Tuning the Innate Immune Response to Cyclic Dinucleotides Using Atomic Mutagenesis

Supporting Information

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1 Experimental section

Enzymatic synthesis, purification and quantification of CDN analogs.[1] Guanosine 5'-triphosphates analogs (500 μM) were incubated with DncV (2.3 μM) in a buffer (0.1 M NaCl, 40 mM Tris pH 7.5 and 10 mM $MgCl_2$) at 37°C for 2–5 h, or room temperature overnight. The reaction mixture was then heated at 90 °C for 5 min and chilled on ice for 15 minutes and filtered through a 0.22 μm filter. The supernatant was separated by Sepax Bio C-18 column (250 × 10mm, 5 μm particle size) with a gradient of 0.5–20% of 10 mM NH_4OAc, pH 7 in MeOH in 20 minutes on an Agilent 1200 or 1260 series HPLC system (Agilent Technologies). Collected HPLC fractions were lyophilized with Labconco FreeZone 2.5 lyophilizer and re-dissolved in autoclaved water. UV spectroscopy was used to determine the concentration of each solution with the following extinction coefficients as previously reported:[1b] 26000 L mol^{-1} cm^{-1} for c-di-GMP (260 nm), 8370 L mol^{-1} cm^{-1} for c-di-^{tz}GMP (333 nm), 7470 L mol^{-1} cm^{-1} for c-di-thGMP (321 nm), 3735 L mol^{-1} cm^{-1} for c-GthGMP (333 nm), and 4185 L mol^{-1} cm^{-1} for c-G^{tz}GMP (333 nm).

Cell cultures. The RAW 264.7 cells were cultured in DMEM (Gibco) supplied with 2 mM L-glutamine, 10% fetal bovine serum (FBS) (Sigma), with or without 1% penicillin and streptomycin. The THP-1 cells were cultured in RPMI (Gibco) supplied with 2 mM L-glutamine, 10% heat-inactivated FBS, 10 mM HEPES (Gibco), 1 mM sodium pyruvate (Gibco), 0.25% glucose (Sigma), 0.05 mM 2-mercaptoethanol (Gibco). The HEK-Blue human type-I IFN reporter cells were cultured in DMEM (Gibco) supplied with 4.5 g/L glucose, 2 mM L-glutamine, 10% heat-inactivated FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL Normocin. The cells were maintained at 37℃ under an atmosphere of 5% CO_2/95% air.

Immunoblotting. RAW 264.7 cells were plated on 24-well plates (5 × 10^5 cells per well) and incubate at 37°C for 48 h. Cells were then transfected with 1–10 μM of CDN in a permeabilization buffer containing 10 μg/mL digitonin, 50 mM HEPES, pH 7, 100 mM KCl, 3 mM $MgCl_2$, 85 mM sucrose, 1 mM ATP, 0.1 mM DTT, 0.2% BSA for 30 mins at 37°C, then incubated with regular growth medium for 2 h. Cells were lysed with NP-40 lysis buffer containing protease inhibitor cocktail (Roche), PhosSTOP (Roche) and PMSF, and total protein was collected and quantified by BCA assay. Protein extracts were resolved by SDS-PAGE with 7.5% gel and transferred to PVDF membrane. Proteins were detected with the following primary antibodies: rabbit anti-pIRF3 monoclonal antibody (Cell Signaling Technology), mouse anti-β actin monoclonal antibody (Sigma-Aldrich).

RT-qPCR. RAW 264.7 cells were plated on 48-well plates (2.5 × 10^5 cells per well) and transfected with CDN using the same method as above after 48 h of incubation at 37°C. Cells were then incubate with regular growth medium for designated time, and total RNA was isolated with TRIzol• reagent and purified with RNeasy mini kit (Qiagen) following the manufacturer's protocol. Following elution, RNA yields were evaluated using a Nanodrop spectrophotometer (Nanodrop technologies). RNA samples were

converted to cDNA with SuperScript III First-Strand Synthesis kit (Invitrogen) with random hexamers following the manufacturer's protocol. Quantitative PCR (SYBR Green) analysis was performed in duplicates on an Applied Biosystems 7300 Real-time PCR system (Invitrogen). Transcription level of IFN-β gene (fwd primer: CAG CTC CAA GAA AGG ACG AAC, rev primer: GGC AGT GTA ACT CTT CTG CAT) of each sample was normalized to housekeeping gene TBP (forward primer: GAAGCTGCGGTACAATTCCAG, reverse primer: CCCC-TTGTACCCTTCACCAAT) and then to untreated samples (UT) using the delta-delta CT method.

Detecting type-I IFN production with reporter 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 phorbol myristate acetate (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, 50 mM HEPES, pH 7, 100 mM KCl, 3 mM $MgCl_2$, 85 mM sucrose, 1 mM ATP, 0.1 mM DTT, 0.2% BSA and then washed and incubated in RPMI medium with 2% FBS at 37 ℃ for 4 h. 50 μL of the cell culture supernatant per well was transferred to 150 μL of HEK-Blue human type-I IFN reporter cells seeded at 50,000 cells/well in a 96-well cell culture plate and incubated at 37℃ 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) prepared according to the manufacturer's instructions. The samples were then incubated at 37℃ for 80 min before absorption was measured at 640 nm with an EnSpire plate reader (PerkinElmer). The absorption signal of each sample was normalized to untreated samples (UT).

2 Supporting figures

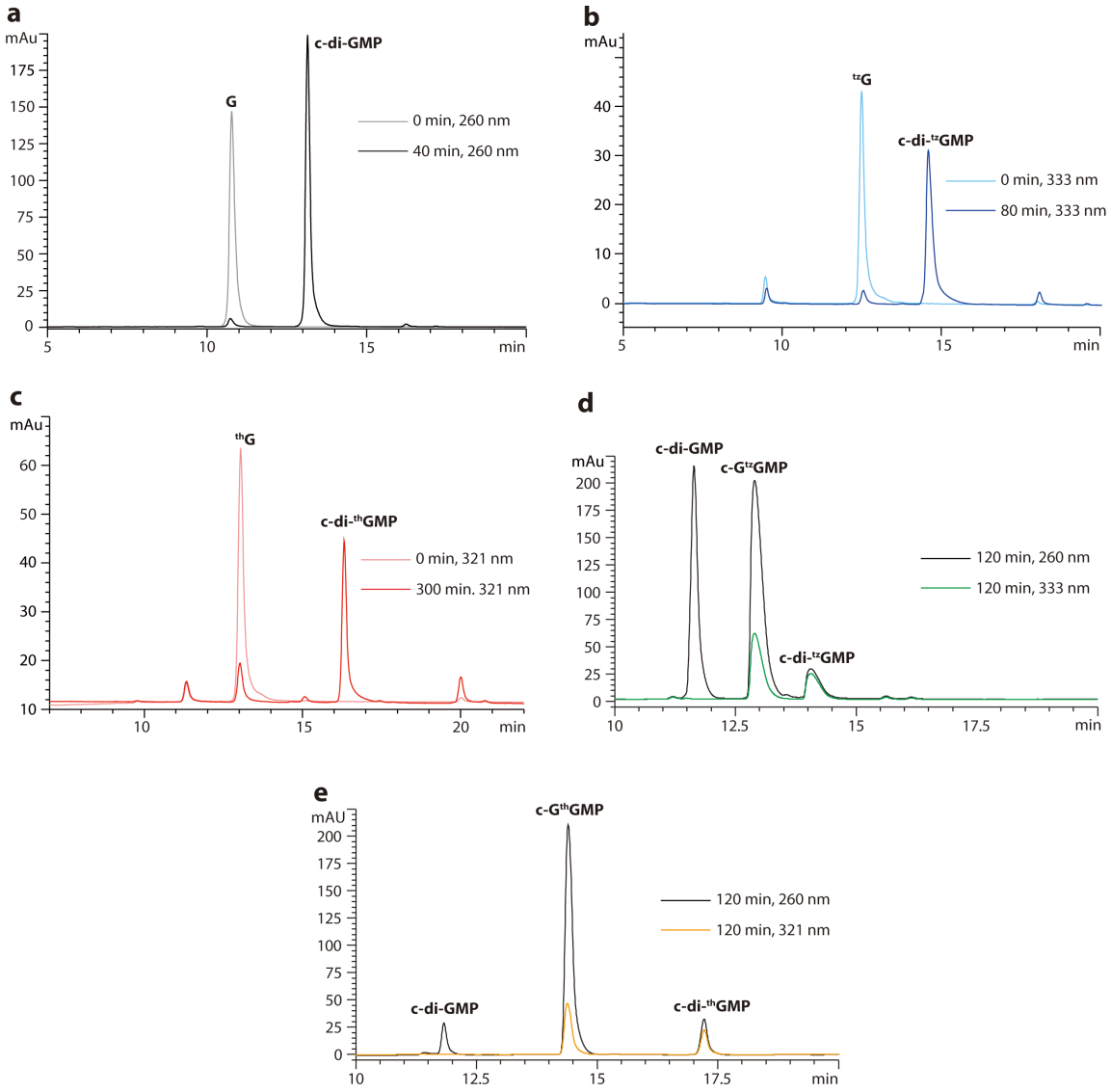

Figure S1. HPLC traces of DncV-mediated CDN syntheses. Reactions were treated with calf intestinal alkaline phosphatase (CIAP) at designated times; the starting materials were therefore presented as G, ^{tz}G and thG. (a–c) HPLC traces of DncV-mediated c-di-GMP, c-di-^{tz}GMP and c-di-thGMP synthesis reactions, respectively, with Sepax Bio C18 analytical column. (d–e) HPLC traces of DncV-mediated c-G^{tz}GMP, c-GthGMP synthesis reactions, respectively, with Sepax Bio C18 semi-prep column.

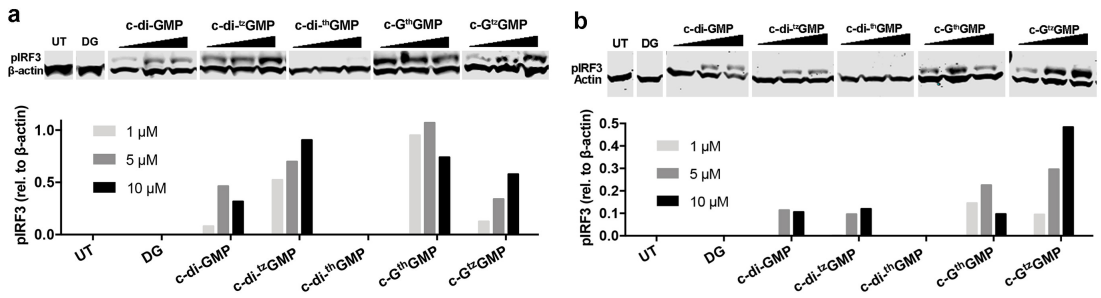

Figure S2. Two independent trials of pIRF3 formation induced by c-di-GMP analogues quantified by western blotting. See experimental section for details.

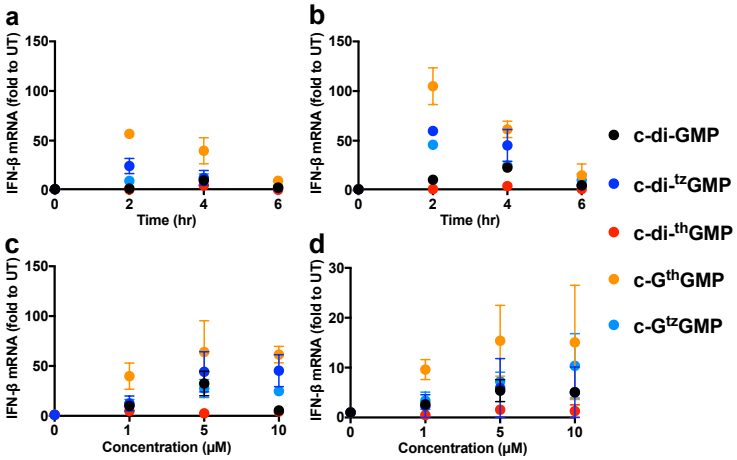

Figure S3. IFN production induced by c-di-GMP and its analogues. RAW 264.7 cells were transfected with 1, 5, 10 μM of c-di-GMP, c-di-^{tz}GMP, c-GthGMP, c-di-GthGMP and c-G^{tz}GMP respectively, and incubated for 2, 4, 6 hours respectively before lysed by TRIzol reagent. RNA purification and RT-qPCR were conducted following the protocol in the Experimental section. (a), (b), IFN response after 2, 4, 6 hours of incubation with 1, 10 μM of CDNs, respectively. (c), (d), IFN response to 1, 5, 10 μM of CDNs after 4, 6 hours of incubation. Assays were done in duplicates. Error bars indicate SD.

3 References

[1] a) K. D. Launer-Felty, S. A. Strobel, *Nucleic Acids Res.* **2018**, *46*, 2765–2776; b) Y. Li, P. T. Ludford, A. Fin, A. R. Rovira, Y. Tor, *Chem. Eur. J.* **2020**, *Accepted Author Manuscript*, doi: 10.1002/chem.202001194.