Cyclic dinucleotides (CDNs) trigger the innate immune response in eukaryotic cells through the stimulator of interferon genes (STING) signaling pathway. To decipher this complex cellular process, a better correlation between structure and downstream function is required. Herein, we report the design and immunostimulatory effect of a novel group of c-di-GMP analogues. By employing an “atomic mutagenesis” strategy, changing one atom at a time, a class of gradually modified CDNs was prepared. These c-di-GMP analogues induce type-I interferon (IFN) production, with some being more potent than c-di-GMP, their native archetype. This study demonstrates that CDN analogues bearing modified nucleobases are able to tune the innate immune response in eukaryotic cells.

The innate immune system of eukaryotes possesses diverse mechanisms for detection of invading pathogens. One of the most fundamental processes relies on cell surface or intracellular receptors that recognize molecular patterns unique to microorganisms. Such pattern recognition receptors (PRRs) are capable of distinguishing pathogen-associated molecular patterns (PAMPs) from the molecular repertoire of endogenous microorganisms. Cyclic dinucleotides (CDNs), which play critical roles in the cGAS-STING and RECON innate immune signaling pathways, have been recognized as PAMPs in recent years.

STING was the first mammalian receptor identified that directly binds CDNs, most notably 2',3'-cGAMP, the product of the cytosolic DNA sensor cGAS. A conformational change upon ligand binding recruits and activates the kinase TBK1 (Figure 1), and phosphorylation of STING by TBK1 facilitates recruitment of transcription factor IRF3. When IRF3 itself gets phosphorylated by TBK1, it forms an activated homodimer that induces expression of type-I interferon (IFN α/β) and other cytokines within the nucleus (Figure 1). In addition to the TBK1–IRF3 pathway, STING can activate other signaling pathways, including NF-κB and STAT6.

Activation of STING by CDN analogues has shown pharmacological promise for improving the efficacy of cancer immunotherapies, including PD1 and CTLA-4 targeted drugs and CAR-T cell therapy. Consequently, medicinal chemistry efforts have sought to develop hydrolysis-resistant CDNs with longer cellular residency time by altering the ribose and/or the phosphate moieties. Chemical and chemoenzymatic approaches have been taken for the preparation of CDN analogues bearing either backbone or nucleobase modification. Analyses of the biological activities of such CDN analogues have provided valuable information on their binding properties to downstream sensors and augmented our knowledge regarding their structure–activity relationships.

In a recent publication, two fluorescent guanosine analogues developed in our laboratory were used to prepare novel emissive CDNs, in which atomic mutagenesis replaces the nucleobase’s imidazole ring with a thiophene or an isothiazole moiety (1G or 2G, respectively, Figure 1). Although this contribution primarily focused on the photophysical properties of the emissive CDNs, these compounds could also provide insight into CDNs and their biological recognition, as the G analogues differ by one atom, and along with native guanosine,

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**Figure 1.** CDN analogues and their immunostimulatory effects.

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thus present a gradually altered purine molecular architecture. To deepen our molecular level understanding of CDN signaling, we analyzed here the immunostimulatory effects of these systematically modified CDNs. We demonstrate that certain analogues can induce type-I IFN production more potently than their native archetype, highlighting potential new approaches to studying and manipulating the eukaryotic innate immune response.

The dimeric and mixed CDN analogues shown in Figure 1 were made from GTP, ^4^GTP and ^4^GTP by using DncV, a promiscuous dinucleotide cyclase from Vibrio cholerae (see Experimental Section and Figure S1 in the Supporting Information for data). The substrates and enzyme were incubated at 37°C for 2–5 h, after which the reaction mixture was heat-inactivated and filtered before subjecting it to reverse-phase HPLC separation and purification. Pure fractions were collected, combined and lyophilized. The CDNs were re-dissolved in water for downstream experiments. To preliminarily determine whether the synthetic c-di-GMP analogues could activate the IFN response in eukaryotic cells, THP-1 cells were treated with 5 μM of c-di-GMP, c-G^β^GMP and c-di-th-GMP. After 4 h incubation, induction of type-I IFN was measured with HEK-Blue IFN α/β reporter cells (see SI for experimental details). c-G^β^GMP induced type-I IFN production with comparable efficiency to c-di-GMP, while c-di-th-GMP showed no activity (Figure 2).

To analyze the immunostimulatory effects of all synthetic CDNs in greater detail, RAW 264.7 cells were treated with various concentrations of c-di-GMP, c-di^4^GMP, c-di^4^GMP, c-G^β^GMP and c-G^β^GMP and the phosphorylation of IRF3 to pIRF3 was evaluated. CDNs were thus transfected into RAW 264.7 murine cells with digitonin as described in previous studies.

Figure 2. Type-I IFN induced by CDNs in THP-1 cells. THP-1 cells were seeded at a density of 100,000 cells/well in a 96-well cell culture plate and differentiated with 25 nM of PMA for approximately 20 h prior to treatment with CDNs. Cells were transfected with 5 μM of CDNs in a permeabilization buffer containing 5 μg/mL of digitonin, then washed and incubated in RPMI medium with 2% FBS at 37°C for 4 h. 50 μL of cell culture supernatant per well was transferred to 150 μL of HEK-Blue IFN α/β reporter cells seeded at 50,000 cells/well in a 96-well cell culture plate and incubated at 37°C overnight. The reporter cells were spun down the next day, and 50 μL of cell culture supernatant per well was transferred to a 96-well plate and added with 150 μL of QUANTI-Blue SEAP detection medium (InvivoGen). The samples were then incubated at 37°C for 1 h 20 min before absorption was measured at 640 nm. The absorption signal of each sample was normalized to untreated samples. Two independent assays were performed in duplicate or triplicate. Error bars indicate SD.

Cells were then lysed with NP-40 buffer 2 h after transfection, and total protein was collected for immunoblotting against phosphorylated IRF3 (pIRF3) and β-actin. No pIRF3 was observed for untreated cells (UT) or digitonin-permeabilized cells (DG; Figure 3). Low concentrations (1 μM) of c-di-GMP did not induce obvious IRF3 activation, while 5 and 10 μM displayed comparable efficiency in inducing IRF3 phosphorylation. Increasing amounts of phosphorylated IRF3 were observed when cells were treated with higher concentrations of c-di-th-GMP and c-G^β^GMP, while no clear dose-response was observed for c-G^β^GMP (Figure 3a,b). The least isomorphic analogue, c-di-th-GMP, did not trigger observable IRF3 activation at any of the concentrations tested. Two other biological replicates produced similar trends (Figure S2).

As most synthetic c-di-GMP analogues activated IRF3, we analyzed their dose and time dependency for inducing IFN-β mRNA production by using RT-qPCR. RAW 264.7 cells were transfected with 1, 5 and 10 μM of CDNs as described above and incubated for 2, 4 and 6 h. Total RNA was isolated and used for RT-qPCR (see the Experimental Section in the Supporting Information). As shown in Figure 4a and b, c-di-GMP induced the most IFN-β mRNA production 4 h post transfection, whereas the highest response was observed after 2 h for c-di-th-GMP, c-G^β^GMP, and c-G^β^GMP. The same trend was observed for all three concentrations of CDNs tested (Figures 4a and 5a,b). The IFN response to c-di-th-GMP was minimal, but c-G^β^GMP showed the highest potency in inducing IFN-β mRNA production (Figures 4a–c and 5a–d) among all CDNs tested. After 2 h of incubation, 5 μM of c-G^β^GMP induced tenfold higher IFN-β mRNA production than c-di-GMP, the native messenger. The differences in activity displayed by the analogues and their dependency on the specific assay used are discussed below.

Apparent STING activation by c-di-GMP analogues that contain unnatural isomorphic nucleobases was assessed here by three methods: type I IFN production measured by a reporter cell line, IRF3 phosphorylation measured by western blotting, and IFN-β mRNA production measured by RT-qPCR. The initial analysis was performed in THP-1, a human cell line, whereas

Figure 3. IRF3 phosphorylation induced by c-di-GMP and its analogues. a) IRF3 phosphorylation induced by c-di-GMP analogues. 1, 5 and 10 μM of each CDN was used to transfect RAW 264.7 cells. Cells were lysed with NP-40 lysis buffer 2 h post transfection, 20 μg of total protein was loaded on SDS-polyacrylamide gel. Proteins were transferred to PVDF membrane after gel electrophoresis, and immunoblotted against pIRF3 and β-actin. b) Quantification of western blot. The y-axis indicates relative intensity of pIRF3 compared to β-actin.
more detailed analyses were performed in RAW 264.7, a murine cell line. Our results show that all analogues except for c-di-GMP stimulated the STING pathway in RAW 264.7 cells. The other three analogues appear to stimulate IRF phosphorylation at comparable or higher levels than the parent c-di-GMP 2 h post transfection.

To quantitatively analyze activation of the STING pathway, CDN-induced, IFN-β production was measured by RT-qPCR in RAW 264.7 cells. As seen in Figure 4, IFN-β induction drops in the order: c-G\(^6\)GMP > c-di-G\(^3\)GMP > c-G\(^4\)GMP > c-di-G\(^7\)GMP > c-di-G\(^5\)GMP, although it is apparent the cellular processes show complex concentration/time dependency. The effect of CDN concentrations above 5 μM plateaued except for c-G\(^4\)GMP. Importantly, however, peak IFN-β responses occurred at different times for different analogues, with the synthetic analogues c-di-G\(^3\)GMP, c-G\(^4\)GMP and c-G\(^5\)GMP inducing earlier and stronger maximum IFN-β response compared to the native c-di-GMP (Figures 4 and S3 a,b). This pattern might result from negative feedback mechanisms of the CDNs-activated STING pathway and type-I IFN signaling.

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Figure 4. a) IFN production induced by c-di-GMP and its analogues. RAW 264.7 cells were transfected with 1, 5, 10 μM of c-di-GMP, c-di-G\(^3\)GMP, c-G\(^4\)GMP, c-di-G\(^5\)GMP and c-G\(^6\)GMP and incubated for 2, 4, 6 h before being lysed by TRIzol. RNA purification and RT-qPCR were conducted following the protocol described in the Experimental Section. b) IFN response after 2, 4, 6 h of incubation with 5 μM of CDNs. c) IFN response to 1, 5, and 10 μM of CDNs after 2 h of incubation. Two independent assays were performed in triplicates (n = 2). Error bars indicate SD.
Conflict of Interest

The authors declare no conflict of interest.

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Cyclic dinucleotides (CDNs) play critical regulatory roles in bacteria and trigger the innate immune response in eukaryotic cells. Here we illustrate that a systematic modification of the nucleobases, rather than the phosphate or sugar moieties, can generate STING agonists that demonstrate strong immunostimulatory effects.

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