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Identification of a *Mycobacterium tuberculosis* Cyclic Dinucleotide Phosphodiesterase Inhibitor

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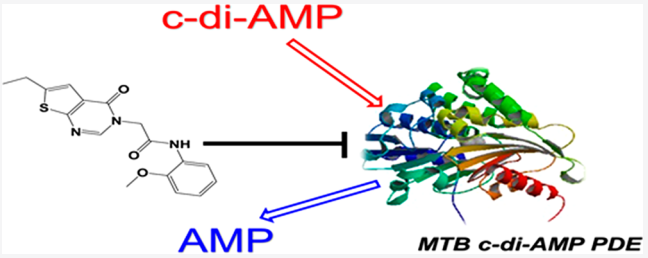
ABSTRACT: Immune cells sense bacteria-derived c-di-GMP and c-di-AMP as well as host-derived cGAMP, which is synthesized by cGAS upon binding to the pathogen's DNA, to mount an immunological response (cytokine production) via the STING-TBK1 pathway. Successful pathogens, such as *Mycobacterium tuberculosis* and group B streptococcus, harbor phosphodiesterases (PDEs) that can cleave bacterial c-di-AMP as well as host-derived cGAMP to blunt the host's response to infection. Selective inhibitors of bacterial cyclic dinucleotide (CDN) PDEs are needed as tool compounds to study the role(s) of CDN PDEs during infection and they could also become bona fide antivirulence compounds, but there is a paucity of such compounds. Using a high-throughput assay, we identified six inhibitors of MTB CDN PDE (CdnP). The most potent inhibitor, **C82** with an IC_{50} of ∼18 μM, did not inhibit the enzymatic activities of three other bacterial CDN PDEs (Ybt, RocR, and GBS-CdnP), a viral CDN PDE (poxin) or mammalian ENPP1.



KEYWORDS: *innate immune system, MTB immune evasion, cyclic dinucleotide, MTB phosphodiesterases (CdnP), antivirulence therapy*

Cyclic dinucleotides (CDNs) have emerged as important second messengers that regulate a dizzying array of processes in all kingdoms. In bacteria, c-di-GMP, c-di-AMP, and 3′3′-cGAMP have been shown to regulate different aspects of bacterial physiology, ranging from biofilm formation and antibiotic resistance to adaptation to different environmental conditions.[1] CDNs cellular levels are maintained by CDN synthesizing enzymes (diadenylate cyclase for c-di-AMP, diguanylate cyclase for c-di-GMP, and cGAMP synthase for 3′3′-cGAMP) and degrading enzyme phosphodiesterase (PDE). Metazoans also make cyclic dinucleotides, such as 2′3′-cGAMP (which contains one noncanonical 2′5′-phosphodiester linkage).[2] 2′3′-cGAMP (referred to as cGAMP hereafter), synthesized by cyclic GMP-AMP synthase (cGAS), is an immune function regulator that signals via the STING-TBK1 pathway.[3] Bacteria-derived cyclic dinucleotides also activate the STING pathway, and hence immune cells are able to sense the presence of intracellular bacteria either by making their own cGAMP upon sensing bacterial DNA in the cytosol or via the binding of bacterial cyclic dinucleotides to STING.[4] Because of the vital role that CDNs play in bacterial physiology and the innate immune system, bacterial CDN synthesizing and degrading enzymes have been targeted for the development of therapeutic agents. Diguanylate cyclase inhibitors with anti-biofilm properties have been reported.[5] Several diadenylate cyclase inhibitors, which have antibacterial properties and also potentiate existing traditional antibiotics, have been identified as well.[6] Our group was the first to report a diadenylate cyclase inhibitor with antibacterial and antibiofilm activity.[7] In contrast to the many reported c-di-GMP or c-di-AMP synthase inhibitors, which have been reported, there is a paucity of identified bacterial CDN phosphodiesterase (PDE) inhibitors.

c-di-AMP is essential for many human pathogens (mainly Gram positive bacteria and mycobacteria) because it regulates key processes such as osmoregulation.[1b,9] For intracellular bacteria, especially those that infect phagocytic cells (such as MTB), the release of bacteria-derived c-di-AMP into the host's cytosol can also elicit the host's immune response via the STING-TBK1 pathway to facilitate pathogen clearance. Successful pathogens, such as MTB and group B streptococcus (which can also exist intracellularly), have developed sophisticated systems to evade regulation while inside phagocytic cells.[10] MTB harbors a c-di-AMP phosphodiesterase (CdnP) that not only hydrolyzes bacteria-derived c-di-AMP to attenuate the activation of STING but also modulates host-derived cGAMP. The mechanism by which MTB CdnP gains access to the host's cGAMP is unknown[10a] (Figure 1a). An analogous pathogen attenuation of host signaling has also been described in group B streptococcus whereby bacterial cyclic dinucleotide phosphodiesterase (membrane anchored and

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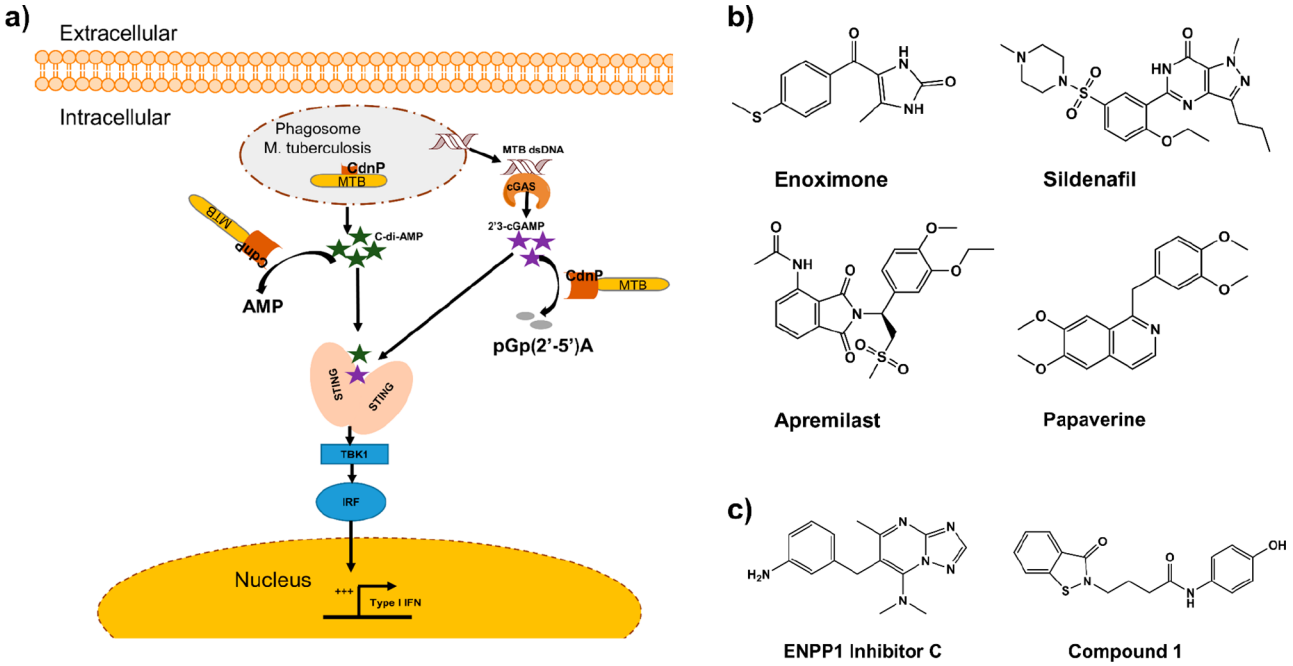

Figure 1. MTB CdnP phosphodiesterase activity inside a macrophage and structures of PDE inhibitors. (a) Schematic illustration of c-di-AMP and cGAMP induction of the type I interferon response via the STING pathway and the attenuation of this response by MTB CDN phosphodiesterase (CdnP) inside a macrophage. (b) Inhibitors of cyclic mononucleotide PDEs. (c) Inhibitors of cyclic dinucleotide PDEs. ENPP1 IC_{50} = 260 nM.[8] IC_{50}s of the commercial PDEs inhibitors indicated in the text.

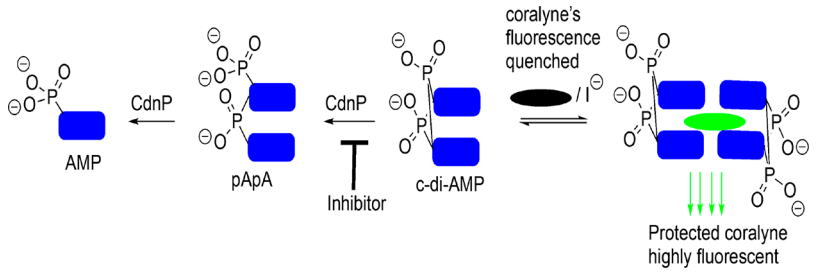

Figure 2. Monitoring of c-di-AMP hydrolysis by MTB CdnP with the coralyne assay. c-di-AMP forms a complex with coralyne resulting in coralyne fluorescence enhancement. MTB CdnP hydrolyzes c-di-AMP into two molecules of AMP (only one is shown in the figure), which does not enhance the coralyne fluorescence.

extracellular) degrades any c-di-AMP that is secreted out of the bacteria, thereby reducing the pathogen-associated molecular pattern (PAMP) level that could be sensed by the host.[10b] Recently, it was disclosed that certain viruses also harbor cyclic dinucleotide phosphodiesterases (such as poxins), which degrade the host cGAMP.[11] Thus, it appears that one of the major ways that pathogens circumvent the immune response is to disable the host's STING signaling response. Consequently, selective cyclic dinucleotide phosphodiesterase inhibitors are needed as tool compounds to decipher the roles of various cyclic dinucleotide phosphodiesterases in bacterial and immune cell physiology. Additionally, these compounds could be developed into antivirulence and/or immune adjuvants or boosters. It is crucial that selective CdnP inhibitors are developed to avoid perturbing the dynamics of commensal or resident bacteria.

Many compounds that inhibit mammalian cGMP or cAMP PDEs have been developed, and some are used in the clinic for diverse diseased states, such as cardiac failure (an example is PDE3 inhibitor enoximone, half maximal inhibitory concentration (IC_{50}) = 10 μM[12]), psoriatic arthritis (an example is PDE4 inhibitor apremilast, IC_{50} = 74 nM[13]), erectile dysfunction (an example is PDE5 inhibitor sildenafil, IC_{50} = 5.22 nM[14]), and vasospasm (an example is PDE10 inhibitor papaverine, IC_{50} = 92.3 nM[15]). See Figure 1b for the structures of PDE inhibitors. As stated earlier, only a handful of compounds that inhibit cyclic dinucleotide phosphodiesterases (for few reported examples, see Figure 1c) have been reported, although it is emerging that these enzymes could also play important roles in various disease progressions.[8,16] The Sintim group reported a benzoisothiazolinone derivative (compound 1) as a selective inhibitor of c-di-GMP PDE RocR.[16a] This compound could inhibit the swarming motility in *Pseudomonas aeruginosa*. In another report, the Sintim group also reported that linear dinucleotides with hydrolysis-resistant phosphodiester linkages could inhibit MTB CdnP, a virulence factor.[10a] A major limitation of these linear dinucleotide analogs is poor cell permeation. Herein, we disclose the identification of a non-nucleotide-based MTB CdnP inhibitor, which does not inhibit cyclic dinucleotide PDEs from other bacteria. Consequently, this compound or more potent analogs thereof could be used as tool compounds to provide insight into how MTB CdnP

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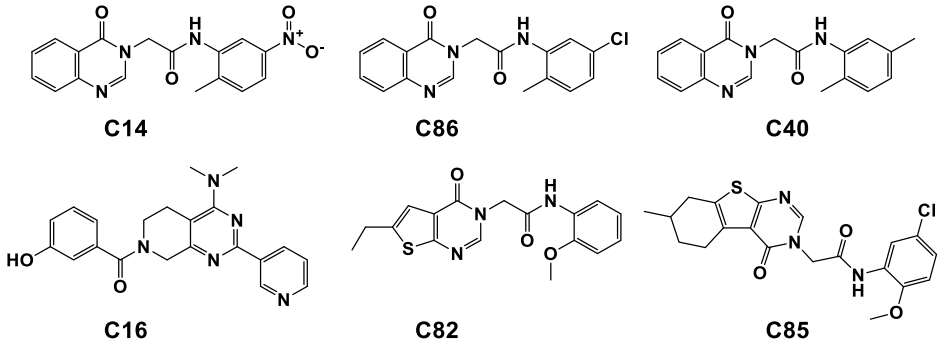

Figure 3. Structures of inhibitors of MTB CdnP, which were identified from the HTS.

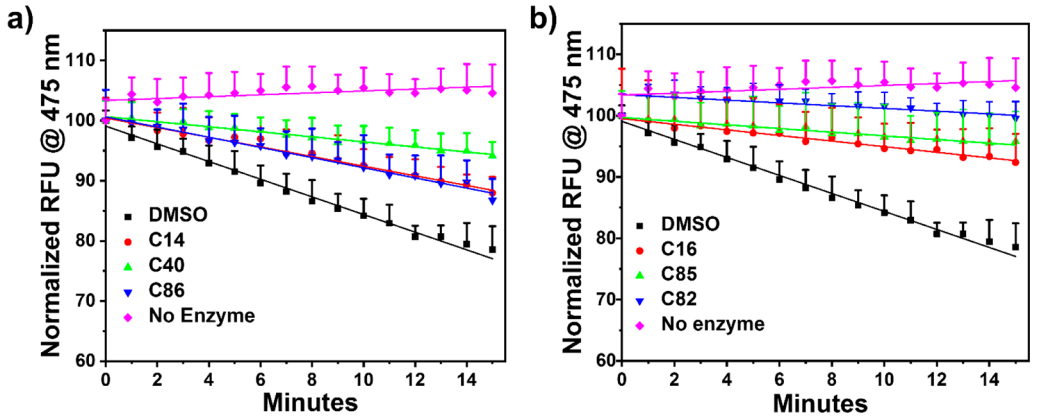

Figure 4. MTB CdnP c-di-AMP hydrolysis in the presence and absence of identified HTS hits visualized with the coralyne. (a) Coralyne assay profiles of MTB CdnP c-di-AMP hydrolysis in the presence of C14, C40, and C86 molecules. (b) Coralyne assay profiles of MTB CdnP c-di-AMP hydrolysis in the presence of C16, C85, and C82 molecules. Assay parameters: 10 μM compounds, 0.5 μM MTB CdnP, and 10 mM KI, reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MnCl_2$). Experiments were conducted in triplicate at 30 °C.

regulates various aspects of infection and may even be developed into antivirulence MTB therapeutics.

RESULTS AND DISCUSSION

The coralyne assay, developed by our group,[17] was used to conduct high-throughput screens for MTB CdnP inhibitors. The assay capitalizes on the fact that c-di-AMP protects coralyne from iodide quenching via the formation of an inclusion complex with c-di-AMP (Figure 2). Following the formation of the c-di-AMP-coralyne complex, coralyne fluorescence emission is greatly enhanced. Since c-di-AMP enhances coralyne fluorescence emission in a concentration-dependent manner (SI, Figure S1a) but AMP does not (SI, Figure S1b), the coralyne assay can be used to track c-di-AMP degradation by MTB CdnP and therefore could be used to identify MTB CdnP inhibitors. Prior to conducting the HTS, the assay was optimized for both kinetics and end-point analysis. Experimental parameters were adjusted to obtain an optimal screening window and, at the same time, to ensure that the MTB CdnP kinetics stayed in the linear range. The screening window was quantified using the Z factor, a screening window coefficient. The Z factor is reflective of both the assay signal dynamic range and the data variation associated with the measurements (eq 1).[18] An ideal assay for HTS ought to have a Z factor of between 1 and 0.5. Hence, we optimized the experimental conditions to obtain ideal assay conditions for the HTS assay. The Z factors for different c-di-AMP concentrations, chosen to be close to the apparent K_m (obtained via the coralyne assay), were computed (SI, Figure S2a,b). The concentration of potassium iodide, KI, which is used as an anion quencher to reduce the fluorescence of the unbound coralyne,[17] was also varied and the Z factor was computed. Optimal assay conditions for both kinetic (Z factor = 0.61) and end-point (Z factor = 0.71) approaches were found to be 70 μM c-di-AMP and 10 mM KI (SI, Figure S2b). Thus, the HTS was conducted with these conditions. A total of 90 000 compounds (Purdue Chemical Genomics Facility compound library), which included the kinase inhibitor library, natural products, and diversity library, were screened for CdnP inhibition. Compounds that exhibited over 40% inhibition were selected as potential MTB CdnP inhibitors (Figures 3 and 4).

Next, we ran HPLC-based enzymatic reactions to confirm the putative CdnP inhibitors identified from the HTS. MTB CdnP was incubated in the presence and the absence of the hit compounds, and reactions were quenched and then analyzed via liquid chromatography. The reactions were analyzed at two different time points (after 3 and 12 h incubations). All of the compounds, except for **C16** and **C40**, showed substantial inhibition after 3 h of incubation; **C86**, **C82**, and **C14** showed the most potent inhibitory effects (Figure 5a). Only **C82** retained any inhibitory effect after an overnight incubation (Figure 5b), and thus we designated it as our most potent MTB CdnP inhibitor. The fact that **C16**, the only molecule lacking the *N*-phenyl-6-oxo-pyrimidine-1-carboxamide moiety, did not show any activity after 3 h of incubation indicates that this moiety is important for inhibitory activity. The lower enzymatic inhibition by **C40**, compared to **C14** and **C86**, indicates that substituents of the phenyl ring impact activity and hence provide

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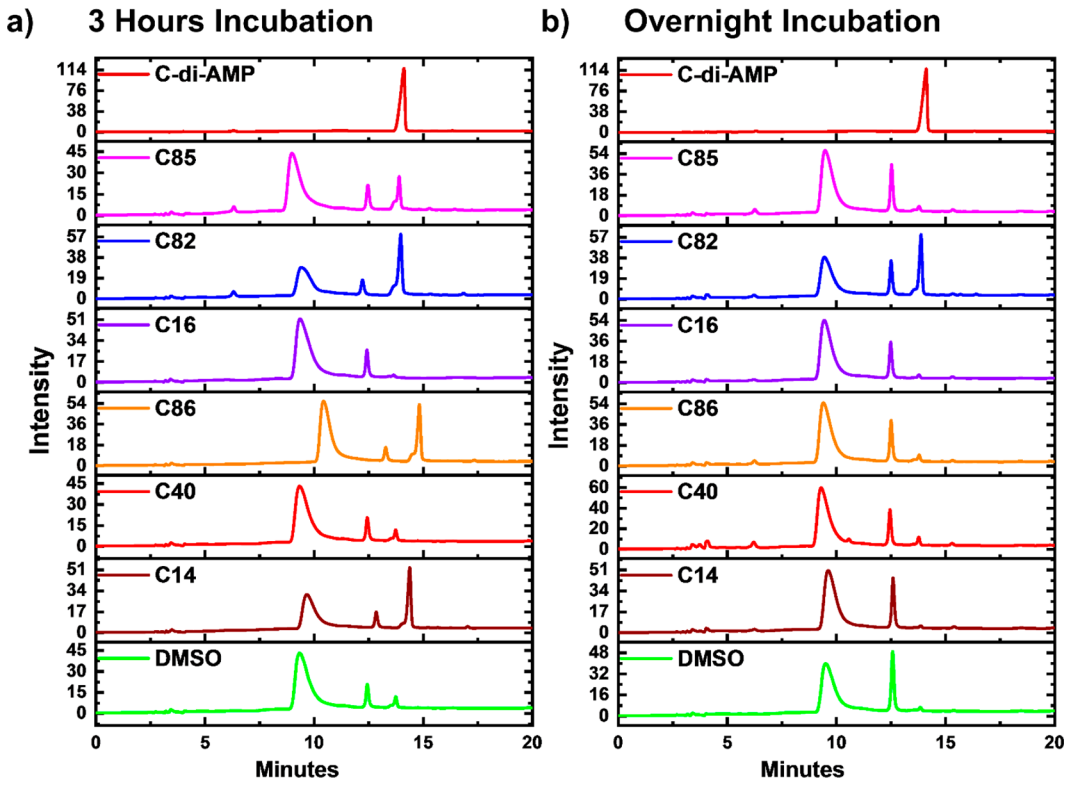

Figure 5. Inhibition of MTB CdnP enzymatic activity by compounds, analyzed via high-performance liquid chromatography (HPLC). (a) HPLC profiles of C-di-AMP cleavage by MTB CndP in the absence and presence of inhibitors after 3 h of incubation. (b) HPLC profiles of C-di-AMP cleavage by MTB CndP in the absence and presence of inhibitors after 12 h of incubation. Experiment conducted with 100 nM MTB CdnP and 70 μM c-di-AMP in 1× reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MnCl_2$) and incubated at 37 °C for the indicated duration. Reactions were analyzed with a COSMOSIL C18-MS-II column.

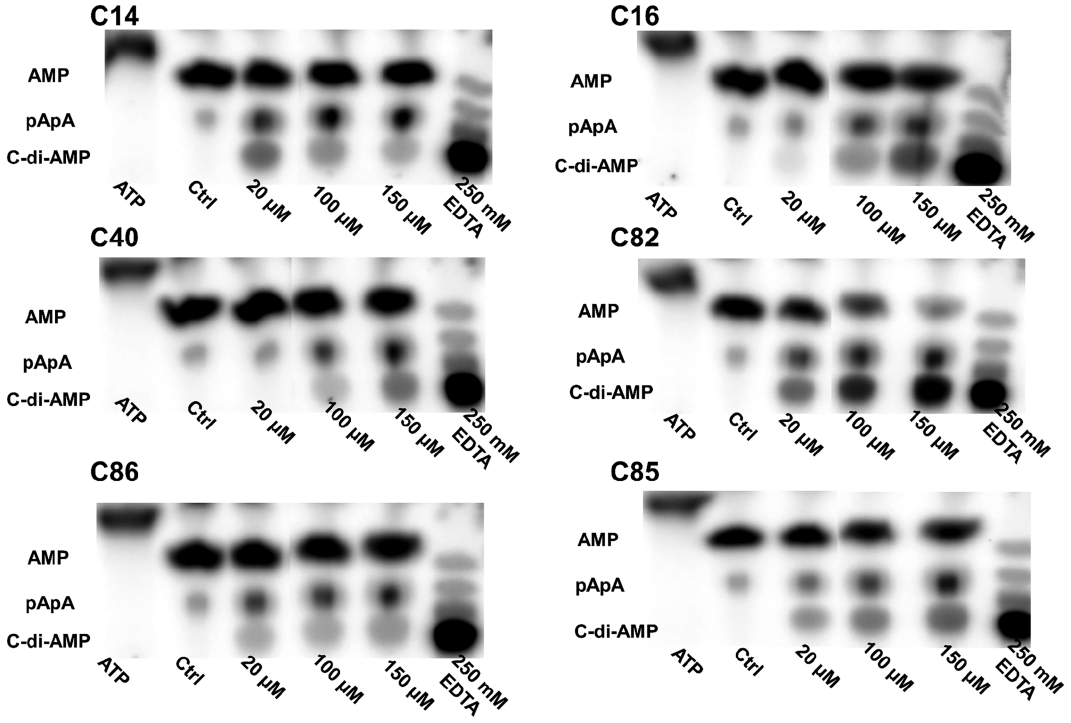

Figure 6. Inhibition of MTB CdnP enzymatic activity by compounds, analyzed via thin-layer chromatography (TLC). Visualization of c-di-AMP cleavage by 0.5 μM MTB CdnP in the presence and absence of varying hit compound concentrations. Ctrl indicates the control group treated with dimethyl sulfoxide (DMSO). The experiment was conducted with 0.5 μM CdnP, 70 μM C-di-AMP, and 6.6 nM ^{32}P-C-di-AMP in 1× reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MnCl_2$) and incubated for 1 h at 37 °C.

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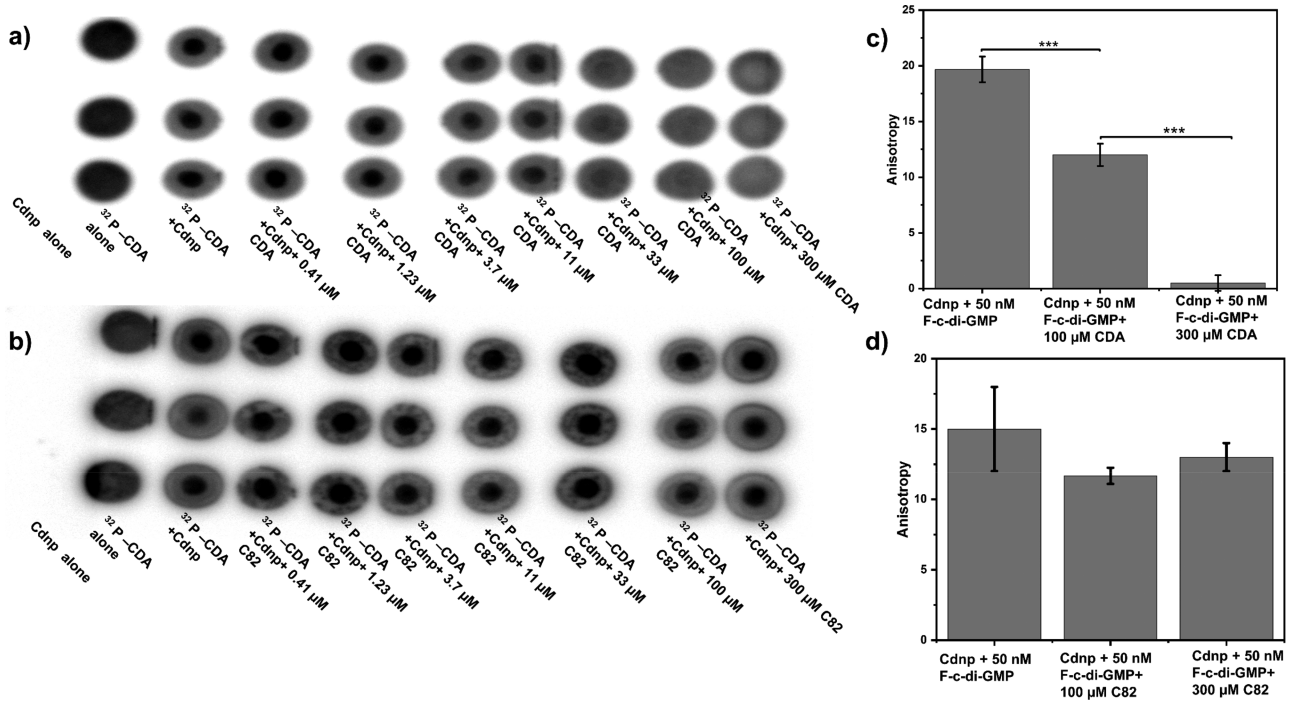

Figure 7. C82 does not displace c-di-AMP from MTB CdnP. (a) Unlabeled c-di-AMP inhibits ^{32}P-c-di-AMP sequestering by MTB CdnP in a concentration-dependent manner. (b) **C82** effects on ^{32}P-c-di-AMP sequestering by MTB CdnP. (a and b) Experiments were conducted with 20 μM MTB CdnP, 1× reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM calcium chloride), and 200 pM ^{32}P c-di-AMP. (c) C-di-AMP significantly attenuates fluorescent c-di-GMP anisotropy in a concentration-dependent manner. (d) **C82** does not attenuate fluorescent c-di-GMP anisotropy. (c and d) Experiments conducted in triplicate with 150 μM MTB CdnP, 1× reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM calcium chloride), and 50 nM F-c-di-GMP. Data are presented as the mean ± standard deviation. CDA = c-di-AMP. ***$p < 0.001$. Statistical analysis was conducted using the student's t test.

a strategy for improving the inhibitor potency in future optimization campaigns.

We further confirmed the inhibitory effects our hit compounds using comparative radioisotope thin layer chromatography (TLC). ^{32}P-c-di-AMP was synthesized by incubating MTB DisA, a c-di-AMP synthase enzyme, with hot ATP (^{32}P-ATP) and cold ATP as previously reported.[19] MTB CdnP reactions were set up in the presence and absence of varying inhibitor concentrations (20 to 150 μM), and c-di-AMP hydrolysis was visualized via TLC. Ethylenediaminetetraacetic acid (ETDA) was used as a surrogate for a potent inhibitor. MTB CdnP enzymatic activity requires a Mn^{2+} cofactor, hence divalent metal chelators such as ETDA can quench the reaction. Therefore, in the presence of ETDA we expected c-di-AMP cleavage to be diminished (Figure 5). All compounds showed concentration-dependent inhibitory activity with **C82** exhibiting the most potent inhibition (Figure 6 and the SI, Figure S4). From the three complementary assays (coralyne, HPLC, and TLC), we concluded that **C82** was the most potent inhibitor. To confirm this, we also determined the IC_{50} of enzyme inhibition by compounds using the safer and more convenient coralyne assay. Consistent with the earlier data, **C82** was the most potent compound. **C82**, **C85**, **C14**, **C40**, **C16**, and **C86** inhibited MTB CdnP enzymatic activity (50 nM MTB CdnP and 80 μM c-di-AMP) with IC_{50} values of 17.5 ± 1.4, 22 ± 1.8, 24.8 ± 1.4, 26.4 ± 1.1, 42.6 ± 1.4, and >60 μM respectively (SI, Figure S3).

To rule out the possibility that the observed CdnP inhibition was due to nonspecific protein denaturation by compounds, we incubated the MTB CdnP enzyme with compounds (50 μM) or sodium dodecyl sulfate (SDS), a detergent that denatures proteins for 1 h. The small molecules were then dialyzed out, c-di-AMP was added to the compound-treated enzyme, and the degree of c-di-AMP hydrolysis by MTB CdnP was evaluated using the recently described STING fluorescence polarization assay, which is a convenient method for monitoring the activities of cyclic dinucleotide metabolism enzymes.[20] Although the treatment of MTB CdnP with SDS completely destroyed the enzymatic activity, the enzyme retained activity after a 1 h incubation with compound **C82**, **C85**, **C14**, **C40**, **C16**, or **C86**, followed by dialysis (SI, Figure S5). Pleasingly, the activities of the enzyme samples treated with the most potent inhibitor, **C82**, were identical to that of the DMSO control (SI, Figure S5b). Thus, we conclude that the inhibition of CdnP by **C82** is not via promiscuous enzyme denaturation. We proceeded to confirm that **C82** does indeed bind to MTB CdnP using an intrinsic fluorescence assay.[21] MTB CdnP intrinsic fluorescence decayed in **C82** in a concentration-dependent manner, confirming that **C82** does bind to MTB CdnP (SI, Figure S6). We asked if **C82** and c-di-AMP shared a common binding site by determining if **C82** could displace bound c-di-AMP from MTB CdnP. To do this, we incubated MTB CdnP with radiolabeled c-di-AMP and tried to displace the bound c-di-AMP with different concentrations of **C82** using the differential radial capillary action of a ligand (DRaCALA) assay.[5b] Although unlabeled c-di-AMP could displace radiolabeled c-di-AMP from MTB CdnP (Figure 7a), **C82**, even at 300 μM, did not displace radiolabeled c-di-AMP from the enzyme (Figure 7b). We also used a secondary fluorescent polarization displacement assay to confirm this finding. Briefly, fluorescently labeled c-di-GMP was incubated with MTB CdnP and treated with different concentrations of **C82** or c-di-AMP in the presence of calcium to inhibit the cleavage of the bound CDN.[10a] Consistent with the DRaCALA

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results, **C82** did not displace the fluorescently labeled c-di-GMP from MTB CdnP, and unlabeled c-di-AMP was able to displace fluorescently labeled c-di-GMP from MTB CdnP in a concentration-dependent manner (Figure 7c,d).

We proceeded to investigate if **C82** was a promiscuous CDN phosphodiesterase (PDE) inhibitor. We screened **C82** against three bacterial CDN PDEs (Ybt, RocR, and GBS-CdnP), one mammalian CDN PDE, ENPP1, and poxin, a viral CDN PDE. Ybt is a *Bacillus subtilis* CDN PDE that hydrolyzes both c-di-AMP and c-di-GMP.[22] RocR is a *P. aeruginosa* c-di-GMP PDE, and GBS-CdnP is a group B streptococcus (GBS) c-di-AMP PDE.[23] GBS-CdnP, just like MTB CdnP, has been shown to dampen STING-dependent type I interferon induction.[23b] Mammalian ENPP1 is capable of degrading both cGAMP and bacterial CDNs.[10a] Consequently, ENPP1 is an excellent target for the development of immunotherapy agents for both cancer and infection management. Poxins (poxvirus immune nuclease) are 2′ 3′-cGAMP-degrading enzymes whose activities result in dampened STING-dependent signaling.[11] For selectivity studies, the assay conditions were optimized to ensure that most of the substrate was not hydrolyzed. **C82** did not inhibit any of these enzymes (SI, Figure S7), so **C82** can be considered to be an MTB CdnP-specific inhibitor (at least when compared to five other cyclic dinucleotide PDEs). Finally, we evaluated **C82** cytotoxicity against mammalian cells. For up to 100 μM, **C82** did not significantly inhibit cell viability (SI, Figure S8).

CONCLUSIONS

Because of the vital roles of CDNs in bacterial cell physiology and the modulation of the innate immune system, CDN metabolizing enzymes are attractive therapeutic targets. Several inhibitors of bacterial CDN synthase enzymes have been reported. In contrast, only one non-nucleotide bacterial CDNs PDE inhibitor has been described. To fill in the gap, we embarked on the search for an MTB CdnP PDE inhibitor, which could be used as a tool compound or a potential therapeutic. To our knowledge, this is the first report of a non-nucleotide MTB CdnP PDE-selective inhibitor. **C82** and analogs thereof, which contain the 2-(4-oxothieno [2,3-*d*]pyrimidin-3(4*H*)-yl)-acetamide or 2-(4-oxoquinazolin-3(4*H*)-yl)acetamide moieties, are excellent starting compounds for developing novel cyclic dinucleotide phosphodiesterase inhibitors.

MATERIALS AND METHODS

Protein Expression and Purification. Overnight cultures of *E. coli* BL21(DE3) containing the plasmid of interest were inoculated in 1 L of terrific broth media supplemented with selection antibiotics and cultured to the exponential phase (OD = 0.6) at 37 °C. MTB CdnP plasmid was a gift from William R. Bishai.[10a] Ybt plasmid was a gift from Zhao-Xun Liang.[24] GBS plasmid was a gift from Pierre-Alexandre Kaminski.[23b] Poxin plasmid was a gift from Philip J. Kranzusch.[11] RocR plasmid was a gift from Zhao-Xun Liang.[25] Cells were then supplemented with 0.5 mM IPTG to induce expression. After the addition of IPTG, cells were incubated at 16 °C for 18 h. The cells were then centrifuged at 4 °C for 25 min, and the pellet was resuspended in lysis buffer (25 mM Tris-HCl, 500 mM NaCl, and 20 mM imidazole, pH 8.2, for MTB CdnP; DisA and YybT, 20 mM Hepes-KOH, pH 7.5, 400 mM NaCl, 30 mM imidazole, 10% glycerol, and 1 mM DTT for poxin) supplemented with 1 mM PMSF. The cells were lysed by sonication and centrifuged at 22 000 rpm for 25 min. The supernatants were passed through HisTrap HP 5 mL columns (GE). Wash buffer (25 mM Tris-HCl, pH 8.2, 500 mM, NaCl, and 50 mM imidazole for MTB CdnP; GBS CdnP and YybT, 20 mM Hepes-KOH, pH 7.5, 1 M NaCl, 30 mM imidazole, 10% glycerol, and 1 mM DTT for poxin) was then passed through the column. The wash step was repeated once. Proteins were eluted with 25 mM Tris-HCl, pH 8.2, 500 mM NaCl, and 200 mM imidazole for MTB CdnP, DisA and YybT, 20 mM Hepes-KOH, pH 7.5, 400 mM NaCl, 300 mM imidazole, 10% glycerol, and 1 mM DTT for poxin. Eluted proteins were then dialyzed, and the concentration was determined by measuring the absorbance at 280 nm.

c-di-AMP Synthesis. c-di-AMP used in this study was enzymatically synthesized using DisA. A 20 mL reaction volume containing 1 mM ATP and 2 μM DisA was set up. The reaction was incubated at 37 °C overnight. The reaction was stopped by denaturing at 95 °C for 5 min and then filtered using a 3 K centrifugal filter (VWR International), and c-di-AMP was purified using HPLC [COSMOSIL C18-MS-II packed column (running buffers: 0.1 M TEAA in water and acetonitrile)]. The gradient is as follows: (0−16 min) 99−87% 0.1 M TEAA, and 1−13% acetonitrile; (16−23 min) 87−10% 0.1 M TEAA and 13−90% acetonitrile; (23−25 min) 10−99% 0.1 M TEAA and 90−1% acetonitrile. Purified c-di-AMP was dried with a speed vacuum, and the residue was then resuspended in water. The c-di-AMP concentration was quantified by measuring the absorbance at 260 nm.

IC_{50} Determination. Reactions of a 20 μL volume containing 50 nM MTB CdnP, 100 μM c-di-AMP, 10 mM KI, 10 μM coralyne in 1× reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MnCl_2$) supplemented with 0.0025% Triton X-100 and varying concentrations of identified hits were set up in a Greiner FLUOTRAC 384 well plate. Experimental parameters were optimized to keep the hydrolysis in the linear range. Real time c-di-AMP hydrolysis kinetics was monitored by measuring the fluorescence intensity at λ_{em} = 475 nm (λ_{ex} = 420 nm) at 2 min intervals for 10 min. The reader (Biotek Cytation 5 multimode reader) chamber temperature was set to 30 °C. The slope of the curve was used to estimate the initial velocity. The experiment was carried out in triplicate. Curves were fitted with eq 1

$$y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{1 + 10^{(\log \text{IC50} - X)n}} \quad (1)$$

where n is the hill coefficient.

Calibration Curve Generation. Coralyne (10 μM) in 1× MTB CdnP reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MnCl_2$) was mixed with varying concentrations of c-di-AMP or AMP, and the fluorescence emission at 475 nm following excitation at 420 nm was measured.

High-Throughput Screening. MTB CndP reaction parameters were optimized to yield a Z factor in the 0.5−1 range. MTB CdnP (0.5 μM) and coralyne (10 μM) were used. The Z factor was computed with eq 2

$$Z = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \quad (2)$$

where σ_p and σ_n are the standard deviations of the positive control and negative control respectively, μ_p is the mean of the positive control, and μ_n is the mean of the negative control.

HTS was conducted at the Chemical Genomics Facility at the Purdue Institute for Drug Discovery. Reactions of 20 μL containing 70 μM c-di-AMP, 10 μM coralyne, 10 mM KI, 5 mM

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$MnCl_2$, 10 μM compound from the library, and 0.5 μM MTB CdnP in reaction buffer (50 mM Tris-HCl, pH 8.0) were set up in the following order. Ten microliters of a reaction mixture (1× reaction buffer, 2× c-di-AMP, 2× coralyne, 2× $MnCl_2$, and 2× KI) was dispensed into Greiner FLUOTRAC 384 plates. Compounds were then dispensed with a robot, and finally 10 μL of an enzyme mixture (2× MTB CdnP in 1× reaction buffer) was added to the plates. Fluorescence readings were taken immediately following the addition of the enzyme for kinetic-based screens. c-di-AMP hydrolysis was monitored by measuring the fluorescence intensity at λ_{em} = 475 nm (λ_{ex} = 420 nm) at 1 min intervals for 15 min. On the other hand, plates were incubated for 30 min at 37 °C. The reactions were then stopped by the addition of 30 mM EDTA, and the end-point fluorescence was measured.

HPLC Analysis. MTB CdnP reactions (70 μM c-di-AMP, 100 nM MTB CdnP, 1× reaction buffer) were set up in the presence and absence of the 20 μM compound and incubated at 37 °C for 3 or 12 h. Reactions were quenched by holding at 95 °C for 5 min. The reaction was then filtered using a 3 K centrifugal filter (VWR International) and analyzed using HPLC as described above.

For GBS CdnP, reactions containing 50 μM c-di-AMP, a 1× reaction buffer (50 mM Tris-HCl, pH 8.0, and 5 mM $MnCl_2$), and 100 nM GBS CdnP were set up in the presence and absence of **C82**. Reactions were incubated at room temperature for 15 min. Reactions were quenched by holding at 95 °C for 5 min. The reaction was then filtered using a 3 K centrifugal filter (VWR International) and analyzed using HPLC as described above.

For poxin, reactions containing 20 μM 2′3 cGAMP (Chemietek), a 1× reaction buffer (20 mM Hepes-KOH, pH 7.5), and 5 nM poxin were set up in the presence and absence of **C82**. Reactions were incubated at room temperature for 20 min. Reactions were quenched by heating at 95 °C for 5 min. The reaction was then filtered using a 3 K centrifugal filter (VWR International) and analyzed using HPLC as described above.

For Yybt, reactions containing 70 μM c-di-AMP, 1× reaction buffer (0.1 M Tri-HCl, pH 8.0, 20 mM KCl, and 0.5 mM $MnCl_2$), and 1 μM Yybt were set up in the presence and absence of **C82**. Reactions were incubated at 37 °C for 60 min and quenched by holding at 95 °C for 5 min. The reaction was then filtered using a 3 K centrifugal filter (VWR International) and analyzed using HPLC as described above.

For RocR, reactions containing 25 μM c-di-GMP, a 1× reaction buffer (100 mM Tris-HCl, pH 8.0, 20 mM KCl and 25 mM $MgCl_2$) and 10 nM RocR were set up in the presence and absence of **C82**. Reactions were incubated at 37 °C for 60 min and quenched by holding at 95 °C for 5 min. The reaction was then filtered using a 3 K centrifugal filter (VWR International) and analyzed using HPLC as described above.

For ENNP1, reactions containing 25 μM cGAMP, a 1× reaction buffer (50 mM Tris, pH 9.5, 250 mM NaCl), and 4.58 nM ENNP1 were set up in the presence and absence of **C82**. Reactions were incubated at 37 °C for 60 min. Reactions were quenched by holding at 95 °C for 5 min, filtered using a 3 K centrifugal filter (VWR International), and analyzed using HPLC as described above.

Radiolabeling Experiments. ^{32}P-c-di-AMP was synthesized by incubating 0.033 μM [α^{32}P]-ATP (PerkinElmer), 3 mM ATP, and 10 μM DisA in a buffer containing 40 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10 mM $MgCl_2$ at 37 °C overnight. The DisA reaction was stopped by heat denaturing. For the inhibition test, 0.5 μM MTB CdnP was incubated with 25 μM c-di-AMP, 3.3 nM ^{32}P-c-di-AMP, and varying concentrations of compounds in the MTB CdnP reaction buffer for 1 h at 37 °C. The reaction mixture (1 μL) was applied to a cellulose TLC plate (EMD Millipore) and left to dry at room temperature.

The TLC plates were developed in saturated $(NH_4)_2SO_4$: 1.5 M KH_2PO_4 buffer. The plates were then dried by heating on a hot plate at 95 °C, followed by overnight exposure on a GE Storage Phosphor screen. The screen was visualized with a Typhoon FLA 9500 biomolecular imager.

Intrinsic Fluorescence. Solutions containing 2 μM MTB CndP and varying concentrations of **C82** in 1× PBS were incubated for 30 min at room temperature. Fluorescence spectra were obtained by measuring the fluorescence intensities in the 310 to 400 nm range following excitation at 290 nm.

Differential Radial Capillary Action of a Ligand Assay (DRaCALA). Reaction mixtures containing 20 μM MTB CdnP, 1× reaction buffer (50 mM Tris-HCl, pH 8.0), 5 mM calcium chloride, 200 pM ^{32}P c-di-AMP, and varying concentrations of c-di-AMP or **C82** (total volume = 20 μL) were set up and incubated at room temperature for 10 min. The reaction mixture (5 μL) was pipetted onto a dry, untreated nitrocellulose membrane (GE Healthcare) and allowed to dry at room temperature. The nitrocellulose membrane was exposed to the GE Storage Phosphor screen for 8 h and then visualized with a TyphoonTM FLA 9500 biomolecular imager.

Fluorescence Polarization Competition Assay. Reaction mixtures containing 150 μM MTB CdnP, 1× reaction buffer (50 mM Tris-HCl, pH 8.0), 5 mM calcium chloride, 50 nM fluorescein labeled c-di-GMP (2′-Fluo-AHC-c-diGMP, Biolog), and varying concentrations of c-di-AMP or **C82** (total volume = 20 μL) were set up and incubated at room temperature for 10 min. The fluorescence polarization and anisotropy were determined as described above.

Cell Viability. Culture medium (200 μL) containing 4000 cells was pipetted into each well of a 96-well plate and incubated at 37 °C with 5% CO_2 for 18 h. The cells were then supplemented with compounds or dimethyl sulfoxide for the control group. The cells were incubated for an additional 24 h, and the cell viability was determined with the CellTiter-Blue Cell Viability Assay. Briefly, 10 μL of the CellTiter-Blue reagent was added to the wells and incubated for 4 h at 37 °C with 5% CO_2, and the cell viability was determined by measuring the fluorescence (excitation at 560 nm and emission at 590 nm) with a Biotek Cytation 5 multimode reader. The culture medium was Dulbecco's Modified Eagle's Medium + 10% fetal bovine serum. The cell lines were MDA-MB-231 and MRC-5.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00444.

Optimization of the coralyne assay for high-throughput screening, inhibition of MTB CdnP by compounds, activity of C82 against other cyclic dinucleotide PDEs, and effect of C82 against mammalian cell lines (PDF)

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Notes

The authors declare no competing financial interest.

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