Tuning the Innate Immune Response to Cyclic Dinucleotides by Using Atomic Mutagenesis

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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.[1] One of the most fundamental processes relies on cell surface or intracellular receptors that recognize molecular patterns unique to microorganisms.[2] Such pattern recognition receptors (PRRs) are capable of distinguishing pathogen-associated molecular patterns (PAMPs) from the molecular repertoire of endogenous host "self" patterns.[3] PRR engagement of PAMPs can then trigger the innate immune response to enhance antimicrobial activity, and further modulate the adaptive immune response.[2–4] 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.[5] STING was the first mammalian receptor identified that directly binds CDNs,[6] most notably 2′,3′-cGAMP, the product of the cytosolic DNA sensor cGAS.[7] A conformational change upon ligand binding recruits and activates the kinase TBK1 (Figure 1),[8] 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).[9] In addition to the TBK1–IRF3 pathway, STING can activate other signaling pathways, including NF-κB and STAT6.[9–10]

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.[11] 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.[12] Chemical and chemoenzymatic approaches have been taken for the preparation of CDN analogues bearing either backbone or nucleobase modification.[13] 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.[13–14]

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 (thG or ^{tz}G, respectively, Figure 1).[15] Although this contribution primarily focused on the photophysical properties of the emissive CDNs,[15c] 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,

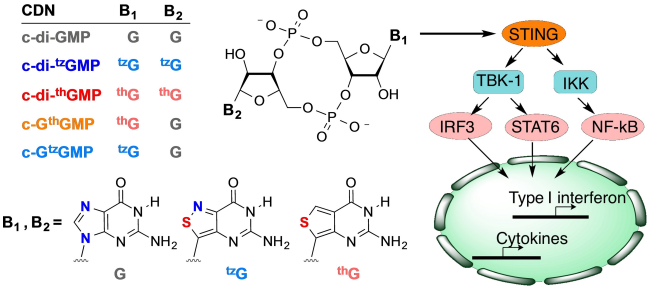

Figure 1. CDN analogues and their immunostimulatory effects.

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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, thGTP and ^{tz}GTP by using DncV, a promiscuous dinucleotide cyclase from *Vibrio cholerae* (see Experimental Section and Figure S1 in the Supporting Information for data).[13b] 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.[15c] 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-GthGMP and c-di-thGMP. 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-GthGMP induced type-I IFN production with comparable efficiency to c-di-GMP, while c-di-thGMP 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-^{tz}GMP, c-di-thGMP, c-GthGMP and c-G^{tz}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.[7b,16]

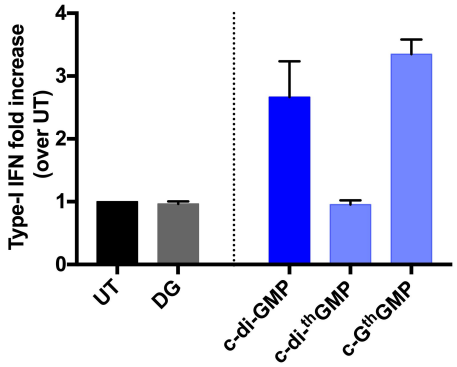

Figure 2. Type-I IFN induced by CDNs in THP-1 cells. THP-1 cells were seeded at a density of 100 000 cells/well in a 96-well cell culture plate and differentiated with 25 nM of PMA for approximately 20 h prior to treatment with CDNs. Cells were transfected with 5 μM of CDNs in a permeabilization buffer 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-^{tz}GMP and c-G^{tz}GMP, while no clear dose-response was observed for c-GthGMP (Figure 3a,b). The least isomorphic analogue, c-di-thGMP, 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-^{tz}GMP, c-GthGMP, and c-G^{tz}GMP. The same trend was observed for all three concentrations of CDNs tested (Figures 4a and S3a,b). The IFN response to c-di-thGMP was minimal, but c-GthGMP showed the highest potency in inducing IFN-β mRNA production (Figures 4a–c and S3a–d) among all CDNs tested. After 2 h of incubation, 5 μM of c-GthGMP 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

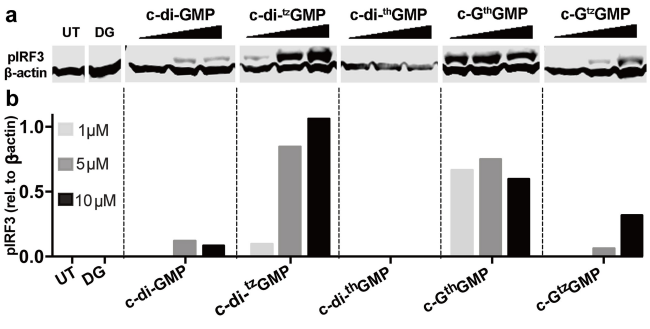

Figure 3. IRF3 phosphorylation induced by c-di-GMP and its analogues. a) IRF3 phosphorylation induced by c-di-GMP analogues. 1, 5 and 10 μM of each CDN was used to transfect RAW 264.7 cells. Cells were lysed with NP-40 lysis buffer 2 h post transfection, 20 μg of total protein was loaded on SDS-polyacrylamide 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 compare to β-actin.

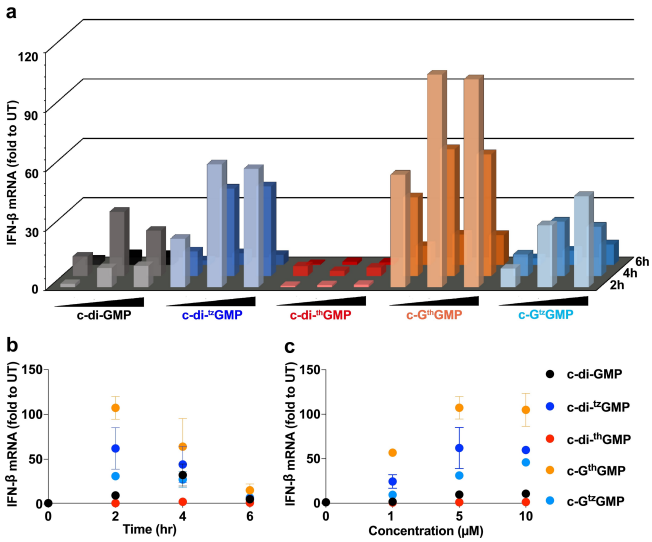

Figure 4. a) IFN production induced by c-di-GMP and its analogues. RAW 264.7 cells were transfected with 1, 5, 10 μM of c-di-GMP, c-di-^{tz}GMP, c-GthGMP, c-di-GthGMP and c-G^{tz}GMP, and incubated for 2, 4, 6 h before being lysed by TRIzol. RNA purification and RT-qPCR were conducted following the protocol 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.

more detailed analyses were performed in RAW 264.7, a murine cell line. Our results show that all analogues except for c-di-thGMP 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 time- and dose-dependent manners. As seen in Figure 4, IFN-β induction drops in the order: c-GthGMP > c-di-^{tz}GMP > c-G^{tz}GMP > c-di-GMP > c-di-thGMP, 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^{tz}GMP. Importantly, however, peak IFN-β responses occurred at different times for different analogues, with the synthetic analogues c-di-^{tz}GMP, c-G^{tz}GMP and c-GthGMP 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.[17] We speculate that rapid and potent IFN induction might concomitantly activate early negative feedback responses, which ultimately result in down tuning IFN-β production.

Among the synthetic CDNs tested in RAW 264.7 cells, the two analogues containing thG, the least isomorphic G surrogate that lacks the basic N7 in the native purine scaffold, displayed dramatically different potency in activating the STING pathway, with c-di-thGMP appearing essentially inactive, while c-GthGMP exerting the strongest effect on IFN-β induction of all analogues tested. This stark difference was also observed in the THP-1 human cell line, although c-GthGMP showed comparable potency to c-di-GMP (Figure 2). Our findings could reflect differences in the assay themselves. RT-qPCR detects IFN-β mRNA levels and not necessarily the translated active protein levels, whereas the reporter assay detects secreted type I interferons, including both IFN α and β. Additionally, the difference between cell lines could be rationalized by the existence of multiple STING alleles in human cells compared to murine cells, which possess different sensitivity to CDNs.[7c,18]

The observed intensity and duration of the cellular signaling response reflect both the affinity of the ligand to STING, as well as its resistance to intracellular degradation processes (assuming negligible differences in transfection efficiencies). It is perhaps not surprising that c-di-thGMP does not serve as a potent STING agonist, as it is the least isomorphic CDN analogue, with two altered purine cores. However, retaining one native G residue, as in c-GthGMP, restores STING activation. Although speculative, this result is consistent with observations made for other asymmetric CDNs, 3′,3′-cGAMP and 2′,3′-cGAMP, that also induce more potent STING activation than c-di-GMP.[8a,12a,19] Either through enhanced binding affinity to STING or potentially increased resistance to hydrolytic degradation, c-GthGMP induces a faster and greater innate immune response relative to the native signal c-di-GMP in murine cells. It would be worthwhile to further investigate the binding affinity of these CDN analogues to different STING variants, to further build correlations with their biological activity. In this context, the intrinsic fluorescence of our modified CDNs could potentially provide an effective tool to facilitate such studies.

Modifying the phosphate and sugar moieties of CDNs has been explored as a strategy to alter the pharmacological potency of STING agonists. Most of the noncognate base-modified CDNs have not been tested in immune response assays.[14f] Here we illustrate that a systematic modification of the nucleobases, rather than the phosphate or sugar moieties, can generate STING agonists that are more potent than c-di-GMP. Particularly intriguing is the high potency of the mixed analogues c-GthGMP and c-G^{tz}GMP, where only one of the native guanosine residues is replaced by an unnatural synthetic C-nucleoside. Recognizing the complexity and intricacies of such cellular pathways, these observations put forth new approaches for the implementation of novel CDN analogues with altered recognition features, where the potency and duration of the triggered cellular immune response can potentially be tuned.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: cyclic dinucleotides · innate immune response · modified nucleosides · STING agonists

[1] R. Medzhitov, C. A. Janeway Jr, *Cell* **1997**, *91*, 295–298.
[2] S. Akira, *Immunol. Rev.* **2009**, *227*, 5–8.
[3] a) S. Gordon, *Cell* **2002**, *111*, 927–930; b) O. Takeuchi, S. Akira, *Cell* **2010**, *140*, 805–820.
[4] A. Iwasaki, R. Medzhitov, *Science* **2010**, *327*, 291–295.
[5] a) M. Motwani, S. Pesiridis, K. A. Fitzgerald, *Nat. Rev. Genet.* **2019**, *20*, 657–674; b) A. P. McFarland, S. Luo, F. Ahmed-Qadri, M. Zuck, E. F. Thayer, Y. A. Goo, K. Hybiske, L. Tong, J. J. Woodward, *Immunity* **2017**, *46*, 433–445; c) A. Ablasser, Z. J. Chen, *Science* **2019**, *363*, eaat8657.
[6] D. L. Burdette, K. M. Monroe, K. Sotelo-Troha, J. S. Iwig, B. Eckert, M. Hyodo, Y. Hayakawa, R. E. Vance, *Nature* **2011**, *478*, 515–518.
[7] a) L. Sun, J. Wu, F. Du, X. Chen, Z. J. Chen, *Science* **2013**, *339*, 786–791; b) J. Wu, L. Sun, X. Chen, F. Du, H. Shi, C. Chen, Z. J. Chen, *Science* **2013**, *339*, 826–830; c) E. J. Diner, D. L. Burdette, S. C. Wilson, K. M. Monroe, C. A. Kellenberger, M. Hyodo, Y. Hayakawa, M. C. Hammond, R. E. Vance, *Cell Rep.* **2013**, *3*, 1355–1361.
[8] a) P. Gao, M. Ascano, T. Zillinger, W. Wang, P. Dai, A. A. Serganov, B. L. Gaffney, S. Shuman, R. A. Jones, L. Deng, G. Hartmann, W. Barchet, T. Tuschl, D. J. Patel, *Cell* **2013**, *154*, 748–762; b) Q. Chen, L. Sun, Z. J. Chen, *Nat. Immunol.* **2016**, *17*, 1142–1149; c) S. Liu, X. Cai, J. Wu, Q. Cong, X. Chen, T. Li, F. Du, J. Ren, Y.-T. Wu, N. V. Grishin, Z. J. Chen, *Science* **2015**, *347*, aaa2630.
[9] S. R. Margolis, S. C. Wilson, R. E. Vance, *Trends Immunol.* **2017**, *38*, 733–743.
[10] a) H. Chen, H. Sun, F. You, W. Sun, X. Zhou, L. Chen, J. Yang, Y. Wang, H. Tang, Y. Guan, W. Xia, J. Gu, H. Ishikawa, D. Gutman, G. Barber, Z. Qin, Z. Jiang, *Cell* **2011**, *147*, 436–446; b) T. Abe, G. N. Barber, *J. Virol.* **2014**, *88*, 5328–5341.
[11] a) J. Fu, D. B. Kanne, M. Leong, L. H. Glickman, S. M. McWhirter, E. Lemmens, K. Mechette, J. J. Leong, P. Lauer, W. Liu, K. E. Sivick, Q. Zeng, K. C. Soares, L. Zheng, D. A. Portnoy, J. J. Woodward, D. M. Pardoll, T. W. Dubensky Jr, Y. Kim, *Sci. Transl. Med.* **2015**, *7*, 283ra252; b) A. Li, M. Yi, S. Qin, Y. Song, Q. Chu, K. Wu, *J. Hematol. Oncol.* **2019**, *12*, 35.
[12] a) L. Corrales, L. H. Glickman, S. M. McWhirter, D. B. Kanne, K. E. Sivick, G. E. Katibah, S. R. Woo, E. Lemmens, T. Banda, J. J. Leong, K. Metchette, T. W. Dubensky Jr., T. F. Gajewski, *Cell Rep.* **2015**, *11*, 1018–1030; b) L. Li, Q. Yin, P. Kuss, Z. Maliga, J. L. Millan, H. Wu, T. J. Mitchison, *Nat. Chem. Biol.* **2014**, *10*, 1043–1048.
[13] a) B. Novotná, L. Vaneková, M. Zavřel, M. Buděšínský, M. Dejmek, M. Smola, O. Gutten, Z. A. Tehrani, M. Pimková Polidarová, A. Brázdová, R. Liboska, I. Štěpánek, Z. Vavřina, T. Jandušík, R. Nencka, L. Rulíšek, E. Bouřa, J. Brynda, O. Páv, G. Birkuš, *J. Med. Chem.* **2019**, *62*, 10676–10690; b) K. D. Launer-Felty, S. A. Strobel, *Nucleic Acids Res.* **2018**, *46*, 2765–2776.
[14] a) C. Wang, M. Sinn, J. Stifel, A. C. Heiler, A. Sommershof, J. S. Hartig, *J. Am. Chem. Soc.* **2017**, *139*, 16154–16160; b) C. A. Shanahan, S. A. Strobel, *Org. Biomol. Chem.* **2012**, *10*, 9113–9129; c) Y. Luo, J. Zhou, S. K. Watt, V. T. Lee, T. K. Dayie, H. O. Sintim, *Mol. BioSyst.* **2012**, *8*, 772–728; d) J. Zhou, Y. Zheng, B. T. Roembke, S. M. Robinson, C. Opoku-Temeng, D. A. Sayre, H. O. Sintim, *RSC Adv.* **2017**, *7*, 5421–5426; e) B. T. Roembke, J. Zhou, Y. Zheng, D. Sayre, A. Lizardo, L. Bernard, H. O. Sintim, *Mol. BioSyst.* **2014**, *10*, 1568–1575; f) P. Clivio, S. Coantic-Castex, D. Guillaume, *Chem. Rev.* **2013**, *113*, 7354–7401.
[15] a) D. Shin, R. W. Sinkeldam, Y. Tor, *J. Am. Chem. Soc.* **2011**, *133*, 14912–14915; b) A. R. Rovira, A. Fin, Y. Tor, *J. Am. Chem. Soc.* **2015**, *137*, 14602–14605; c) Y. Li, P. T. Ludford III, A. Fin, A. R. Rovira, Y. Tor, *Chem. Eur. J.* **2020**, *27*, 6076–6084.
[16] S. E. Girardin, I. G. Boneca, L. A. M. Carneiro, A. Antignac, M. Jéhanno, J. Viala, K. Tedin, M. Taha, A. Labigne, U. Zäthringer, A. J. Coyle, P. S. DiStefano, J. Bertin, P. J. Sansonetti, D. J. Philpott, *Science* **2003**, *300*, 1584–1587.
[17] a) H. Konno, K. Konno, G. N. Barber, *Cell* **2013**, *155*, 688–698; b) R. A. Porritt, P. J. Hertzog, *Trends Immunol.* **2015**, *36*, 150–160.
[18] a) P. Gao, T. Zillinger, W. Wang, M. Ascano, P. Dai, G. Hartmann, T. Tuschl, L. Deng, W. Barchet, D. J. Patel, *Cell Rep.* **2014**, *8*, 1668–1676; b) G. Yi, V. P. Brendel, C. Shu, P. Li, S. Palanathan, C. Cheng Kao, *PLoS One* **2013**, *8*, e77846.
[19] X. Zhang, H. Shi, J. Wu, X. Zhang, L. Sun, C. Chen, Z. J. Chen, *Mol. Cell* **2013**, *51*, 226–235.

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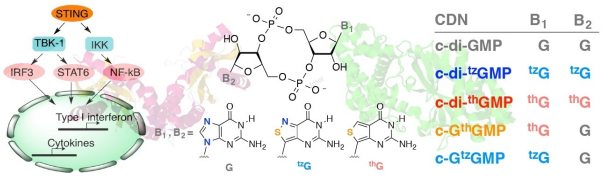

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Tuning the Innate Immune Response to Cyclic Dinucleotides by Using Atomic Mutagenesis

Back to base: 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.