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Identification of small molecules inhibiting diguanylate cyclases to control bacterial biofilm development

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Abstract

Biofilm formation by pathogenic bacteria is an important virulence factor in the development of numerous chronic infections, thereby causing a severe health burden. Many of these infections cannot be resolved, as bacteria in biofilms are resistant to the host's immune defenses and antibiotic therapy. An urgent need for new strategies to treat biofilm-based infections is critically needed. Cyclic di-GMP (c-di-GMP) is a widely conserved second-messenger signal essential for biofilm formation. The absence of this signalling system in higher eukaryotes makes it an attractive target for the development of new anti-biofilm agents. In this study, the results of an *in silico* pharmacophore-based screen to identify small-molecule inhibitors of diguanylate cyclase (DGC) enzymes that synthesize c-di-GMP are described. Four small molecules, LP 3134, LP 3145, LP 4010 and LP 1062 that antagonize these enzymes and inhibit biofilm formation by *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in a continuous-flow system are reported. All four molecules dispersed *P. aeruginosa* biofilms and inhibited biofilm development on urinary catheters. One molecule dispersed *A. baumannii* biofilms. Two molecules displayed no toxic effects on eukaryotic cells. These molecules represent the first compounds identified from an *in silico* screen that are able to inhibit DGC activity to prevent biofilm formation.

Keywords

biofilm; anti-infective; small molecule antagonist; *Pseudomonas*; *Acinetobacter*; c-di-GMP

Introduction

Biofilms are surface-associated bacterial conglomerates that, according to the National Institutes of Health, are associated with 80% of infections (Hall-Stoodley et al. 2004). In the US alone biofilms have been blamed for causing millions of infections, and the associated costs to treat such infections exceed \$1 billion in expenses (Wolcott et al. 2010). The biofilm

mode of growth is associated with increased intrinsic antibiotic tolerance compared to planktonic cells due to a number of factors, including the physical barrier of the matrix, induction of specific genetic pathways and the increase in the prevalence of persister cells (Costerton et al. 1995; Anderl et al. 2000). The matrix provides protection for the biofilm from the host immune system by preventing antibody recognition and engulfment by phagocytic cells (Davies 2003; Mah et al. 2003; Hall-Stoodley & Stoodley 2009).

Biofilms interfere with clinical therapy for chronic, persistent and wound-related infections on various indwelling medical devices (Fux et al. 2005; Hall-Stoodley & Stoodley 2009). Biofilms also trigger inflammation and impair the wound-healing process (Wolcott et al. 2010). Often, the only effective treatment option for biofilm-based chronic wound infections is to amputate the infected limb (Jeys & Grimer 2009). All of these factors pose significant challenges to clear infections and compel the development of new methods designed to inhibit bacterial biofilm formation.

Recently, the second messenger molecule cyclic di-GMP (c-di-GMP) has emerged as an important signal-controlling biofilm formation in a majority of bacteria (Romling et al. 2005; Jenal & Malone 2006; Ryan et al. 2006; Cotter & Stibitz 2007; Tamayo et al. 2007). Synthesis of c-di-GMP occurs *via* diguanylate cyclases (DGC) encoding of GGDEF domains while degradation of c-di-GMP occurs *via* phosphodiesterase (PDE) encoding either an EAL or HD-GYP (Ryjenkov et al. 2005; Schmidt et al. 2005; Dow et al. 2006; Ryan et al. 2006). Sequence analysis of bacterial genomes reveals that most prominent human pathogens encode enzymes predicted to be involved in c-di-GMP signaling, highlighting the significance of this novel second messenger in bacteria (Galperin 2004). More importantly, the enzymatic mechanism of DGCs and PDEs is highly conserved, and the enzymes from different bacterial species are able to cross complement mutations in one another as demonstrated by complementation studies between *Salmonella enterica* and *Yersinia pestis* (Simm et al. 2005). For example, the unrelated DGC, *hmsT*, from *Y. pestis* was able to complement a mutation in the DGC, *adrA*, in *Salmonella enterica* (Simm et al. 2005) despite sharing no homology outside of the DGC domain.

Due to the highly conserved nature of c-di-GMP signalling systems in bacteria, and the mounting evidence for their role in modulating biofilm formation, targeting c-di-GMP signaling systems, therefore, provides an attractive approach to abolish biofilm formation (Navarro et al. 2009).

Because c-di-GMP is not necessary for bacterial growth, small molecules that lower c-di-GMP would not select for resistant organisms compared to traditional antibiotics that are either bacteriostatic or bactericidal. In addition, since c-di-GMP molecules are not encoded in higher eukaryotic organisms, small molecules inhibiting this signal would be predicted to be less toxic to the infected host. Only a few efforts to target c-di-GMP signaling as a means to prevent formation of biofilm have been described, but these efforts do not directly interfere with DGC activity (Newell et al. 2009, 2011; Antoniani et al. 2010). Currently, only two chemical inhibitors have been identified that inhibit DGC activity, reduce biofilm formation and significantly reduce the intracellular concentration of c-di-GMP in bacteria (Sambanthamoorthy et al. 2012).

Here, the authors to the repertoire of small molecules inhibiting DGCs by reporting identification of four small molecules from a 3D pharmacophore-based *in silico* screening approach. These four molecules inhibited DGC enzymes WspR and tDGC from *Pseudomonas aeruginosa* and *Thermotoga maritima* and exhibited anti-biofilm activity against *A. baumannii* and *P. aeruginosa*. All four molecules were able to disperse preformed biofilms of *P. aeruginosa*, but only one was able to disperse *A. baumannii* biofilms significantly. One compound, LP-3134, was able to affect the initial adherence of *P. aeruginosa* to a silicone surface and significantly impair the development of the biofilm of *P. aeruginosa* in a urinary catheter.

The four DGC inhibitors identified in this study will, thereby, serve as a foundation to develop efficacious and potent inhibitors of DGC enzymes to abolish the bacterial biofilm development in both medical and industrial settings.

Materials and methods

Bacteria and media

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli*, *Thermotoga maritima* and *Pseudomonas aeruginosa* cells were grown at 37 °C with constant aeration in Luria Bertani broth (LB). *Acinetobacter baumannii* cells were grown at 37 °C with constant aeration in Brain Heart Infusion broth (BHI). For expression studies, isopropyl β -D-1-thiogalactopyranoside (IPTG) was used at concentrations of 100 $\mu\text{g ml}^{-1}$. When necessary, antibiotics were used at concentrations of 50 or 100 $\mu\text{g ml}^{-1}$.

In silico virtual screening for potential candidates of selective DGC inhibitors

A 2D pharmacophore generated based on the interaction of guanine base with PleD from *Caulobacter crescentus* is shown in Figure 1a and a second pharmacophore containing two of the hydrogen bonds found in guanine base and attached to a five-membered ring is shown in Figure 1b. Using queries derived from these two 2D pharmacophores, a focused library from the database of commercially available millions of compounds was generated. *In silico* screening of this focused library was performed using the amino acid residues in the active site of the published crystal structure (Pubmed: 15569936) that are within 6.5 Å from the GMP part of bound c-di-GMP. During the *in silico* screening, the 3D pharmacophore features of the active site such as the size of the active site and other potential as well as guanine-specific interactions were included. The matching between features in the pharmacophore and the small molecule in the database is within a root-mean square deviation of 1 Å. The complex of PleD and the identified lead inhibitors were further refined and energy minimized to generate the final inhibitor-PleD complex. All calculations were carried out using the MOE (Molecular Operating Environment, Chemical computing group, Quebec, Canada) software and the electrostatic interactions were calculated using the 'R-Field' option in MOE. Based on the binding energy between each compound with the binding site of PleD and on the differences in the exposed solvent-accessible surface areas of bound and unbound conformation of each compound, 500 top-ranking compounds were selected. Chemoinformatics filters such as logP, number of rotatable bonds and visually checking the position and orientation of these 500 top-ranking compounds with respect to

those of the GTP bound to the active site of PleD were used to select a list of compounds for biological assays.

Protein production

T. maritima DGC tDGC-R158A was amplified, cloned and expressed in *E. coli* BL21 (DE3) cells (Invitrogen, CA, USA). The full-length DNA sequence of tDGC was synthesized and inserted into NdeI and XhoI sites of an expression plasmid pET 21b (Genescript, NJ, USA), resulting in strain pET21bTD. A 6× Histag was added at the C-terminal of the protein to enable purification. *E. coli* BL21 (DE3) carrying the expression plasmid (pET28b (+) with tDGC) was grown in LB medium supplemented with kanamycin (30 µg ml⁻¹) at 37 °C. When the OD at 600 nm reached 0.8, 0.8 mM IPTG was added to induce protein expression at 25 °C for 4 h. For lysis, the bacteria were pelleted by centrifuging at 2000 rpm for 10 min. The cell pellet was re-suspended in 20 ml of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5% glycerol, 1 mM β-mercaptoethanol and 1 mM phenylmethanesulfonylfluoride, and the cells were lysed by passage through a French pressure cell (three times 30 s). The suspension was clarified by centrifugation for 10 min at 5000 g. The supernatant was then further clarified by ultracentrifugation (100,000 g, 1 h). For purification using Hi Trap IMAC FF column (GE Healthcare, PA, USA), the supernatant was loaded onto Ni-NTA affinity resin, washed with W1 buffer (containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5% glycerol, 1 mM β-mercaptoethanol and 20 mM imidazole), and eluted with an imidazole gradient from 20 to 500 mM in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5% glycerol and 1 mM β-mercaptoethanol. All protein purification steps were carried out at 4 °C.

WspR, a DGC from *P. aeruginosa*, was amplified, cloned and expressed in *E. coli* BL21 (DE3) cells (Invitrogen). The procedure followed that previously reported by De et al. (2008). Specifically, the full-length DNA sequence of WspR was synthesized and inserted into NdeI and XhoI sites of an expression plasmid pET 21b (Genescript) resulting in strain pET21bWsp. A 6×-His tag was added at the C-terminal of the protein. Transformed *E. coli* BL21 (DE3) cells were grown in LB medium supplemented with 100 µg ml⁻¹ ampicillin at 37°C. At a cell density corresponding to an absorbance of 1.0 at 600 nm, the temperature was reduced to 18°C and the protein production was induced with 1 mM IPTG for 12–16 h. Cells were collected by centrifugation and then re-suspended in 25 mM Tris-HCl buffer containing 500 mM NaCl, 20 mM imidazole and 5 mM 2-mercaptoethanol (pH 8.0). After cell lysis by sonication, cell debris was removed by centrifugation at 40,000 g for 60 min at 4°C. The enzyme was purified by Hi Trap IMAC FF column (GE Healthcare) by elution with 500 mM imidazole in the above buffer. Further purification used SEC column Superdex 200 HR 26/60 (GE Healthcare) by using 25 mM Tris-HCl, 100 mM NaCl, and 1 mM DTT (pH 7.4) as equilibration and running buffer. Fractions containing WspR (MW 39 k Da) were pooled and concentrated using centricon Spin column (30 k Da cutoff).

Measurement of *in vitro* DGC activity

The ability of compounds to inhibit DGC activity was determined using the EnzChek Pyrophosphate Assay (Invitrogen), as previously described (Sambanthamoorthy et al. 2012) to allow high-throughput measurements.

Assessment of biofilm formation

Biofilm formation was measured under both static and flow conditions. For the static condition, a quantitative crystal violet assay was used on polystyrene 96-well and MBEC plates (Biosurface Technologies, MT, USA) as described previously (Harrison et al. 2005; Sambanthamoorthy et al. 2008). Three independent experiments were performed for each of these assays. For biofilm experiments under flow conditions, biofilms were grown in disposable flow cells (Stovall Life Science, NC, USA) as previously described (Sambanthamoorthy et al. 2008). Biofilm formation on the flow cell was imaged both macroscopically and microscopically at 24 and 48 h. Three sections of the flow cell chosen randomly were imaged and representative images are shown. Each section represents dimensions of 250 μm by 250 μm with a resolution of 512 by 512 pixels and shows the same depth. Cross sections of each section were performed at 0.5–1 μm for different pathogens.

Microscopy

For CLSM analysis of biofilms, the medium flow was stopped and the fluorescent dyes SYTO-9 and propidium iodide (Molecular Probes, OR, USA) were injected into the flow cell chamber and incubated for 30 min in the dark. Confocal microscope images were acquired using a Carl Zeiss PASCAL Laser Scanning Microscope (Carl Zeiss, Jena, Germany) equipped with a 63 $\times$ /1.4 numerical aperture Plan-Apochromat objective. The SYTO-9 and propidium iodide fluorophores were excited with an argon laser at 488 nm and the emission band-pass filters used for SYTO-9 and propidium iodide were 515 ± 15 nm and 630 ± 15 nm, respectively. CLSM z-stack image analysis and processing were performed using Carl Zeiss LSM 5 PASCAL Software (Version 3.5, Carl Zeiss). Image stacks of biofilms were acquired from at least three distinct regions on the flow cell. Biofilm thickness was measured starting from the z-section at the interface of flow cell/biofilm to the z-section at the top of the biofilm surface containing <5% of total biomass.

Biofilm dispersal

For biofilm dispersal experiments, overnight-grown cultures of *P. aeruginosa* were standardized to 0.1 OD₅₉₅ and 165 μl were transferred to the wells of a MBEC microtiter plate which was then covered by the MBEC lid. Biofilms were grown on the MBEC pegs under shaking conditions for 24 h. The lid was removed and transferred to a new plate in which the wells had been filled with a 100 μM concentration of compounds LP 3134 and LP 3145. The pegs were immersed for 30 min and the lid was then transferred and gently washed twice with 200 μl of phosphate-buffered saline (PBS) to remove non-adherent cells. Adherent biofilms on the pegs were fixed with 200 μl of 100% ethanol prior to staining for 2 min with 200 μl of 0.41% (wt/vol) crystal violet in 12% ethanol (Biochemical Sciences, NJ, USA). The pegs were washed several times with PBS to remove excess stain. Quantitative assessment of biofilm formation was obtained by immersing the pegs in a sterile polystyrene microtiter plate containing 200 μl of 100% ethanol, incubating at room temperature for 10 min and determining the absorbance at 595 nm using a Spectra-Max M5 microplate spectrophotometer system (Molecular Devices, CA, USA). The results were interpreted by comparing the effects of compounds on treated bio-films with the untreated biofilms of *P.*

aeruginosa. Experiments were performed in triplicates and three independent experiments were performed for each of these assays.

Assessment of molecules impacting adhesion in catheters

The adherence assay measures bacterial adherence to a catheter pre-coated with plasma. This assay was performed as previously described (Sambanthamoorthy et al. 2008). Briefly, overnight-grown cultures of *P. aeruginosa* were standardized to an OD₆₅₀ of 0.1. 5cc 14 Fr silicone catheters (Bard, GA, USA) were cut to a length of 0.5 cm and pre-coated overnight with human plasma (Sigma, MO, USA). The catheters were transferred to appropriate *P. aeruginosa* cultures in a 24-well plate and incubated at 37 °C for 1 h either in the presence or absence of the DGC inhibitors. The catheters were removed using sterile forceps and washed three times in sterile PBS. After washing, the catheters were placed in 100% ethanol for 10 min and stained with crystal violet for 2 min. The catheters were washed several times in PBS, destained by immersing in 100% ethanol and the absorbance at 595 nm was determined using a Spectra-Max M5 microplate spectrophotometer system. Three independent experiments were performed for each of these assays. The mean and standard errors were calculated for the adherence of each strain.

Assessment of biofilm formation in catheters

14-French Bard urinary catheters were cut into 1 cm pieces and placed in 24 well plates. A standardized overnight culture of *P. aeruginosa* was inoculated into the well and incubated overnight at 37 °C either in the presence or absence of the DGC inhibitors. Cultures were removed and catheters were gently washed twice with PBS to remove non-adherent cells. Adherent biofilms on the catheters were fixed with 100% ethanol prior to staining for 10 min with 200 µl of 0.41% (wt./vol.) crystal violet in 12% ethanol. Catheters were washed several times with PBS to remove excess stain. Quantitative assessment of biofilm formation was obtained by moving the catheters to a sterile polystyrene microtiter plate containing 200 µl of 100% ethanol and incubating at room temperature for 10 min to elute the stain. The absorbance at 595 nm was determined using a SpectraMax M5 microplate spectrophotometer system.

Cell viability assay

HEK-293 (keratinocytes) and Raw264.7 cells (obtained from ATCC) were used in this study. The cytotoxicity of compounds in Raw264.7 cells was evaluated by a Lactate dehydrogenase (LDH) cytotoxicity assay. The LDH cytotoxicity assay was performed according to the manufacturer's guidelines (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega, WI, USA).

Measurement of intracellular c-di-GMP concentration in vivo

Lead compounds identified from the chemical screen were evaluated for their ability to inhibit c-di-GMP production *in vivo*. A high-performance liquid chromatography-mass spectrometry (LC-MS-MS) assay was performed to determine *in vivo* c-di-GMP inhibition as previously described (Bobrov et al. 2011). Briefly, bacteria were grown in 2.0 ml of LB medium either in the absence or presence of the lead compounds from an overnight

inoculum to an optical density of 1.0 at 595 nm. The cells were centrifuged at 12,000 rpm for 30 s and washed with 300 μ l of methyl alcohol/acetonitrile/formic acid buffer. The cells were placed at -20°C for 30 min and centrifuged at 15,000 rpm for 5 min. The supernatant was analysed by LC-MS-MS (Waters Corporation, Massachusetts, USA). All compounds were analysed in triplicate.

Statistical analysis

Statistical significance was determined using a paired one-tailed Student's *t* test based on the hypothesis that the lead compounds would lower the activity of DGC enzymes, biofilm formation and bacterial adhesion.

Results

Identification of DGC inhibitors from *in silico* screening

The number of selected compounds in the guanine/oroidin-moiety-based focused library was around 15,000. Docking of these compounds and scoring of the docked ligand–protein complexes led to the formation of 292 compounds for biological assays (Table 2). Based on availability, 250 of these compounds were purchased for further analysis. For experimental testing of inhibitors, the DGC enzyme PleD from *Caulobacter crescentus* was not used due to a loss of activity following purification. Therefore, the compounds were tested for the ability to inhibit DGC activity using the recombinant DGC tDGC from *Thermotoga maritima* in an *in vitro* enzyme assay. Briefly, the conversion of GTP to c-di-GMP by DGCs produces pyrophosphate, which was monitored using the EnzCheck Pyrophosphate Assay (Invitrogen). The assay was slightly modified to allow screening in a high-throughput manner and determined the percentage inhibition compared with untreated enzyme of each compound. Four of the 250 test compounds, namely LP 3134, LP 3145, LP 4010 and LP 1062, significantly reduced the activity of tDGC at concentrations ranging from 12.5 to 200 μM (Table 3).

Furthermore, to test if the compounds functioned as general DGC inhibitors and were not limited to inhibition of tDGC, the inhibition of the well-studied DGC WspR from *P. aeruginosa* was examined. This analysis revealed that all four compounds reduced WspR activity (Table 3), suggesting that these four compounds are general inhibitors of DGC enzymes. The four compounds also did not significantly deter bacterial growth (data not shown). The chemical structures and names of the inhibitors of DGC are indicated in Figure 2.

The four inhibitors of DGC prevent biofilm formation by *P. aeruginosa*

The four inhibitors of DGC were analysed for anti-biofilm activity against *P. aeruginosa* strain PAO1 using a static MBEC biofilm assay. All the four DGC inhibitors significantly inhibited biofilm formation ($p < 0.0012$) by *P. aeruginosa* (Figure 3). Next, the anti-biofilm activities of selected lead compounds under fluid flow were examined. For these experiments, compounds LP 3134 and LP 3145 were chosen to be evaluated for anti-biofilm activities in a continuous flow cell biofilm reactor. In this assay, the biofilm development on a glass surface was monitored under a constant flow of fresh growth medium supplemented

with or without the test compound. This method is more physiologically relevant as it closely mimics natural biofilms that might form in environmental reservoirs or during infection of a human host. The biofilm inhibition or reduction of PAO1 strain in the absence and presence of 200 μ M of LP 3134 and LP 3145 was determined. Representative images depicting the coverage of the biofilm are shown in Figure 4. The experiment was repeated three times. Both LP 3134 and LP 3145 showed a significant reduction of biofilm formation in the flow cell system (Figure 4).

LP 3134 and LP 3145 reduces biofilm formation by *A. baumannii*

To examine if the inhibitors of DGC can reduce biofilm formation in a different pathogen, the inhibition of DGC activity against *A. baumannii* was evaluated. This pathogen is multi-drug resistant and chronically colonizes tissue wounds as biofilms (Dallo & Weitaio 2010; Murphy et al. 2011). All four inhibitors of DGC were able to significantly reduce biofilm formation by *A. baumannii* in the MBEC biofilm formation assay (Figure 3). Similar to the analysis of *P. aeruginosa*, the ability of LP 3134 and LP 3145 to inhibit biofilm of *A. baumannii* under flow conditions was determined. Both LP 3134 and LP 3145 substantially reduced the biofilms of *A. baumannii* compared to the untreated control (Figure 4).

DGC inhibitors disperse established *P. aeruginosa* and *A. baumannii* biofilms

For all the biofilm experiments described thus far, the inhibitors were added concurrently with inoculation of the bacteria. To determine if the lead compounds could disperse established biofilms, *P. aeruginosa* biofilms were grown on MBEC pegs for 24 h. The pegs were removed, washed in PBS and transferred to new plates with lead compounds at 100 μ M in fresh medium for 1 and 24 h. The pegs were removed and the amount of dispersal from the pegs was determined by quantifying the biofilm remaining on the pegs after treatment. All four DGC inhibitors dispersed *P. aeruginosa* biofilms when compared with the DMSO controls (Figure 5). A similar experiment was performed to determine if the DGC inhibitors could disperse preformed *A. baumannii* biofilms, but surprisingly activity was only observed with LP 3134 (Figure 5).

LP 3134 inhibits *P. aeruginosa* adherence to a surface

The first step in biofilm development is primary adhesion of the bacteria to a surface. An adhesion experiment was done to measure the ability of cells to attach to surfaces in the presence of DGC inhibitors (Figure 6). This was done by incubating the bacteria only in the presence of the surface for 1 h and it was assumed that any surface-associated biological material during this short time frame was due to attachment rather than biofilm development. Silicone surfaces were chosen to be examined due to extensive usage of silicone as a catheter material. When *P. aeruginosa* was grown in the presence of the four DGC inhibitors, only compound LP 3134 interfered significantly in the initial adherence of *P. aeruginosa* to surfaces. In contrast, no adhesion defect was observed for *A. baumannii* when grown in the presence of the four DGC inhibitors (data not shown).

LP 3134 and LP 3145 reduce the biofilm formation on urethral catheters

To test the effect of LP 3134 and LP 3145 on medically relevant objects, *P. aeruginosa* was grown on 14-French urethral catheters in the presence and absence of LP 3134 and LP 3145. The biofilm formed by *P. aeruginosa* was prevalent as thick patches along the growth surface. Both LP 3134 and LP 3145 reduced biofilm formation on the catheters (Figure 7). Given the importance of *P. aeruginosa* implicated in urinary tract infections and biofilm development on urinary catheters, these results have the potential for practical applications.

LP 3134 exhibits druggable properties

Compound LP 3134 was examined for properties considered advantageous for subsequent development as a drug candidate. Based on the chemical analysis of known small molecule drugs, Lipinski et al. (1997) developed a set of rules, known as Lipinski's Rule of 5 that describe the most desirable properties for drug development. Molecules LP 3134, LP 3145 and LP 1062 only violate the molecular weight condition of the Lipinski rules as the molecular weights of these compounds are little more than 500 Da. Compound LP 4010 appeared to have no violation of the Lipinski Rule of 5.

Likewise, the DGC inhibitors were tested to determine if they were toxic to eukaryotic cells. Cell viability assays were performed using keratinocytes and LDH to assess the toxicity of compounds to eukaryotic cells. Compounds were administered to cultured human keratinocytes and cytotoxicity assays were performed. Only compounds LP 3134 and LP 4010 demonstrated no cytotoxicity to keratinocytes (data not shown). In addition, a non-radioactive cytotoxicity colorimetric assay was performed to quantitatively measure LDH. Again, of the four compounds, LP 3134 displayed toxic effects only at 300 μ M whereas LP 4010 showed slight toxic effects starting at a concentration of 200 μ M. Both LP 3145 and LP 1062 were toxic at all the concentrations tested (Figure 8).

Discussion

Here, four novel small molecules that inhibit DGC enzymes are described. It is now apparent that c-di-GMP is a central regulator of the prokaryote biofilm lifestyle and mounting evidence also links this molecule to virulence factor expression. Therefore, c-di-GMP presents a new target for the development of antimicrobial strategies.

The results indicate that compound LP 3134 is the most promising candidate, as it possesses broad-spectrum activity, inhibiting DGC activity from enzymes originating from different bacteria. It also inhibited the biofilm development of both *P. aeruginosa* and *A. baumannii* under static and flow conditions. This result is critical because flow cell biofilm assays are generally thought to more closely mimic physiologically relevant conditions than microtiter-based biofilm assays where the medium is not replenished and the culture grows to stationary phase, ultimately using up all of the available nutrient resources leading to less reproducible results.

Here, the catalytic domain of DGC (residues: 286–454) of the published crystal structure of the full-length DGC PleD from *C. crescentus* was used for the *in silico* screening (PDB ID: 1W25; <http://www.rcsb.org>). This domain is very specific to GMP. In this crystal structure, a

c-di-GMP molecule was bound to the active site. The reason for the specificity of the guanine base is due to the three hydrogen bonds: (1) between the N3 of the guanine base with the NH₂ of N335, (2) between the N2 and the side chain carbonyl group of N335 and (3) between the N1 of the base and oxygen of the side-chain carboxyl group of D344. In addition, one of the non-ester oxygen atoms of the phosphate group in the bound c-di-GMP forms a hydrogen bond with the backbone NH of G369. It appears the active site has space for binding to one of the GMPs before and after the formation of a c-di-GMP molecule. Since the mechanism of catalysis is not known at the atomic level and only one of the GMPs of c-di-GMP is bound to the active site, for the development of a 3D pharmacophore, the authors focused on the specificity of guanine base interactions with PleD as found in the crystal structure. Here, a 3D pharmacophore-based *in silico* screening/docking of a focused library containing 'guanine-like' small organic compounds was used for identification of potential lead inhibitors against the GTP binding site of DGC.

Figure 1c shows the amino acid residues involved in the binding of compound LP 3134, which makes four hydrogen bonds with the PleD GTP binding site predicted by the *in silico*/docking studies. The three hydrogen bonds from the six-membered ring containing three hydroxyl groups are similar to that of the three hydrogen bonds between GMP and PleD as discussed earlier. The fourth hydrogen bond is between the only oxygen of the fused rings and the backbone N-H of R366. The hydrophobic side chain of L337 interacts favorably with the six-membered ring containing the three hydroxyl groups. In the case of compound LP 4010, the linker atoms N and the carbonyl group closer to the five-membered ring form hydrogen bonds with N335. The hydroxyl group *ortho* to the carbon connecting the rings and the hydroxyl group *ortho* to the carbon containing a flexible R group each form a hydrogen bond with the side chain NH₂ and C=O of N335, respectively, whereas in compound LP 3145 the oxygen atom of the carbonyl group in the ring and the adjacent hydroxyl group in the same ring each form hydrogen bonds with the side chain NH₂ and C=O of N335, respectively.

The compounds LP 3145, LP 4010 and LP 1062 form only two hydrogen bonds with N335 of PleD, rather than three hydrogen bonds, as observed for the guanine base of GMP, as well as compound LP 3134. Thus, the predicted positions and orientations of the four chemically different lead inhibitors in the GTP binding site of PleD could help further for lead optimization of these compounds and develop into potent inhibitors against PleD.

The strain of *P. aeruginosa* used in this study, PAO1, encodes over 30 distinct DGC enzymes. Therefore, it is hypothesized that these four compounds must be able to inhibit multiple DGC enzymes in the bacterium. Although the assays used in the initial steps of the screening strategy do not directly detect concentrations of intracellular c-di-GMP, they can measure the activity of DGC which regulates biofilm formation. Utilizing two different DGCs (tDGC-R158A and WspR) in the pyrophosphate assay was an additional asset, since the aim was to identify molecules that are active against more than one specific DGC.

An attempt was made to measure a reduction in the intracellular concentration of c-di-GMP in *A. baumannii* and *P. aeruginosa* when exposed to the inhibitors, but this was not

successful in detecting c-di-GMP in the wild strains. A lack of detection of c-di-GMP using LC-MS-MS is not uncommon (Edmunds et al. 2013).

Regardless of whether or not the inhibitors of DGCs identified here reduce intracellular c-di-GMP, these compounds exhibited significant anti-biofilm properties. LP 3134 inhibited biofilm formation by *P. aeruginosa* at every step, including inhibiting initial attachment, development of biofilm and promoting dispersion. There is growing evidence demonstrating that reduced c-di-GMP levels promote dispersion from a biofilm. For example, exposure of *P. aeruginosa* to starvation conditions triggers biofilm dispersal (Gjermansen et al. 2005; Schleheck et al. 2009). This dispersion required the PDE DipA and a chemotaxis protein, BdlA that responds to c-di-GMP (Morgan et al. 2006). Furthermore, it has been shown that LapD, a c-di-GMP effector protein in *P. fluorescens*, triggers dispersion from a surface under low levels of c-di-GMP by triggering proteolysis of LapA from the cell surface (Monds et al. 2007; Newell et al. 2009). These results suggest that a decrease in levels of c-di-GMP may be a signal for dispersion of biofilm. Therefore, it is not surprising that all four DGC inhibitors identified dispersed established biofilms of *P. aeruginosa*.

Recent studies demonstrating bacterial pathogens capable of forming biofilms in the host organs and indwelling medical devices *in vivo* using relevant animal models have been reported, thereby suggesting a role for this mode of existence during human infections (Hall-Stoodley et al. 2006; Sloan et al. 2007; Stoodley et al. 2008, 2010; Chauhan et al. 2012). In addition, formation of bacterial biofilm is also responsible for significant industrial economic loss and high morbidity and mortality in medical settings. The present results show that LP 3134 impacts the development of biofilm on silicone urinary catheters, thereby opening the possibility of using it to modify materials for the construction of anti-biofilm catheters and related implantable biomaterial. Given its broad-spectrum activity against two different DGCs, it is expected that LP 3134 will exhibit anti-biofilm activity against catheter-related biofilm pathogens such as *E. coli* and *Klebsiella pneumoniae* since they encode a significant number of GGDEF domains (Trautner & Darouiche 2004; Jacobsen et al. 2008; Stahlhut et al. 2012). Furthermore, such compounds may also be used in the future to eradicate biofilms formed in the organs of the mammalian host.

Recently, using a whole cell luminescence-based screen, Sambanthamoorthy et al. (2012) reported the first ever small molecule inhibitors of DGC that inhibited the formation of biofilm and decreased the intracellular levels of c-di-GMP by direct inhibition of DGC enzymes. In this report, an *in silico*-based approach to identify additional novel and chemically different sets of small molecules from a focused library containing ‘guanine-like’ commercially available compounds was used that can reduce the formation of biofilm by directly inhibiting DGC enzymes. Therefore, these molecules broaden the new class of anti-biofilm compounds that function by inhibiting the DGC enzymes.

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Figure 1.

(a) represents the 2-D pharmacophore generated based on the interaction of guanine base with PleD; (b) shows a second pharmacophore based on the oroidin template containing some of the features of a guanine base; (c) shows the binding site of the compound LP 3134 (shown in ball-and-stick model with atom-based colour coding) in PleD (shown in stick model with atom-based colour coding). The dashed line represents a hydrogen bond.

Figure 2.

The chemical names, structure and molecular weights of the inhibitors of DGC. **LP 3134** = N'-((1E)-{4-ethoxy-3-[(8-oxo-1,5,6,8-tetrahydro-2H-1,5-methanopyrido[1,2-a][1,5]diazocin-3(4H)-yl)methyl]phenyl)methylene)-3,4,5-trihydroxybenzohydrazide. **LP 3145** = 1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-2,2'-binaphthalene-8,8'-dicarbaldehyde. **LP 4010** = benzenesulfonylamide,4-amino-N-methyl-N-[3-(3,4,7,8-tetrahydro-2,4-dioxo-2H-thiopyrano[4,3-d]pyrimidin-1(5H)-yl)propyl]. **LP 1062** = (E)-1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one. The molecular weights of the four compounds are 518.22, 518.5, 404.10 and 516.54 kDa for LP 3134, LP 3145, LP 4010 and LP 1062, respectively.

Figure 3.

The ability of the four inhibitors of DGC at a concentration of 200 μ M to reduce the formation of biofilm in *P. aeruginosa* and *A. baumannii*. The treated cells were statistically different from the DMSO controls. This experiment was repeated three times for each treatment and the histogram displays the average biofilm biomass with the associated SD (* $p < 0.05$).

Figure 4.

CLSM images of the biofilm. *P. aeruginosa* and *A. baumannii* grown in the presence and absence of 200 μ M LP 3134 and LP 3145 were imaged 48 h post inoculation of flow cells. The panels on the left are an overlay of multiple slices, and the side frames of the panels on the right show the z-stack showing the thickness and the architecture of the biofilm. The line in the z-stack indicates the level at which the photograph of the x-y plane was taken. Photographs were taken at a magnification of $\times 600$.

Figure 5.

The ability of the four inhibitors of DGC to disperse the formation of biofilm in *P. aeruginosa* and *A. baumannii* with and without inhibitors at a concentration of 200 μ M. This experiment was repeated three times for each treatment and the histogram displays the average biofilm biomass with the associated SD. *Indicates statistically significant differences.

Figure 6.

The ability of LP 3134 to reduce initial adherence of *P. aeruginosa* on silicone catheters with and without inhibitors at a concentration of 200 μ M. The results represent the mean $\pm$ the SEM of three independent experiments. The Student's paired *t* test was used to compare the treated and non-treated cells. *Denotes statistical significance of $p < 0.05$.

Figure 7.

The ability of LP 3134 and LP 3145 to reduce *P. aeruginosa* biofilms on silicone catheters with and without inhibitors at a concentration of 200 μ M. The results represent the mean $\pm$ SEM of three independent experiments. The Student's paired *t* test was used to compare the treated and non-treated catheters. *Denotes statistical significance of $p < 0.05$.

Figure 8.

Toxicity testing of the four inhibitors of DGC in mammalian cells. Raw264.7 cells were treated as indicated and viability was measured at 24 h, following the directions of manufacturer.

Table 1

Strains and plasmids used in the study.

Strain or plasmid	Description	Source
Strains		
<i>Pseudomonas aeruginosa</i> PA01	Wild type strain	Stover et al. (2000)
<i>Acinetobacter baumannii</i> 5711	Wild type strain (wound isolate)	Zurawski et al. (2012)
<i>Thermotoga maritima</i>	Wild type strain	Rao et al. (2009)
<i>E. coli</i> 21 (DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dck</i> (DE3)	Invitrogen
Plasmids		
pET21bWsp	WspR purification plasmid	This study
pET21bTD	tDGC purification plasmid	This study
Primers		
<i>wspR</i> _F	GAAGGAGATATACATATGCACAACCCATG	This study
<i>wspR</i> _R	GTGGTGGTGGTGCTCGAGGCCCGCCGGGGCCGGC	This study
<i>tDGC</i> _F	GCCGCTATTCTTCGAACTG	This study
<i>tDGC</i> _R	AAATTCATCGCCACCATAGC	This study

Table 2Final results of *in silico* screening.

Commercial library	Company	Identified compounds
Guanine-based library	ChemDiv	48
Natural product library	ChemDiv	100
Natural product library	Tim-Tech	50
Synthetic compound library	Tim-Tech	51
Synthetic compound library	Anamine	43
Total	3	292

Table 3

Representative inhibition assays.

Compound	% Inhibition of tDGC-R158A	IC ₅₀ (μM) for WspR	Confidence interval for WspR (μM)
LP-3134	72.1 (at 100 μM)	44.9	33.5–56.2
LP-3145	28.0 (at 50 μM)	70.93	61.1–80.7
LP-4010	20.5 (at 200 μM)	102.4	91.7–113.0
LP-1062	26.8 (at 50 μM)	73.1	59.3–86.9

Notes: The inhibition of the DGCs WspR from *P. aeruginosa* and tDGC-R158A from *T. maritima* at varying inhibitor concentrations is shown for all four molecules.