeruginosa*. No difference in growth was observed when *P. aeruginosa* PA14 was grown in the presence of DMSO, Eb or EbO indicating that Eb and EbO did not interfere with essential processes (Figure S5).

We tested the *in vivo* efficacy of Eb and EbO to reduce DGC-mediated swimming repression in *P. aeruginosa*. We systematically tested *P. aeruginosa* PA14 overexpressing each of the 17 GGDEF-containing proteins, 5 EAL domain proteins and 16 GGDEF-EAL domain proteins for their effect on swimming (Figure S6).⁶ Overexpression of PA0847,

PA1107, PA1120 (TpbB),⁴² PA1727, PA3702 (WspR),⁷ PA4332 (SadC),⁴³ and PA5487 with 1 mM IPTG greatly reduced the swim radius of *P. aeruginosa* PA14 to an extent similar to the complete loss of motility observed in a *fliC* deletion mutant (which lacks flagella) (Figure S6). Since each DGC has different activity, we empirically determined the minimal IPTG concentration required to repress swimming for each DGC (Figure S6). When the indicated IPTG concentration was added to induce DGC gene expression, swimming motility for each of these strains was significantly reduced (Figure 6A white and gray bars). Addition of Eb or EbO to the media containing the appropriate IPTG concentration led to significant increases in swimming diameter compared to the DMSO control for PA1107 (3.4- and 4-fold), PA1120 (2.5- and 3.4-fold), PA1727 (1.1- and 1.3-fold) and WspR (2- and 2.5-fold), while there was no change in swimming motility for PA14 overexpressing PA0847 (Figure 6A blue and red bars). These results indicate that Eb and EbO can inhibit the activity of some, but not all, DGCs in live *P. aeruginosa* cells. When the location of the cysteine residues in both Eb-sensitive and Eb-resistant DGCs were examined bioinformatically, no pattern was observed to suggest that cysteines in specific locations of the protein were critical to Eb activity (Figure S7). The inability of Eb and EbO to reverse the effects of PA0847 are worth emphasizing: if Eb or EbO promoted motility by altering proteins involved in flagellar synthesis or function, then swimming would be reversed for all DGC overexpression strains including PA0847 (Figure 6A). We were interested in the relative activity of Eb and EbO. Swimming by PA14 overexpressing PA3702 was measured in the presence of different concentrations of Eb or EbO (Figure 6B). Interestingly, EbO was able to reverse cdiGMP-mediated repression of swimming at a lower concentration suggesting greater *in vivo* efficacy as compared to Eb (Figure 6B). Together, these results suggest that Eb and EbO are able to inhibit DGCs *in vivo* to reverse DGC-mediated repression of swimming motility.

P. aeruginosa PA14 biofilm formation is induced by increased expression of the *pel* operon and allosteric activation of PelD by cdiGMP.^{8, 10, 44} Expression of the *pel* operon can be activated through two mechanisms: DGC-dependent transcription activation or DGC-independent reduction in translational repression.^{8, 10, 45} DGC-dependent transcriptional activation occurs through the production of cdiGMP, which then binds to FleQ and changes the conformation of FleQ to activate *pel* transcription.^{8, 45} Alternatively, a *retS* mutation enhances the steady state transcript level of the *pel* operon independently of DGCs and leads to enhanced biofilm formation that is also dependent on the *pel* operon.⁴⁴ Both of these mechanisms, however, require cdiGMP binding to PelD.¹⁰ *In vitro*, we observed that Eb and EbO reduced DGC activity and inhibited cdiGMP binding to PelD (Figure 4). Since there is no known enzymatic activity of PelD, we asked whether Eb or EbO could reduce biofilm formation *in vivo* through either inhibition of DGC activity or cdiGMP binding to PelD. To address this question, we used a PA14 strain carrying a plasmid encoding an IPTG inducible WspR. Such induction resulted in an ~5-fold increase in the level of biofilm, as quantified by crystal violet assays. We examined whether Eb and EbO could reverse this effect, as would be expected for inhibition of WspR.⁴⁶ We observed that when Eb or EbO was added, biofilm formation was significantly reduced by ~40% compared to the DMSO control (Figure 7A). These data suggest that Eb and EbO are able to lower DGC-mediated biofilm formation. To determine whether Eb or EbO antagonized PelD to contribute to the reduction

in biofilm formation described above, we measured DGC-independent biofilm formation in PA14 *retS* (Figure 7B). Neither Eb nor EbO reduced biofilm formation (Figure 7B). This suggests that, despite *in vitro* data that indicates that Eb is able to inhibit cdiGMP binding by PelD, Eb is unable to inhibit PelD activity *in vivo*. These results imply that Eb modification mimics the activated state of PelD and that Eb is an agonist for PelD. These findings are important because they indicate that the molecular targets of Eb or EbO *in vivo* are DGCs; if Eb or EbO were interfering with processes downstream of DGCs (such as polysaccharide synthesis or export) then biofilm formation would have been reduced in the Eb-treated PA14 *retS* strain. Together, these flagella motility and biofilm results indicate that Eb is membrane permeable and can inhibit DGCs *in vivo*.

Discussion

Here we are introducing a novel HTS for inhibitors of protein-ligand interactions based on DRaCALA.^{22, 23} Our assay most closely resembles SPA that also measures protein-ligand binding.¹⁷ The advantage of DRaCALA is that the readout is based on radiation and bypasses interference from inherent colorimetric or fluorometric properties of compounds within chemical libraries.^{19, 20} There are several limitations in the use of DRaCALA. First, DRaCALA utilizes radiation, which requires specialized safety and monitoring. This particular problem seems to be common between SPA and DRaCALA-based assays. Second, DRaCALA is limited to protein interactions with ligands that can be mobilized on nitrocellulose. Fluorescent labels typically result in the immobilization of the tagged molecule, limiting the use of non-radioactive probes.²³ The throughput of DRaCALA-based HTS is currently limited since the assay is not yet fully automated. However, DRaCALA based-HTS assays hold promise for future automation through robotic adaptation, which would dramatically increase their throughput and utility. We believe DRaCALA-based HTS presents a new approach to identify drug-like molecules that can probe biological systems by inhibiting protein-ligand interactions.

We and several other laboratories have shown that Eb can covalently modify cysteines residues in proteins *in vitro*. However, Eb only acts on a subset of DGCs *in vivo*. What is the basis for the selectivity of DGC inhibition? We propose that the unique activity of Eb on DGC depends on the proximity of cysteines to the cdiGMP binding pocket. WspR has two cysteines at positions 49 and 240. Cys240 is directly adjacent to the RxxD I-site that binds cdiGMP in the catalytic domain.³³ Modification of Cys240 by Eb directly occludes cdiGMP binding and mediates inhibition of DGC activity. Cysteine residues are found in every DGC. For example, three other DGCs, PA1107, PA1120, and PA1727, have cysteines proximal to the I-site and are susceptible to inhibition by Eb (Figure 6 and Supplemental Figure 7). In contrast, our results for PA0847 suggest that either the I-site of PA0847 is insensitive to Eb inhibition or none of the cysteines in this protein are located in positions that would enable ebselen-mediated inhibition. One possible molecular explanation for the resistance of PA0847 to Eb is that the I-site in PA0847 has a proline residue between the arginine and aspartate of the RxxD motif. Two other pseudomonas DGCs (PA1181 and PA3343) also have a proline between the RxxD motif. Future studies will test whether the proline within the I-site render DGCs refractory to Eb inhibition.

Since Eb can target the cdiGMP binding site in PelD, it represents a lead for modification that could lead to analogues that antagonize PelD function. These modified analogues would have the advantage in inhibiting a single target that is indispensable for biofilm formation in PA14.¹⁰ Combining compounds that reduce biofilm formation together with traditional antibiotics is likely a synergistic strategy that can be more effective in treating biofilm-mediated infections. Furthermore, developing small molecules using a DRaCALA-based approach that abrogate cdiGMP binding to other receptors such as Alg44 and inhibit their function would reduce the burden of bacterial infection associated with alginate production and provide promise in the development of novel therapeutics.⁹

Conclusions

In summary, our results demonstrate that DRaCALA-based HTS is a novel approach for identification of small molecule inhibitors of cdiGMP binding to receptor proteins. Using this approach, we identified ebselen as an inhibitor of cdiGMP binding to DGCs and an antagonist WspR enzymatic activity. Treatment of *P. aeruginosa* with Eb and EbO reversed cdiGMP regulated phenotypes, including motility and biofilm formation. This work represents a proof-of-principle for the use of DRaCALA-based HTS for the identification of chemical inhibitors of biological systems.

METHODS

Materials

National Clinical Collection library 1 was purchased from Evotec. Eb and EbO were repurchased from Cayman Chemical Co. and MP Biomedicals. Compounds were dissolved in DMSO, aliquoted and stored at -20 °C until further use. Radionuclides were purchased from Perkin Elmer. Nitrocellulose was purchased from Whatman. TLC plates were purchased from EMD Millipore. CdiGMP was purchased from Axxora.

Bacterial strains and growth conditions

Pseudomonas aeruginosa PA14 strains and plasmids are listed in Supplemental Table 1. Strains were grown shaking in LB at 37 °C unless otherwise specified. PA14 with pMMB plasmids were grown with gentamicin (75 µg/mL).

Protein purification

Protein was purified as described previously.^{9, 10, 22, 31}

Differential radial capillary action of ligand assay

DRaCALA was performed as described previously.²²

HTS-DRaCALA

HTS-DRaCALA is based on methods described above.²² Compound library or cdiGMP competitor control was suspended in 20 µl of binding buffer (10 mM Tris, pH 8.0, 100 mM NaCl and 5 mM MgCl₂). Protein (Alg44 or PelD) was dispensed into each well using a Biotek MultiFlo liquid dispenser (Vermont, USA), mixed and incubated for 5 minutes at

room temperature. ^{32}P -cdiGMP (10 μL of 4 nM ligand) was dispensed to each well, mixed and incubated for 5 minutes at room temperature. Reactions (1 μL) were then pin transferred to dry nitrocellulose using a 384 Slot Pin Multi-Blot Replicator (V & P Scientific, California, USA). Membranes were allowed to dry completely and then imaged and analyzed as described.²²

WspR activity assay

WspR activity was assayed by thin layer chromatography (TLC)⁴⁷ and analyzed as described.³¹

Liquid chromatography mass spectrometry (LCMS)

WspR (5 μM) was treated with Eb or EbO (50 μM), incubated at room temperature for ten minutes, and centrifuged over a 3 kD molecular weight cut off filter to separate protein from small molecules. WspR protein samples were loaded into an Agilent Poroshell C3 HPLC column (2.1 $\times$ 100 mm) and eluted with a linear gradient of 15–55% solvent B in 15 min, followed by 5 min wash at 80% B and equilibration at 15% B for 10 min. Solvent A is 5% ACN in water with 0.1% formic acid, and solvent B is 95% ACN in water with 0.1% formic acid. Spectra of m/z 400–2000 were acquired with an LTQ Orbitrap XL mass spectrometer with resolution of 60,000 at m/z 400. After acquisition, mass spectra over the chromatography peak were averaged. Averaged spectrum was deconvoluted using MagTran 1.03.⁴⁸ Eb and EbO were analyzed the same way except using a Zorbax StableBond C18 (1.0 $\times$ 75 mm) column with HPLC gradient starting at 0% B.

Growth curves

P. aeruginosa PA14 were grown in LB overnight at 37 °C with shaking. Bacteria were then subcultured in LB with either 2% DMSO alone, 50 μM Eb, or 50 μM EbO. Cultures were grown with shaking at 37 °C for 16 hours with absorbance read at 600 nm every five minutes using a spectrophotometer.

Swim motility assay

Swimming motility was assayed by spotting 3×10^6 CFUs from an overnight culture onto 0.3% agar LB plates containing the indicated concentration of IPTG, DMSO, Eb, EbO and appropriate antibiotics. Plates were incubated at 30 °C in a humidified chamber for 18 hours and imaged using a FujiFilm LAS-3000. Swim diameter was determined using MultiGauge software.

Crystal Violet pellicle assay

Assay was performed as described previously.⁴⁶ Following growth with indicated concentration of inhibitor and IPTG, media was decanted. The attached biofilms were washed and stained with 0.1% crystal violet.¹⁰ Excess dye was removed by washing. Retained dye was dissolved in 30% acetic acid and read with a spectrophotometer at 590 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic of DRaCALA-HTS. + indicates the addition of unlabeled cdiGMP competitor. – indicates the addition of no competitor.

Figure 2.

Validation of DRaCALA-HTS. (A) Image of 384 well DRaCALA spots containing the indicated unlabeled cdiGMP competitor. (B) Fraction bound was calculated for image in (A). (C) Image of 384 well DRaCALA spots containing the indicated concentrations of Alg44. (D) Fraction bound was quantified for each well in (C) and plotted against protein concentration.

Figure 3.

DRaCALA-HTS identifies ebselen as an inhibitor of PelD cdiGMP interactions. (A) Image of DRaCALA spots against NCC1 compound library. Control well containing unlabeled cdiGMP competitor is presented in green box and well containing ebselen is presented in red box. (B) Fraction bound was quantified for each well and plotted against well position. Red line indicates average fraction bound for the plate. Green lines represent 3 standard deviation cutoff set for identification of leads. Fraction bound of positive control is marked by green arrow and fraction bound of ebselen is marked by red arrow.

Figure 4.

Eb and EbO inhibit cdiGMP signaling in vitro. (A) Chemical structure of Eb and EbO. (B) Fraction ^{32}P -cdiGMP bound was determined using DRaCALA for the indicated proteins in DMSO (black bars), Eb (blue bars) and EbO (red bars). (C) Concentration dependence of inhibition of PelD binding to cdiGMP by Eb (blue) or EbO (red) concentrations. (D) Fraction ^{32}P -cdiGMP bound by WspR in the presence of DMSO (black), Eb (blue), and EbO (red) after incubation for 0 minutes (closed) and 30 minutes (open). (E) WspR velocity was assayed by TLC in the presence of 10 μM GTP and specified inhibitor (50 μM). (F) WspR velocity was assayed at varying substrate concentrations at the specified Eb concentration.

Figure 5.

Effect of Eb on WspR activity and molecular weight. (A) WspR activity in the presence of DMSO, Eb or EbO with or without DTT. (B–D) Mass spectra of WspR incubated with (B) DMSO, (C) Eb, (D) EbO (50 μM) in the presence or absence of 1 mM DTT. (E–H) DGC activity of (E) WT (F) C49A (G) C240A and (H) AxxA WspR in the presence of DMSO, Eb, EbO or cdiGMP (50 μM). All data represent the average of at least three independent experiments and error bars are $\pm$ s.e.m. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0005$.

Figure 6.

Eb and EbO reverse cdiGMP-mediated repression of flagella motility. (A) Swim diameter was assessed for PA14 pMMB-PA0847, pMMB-PA1107, pMMB-PA1120, pMMB-PA1727, and pMMB-WspR with and without (white) IPTG. Swimming for induced strains was measured in the presence of DMSO (gray), Eb (50 μM; blue), or EbO (50 μM; red). (B) Swimming for PA14 pMMB-PA3702 was measured in the presence of IPTG and varying concentrations of either Eb (blue) or EbO (red). All data represent the average of at least three independent experiments and error bars are $\pm$ s.e.m. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0005$.

Figure 7.

Eb and EbO reduce DGC-dependent biofilm formation. (A) Biofilm formation of PA14 pMMB-WspR without IPTG (white) and with IPTG and DMSO (gray), Eb (blue) or EbO (red) by crystal violet pellicle assay after static incubation at 30 °C for 16 hours. (B) Biofilm formation for PA14 $\Delta retS$ in the presence of DMSO (black), Eb (blue), or EbO (red) was measured as described above. All data represent the average of at least three independent experiments and error bars are $\pm$ s.e.m. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0005$.