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Article

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Design, synthesis, and biological evaluation of novel cyclic adenosine-inosine monophosphate (cAIMP) analogs that activate stimulator of interferon genes (STING)

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ABSTRACT

We describe novel STING-activating cyclic dinucleotides whose constituent nucleosides are adenosine and inosine, and that vary by ribose substitution, internucleotide linkage position and phosphate modification. In mammalian cells *in vitro*, some of these cAIMP analogs induce greater STING-dependent IRF and NF-κB pathway signaling than do the reference agonists for murine (DMXAA) or human (2',3'-cGAMP) STING. In human blood *ex vivo*, they induce type I interferons (IFNs) and pro-inflammatory cytokines: for the former, 3',3'-cAIMP (**9**; EC_{50}: 6.4 μM), and analogs **52-56** (EC_{50}: 0.4 μM to 4.7 μM), which contain one or two 2'-fluoro-2'-

deoxyriboses and/or bis-phosphorothioate linkages, are more potent than 2',3'-cGAMP (EC_{50}: 19.6 μM). Interestingly, **9** induces type I IFNs more strongly than do its linkage isomers 2',3'-cAIMP (**10**), 3',2'-cAIMP (**23**) and 2',2'-cAIMP (**27**). Lastly, some of the cAIMP analogs are more resistant than 2',3'-cGAMP to enzymatic cleavage *in vitro*. We hope to exploit our findings to develop STING-targeted immunotherapies.

INTRODUCTION

Stimulator of interferon genes (STING) is an adaptor protein that is paramount in innate immunity, especially in orchestrating the body's responses to cytoplasmic DNA, whether pathogen DNA (*e.g.* viral or microbial), self DNA (*e.g.* mitochondrial or junk) or tumor DNA. It is widely expressed in immune cells (*e.g.* dendritic cells, macrophages and B cells) and endothelial cells, among others. Activation of STING leads to production of type I interferons such as IFN-α and IFN-β, via the interferon regulatory factor (IRF) pathway; and to production of pro-inflammatory cytokines such as tumor-necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β), via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. Human STING has four known natural agonists, which correspond to the four known naturally occurring cyclic dinucleotides (CDNs; Figure 1). Three of these CDNs (c-diGMP, c-diAMP and cGAMP) are released by microbes into host cells during infection[1]. However, the fourth, 2',3'-cGAMP, is produced by the DNA sensor cyclic GMP-AMP synthase (cGAS) in mammalian cells after it detects self or foreign DNA in the cytoplasm (see, for example: Ablasser *et al.*[2] and Zhang *et al.*[3]). Microbial CDNs contain a (3',5')(3',5') phosphodiester linkage (denoted as "3',3'"), whereas the mammalian CDN contains a (2',5')(3',5') linkage (denoted as "2',3'").

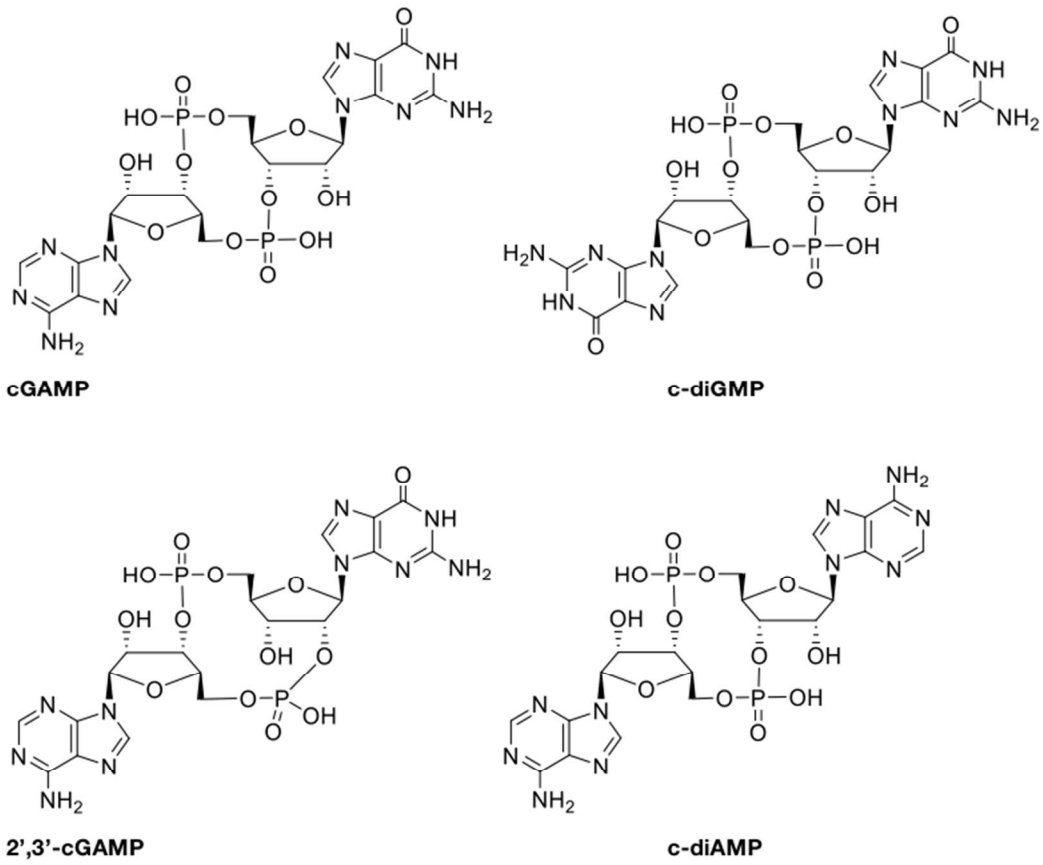


Figure 1. The known natural agonists of STING are the four known natural CDNs. Clockwise from top left: the microbial (3',3') CDNs cGAMP, c-diGMP and c-diAMP, and the mammalian CDN 2',3'-cGAMP.

Since its discovery in 2008[4], STING has been widely imputed in infectious diseases, cancer and auto-immunity[5]. Accordingly, it is attracting increasing attention as a potential immunotherapy target. Synthetic STING agonists that exhibit favorable drug-like properties are currently being developed as adjuvants, anti-tumor agents and other immunotherapy agents. In fact, a CDN STING agonist has very recently entered Phase I clinical trials for monotherapy of diverse solid tumors[6].

Herein we report the design, synthesis, and biological screening of novel CDNs that activate STING signaling. In the context of immunotherapy drug development, we sought to develop CDNs that could offer certain advantages over natural CDNs in terms of STING-dependent cytokine induction potency and selectivity, and drugability. Unlike natural CDNs, whose constituent nucleosides are guanosine and/or adenosine, the CDNs we describe here contain one adenosine nucleoside and one inosine nucleoside. Beginning with the parent molecule 3',3'-cAIMP (**9**), we designed, synthesized and biologically tested eleven cAIMP analogs that vary by sugar (ribose, 2'-deoxyribose or 2'-fluoro-2'-deoxyribose) and by internucleotide linkage position (2',2'; 2',3'; 3',3'; or 3',2') and phosphate modification (bis-phosphodiester or bis-phosphorothioate). We screened all eleven analogs for STING-dependent activities *in vitro* in two mammalian cell lines: RAW 264.7 murine macrophages (for induction of the IRF pathway) and THP-1 human leukemic monocytes (for induction of the IRF and NF-κB pathways). We also screened these analogs for *ex vivo* production of type I IFNs and pro-inflammatory cytokines in human blood from healthy donors. Lastly, we explored the resistance of the cAIMP analogs to cleavage *in vitro* by two enzymes: nuclease P1 (NP1) and snake-venom phosphodiesterase (SVPD). We summarize our findings and discuss our preliminary structure-activity relationship (SAR) observations on these cAIMP analogs, highlighting those features that appear to enhance STING-pathway activity and/or resistance to enzymatic cleavage relative to 2',3'-cGAMP.

RESULTS

Design and synthesis of cAIMP analogs

We began our study by reflecting on the fact that all four known natural CDNs are based on the purine bases guanosine and/or adenosine. We decided to explore how using another purine

nucleoside, inosine, in CDNs might influence STING-pathway agonism. Thus, we designed, synthesized and screened eleven cAIMP analogs, whose structures are summarized in Figure 2 and Table 1. Unless otherwise specified, the cAIMP analogs of the present study were synthesized using methods described, or adapted from those described, in the following references: Hyodo *et al.*[7], Gaffney *et al.*[8], Rammler *et al.*[9], Shanahan *et al.*[10], Zhou *et al.*[11] and Ora *et al.*[12].

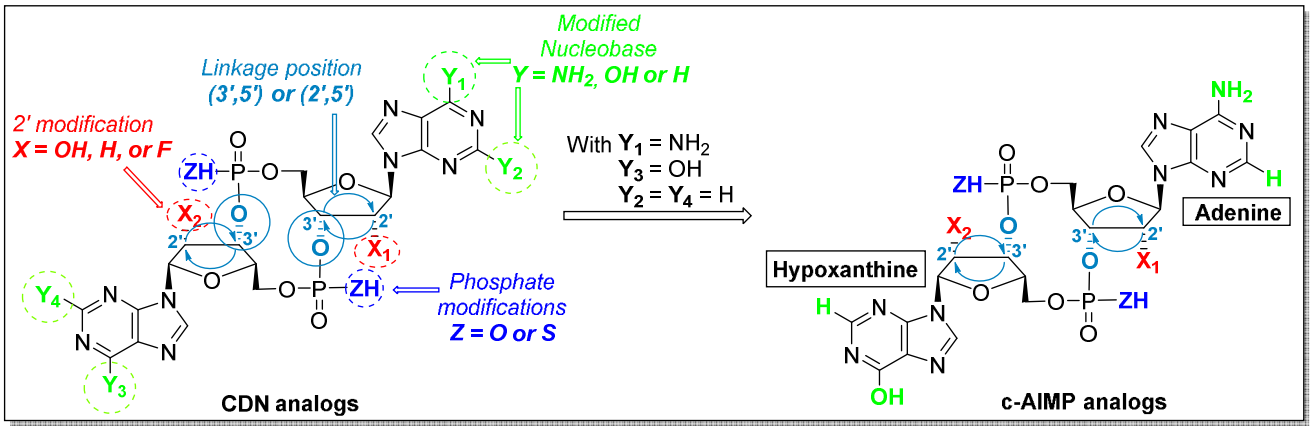


Figure 2. General structure of the cAIMP analogs of the present study.

Table 1. Structures of the cAIMP analogs of the present study

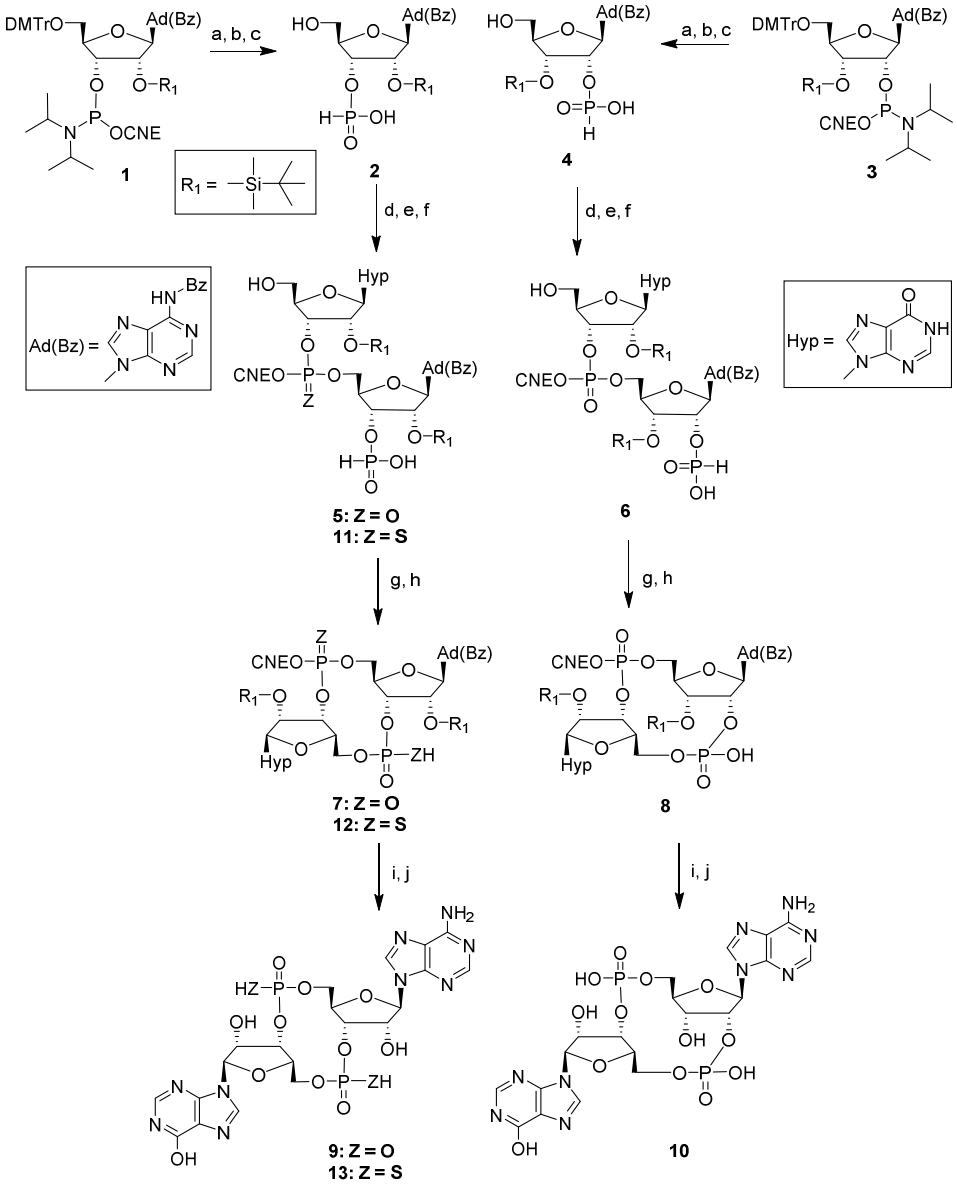
Compound	Linkage	X_1	X_2	Z
9	3',3'	OH	OH	O
10	2',3'	OH	OH	O
23	3',2'	OH	OH	O
27	2',2'	OH	OH	O
13	3',3'	OH	OH	S

51	3',3'	H	H	O
52	3',3'	F	F	O
53	3',3'	F	F	S
54	3',3'	H	F	O
55	3',3'	F	H	O
56	3',3'	F	H	S

We started by preparing **9**, its linkage isomer **10** and its bis-phosphorothioate analog 3',3'-cAIM(PS)$_2$ (**13**) (Scheme 1). Synthesis began with the commercially available compounds N^6-Bz-2'-*O*-TBDMS-5'-*O*-DMTr adenosine phosphoramidite (**1**) or N^6-Bz-3'-*O*-TBDMS-5'-*O*-DMTr adenosine phosphoramidite (**3**). Phosphoramidites **1** and **3** were converted to their corresponding *H*-phosphonates and subsequently detritylated with dichloroacetic acid to give the 3'-*H*-phosphonate **2** and the 2'-*H*-phosphonate **4**, respectively. Then, 3'-*O*-TBDMS-5'-*O*-DMTr inosine phosphoramidite was coupled with the 5'-*O*-free alcohol of **2** or **4**, using an ACN solution of Activator 42® (0.1 M) as promoter in the presence of 3 Å molecular sieves (MS 3 Å). The resulting phosphite products were oxidized with either 5.5 M *tert*-butyl hydroperoxide (*t*BuOOH) in decane, or sulfurized phenylacetyl disulfide (PADS). The 5'-*O*-DMTr group in each compound was removed by treatment with a 20% solution of dichloroacetic acid in dichloromethane to give the linear dimers **5** and **11** (from **2**), and **6** (from **4**). The dimers were then cyclized in a high-dilution mixture of 5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane (DMOCP) in dry pyridine, and then treated with either iodine and water (for bis-phosphodiester)

or elemental sulfur (for bis-phosphorothioate diester) to provide the corresponding fully protected CDNs **7**, **8** and **12**. Finally, these CDNs were deprotected by treatment with 33% methylamine in EtOH at 50 °C to remove the benzoyl (Bz) and cyanoethyl protecting groups, and then treated with $Et_3N{\cdot}3HF$ in pyridine, and triethylamine, to remove the *tert*-butyldimethylsilyl (TBDMS) protecting groups, to give the target compounds **9**, **10** and **13**.

Scheme 1. Preparation of the cAIMP analogs **9**, **10** and **13**[a]

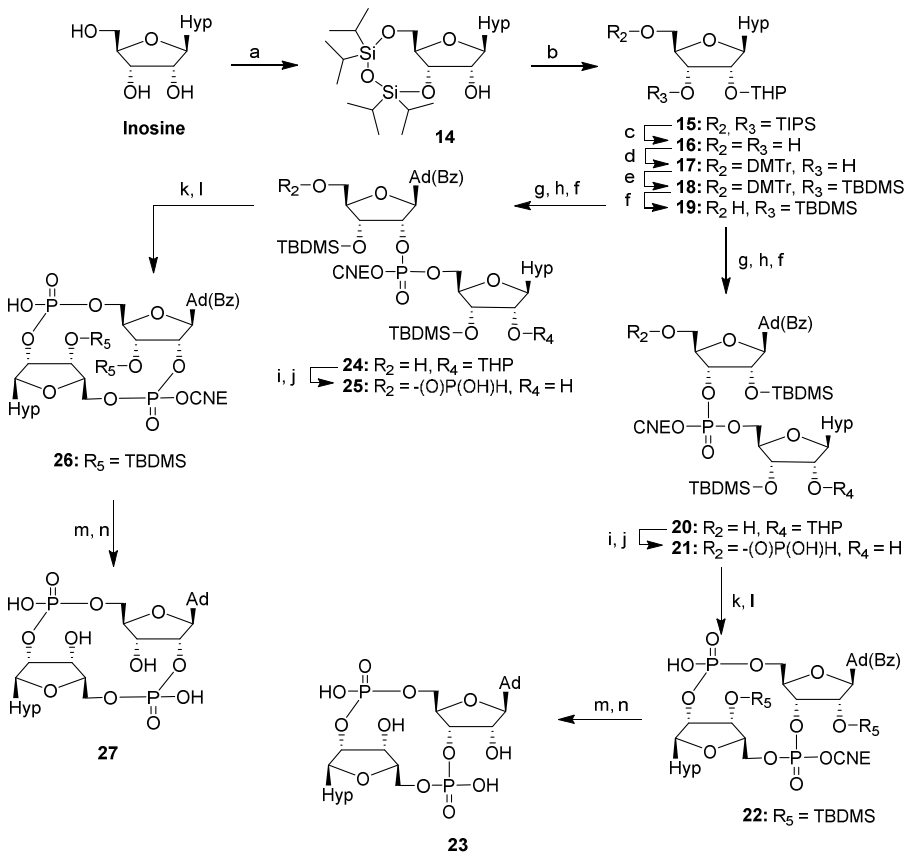


[a]Reagents and conditions: (a) H_2O, pyridinium trifluoroacetate (Pyr·TFA), dry acetonitrile (ACN), RT; (b) *tert*butyl amine (*t*$BuNH_2$); (c) dichloroacetic acid (DCA), H_2O, dichloromethane (CH_2Cl_2); (d) 5'-*O*-DMTr-2'-*O*-TBDMS-inosine phosphoramidite, Activator 42®, ACN, 3 Å molecular sieves (MS); (e) 5.5 M *tert*-butyl hydroperoxide (*t*BuOOH) in decane for Z = O, or phenylacetyl disulfide (PADS) for Z = S; (f) DCA, H_2O, CH_2Cl_2; (g) 5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane (DMOCP), pyridine (Pyr); (h) iodine (I_2), H_2O for Z = O or S_8

for Z = S; (i) methylamine ($MeNH_2$) 33% in ethanol (EtOH), RT or 50 °C; and (j) triethylamine (Et_3N), pyr. $Et_3N \cdot 3HF$.

To synthesize the next two analogs (Scheme 2), the cAIMP linkage isomers (3',2')cAIMP (**23**) and (2',2')cAIMP (**27**), we began by simultaneously protecting the hydroxy groups in the 3' and 5' positions of commercially available inosine via treatment with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (TIPSCl)[13, 14]. The resulting protected nucleoside **14** was treated with 3,4-dihydro-2*H*-pyran and pyridinium *p*-toluenesulfonate (PPTS) in dry CH_2Cl_2 to give the corresponding fully protected inosine intermediate **15**, which was subsequently treated with tetrabutylammonium fluoride (TBAF) on silica gel in tetrahydrofuran (THF) at room temperature to afford 2'-*O*-THP-inosine (**16**). After sequential protection of the 5' position with DMTr and the 3' position with TBDMS, the 5' position of the resulting intermediate was deprotected in acidic media to afford compound **19**. Coupling of **19** with phosphoramidites **1** or **3** gave the dimers **21** or **25**, respectively. These dimers were then converted into their corresponding 5-*H*-phosphonates and deprotected in acid media to remove the THP groups. Finally, the resulting intermediates were cyclized and deprotected as described for preparation of **9**, to give **23** and **27**.

Scheme 2. Preparation of the cAIMP analogs **23** and **27**[a]

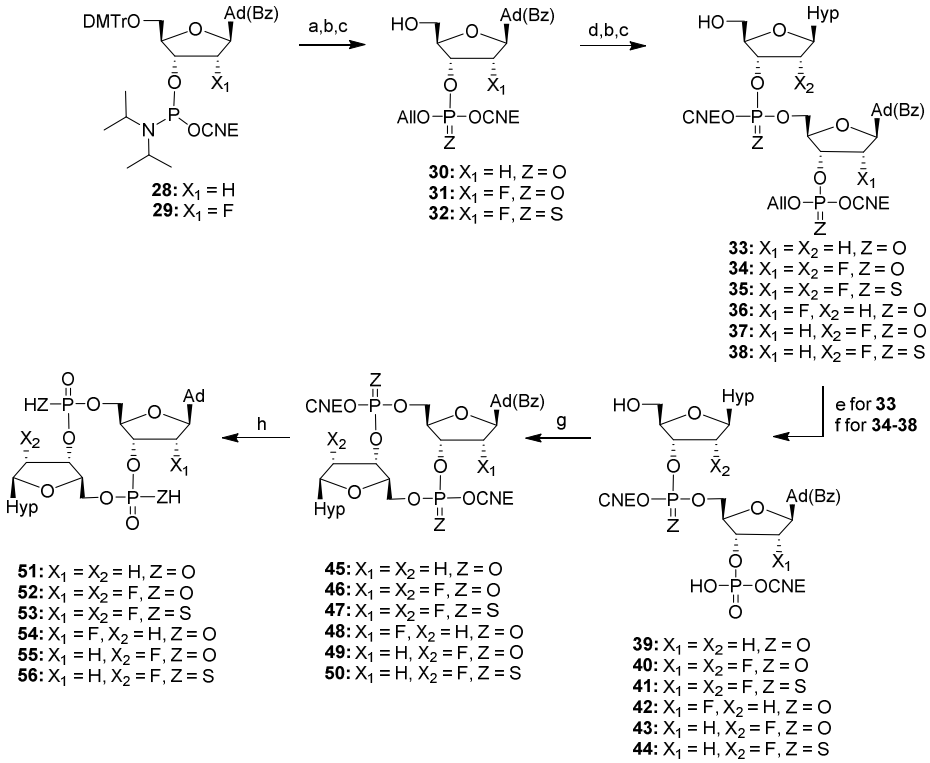


[a]Reagents and conditions: (a) $TIPSCl_2$, Pyr; (b) 3,4-dihydro-2*H*-pyran, PPTS, dry CH_2Cl_2; (c) TBAF on silica gel, THF; (d) DMTr-Cl, Pyr/CH_2Cl_2 (4:1); (e) TBDMS-Cl, imidazole, Pyr; (f) $ZnBr_2$ (0.5 M) *i*PrOH/CH_2Cl_2 (1:1); (g) adenosine 3'- or 2'-*O*-phosphoramidite, Activator 42®, ACN, 3 Å MS; (h) 5.5M *t*BuOOH in decane; (i) diphenylphosphite, pyr. TEAA 0.1 M; (j) DCA 10% in CH_2Cl_2; (k) DMOCP, Pyr; (l) I_2, H_2O; (m) 33% $MeNH_2$ in EtOH, RT or 50 °C; and (n) triethylamine (Et_3N), pyr. $Et_3N{\cdot}3HF$.

Finally, we synthesized (Scheme 3) c-(dAMP-dIMP) (**51**), c-(2'FdAMP-2'FdIMP) (**52**), c-[2'FdAMP(S)-2'FdIMP(S)] (**53**), c-(dAMP-2'FdIMP) (**54**), c-(2'FdAMP-dIMP) (**55**) and c-[2'FdAM(PS)-dIM(PS)] (**56**), all of which, relative to cAIMP, differ by the 2'-substitution at

either or both sugars, and by the internucleotide linkage position and phosphate substitution. Thus, inosine phosphoramidites **28** and **29** were each condensed with the 5'-*O*-free nucleoside allyl phosphotriesters **30-32**, using the standard procedure, to afford the corresponding linear dimers **33-38**. Subsequently, the allyl group in each dimer was removed by treatment of sodium iodide in acetone (for compound **33**), or tetrakis(triphenylphosphine)palladium(0) and *N*-methylaniline in THF (for compounds **34-38**). The resulting products were cyclized with DMOCP in dry pyridine. Finally, the cyclic compounds **45-50** were fully deprotected by successive treatment with 33% methylamine in ethanol to afford the desired analogs **51-56**. Due to their high polarity, these compounds had to be purified using reverse-phase HPLC.

Scheme 3. Preparation of cAIMP analogs **51**-**56**[a]



[a]Reagents and conditions: (a) allyl alcohol, Activator 42®, ACN, 3 Å MS; (b) 5.5 M *t*BuOOH) in decane for Z = O or PADS for Z = S; (c) DCA, H_2O, CH_2Cl_2; (d) 5'-*O*-DMTr-2'-X_2-inosine phosphoramidite, Activator 42®, ACN, 3 Å MS; (e) NaI, acetone, reflux, 2 h; (f) tetrakis(triphenylphosphine)palladium(0), *N*-methylaniline, THF; (g) 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT), pyridine; (h) 33% $MeNH_2$ in EtOH.

Biological Evaluation.

***In vitro* screening of cAIMP analogs in a murine macrophage cell line**

We first screened the cAIMP analogs for IRF pathway induction in a murine immune cell line that expresses STING, and then compared their activities to that of the reference agonists for murine (DMXAA[15,16]) and human (2',3'-cGAMP) STING. We used the murine macrophage reporter cell line RAW-Lucia™ ISG, which reports on IRF pathway activation as an indirect measure of type I IFN induction. Each cAIMP analog induced IRF activation to distinct levels (Table 2). Interestingly, compared to DMXAA (EC_{50}: 35.2 μM) and 2',3'-cGAMP (EC_{50}: 18.8 μM), most of the cAIMP analogs gave a superior IRF response, with **53** (EC_{50}: 0.6 μM) and **56** (EC_{50}: 2.2 μM) exhibiting strikingly higher activity. We next screened the analogs in the corresponding STING knockout cell line, RAW-Lucia™ ISG-KO-STING. As expected, none of them exhibited any IRF activity in the knockout cells (data not shown). Together, these findings indicate that in a murine immune cell line, the cAIMP analogs trigger STING-dependent IRF pathway induction and that their activities differ to that of DMXAA and 2',3'-cGAMP.

Table 2. Biological activities of the cAIMP analogs of the present study, and of DMXAA and 2',3'-cGAMP[a,b]

Assay	RAW-Lucia™ ISG		THP1-Dual™				Blood	
Readout	IRF		IRF		NF-κB		Type I IFNs	
Compound	EC_{50} (μM)[a]	E_{max} (%)[b]	EC_{50} (μM)[a]	E_{max} (%)[b]	EC_{50} (μM)[a]	E_{max} (%)[b]	EC_{50} (μM)[c]	E_{max} (%)
9	8.4 ± 5.7	107.0 ± 24	5.1 ± 1.4	134 ± 13	15.9 ± 4.1	97 ± 21	6.4 ± 2.3	96 ± 8
10	15.9 ± 12.2	85.0 ± 2	15.1 ± 0.8	126 ± 2	60.6 ± 2.6	116 ± 1	11.2 ± 3.8	101 ± 13
23	6.1 ± 4.8	85 ± 9	12.5 ± 0.3	141 ± 1	54.3 ± 12.1	100 ± 9	15.5 ± 4.9	104 ± 13
27	17.4 ± 12.3	95 ± 1	17.9 ± 1.2	115 ± 2	110.9 ± 10.2	162 ± 15	20.5 ± 5.5	100 ± 17
13	4.0 ± 2.7	115 ± 30	1.6 ± 1.3	94 ± 7	7.8 ± 1.3	100 ± 24	10.6 ± 3.9	104 ± 9
51	11.6 ± 4.3	91 ± 1	10 ± 4.1	133 ± 5	62.3 ± 18.7	109 ± 12	9.9 ± 2.5	108 ± 12
52	5.1 ± 0.1	90 ± 14	1.1 ± 0.4	79 ± 4	15.4 ± 3.2	99 ± 23	0.7 ± 0.1	101 ± 4
53	0.6 ± 0.4	104 ± 5	0.3 ± 0.2	81 ± 15	1.6 ± 0.3	94 ± 26	0.4 ± 0.1	103 ± 3
54	9.1 ± 7.1	99 ± 7	1.4 ± 0.4	91 ± 1	25.5 ± 0.5	93 ± 8	3.6 ± 1.1	98 ± 8
55	5.0 ± 2.6	93 ± 36	2.3 ± 0.9	81 ± 3	32.3 ± 10.2	105 ± 18	2.8 ± 0.9	103 ± 4
56	2.2 ± 0.5	83 ± 1	1.9 ± 1.7	87 ± 24	9.9 ± 0.2	106 ± 25	4.7 ± 2.1	103 ± 5
DMXAA	35.2 ± 14.1	68 ± 40	n.t.	n.t.	n.t	n.t.	n.t	n.t.
2',3'-cGAMP	18.8 ± 9.7	100	7.2 ± 3.2	100	39.1 ± 22.6	100	19.6 ± 6.7	100

a. Each value is the mean ± SD from three independent experiments. b. E_{max} values are reported as a mean percentage ± SD relative to the corresponding 2',3'cGAMP response from three independent experiments. c. Each value is the mean ± SD from three independent experiments on a total of fifteen healthy donors.

In vitro screening of cAIMP analogs in a human monocyte cell line

We next evaluated the ability of the cAIMP analogs to induce both the IRF and the NF-κB pathways in a human immune cell line that expresses STING, and then compared their activities to that of 2',3'-cGAMP (in this case we did not test DMXAA, as it does not bind to human STING[16]). We employed the THP-1 human monocyte reporter cell line THP1-Dual™, which uses a dual-reporter system to report on IRF activation as an indirect measure of type I IFN induction, and NF-κB activation as an indirect measure of pro-inflammatory cytokine induction. Each cAIMP analog activated both pathways to varying degrees (Table 2). In terms of IRF activity relative to that of 2',3'-cGAMP (EC_{50}: 7.2 μM), six of the cAIMP analogs (**13** and **52-56**) exhibited greater than threefold activity, whereas the remaining five showed roughly similar activity. Alternatively, for NF-κB pathway activation, **13**, **53** and **56** were also more active (> threefold) than 2',3'-cGAMP (EC_{50}: 39.1 μM), with **53** showing nearly 20-fold greater activity; in contrast, the remaining analogs had similar or lower activity than 2',3'-cGAMP (in all cases, EC_{50}: *ca.* 15.4 μM to 110.9 μM). Together, these findings indicate that in a human immune cell line, the cAIMP analogs induce the IRF and the NF-κB pathways and that some of them are more active than 2',3'-cGAMP at activating either or both pathways.

Ex vivo screening of cAIMP analogs in human blood

Having demonstrated that the cAIMP analogs trigger STING signaling *in vitro*, we then assessed their cytokine-induction activities in human blood *ex vivo*. To this end, we tested blood samples collected from healthy donors (three experiments; five donors per experiment) by incubating them with either a cAIMP analog or 2',3'-cGAMP at a range of CDN concentrations, and then evaluating the induction of type I IFNs (IFN-α and IFN-β), type III interferons (IL-28 a/b), interleukin 1 (IL-1) and interleukin 6 (IL-6). Figure 3A shows dose-response curves for these activities for **9**, as a representative example. However, the only activity for which we were able to calculate EC_{50} values was type I IFN induction (see Table 2 and Figure 3B), as the other activities were characterized by a lack of saturating responses, even at the highest test dose. In terms of inducing type I IFNs, every one of the cAIMP analogs showed either significantly greater (up to ~ 45-fold; $p < 0.05$), or similar, activity relative to 2',3'-cGAMP (EC_{50}: 19.6 μM ± 6.7 μM) (Table 2 and Figure 3B). The most active analogs were **53** (EC_{50}: 0.4 μM ± 0.1 μM; $p = 0.003$) and **52** (EC_{50}: 0.7 μM ± 0.1 μM; $p = 0.002$), which also gave the strongest IL-1 production (see Figure 3C). In contrast, **9**, whose EC_{50} for type I IFN induction activity (6.4 μM ± 2.3 μM; $p = 0.046$) was three times lower than that of 2',3'-cGAMP, was highly selective for this activity over induction of IL-1 (see Figure 3C). Together, the above findings indicate that in whole blood from healthy human donors, the cAIMP analogs induce type I IFNs and other cytokines to varying degrees and that they exhibit distinct cytokine induction activities to that of 2',3'-cGAMP. Importantly, some of these analogs preferentially induce type I IFNs over IL-1, whereas others give a more balanced response.

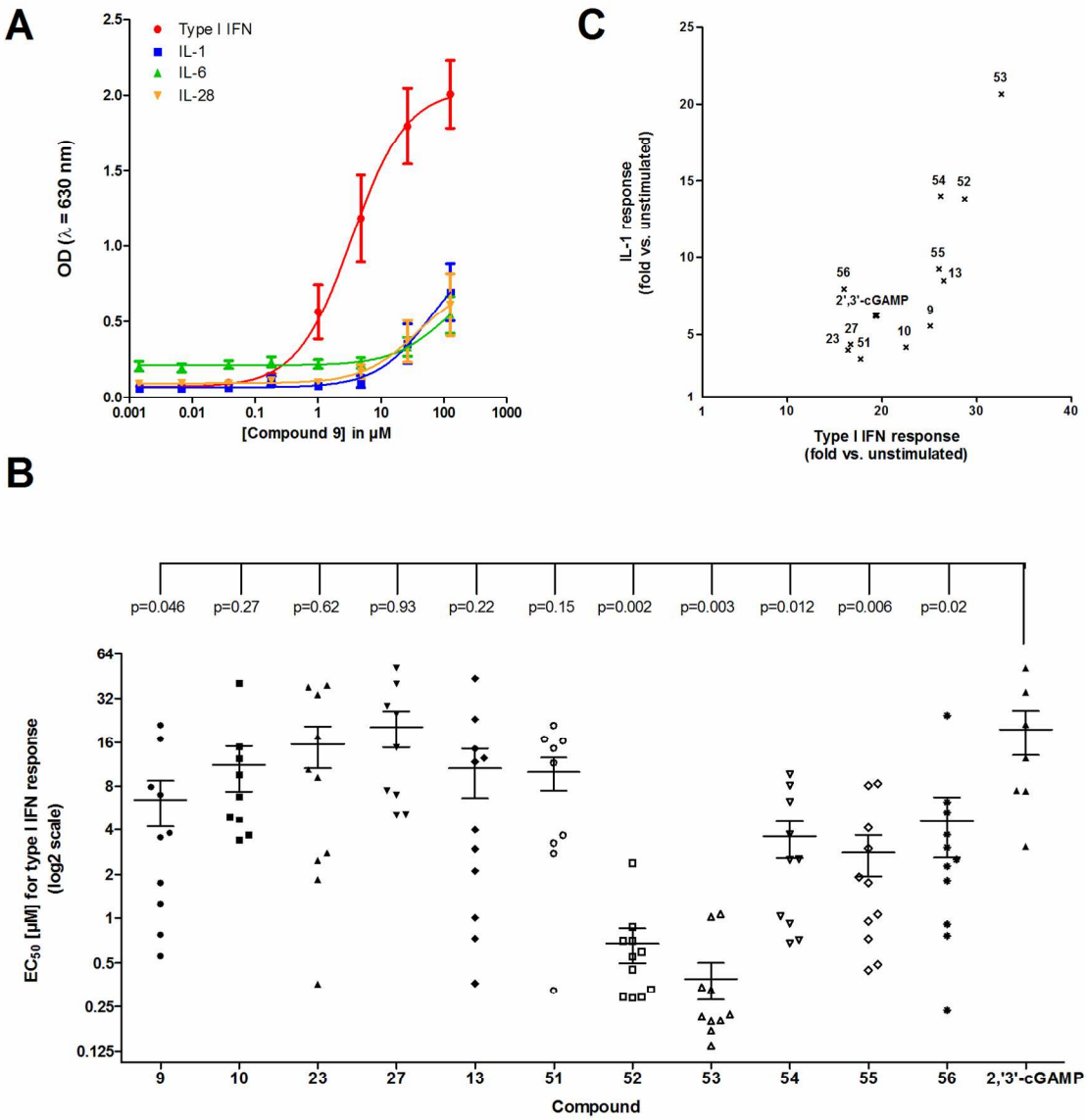


Figure 3. *Ex vivo* cytokine induction in human blood. (A) Mean cytokine induction for stimulation of blood with **9**. Dose-response curves for production of type I IFNs, IL-6, IL-28 or IL-1 were calculated using GraphPad Prism software. (B) Plot of EC_{50} values for induction of type I IFNs for the indicated compounds (points: values for individual donors; bars: mean values ± SEM acquired from three independent experiments). Significance ($p < 0.05$) was determined by a two-tailed, unpaired *t*-test relative to 2',3'-cGAMP. (C) Type I IFN induction vs. IL-1

induction at a CDN concentration of 10 μg/mL. Each value represents the mean from a total of fifteen donors (in three experiments), expressed as fold induction (normalized to the mean value for unstimulated cells).

Enzymatic stability

As previously mentioned, we are endeavoring to design and synthesize STING-activating CDNs that can offer advantages over natural CDN STING agonists, not only in terms of activity, but also as concerns drugability. Considering that CDNs can be degraded by circulating and cytoplasmic enzymes such as nucleases and phosphodiesterases (see, for example: Shanahan *et al.*[17]), which can ultimately influence their PK, we subjected our cAIMP analogs and 2',3'-cGAMP to a preliminary *in vitro* enzyme cleavage assay. To this end, we incubated each analog for up to 2 h with either nuclease P1 (NP1) or snake venom phosphodiesterase (SVPD), both of which cleave CDNs at their phosphodiester linkages[18, 19]. To measure the resistance of each CDN to each enzyme, we monitored the reactions by HPLC. In terms of resistance to NP1, some of the cAIMP analogs were more resilient than 2',3'-cGAMP (83% remaining): in fact, **13**, **52** and **53** did not show any signs of degradation (Figure 4, top). The remaining analogs showed a similar level of resistance to that of 2',3'-cGAMP, except for **9**, **23** and **51**, which had been totally, or near totally, degraded within 2 hours, exhibiting half-lives ($T_{1/2}$) of 23.1 minutes (**9**), 7.1 minutes (**23**) and 5.7 minutes (**51**). Regarding resistance to SVPD, all of the cAIMP analogs exhibited either markedly greater (**13**, **51**, **52**, **53**, **55** and **56**), or similar, stability relative to 2',3'-cGAMP. Interestingly, even after 2 hours incubation, **53** and **56** had not undergone any degradation (Figure 4). These results reveal that some of the cAIMP analogs are more stable than 2',3'-cGAMP to cleavage by NP1 and/or SVPD.

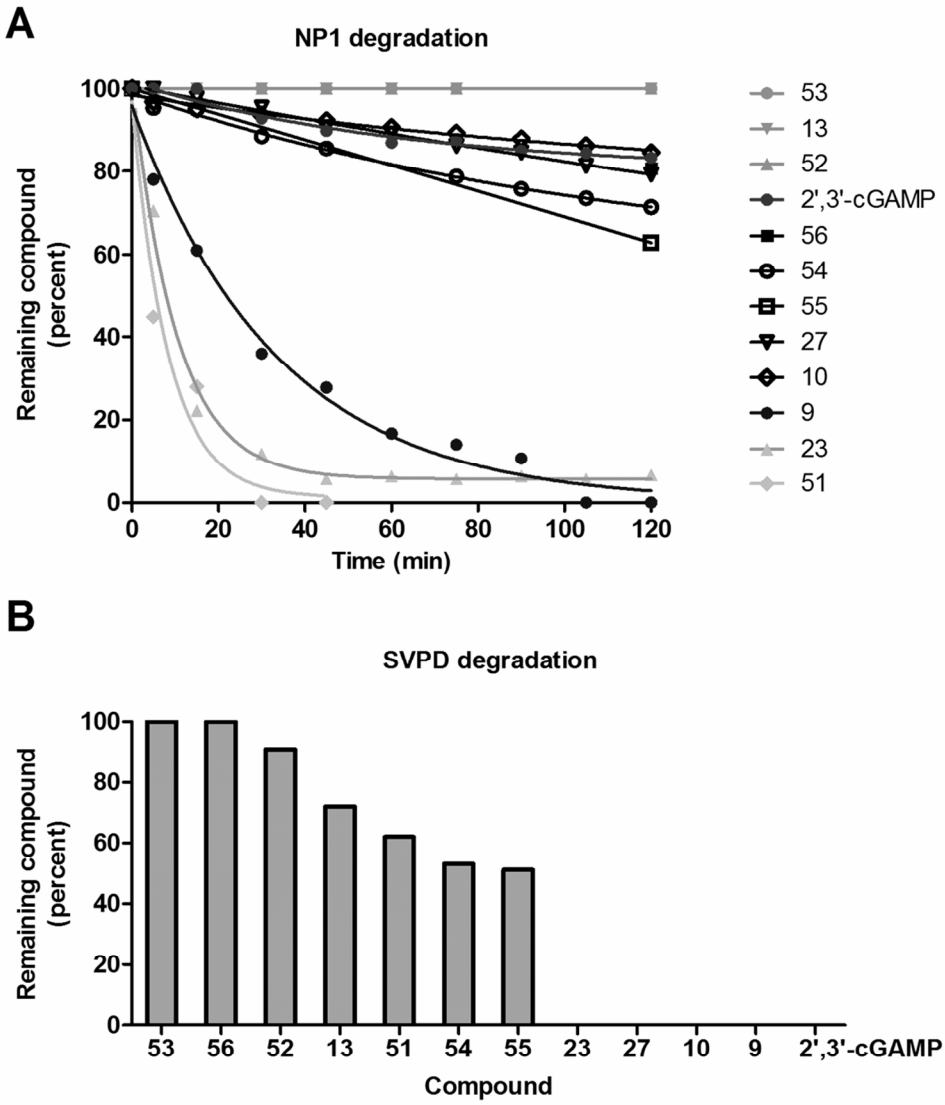


Figure 4. *In vitro* enzymatic cleavage resistance assays. The cAIMP analogs and 2',3'-cGAMP were each incubated for 2 h with either (A) nuclease P1 (NP1) or (B) snake venom phosphodiesterase (SVPD). The amount of remaining CDN was determined by HPLC (% peak area).

DISCUSSION

We provide here a preliminary SAR analysis for the cAIMP analogs. Based on the ensemble of biological results, we compare these analogs to the parent compound, **9**, based on each structural parameter explored. We also consider the activities of all the analogs relative to those of 2',3'-cGAMP.

Based on literature precedent describing substantial differences in the STING-binding activity and modes of cGAMP linkage isomers[3, 20, 21], we sought to evaluate the role of the internucleotide linkage position in the four linkage isomers of cAIMP (**9**, **10**, **23** and **27**). Intriguingly, whereas 2',3'-cGAMP has been described to bind to STING approximately 300 times more strongly than does 3',3'-cGAMP[3], our screening results suggest that **10** and **9** induce STING signaling to similar levels. We did observe that **9** was generally more active *in vitro* and gave slightly stronger induction of type I IFNs in blood. Extending this analysis to the remaining cAIMP linkage isomers **23** and **27**, we noted that all four linkage isomers gave comparable results for induction of IL-1 in blood but differed in their ability to induce type I IFNs. Specifically, **9** was the strongest inducer of type I IFNs, especially relative to **27**. Thus, in terms of internucleotide linkage geometry, it appears that AI CDNs do not exhibit the same SAR as the corresponding (naturally occurring) cGAMP linkage isomers. Accordingly, for the future design of CDN STING agonists, the choice of internucleotide linkage should account for the two constituent bases, and *vice versa*.

In terms of enzymatic degradation, **10** and 2',3'-cGAMP were similarly more resistant than **9** to NP1, but all three CDNs were susceptible to hydrolysis by SVPD, which suggests that the linkage position influences stability in an enzyme-specific fashion. In line with these findings, Danilchanka and Mekalanos[22], and Gao *et al.*[23], have previously reflected on the possible

existence, in eukaryotic cells, of linkage-specific phosphodiesterases that could distinguish between self (*i.e.* 2',3'-cGAMP) and foreign (*i.e.* 3',3') CDNs.

Considering the ribose modifications, we first observed that **9** and **51** generally had similar activity *in vitro*, although the former gave mildly higher induction of type I IFNs in blood (based on EC_{50}). In terms of enzymatic stability, although both CDNs had been totally degraded by NP1 within 2 hours, **51** did show a fourfold shorter half-life. Interestingly, Li *et al.* reported that 2',3'-cGAMP and its mono-2'-deoxyribose analog 2',3'-cdGAMP underwent similar levels of degradation ($T_{1/2}$ = *ca.* 1 hour for both compounds) by ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1)[24]; however, the authors did not test the corresponding bis-2'-deoxyribose analog. Nevertheless, whereas **9** had also been degraded by SVPD within 2 hours, **51** was partially resistant, having undergone less than 40% degradation. Thus, the bis-2'-deoxyribose substitution appears to increase or decrease the resistance of cAIMP to enzymatic cleavage on an enzyme-specific basis.

We observed that the cAIMP analogs containing at least one 2'-fluoro-2'-deoxyribose sugar (**52-56**) were typically among the most active compounds in all assays. Specifically, in blood the bis-2'-fluoro-2'-deoxyribose analogs **52** and **53** were markedly more active for induction of type I IFNs and of IL-1 than were the mono-2'-fluoro-2'-deoxyribose compounds **54-56**, which in turn were more active than all the remaining (*i.e.* non-fluoro) analogs for induction of type I IFNs. Likewise, **52** and **53** were also among the most resistant analogs to enzymatic degradation and were more resistant than **54** and **55**. Interestingly, **54** and **55**, which differ only by the location of the 2'-fluoro group (2'-FdI vs. 2'-FdA, respectively), exhibited similar activities in nearly all of the assays, which suggests that this moiety enhances STING-pathway signaling and enzymatic stability relative to **9** irrespective of which nucleoside is modified. Regardless, we

cannot yet conclude whether the apparently superior activity of these fluoro-cAIMP analogs relative to both **9** and 2',3'-cGAMP is due to superior enzymatic stability, stronger STING binding, greater cellular uptake (*e.g.* owing to the lipophilicity of the 2'-F atoms relative to the corresponding 2'-OH groups in **9**), or a combination of these or other factors known to be conferred by fluorine atoms[25].

In oligonucleotide chemistry, replacement of phosphodiester linkages with phosphorothioate linkages is a well-known strategy for lengthening the half-life of drugs *in vivo*, as it confers partial or total resistance to enzymatic cleavage (for a review on the PK of phosphorothioate oligonucleotides, see, for example: Srinivasan *et al.*[26]). Furthermore, phosphorothioate analogs of oligonucleotides have been reported to exhibit distinct activities relative to their parent (phosphodiester) compounds. In fact, phosphorothioate CDNs have been described to be more stable to enzymatic resistance than their corresponding phosphodiester CDNs *in vitro*[24]; to induce more STING-dependent production of type I IFNs *in vitro* in monocytes[27], lung fibroblasts[24] and modified HEK293 cells[24]; and to provide greater efficacy as STING agonists for immunotherapy of cancer in mouse tumor models *in vivo*[27]. Consistent with these and other reports on the relative activity of phosphorothioate CDNs, in our study all three phosphorothioate analogs (**13**, **53** and **56**) were more active than their corresponding phosphodiester analogs (**9**, **52** and **55**, respectively) for cytokine induction *in vitro*. The cytokine induction activity of CDNs depends partly on their stability to enzymatic cleavage. For example, McWhirter *et al.* reported that c-diGMP induced IFN-β in bone marrow-derived macrophages, but the product resulting from hydrolysis of c-diGMP by SVPD was inactive[28]. Similarly, Li *et al.* attributed the superior *in vitro* cytokine induction capacity of 2',3'-cGAM(PS)$_2$ relative to 2',3'-cGAMP exclusively to the greater resistance of the former to phosphodiesterase digestion, rather than to superior

STING binding, as the two compounds exhibited similar affinities in a STING-binding assay[24]. Consistent with this premise, we also observed that our phosphorothioate analogs were among the most stable to enzymatic degradation. However, in the present work we did not directly measure the STING-binding affinity of the cAIMP analogs and thus, cannot speculate on this parameter. Regardless of whether the superior *in vitro* activity of the phosphorothioates relative to the phosphodiesters was indeed due to the greater enzymatic stability of the former, we cannot yet explain why this advantage did not consistently translate to better cytokine induction activity in blood. For example, concerning type I IFN induction, although **53** was slightly more active than **52**, **13** and **56** were actually slightly less active than **9** and **55**, respectively.

A crucial factor to consider when assessing the activity of phosphorothioate CDNs is stereochemical purity: since each of the two internucleotide linkages in a CDN can exist as either of two diastereomers (Rp or Sp), each CDN can theoretically exist as four possible stereoisomers ([Rp, Rp]; [Rp, Sp]; [Sp, Sp]; or [Sp, Rp]). Interestingly, Corrales *et al.* reported that for induction of type I IFNs in THP1 cells by bis-phosphorothioate CDNs such as 2',3'-cdiAM$(PS)_2$, each Rp,Rp stereoisomer was more active than its corresponding Rp,Sp stereoisomer.[27] In our study, we screened **13**, **53** and **56** as mixtures of their constituent stereoisomers.

The results for cytokine induction in blood demonstrated that the cAIMP analogs vary in their relative abilities to drive STING-dependent induction of type I interferons and of IL-1. For drug development, such differences might ultimately represent a therapeutic advantage according to the desired activity: for example, for selective induction of type I IFNs (as with **9** and **10**), or for a more balanced response between type I IFNs and IL-1 (as with **52-54**).

Not only did we observe that type I IFN induction activity in blood differed with each CDN, but we also observed that it varied widely by donor. Interestingly, this variability was the

smallest for the two most active CDNs (**52** and **53**). There is some precedent for such heterogeneity in assays of STING-activating compounds. Fu *et al.* stimulated PBMCs from human donors with different CDN STING agonists, and found that production of IFN-β and TNF-α varied not only by CDN, but also by each donor's STING (*Tmem173*) genotype[29]. Among the four genotypes in their study, they observed the weakest cytokine induction for the heterozygous variant R232H/R71H-G230A-R293Q[30]. Given their findings, and the variability that we observed in the present study, one can reason that the therapeutic utility of a STING-modulating drug might ultimately have to be ascertained on a patient-by-patient basis: for example, by using a blood-based companion diagnostic test.

CONCLUSIONS

We have reported the design, synthesis and biological screening of a set of novel STING-activating cAIMP analogs. Compared to the reference agonists for murine (DMXAA) and human (2',3'-cGAMP) STING, some of these cAIMP analogs trigger greater induction of the IRF and/or NF-κB pathways *in vitro* in human and murine immune cell lines. Similarly, these cAIMP analogs induce cytokine production *ex vivo* in human blood from healthy donors, with some of them, such as **9**, showing selective induction of type I IFNs over IL-1. In contrast to literature reports that 2',3'-cGAMP is a dramatically stronger STING agonist than is its linkage isomer 3',3'-cGAMP, we did not observe any marked difference in activity between **10** and **9** in most of our assays, although in human blood, **9** did demonstrate slightly stronger induction of type I IFNs relative to all three of its linkage isomers. Provisional SAR analysis suggests that the combination of adenosine and inosine as constituent nucleosides, the 3',3' internucleotide linkage geometry, the presence of one or two 2'-fluoro-2'-deoxyribose sugars and, in some

cases, a bis-phosphorothioate linkage, each seems to enhance STING activation potency relative to 2',3'-cGAMP. However, the advantages of each of these structural features are not necessarily additive. Moreover, the 2'-deoxyribose and 2'-fluoro-2'-deoxyribose sugars, and the bis-phosphorothioate linkage, also seem to confer resistance to cleavage by certain phosphodiesterases. It would be interesting to ascertain how these same features influence the PK/PD of such compounds *in vivo*. Accordingly, we are currently evaluating these cAIMP analogs in healthy animals and in animal models of disease, with the aim of identifying viable preclinical candidates for STING-based immunotherapy of cancer, infectious diseases and other disorders.

EXPERIMENTAL SELECTION

Chemistry Materials, General Procedures, and Methods. Anhydrous solvents were purchased from Sigma-Aldrich and all reagents for nucleoside and nucleotide synthesis were purchased from Sigma-Aldrich, TCI, Carbosynth or ChemGenes. Unless noted otherwise, all commercially obtained solvents and reagents were used directly. Moisture- or air-sensitive reactions were run under argon atmosphere in oven-dried (120 °C) glassware and in the presence of activated 3Å molecular sieves. Amidite coupling reactions and cyclizations were done in anhydrous acetonitrile or pyridine under dry argon or nitrogen. The starting materials for all reactions in dry pyridine were dried by concentration (three times) from pyridine. Preparative silica-gel flash chromatography was done on Fluka 60 Å high-purity grade or Merck Grade 9385 silica, using gradients of methanol in dichloromethane. LC/ES-MS was performed on an Agilent 1290 Infinity UHPLC system coupled to an Agilent 1260 Infinity Diode Array Detector (DAD) and an Agilent 6130 Quadrupole mass spectrometer equipped with an electrospray ionization

source (ESI) and controlled by Chemstation software. The LC system was equipped with an Aquity CSH C18 column (50 × 2.1 mm 1.7 µm) using gradients of 10 mM aq. triethylammonium sodium bicarbonate and acetonitrile at a flow-rate of 300 µl/min. The UV detection wavelength was 254 nm. The mass spectrometer was run in positive and negative ESI modes. Purity for all final compounds was confirmed to be greater than 95% by LC-MS. Preparative HPLC was done on a Waters preparative 150Q HPLC system with monitoring at 254 nm on a SunFire Prep C18 column (5 µm OBD; 30 x 150 mm) using gradients of 10 mM aq. triethylammonium bicarbonate and acetonitrile at a flow-rate of 100 mL/min. ^{1}H-NMR and ^{31}P-NMR spectra were acquired on a Bruker Fourier 300 at room temperature and are reported in ppm. Molecular sieves (3 Å; Aldrich) were employed after drying the commercially supplied product at 250 °C for 12 h under vacuum. Exact mass characterization was done on a Waters ESI-QTOF operating in negative-ion acquisition mode. The compounds were dissolved in a 1:1 (v/v) mixture of water (0.1% formic acid) and acetonitrile. Flow Injection Analysis (FIA) was performed at a rate of 0.15 mL/min.

2'-*O*-(TBDMS)-3'-*O*-(H-phosphonate)-N^6-(Bz)adenosine (2). The adenosine phosphoramidite **1** (11.7 g, 14.73 mmol) was dissolved in a solution of ACN (50 mL). To the solution were added water (0.53 mL, 29.46 mmol) and pyridine·TFA (3.41 g, 17.67 mmol), and the resulting mixture was stirred for 15 min at RT. Then, *tert*-butylamine was added, the solution was stirred for 15 min, and the solvents were removed *in vacuo*. The residue was treated with a 3% solution of DCA in CH_2Cl_2 (100 mL) and water (10 equiv.) for 15 min. The reaction was quenched with MeOH and pyridine. The solvents were removed *in vacuo*, and the residue was purified by silica-gel column chromatography, using CH_2Cl_2/EtOH/MeOH (80/10/10) as eluent, to give **2** (6.8 g; 84% yield). LC-MS: Rt = 4.41 min, m/z = 550 $[M+H]^+$, m/z = 548 $[M-H]^-$.

3'-*O*-(TBDMS)-2'-*O*-(H-phosphonate)-N^6-(Bz)adenosine (4). The adenosine phosphoramidite **3** was subjected to a similar procedure to that described for preparation of **2**, to give **4** (1.10 g; 94% yield). LC-MS: Rt = 4.47 min, m/z = 550 $[M+H]^+$, m/z = 548 $[M-H]^-$.

[2'-*O*-(TBDMS)-3'-*O*-(CE)phosphotriester-inosine]-(3',5')-[2'-*O*-(TBDMS)-3'-*O*-(*H*-phosphonate)-N^6-(Bz)adenosine] (5). To a solution of compound **2** (2.18 g, 3.97 mmol) in a solution of Activator 42® (0.1 M in ACN) (79.4 mL, 7.94 mmol) in the presence of molecular sieves (3 Å) was added, in one portion, the commercial available inosine phosphoramidite (9.21 g, 10.4 mmol). The mixture was stirred for 30 min at RT. Then, a 5.5 M solution of *tert*-butyl hydroperoxide in decane (1.4 mL, 7.7 mmol) was added to the mixture, which was stirred for 40 min. The solution was filtered, and the molecular sieves were washed with CH_2Cl_2. The filtrate was concentrated *in vacuo*. The residue was treated with 3% DCA in CH_2Cl_2 (65 mL) in the presence of water (10 equiv.) for 15 min. The reaction was quenched with MeOH and pyridine. The solvents were removed *in vacuo,* and the residue was purified by silica-gel column chromatography, using CH_2Cl_2/MeOH (5% to 20%) as eluent to give **5** (3.27 g; 78% yield). LC-MS: Rt = 5.22 min, m/z = 1048 $[M+H]^+$, m/z = 1046 $[M-H]^-$.

[2'-*O*-(TBDMS)-3'-*O*-(CE)phosphotriester-inosine]-(3',5')-[3'-*O*-(TBDMS)-2'-*O*-(*H*-phosphonate)-N^6-(Bz)adenosine] (6). Compound **4** was reacted with inosine phosphoramidite using a similar procedure to that described for preparation of **5**, to give **6** (410 mg; 44% yield). Rt = 4.70 min, m/z = 1048 $[M+H]^+$, m/z = 1046 $[M-H]^-$.

(3',3')Cyclic-[2'-*O*-(TBDMS)-3'-*O*-(phosphodiester)-N^6-(Bz)adenosine]-[2'-*O*-(TBDMS)-3'-*O*-(CE)phosphotriester-inosine] (7). Compound 5 (3.27 g, 3.12 mmol) was coevaporated with dry pyridine three times. The residue was suspended in dry pyridine (67 mL), and to the resulting solution was added 5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane (DMOCP)

(1.99 g, 10.8 mmol). The resulting mixture was stirred at RT for 3 h. Then, iodine (0.99 g, 3.92 mmol) and water (30 equiv.) were added to the mixture. After 10 min, aq. $NaHSO_3$ (0.15%) (670 mL) was added until complete discoloration was observed, and then aq. $NaHCO_3$ was added. The aqueous layer was extracted three times with a 1:1 (v/v) mixture of EtOAc/Et_2O (800 mL). The organic layers were pooled, dried over $MgSO_4$, and filtered. The solvent was removed *in vacuo* to give **7** (3.01 g, 92% yield). This crude intermediate was used in the next step without any further purification. LC-MS: Rt = 5.41 min, m/z = 1046 $[M+H]^+$, m/z = 1044 $[M-H]^-$.

(2',3')cyclic-[3'-*O*-(TBDMS)-2'-*O*-(phosphodiester)-N^6-(Bz)adenosine]-[2'-*O*-(TBDMS)-3'-*O*-(phosphodiester)-inosine] (8). Compound **6** was subjected to a similar procedure to that described for preparation of **7**, to give **8** (364 mg; 89% yield). LC-MS: Rt = 5.66 min, m/z = 1046 $[M+H]^+$, m/z = 1044 $[M-H]^-$.

cAIMP (9). Compound **7** (4.43 g, 4.24 mmol) was treated with a solution of methylamine 33% in EtOH (190 mL), and the resulting mixture was stirred at 50 °C for 4 h. The reaction mixture was concentrated, and the resulting residue was dried *in vacuo*. The dried material was dissolved in dry pyridine (14 mL) and treated with $Et_3N{\cdot}3HF$ (14.0 mL, 85.89 mmol). The mixture was stirred at 50 °C for 3 h. Then, the pH of the solution was adjusted to 6 with 1M TEAA (75 mL). The solution was concentrated *in vacuo* (20 mL), and then treated with cooled acetone (210 mL) to provide a solid precipitate. The crude product was filtered, and then purified by preparative HPLC using a C_{18} Sunfire column (30 x 150 mm, 5 µm) with aq. ammonium formate/ACN as eluent. The fractions containing the desired compound were pooled, and the resulting solution was concentrated and lyophilized. The ammonium salt was exchanged on sodium Amberlite column to give the sodium salt of **9** (0.87 g; 32% yield). LC-MS: Rt = 2.72 min, m/z = 660 $[M+H]^+$, m/z = 658 $[M-H]^-$. ^1H NMR (D_2O, 300 MHz) δ (ppm) 8.34 (s, 1H), 8.27 (s, 1H), 8.10

(s, 1H), 7.86 (s, 1H), 5.94 (s, 2H), 5.06-4.80 (m, 4H), 4.42 (m, 4H), 4.03 (m, 2H). ^{31}P NMR (D_2O, 202 MHz) δ (ppm) -0.88 and -1.95. Exact MS (ESI-TOF for $C_{20}H_{22}N_9O_{13}P_2$ $[M-H]^-$ calculated 658.0812; observed mass: 658.0801.

(2',3')cAIMP (10). Compound **8** was subjected to a similar procedure to that described for preparation of **9**, to give **10** (50 mg; 19% yield). LC-MS: Rt = 1.53 min, m/z = 660 $[M+H]^+$, m/z = 658 $[M-H]^-$. ^{1}H NMR (D_2O, 300 MHz) δ (ppm) 8.50 (s, 1H), 8.14 (s, 1H), 8.13 (s, 1H), 8.09 (s, 1H), 6.24 (d, 1H), 6.08 (s, 1H), 5.20 (m, 1H), 4.83-4.73 (m, 2H), 4.58 (d, 1H), 4.40 (m, 2H), 4.26 (m, 2H), 4.04 (m, 2H). ^{31}P NMR (D_2O, 202 MHz) δ (ppm) -1.03 and -1.93. Exact MS (ESI-TOF for $C_{20}H_{22}N_9O_{13}P_2$ $[M-H]^-$ calculated 658.0812; observed mass: 658.0810.

[2'-*O*-(TBDMS)-3'-*O*-(CE)phosphorothioate-triester-inosine]-(3',5')-[2'-*O*-(TBDMS)-3'-*O*-(*H*-phosphonate)-N^6-(Bz)adenosine] (11). To a solution of **2** (2.78 g, 5.06 mmol) in a solution of Activator 42® (0.1 M in ACN) (101 mL, 10.1 mmol) in the presence of molecular sieves (3 Å) was added, in one portion, inosine phosphoramidite (7.22 g, 8.1 mmol). The mixture was stirred for 30 min at RT. Then, a solution of phenylacetyl disulfide (PADS) (3.1 g, 10.0 mmol) in dry pyridine (50 mL) was added, and the mixture was stirred at RT for 45 min. The resulting solution was filtered, and the molecular sieves were washed with CH_2Cl_2. The filtrate was concentrated *in vacuo* and coevaporated with ACN three times. The residue was treated with 3% DCA in CH_2Cl_2 (65 mL), in the presence of water (10 equiv.), for 15 min. The reaction was quenched with MeOH and pyridine. The solvents were removed *in vacuo*, and the residue was purified by silica-gel column chromatography, using CH_2Cl_2/MeOH (5% to 20%) as eluent, to give **11** (4.94 g; 91% yield). LC-MS: Rt = 5.42 and 5.52 min, m/z = 1064 $[M+H]^+$, m/z = 1062 $[M-H]^-$.

(3',3')Cyclic-[2'-*O*-(TBDMS)-3'-*O*-(phosphorothioate-diester)-N^6-(Bz)adenosine]-[2'-*O*-(TBDMS)-3'-*O*-(CE)phosphorothioate-triester-inosine] (12). Compound **11** (4.94 g, 4.65 mmol) was coevaporated with dry pyridine three times. The residue was suspended in dry pyridine (286 mL), and to the solution was added DMOCP (2.4 g, 13.0 mmol). The resulting mixture was stirred at RT for 2 h. Then, elemental sulfur (0.51 g, 15.9 mmol) was added, and the mixture was stirred at RT for 45 min. Then, the mixture was concentrated *in vacuo,* coevaporated with toluene three times, precipitated in ACN to remove the excess sulfur. The solvents were removed *in vacuo* to give **12** (3.18 g; 64% yield). This crude intermediate was used in the next step without any further purification. LC-MS: Rt = 5.61 and 5.71 min, m/z = 1078 $[M+H]^+$, m/z = 1076 $[M-H]^-$.

c-AIM(PS)$_2$ (13). Compound **12** (1.25 g, 1.16 mmol) was treated with 33% methylamine in EtOH (85 mL), and the resulting mixture was stirred at 50 °C for 3 h. The mixture was concentrated, and the resulting residue was dried *in vacuo*. The solid was dissolved in dry pyridine (8 mL), and the solution was heated to 50 °C, and then treated simultaneously with Et_3N (7.2 mL, 51.66 mmol) and $Et_3N{\cdot}3HF$ (4.2 mL, 25.77 mmol). The mixture was stirred for 5 h. The resulting solution was cooled to RT and treated with a large volume of acetone (100 mL) to yield a precipitate, which was then filtered, and washed with cold acetone. The crude product purified by preparative HPLC, using a C_{18} Sunfire column (30 x 150 mm, 5 μm) with TEAB (aq.)/ACN as eluent. The fractions containing the desired compound were pooled, and the solution was concentrated and lyophilized. The triethylammonium salt was exchanged on a sodium Amberlite column to provide the sodium salt of **13** (0.287 g; 40% yield). LC-MS: Rt = 3.45 min, m/z = 692 $[M+H]^+$, m/z = 690 $[M-H]^-$. ^1H NMR (D_2O, 300 MHz) δ (ppm) 8.54 (s, 1H), 8.37 (s, 1H), 8.22 (s, 1H), 7.97 (s, 1H), 6.65 (dd, 1H), 6.15 (dd, 1H), 4.60-4.50 (m, 4H),

4.45 (m, 4H), 4.05 (m, 2H). ^{31}P NMR (D_2O, 202 MHz) δ (ppm) 54.60, 54.40 and 54.11, 54.07. Exact MS (ESI-TOF for $C_{20}H_{22}N_9O_{11}P_2S_2$ $[M-H]^-$ calculated 690.0355; observed mass: 690.0351.

(5',3')-*O*-(TIPS)inosine (14). To a solution of inosine (10.0 g, 37.2 mmol) in dry pyridine (200 mL) was added $TIPSCl_2$ (14.1 g, 44.7 mmol). The solution was stirred for 18 h at RT. Then, the reaction was quenched with MeOH (50 mL), and the solvents were removed *in vacuo*. The residue was dissolved in EtOAc, and then washed with saturated aq. $NaHCO_3$, water and brine. The organic layer was dried over $MgSO_4$, filtered, and then concentrated *in vacuo*. The crude compound was purified by silica-gel column chromatography, using DCM/MeOH as eluent, to give **14** (13.5 g; 70% yield). LC-MS: Rt = 5.32 min, m/z = 511 $[M+H]^+$, m/z = 509 $[M-H]^-$. ^{1}H NMR ($CDCl_3$-d_1, 300 MHz) δ (ppm) 13.04 (s, 1H), 8.10 (s, 1H), 8.03 (s, 1H), 5.99 (s, 1H), 4.93 (t, 1H), 4.50 (d, 1H), 4.11 (m, 3H), 1.83 (m, 4H), 1.09 (m, 32H).

(5',3')-*O*-(TIPS)-2'-*O*-(Thp)inosine (15). To a solution of **14** (5 g, 9.79 mmol) in dry DCM (75 mL) were added 3,4-dihydro-2H-pyran (24.7 g, 293.7 mmol) and PPTS (7.38 g, 29.37 mmol). The solution was stirred for 18 h at RT, and then quenched with saturated aq. $NaHCO_3$. The different layers were separated, and the organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and then concentrated *in vacuo*. The crude compound was purified by silica-gel column chromatography, using DCM/MeOH as eluent, to give **15** (4.71 g; 80% yield). LC-MS: Rt = 6.72 min, m/z = 595 $[M+H]^+$, m/z = 593 $[M-H]^-$. 1H NMR (CDCl3-d1, 300 MHz) δ (ppm) 12.89 (d, 1H), 8.12 (s, 1H), 8.09 (s, 1H), 8.04 (d, 1H), 6.05 (d, 1H), 5.07 (t, 1H), 4.76 (m, 1H), 4.53 (m, 2H), 4.40-4.05 (m, 5H), 1.85-1.55 (m, 10H), 1.04 (m, 32H).

2'-*O*-(Thp)inosine (16). To a solution of **15** (4.71 g, 7.92 mmol) in THF (100 mL) was added TBAF on silica gel (10.56 g, 15.84 mmol). The solution was stirred for 3 h at RT. Then, the

reaction was filtered, the silica gel was washed with THF, and the filtrate was concentrated *in vacuo* to give **16** (2.7 g; 96% yield). The crude compound was used was used in the next step without any further purification. LC-MS: Rt = 4.32 min, m/z = 353 $[M+H]^+$, m/z = 351 $[M-H]^-$.

5'-*O*-(DMTr)-2'-*O*-(Thp)inosine (17). To a solution of **16** (2.70 g, 7.66 mmol) in dry pyridine (40 mL) was added dropwise a solution of DMTrCl (2.17 g, 6.42 mmol) in DCM (5 mL). The solution was stirred at RT for 2 h. Then, the reaction was quenched with 5% aq. $NaHCO_3$ (110 mL), and the aqueous layer was extracted with DCM three times. The organic layers were pooled, and the solution was dried over $MgSO_4$, filtered, and then concentrated *in vacuo*. The residue was purified by silica-gel column chromatography, using 1% pyridine in DCM/MeOH as eluent, to give **17** (4.78 g; 95% yield). LC-MS: Rt = 6.80 min, m/z = 655 $[M+H]^+$, m/z = 653 $[M-H]^-$. ^1H NMR ($CDCl_3$-d_1, 300 MHz) δ (ppm) 12.80 (s, 1H), 8.02 (s, 1H), 8.00 (s, 1H), 7.71 (m, 2H), 7.73 (m, 2H), 7.24 (m, 9H), 6.20 (d, 1H), 5.07 (t, 1H), 4.83 (m, 1H), 4.35 (m, 2H), 3.78 (s, 6H), 3.45-3.30 (m, 4H), 1.77-1.56 (m, 6H).

5'-*O*-(DMTr)-3'-*O*-(TBDMS)-2'-*O*-(Thp)inosine (18). To a solution of **17** (4.78 g, 7.30 mmol) in dry pyridine (40 mL) were added imidazole (1.29 g, 18.98 mmol) and TBDSMCl (1.43 g, 9.49 mmol). The reaction was stirred for 18 h at RT. Then, the solution was diluted with DCM (100 mL), and washed with saturated aq. $NaHCO_3$, water and brine. The organic layer was dried over $MgSO_4$, filtered, and then concentrated *in vacuo*. The residue was purified by silica-gel column chromatography, using 1% pyridine in DCM/MeOH as eluent, to give **18** (5.12 g; 91% yield). LC-MS: Rt = 8.15 min, m/z = 769 $[M+H]^+$, m/z = 767 $[M-H]^-$. 1H NMR ($CDCl_3$-d_1, 300 MHz) δ (ppm) 12.70 (s, 1H), 8.02 (s, 1H), 7.97 (s, 1H), 7.40 (m, 2H), 7.26 (m, 11H), 6.09 (d, 1H), 4.80 (m, 1H), 4.36 (m, 1H), 4.16 (m, 2H), 3.72 (s, 6H), 3.45-3.26 (m, 4H), 1.61-1.37 (m, 8H), 0.81 (s, 9H), 0.09 (dd, 6H).

3'-*O*-(TBDMS)-2'-*O*-(Thp)inosine (19). Compound **18** (4.93 g, 6.41 mmol) was dissolved in a 0.5 M solution of $ZnBr_2$ in 1:1 DCM/*i*PrOH (40 mL, 19.3 mmol). The solution was stirred for 40 min at RT, and then neutralized with 1N $NaHCO_3$ solution. The different layers were separated, and the organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and then concentrated *in vacuo*. The residue was purified by silica-gel column chromatography, using DCM/MeOH as eluent, to give **19** (2.68 g; 90% yield). LC-MS: Rt = 6.22 min, m/z = 467 $[M+H]^+$, m/z = 465 $[M-H]^-$. NMR ($CDCl_3$-d_1, 300 MHz) δ (ppm) 12.09 (s, 1H), 7.97 (s, 1H), 7.97 (s, 1H), 7.82 (s, 1H),), 6.34 (d, 1H), 4.56 (t, 1H), 4.36 (m, 1H), 4.04 (m, 1H), 3.65-3.46 (m, 5H), 1.76-1.52 (m, 6H), 0.89 (s, 9H), 0.08 (dd, 6H).

[2'-*O*-(TBDMS)-3'-*O*-(CE)phosphotriester-N^6-(Bz)adenosine]-(3',5')-[3'-*O*-(TBDMS)-2'-*O*-(Thp)inosine] (20). Intermediate **19** was reacted with the commercially available 3'-*O*-phosphoramidite of adenosine using a similar procedure to that described for preparation of **5**, to give **20** (1.9 g; 64% yield). Rt = 6.99 min, m/z = 1068 $[M+H]^+$, m/z = 1066 $[M-H]^-$.

[5'-*O*-(H-phosphonate)-2'-*O*-(TBDMS)-3'-*O*-(CE)phosphotriester-N^6-(Bz)adenosine]-(3',5')-[3'-*O*-(TBDMS)-2'-*O*-(Thp)inosine] (21). To a solution of **20** (1.9 g, 1.78 mmol) in dry pyridine (20 mL) was added diphenylphosphite (1.25 g, 5.34 mmol). The reaction was stirred for 2 h at RT. To the reaction was then 0.1 M TEAA (53 mL, 5.34 mmol). The reaction was stirred for 45 min at RT. Then, the solvents were removed *in vacuo* and the residue was dissolved in DCM (100 mL). The organic layer was washed with aq. $NaHCO_3$ (5%) solution, water and brine, dried over $MgSO_4$, filtered, and then concentrated *in vacuo*. The crude intermediate was treated with 10% DCA in DCM (50 mL). The reaction was stirred for 2 h at RT. Then, the reaction was neutralized by addition of pyridine (17 mL). The solvents were removed *in vacuo* to give **21** (2.2

g; 100% yield). This crude intermediate was used in the next step without any further purification. LC-MS: Rt = 7.38 min, m/z = 1048 $[M+H]^+$, m/z = 1046 $[M-H]^-$.

(3',2')Cyclic-[2'-*O*-(TBDMS)-3'-*O*-(CE)phosphotriester-N^6-(Bz)adenosine]-[2'-*O*-(phosphodiester)-3'-*O*-(TBDMS)inosine] (22). Compound **21** was subjected to a similar procedure to that described for preparation of **7**, to give **22** (1.73 g; 83% yield). LC-MS: Rt = 7.20 min, m/z = 1046 $[M+H]^+$, m/z = 1044 $[M-H]^-$.

3',2'c-AIMP (23). Intermediate **22** was subjected to a similar procedure to that described for preparation of **9**, to provide **23** (50 mg; 19% yield). LC-MS: Rt = 1.53 min, m/z = 660 $[M+H]^+$, m/z = 658 $[M-H]^-$. ^{1}H NMR (D_2O, 300 MHz) δ (ppm) 8.50 (s, 1H), 8.14 (s, 1H), 8.13 (s, 1H), 8.09 (s, 1H), 6.24 (d, 1H), 6.08 (s, 1H), 5.20 (m, 1H), 4.83-4.73 (m, 2H), 4.58 (d, 1H), 4.40 (m, 2H), 4.26 (m, 2H), 4.04 (m, 2H).^{31}P NMR (D_2O, 202 MHz) δ (ppm) -1.16 and -2.37. Exact MS (ESI-TOF for $C_{20}H_{22}N_9O_{13}P_2$ $[M-H]^-$ calculated 658.0812; observed mass: 658.0811.

[3'-*O*-(TBDMS)-2'-*O*-(CE)phosphotriester-N^6-(Bz)adenosine]-(3',5')-[3'-*O*-(TBDMS)-2'-*O*-(Thp)inosine] (24). Compound **19** was reacted with the commercially available 2'-*O*-phosphoramidite of adenosine using a similar procedure to that described for preparation of **5**, to give **24** (1.9 g; 60% yield). Rt = 7.08 min, m/z = 1068 $[M+H]^+$, m/z = 1066 $[M-H]^-$.

[5'-*O*-(H-phosphonate)-3'-*O*-(TBDMS)-2'-*O*-(CE)phosphotriester-N^6-(Bz)adenosine]-(3',5')-[3'-*O*-(TBDMS)-2'-*O*-(Thp)inosine] (25). To a solution of **24** (1.9 g, 1.78 mmol) in dry pyridine (20 mL) was added diphenylphosphite (1.25 g, 5.34 mmol). The reaction was stirred at RT for 2 h. It was treated with 0.1 M TEAA (53 mL, 5.34 mmol) and then stirred for another 45 min. Then, the solvents were removed *in vacuo* and the residue was dissolved in DCM (100 mL). The organic layer was washed with 5% aq. $NaHCO_3$, water and brine, dried over $MgSO_4$, filtered, and then concentrated *in vacuo*. The crude intermediate (2 g) was treated with 10%

DCA in DCM (50 mL, 85.0 mmol). The reaction was stirred at RT for 2 h. Then, the reaction was neutralized by addition of pyridine (17 mL, 177.0 mmol). The solvents were removed *in vacuo* to give **25** (1.8 g; 100% yield). This intermediate was used in the next step without any further purification. LC-MS: Rt = 5.39 min, m/z = 1048 $[M+H]^+$, m/z = 1046 $[M-H]^-$.

(2',2')Cyclic-[3'-*O*-(TBDMS)-2'-*O*-(CE)phosphotriester-N^6-(Bz)adenosine]-[2'-*O*-(phosphodiester)-3'-*O*-(TBDMS)inosine] (26). Intermediate **25** was subjected to a similar procedure to that described for preparation of **7**, to give **26** (1.5 g; 75% yield). LC-MS: Rt = 5.41 min, m/z = 1046 $[M+H]^+$, m/z = 1044 $[M-H]^-$.

2',2'c-AIMP (27). Intermediate **26** was subjected to a similar procedure to that described for preparation of **9**, to give **27** (17.5 mg; 22% yield). LC-MS: Rt = 1.35 min, m/z = 660 $[M+H]^+$, m/z = 658 $[M-H]^-$. ^{1}H NMR (D_2O, 300 MHz) δ (ppm) 8.44 (s, 1H), 8.13 (s, 1H), 8.08 (s, 1H), 8.07 (s, 1H), 6.20 (d, 1H), 6.07 (d, 1H), 5.22 (m, 1H), 4.91 (m, 1H), 4.79 (m, 2H), 4.63 (m, 1H), 4.56 (m, 1H), 4.22 (m, 2H), 4.07 (m, 2H). ^{31}P NMR (D_2O, 202 MHz) δ (ppm) -0.99 and -1.59. Exact MS (ESI-TOF for $C_{20}H_{22}N_9O_{13}P_2$ $[M-H]^-$ calculated 658.0812; observed mass: 658.0797.

3'-*O*-[(Allyl,CE)phosphotriester]-N^6-(Bz)-2'-deoxyadenosine (30). The commercially available phosphoramidite **28** (3.0 g, 3.49 mmol) was coevaporated with dry ACN three times, and the resulting solid was dissolved in a solution of Activator 42® (70.0 mL, 7.00 mmol) in the presence of molecular sieves (3 Å). To the solution was added allyl alcohol (0.480 mL, 7.00 mmol), and the resulting mixture was stirred for 30 min. Then, 5.5 M *tert*-butyl hydroperoxide in decane (1.14 ml, 9.09 mmol) was added, and the mixture was stirred for 40 min. The solution was filtered, the molecular sieves were washed with DCM, and the filtrate was concentrated *in vacuo*. The residue was treated with 3% DCA in CH_2Cl_2 (50 mL), in the presence of water (10 equiv.), for 15 min. The reaction was quenched with MeOH and pyridine. The solvents were

removed *in vacuo*, and the residue was purified by silica-gel column chromatography, using CH_2Cl_2/MeOH (5% to 20%) as eluent, to give **30** (1.84 g; 95% yield). LC-MS: Rt = 4.31 min, m/z = 529 $[M+H]^+$, m/z = 527 $[M-H]^-$.

3'-*O*-(Allyl,CE)phosphotriester-N^6-(Bz)-2'-deoxy-2'-fluoroadenosine (31). The commercially available phosphoramidite **29** (10.0 g, 11.0 mmol) was coevaporated with dry ACN three times, and the resulting solid was dissolved in a solution of Activator 42® (0.1 M in ACN) (228.0 mL, 22.8 mmol) in the presence of molecular sieves (3 Å). To the solution was added allyl alcohol (1.60 mL, 23.53 mmol), and the resulting mixture was stirred for 30 min. Then, 5.5 M *tert*-butyl hydroperoxide in decane (2.9 ml, 23.0 mmol) was added, and the mixture was stirred for 40 min. The solution was filtered, the molecular sieves were washed with DCM, and the filtrate was concentrated *in vacuo*. The residue was treated with 3% DCA in CH_2Cl_2 (280 mL), and water (10 equiv.), for 15 min. The reaction was quenched with MeOH and pyridine. The solvents were removed *in vacuo*, and the residue was purified by silica-gel column chromatography, using CH_2Cl_2/MeOH (5% to 20%) as eluent, to give **31** (6.20 g; 99% yield). LC-MS: Rt = 4.50 min, m/z = 547 $[M+H]^+$, m/z = 545 $[M-H]^-$.

3'-*O*-(Allyl,CE)phosphorothioate-triester-N^6-(Bz)-2'-deoxy-2'-fluoroadenosine (32). The commercially available phosphoramidite **29** (10.0 g, 11.0 mmol) was coevaporated with dry ACN three times, and the resulting solid was dissolved in a solution of Activator 42® (0.1 M in ACN) (228.0 mL, 22.8 mmol) in the presence of molecular sieves (3 Å). To the solution was added allyl alcohol (1.60 mL, 23.53 mmol) and the resulting mixture was stirred for 30 min. The mixture was concentrated *in vacuo* and the residue was dissolved in 0.2 M PADS in dry pyridine (114 mL). The mixture was stirred at RT for 45 min. The solution was filtered, the molecular sieves were washed with CH_2Cl_2, and the filtrate was concentrated *in vacuo* and then,

coevaporated with ACN three times. The residue was treated with 3% DCA in CH_2Cl_2 (280 mL), and water (10 equiv.), for 15 min. The reaction was quenched with MeOH and pyridine. The solvents were removed *in vacuo*, and the residue was purified by silica-gel column chromatography, using CH_2Cl_2/MeOH (5 to 20%) as eluent, to give **32** (4.55 g; 71% yield). LC-MS: Rt = 5.06 min, m/z = 563 $[M+H]^+$, m/z = 561 $[M-H]^-$.

[3'-*O*-(CE)phosphotriester-2'-deoxyinosine]-(3',5')-[3'-*O*-(Allyl,CE)phosphotriester-N^6-(Bz)-2'-deoxyadenosine] (33). To a solution of **30** (1.29 g, 1.72 mmol) in a solution of Activator 42® (0.1 M in ACN) (34.0 mL, 3.40 mmol) in the presence of molecular sieves (3 Å), was added, in one portion, the commercially available phosphoramidite of deoxyinosine (1.0 g, 1.89 mmol). The mixture was stirred for 30 min at RT. Then, 5.5 M *tert*-butyl hydroperoxide in decane (0.55 mL, 4.47 mmol) was added, and the mixture was stirred for 40 min. The resulting solution was filtered, the molecular sieves were washed with CH_2Cl_2, and the filtrate was concentrated *in vacuo*. The residue was treated with 3% DCA in CH_2Cl_2 (50 mL) and water (10 equiv.), for 15 min. The reaction was quenched with MeOH and pyridine. The solvents were removed *in vacuo*, and the residue was purified by silica-gel column chromatography, using CH_2Cl_2/MeOH (5% to 20%) as eluent, to provide **33** (0.50 mg; 32% yield). LC-MS: Rt = 5.28 min, m/z = 896 $[M+H]^+$, m/z = 894 $[M-H]^-$.

[3'-*O*-(CE)phosphotriester-2'-deoxy-2'-fluoroinosine]-(3',5')-[3'-*O*-(Allyl,CE)phosphotriester-N^6-(Bz)-2'-deoxy-2'-fluoroadenosine] (34). Intermediate **31** was reacted with the commercial phosphoramidite of 2'-deoxy-2'-fluoroinosine using a similar procedure to that described for preparation of compound **33**, to give **34** (2.58 g; 51% yield). Rt = 4.39 min, m/z = 932 $[M+H]^+$, m/z = 930 $[M-H]^-$.

[3'-*O*-(CE)phosphorothioate-triester-2'-deoxy-2'-fluoroinosine]-(3',5')-[3'-*O*-(Allyl,CE)phosphorothioate-triester-*N*^{6}-(Bz)-2'-deoxy-2'-fluoroadenosine] (35). Compound **32** (3.2 g, 5.71 mmol) was coevaporated with dry ACN three times, and the resulting solid was dissolved in a solution of Activator 42® (0.1 M in ACN) (110.0 mL, 11.0 mmol) in the presence of molecular sieves (3 Å). To the solution was added the commercial phosphoramidite of 2'-deoxy-2'-fluoroinosine (5.7 g, 7.4 mmol) and the resulting mixture was stirred for 30 min. The mixture was concentrated *in vacuo*, and the residue was dissolved in 0.2 M PADS in dry pyridine (57 mL). The mixture was stirred at RT for 45 min. The solution was filtered, and the molecular sieves were washed with CH_2Cl_2. The filtrate was concentrated *in vacuo* and then, coevaporated with ACN three times. The residue was treated with 3% DCA in CH_2Cl_2 (280 mL), in the presence of water (10 equiv.), for 15 min. The reaction was quenched with MeOH and pyridine. The solvents were removed *in vacuo*, and the residue was purified by silica-gel column chromatography, using CH_2Cl_2/MeOH (5% to 20%) as eluent, to give **35** (4.45 g; 81% yield). LC-MS: Rt = 6.20 min, m/z = 964 $[M+H]^+$, m/z = 962 $[M-H]^-$.

[3'-*O*-(CE)phosphotriester-2'-deoxyinosine]-(3',5')-[3'-*O*-(Allyl,CE) phosphotriester-*N*^{6}-(Bz)-2'-deoxy-2'-fluoroadenosine] (36). Intermediate **31** was reacted with the commercially available phosphoramidite of 2'-deoxyinosine using a similar procedure to that described for preparation of **33**, to give **36** (12.0 g; 64% yield). Rt = 4.12 min, m/z = 914 $[M+H]^+$, m/z = 912 $[M-H]^-$.

[3'-*O*-(CE)phosphotriester-2'-deoxy-2'-fluoroinosine]-(3',5')-[3'-*O*-(Allyl,CE) phosphotriester-*N*^{6}-(Bz)-2'-deoxyadenosine] (37). Intermediate **30** was reacted with the commercially available phosphoramidite of 2'-deoxy-2'-fluoroinosine using a similar procedure

to that described for preparation of **33**, to give **37** (3.08 g; 64% yield). Rt = 4.22 min, m/z = 914 $[M+H]^+$, m/z = 912 $[M-H]^-$.

[3'-*O*-(CE)phosphorothioate-triester-2'-deoxyinosine]-(3',5')-[3'-*O*-(Allyl,CE)phosphorothioate-triester-N^6-(Bz)-2'-deoxy-2'-fluoroadenosine] (38). Intermediate **32** was reacted with the commercially available phosphoramidite of 2'-deoxyinosine using a similar procedure to that described for preparation of **35**, to give **38** (163 mg; 44% yield). LC-MS: Rt = 5.02 and 5.10 min, m/z = 914 $[M+H]^+$, m/z = 912 $[M-H]^-$.

[3'-*O*-(CE)phosphotriester-2'-deoxyinosine]-(3',5')-[3'-*O*-(CE)phosphodiester-N^6-(Bz)-2'-deoxyadenosine] (39). To a solution of **33** (0.50 g, 0.55 mmol) in acetone (20 mL) was added sodium iodide (0.83 g, 5.58 mmol), and the resulting suspension was stirred under reflux for 2 h. The resulting colorless precipitate was collected by filtration, and then washed with chilled acetone to give **39** (395 mg; 83% yield). This compound is highly hygroscopic and thus, was immediately used in the next step. LC-MS: Rt = 3.35 min, m/z = 856 $[M+H]^+$, m/z = 854 $[M-H]^-$.

[3'-*O*-(CE)phosphotriester-2'-deoxy-2'-fluoroinosine]-(3',5')-[3'-*O*-(CE)phosphodiester-N^6-(Bz)-2'-deoxy-2'-fluoroadenosine] (40). To a solution of **34** (3.36 g, 3.61 mmol) in dry THF (30 mL) were added *N*-methyl aniline (0.90 g, 8.45 mmol) and tetrakis(triphenylphosphine)palladium(0) (0.65 g, 0.56). The resulting suspension was stirred at RT for 15 min. Then, the solvent was removed *in vacuo*, and the residue was triturated with diethyl ether. The resulting colorless precipitate was collected by filtration, and then washed with chilled diethyl ether. The crude product was purified by silica-gel column chromatography, using DCM/MeOH (0% to 10%) as eluent, to give **40** (2.40 g; 96% yield). LC-MS: Rt = 3.58 and 3.59 min, m/z = 892 $[M+H]^+$, m/z = 891 $[M-H]^-$.

[3'-*O*-(CE)phosphorothioate-triester-2'-deoxy-2'-fluoroinosine]-(3',5')-[3'-*O*-(CE)phosphorothioate-diester-N^6-(Bz)-2'-deoxy-2'-fluoroadenosine] (41). Intermediate **35** was subjected to a similar procedure to that described for **40**, to give **41** (1.81 g; 34% yield). LC-MS: Rt = 4.25 and 4.55 min, m/z = 924 $[M+H]^+$, m/z = 922 $[M-H]^-$.

[3'-*O*-(CE)phosphotriester-2'-deoxy-2'-fluoroinosine]-(3',5')-[3'-*O*-(CE)phosphodiester-N^6-(Bz)-2'-deoxy-adenosine (42). Intermediate **36** was subjected to a similar procedure to that described for preparation of **40**, to give **42** (11.7 g; 99% yield). LC-MS: Rt = 2.92 min, m/z = 874 $[M+H]^+$, m/z = 872 $[M-H]^-$

[3'-*O*-(CE)phosphotriester-2'-deoxyinosine]-(3',5')-[3'-*O*-(CE)phosphodiester-N^6-(Bz)-2'-deoxy-2'-fluoroadenosine (43). Intermediate **37** was subjected to a similar procedure to that described for preparation of **40**, to give **43** (2.74 g; 93% yield). LC-MS: Rt = 3.11 min, m/z = 874 $[M+H]^+$, m/z = 872 $[M-H]^-$

[3'-*O*-(CE)phosphorothioate-triester-2'-deoxyinosine]-(3',5')-[3'-*O*-(CE)phosphorothioate-diester-N^6-(Bz)-2'-deoxy-2'-fluoroadenosine] (44). Intermediate **38** was subjected to a similar procedure to that described for preparation of **40**, to give **44** (105 mg; 67% yield). LC-MS: Rt = 3.76 and 3.87 min, m/z = 906 $[M+H]^+$, m/z = 904 $[M-H]^-$.

(3',3')Cyclic-[3'-*O*-(CE)phosphotriester-N^6-(Bz)-2'-deoxyadenosine]-[3'-*O*-(CE)phosphotriester-2'-deoxyinosine] (45). Compound **39** (0.39 g, 0.46 mmol) was coevaporated with dry pyridine three times and then, with dry ACN. The residue was suspended in THF (80 mL), and to the resulting heterogeneous mixture were successively added *N*-methylimidazole (0.36 mL, 4.61 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (1.39 g, 4.61 mmol). The resulting mixture was stirred at 25 °C for 18 h. Then, the solvent was removed *in vacuo* and the residue was triturated with EtOAc. The resulting colorless precipitate was

collected by filtration and washed with chilled EtOAc to provide crude **45** (0.38 g; 99% yield). This compound was used in the next step without any further purification. LC-MS: Rt = 3.91 min, m/z = 838 $[M+H]^+$, m/z = 836 $[M-H]^-$.

(3',3')Cyclic-[3'-*O*-(CE)phosphotriester-N^6-(Bz)2'-deoxy-2'-fluoroadenosine]-[3'-*O*-(CE)phosphotriester-2'-deoxy-2'-fluoroinosine] (46). Compound **40** (2.40 g, 2.69 mmol) was coevaporated with dry pyridine three times. The residue was suspended in dry pyridine (528 mL), and to the resulting solution was added 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) (4.0 g, 13.0 mmol). The resulting mixture was stirred at 25 °C for 3 h. Then, the solvent was removed *in vacuo* to give crude **46** (2.11 g; yield 90%). This compound was used in the next step without any further purification. LC-MS: Rt = 4.21 and 4.42 min, m/z = 874 $[M+H]^+$, m/z = 872 $[M-H]^-$.

(3',3')Cyclic-[3'-*O*-(CE)phosphorothioate-triester-N^6-(Bz)2'-deoxy-2'-fluoroadenosine]-[3'-*O*-(CE)phosphorothioate-triester-2'-deoxy-2'-fluoroinosine] (47). Intermediate **41** was subjected to a similar procedure to that described for preparation of **46**, to give **47** (35 mg; 36% yield). LC-MS: Rt = 5.00 and 5.32 min, m/z = 906 $[M+H]^+$, m/z = 904 $[M-H]^-$.

(3',3')Cyclic-[3'-*O*-(CE)phosphotriester-N^6-(Bz)2'-deoxyadenosine]-[3'-*O*-(CE)phosphotriester-2'-deoxy-2'-fluoroinosine] (48). Intermediate **42** was subjected to a similar procedure to that described for preparation of **46**, to give **48** (10.25 g; 88% yield). LC-MS: Rt = 3.59 and 3.80 min, m/z = 856 $[M+H]^+$, m/z = 854 $[M-H]^-$.

(3',3')Cyclic-[3'-*O*-(CE)phosphotriester-N^6-(Bz)2'-deoxy-2'-fluoroadenosine]-[3'-*O*-(CE)phosphotriester-2'-deoxyinosine] (49). Intermediate **43** was subjected to a similar procedure to that described for preparation of **46**, to give **49** (2.08 g; 72% yield). LC-MS: Rt = 3.59 and 3.80 min, m/z = 856 $[M+H]^+$, m/z = 854 $[M-H]^-$.

(3',3')Cyclic-[3'-*O*-(CE)phosphorothioate-triester-N^6-(Bz)2'-deoxy-2'-fluoroadenosine]-[3'-*O*-(CE)phosphorothioate-triester-2'-deoxyinosine] (50). Intermediate **44** was subjected to a similar procedure to that described for preparation of **46**, to give **50** (50 mg; 48% yield). LC-MS: Rt = 4.71 and 4.85 min, m/z = 888 $[M+H]^+$, m/z = 886 $[M-H]^-$.0

c-(dAMP-dIMP) (51). Compound **45** (0.38 g, 0.46 mmol) was treated with 33% methylamine in EtOH (80 mL), and the resulting mixture was stirred at 50 °C for 2 h. The reaction mixture was concentrated, and the resulting residue was precipitated in cold acetone. The solid was filtered, washed with acetone, and purified by preparative HPLC using a C_{18} Sunfire column (30 x 150 mm, 5µm) and TEAB (aq.)/ACN as eluent. The fractions containing the desired compound were pooled and lyophilized. The triethylammonium salt was exchanged on a sodium Amberlite column to give the sodium salt of **51** (90 mg; 31%). LC-MS: Rt = 2.47 min, m/z = 628 $[M+H]^+$, m/z = 626 $[M-H]^-$. ^{1}H NMR (DMSO-*d6*, 300 MHz) δ (ppm) 8.37 (s, 1H), 8.32 (s, 1H), 8.13 (s, 1H), 8.05 (s, 1H), 7.26 (sl, 2H), 6.28 (m, 2H), 4.68 (m, 2H), 4.08 (m, 2H), 3.85 (m, 2H), 2.83 (m, 2H). ^{31}P NMR (D_2O, 202 MHz) δ (ppm) -0.81 and -1.29. Exact MS (ESI-TOF for $C_{20}H_{22}N_9O_{11}P_2$ $[M-H]^-$ calculated 626.0914; observed mass: 626.0914.

c-(2'FdAMP-2'FdIMP) (52). Compound **46** was subjected to a similar procedure to that described for preparation of **51**, to give **52** (104 mg; 10% yield). LC-MS: Rt = 2.78 min, m/z = 664 $[M+H]^+$, m/z = 662 $[M-H]^-$. ^{1}H NMR (D_2O, 300 MHz) δ (ppm) 8.37 (s, 1H), 8.35 (s, 1H), 8.14 (s, 1H), 7.95 (s, 1H), 6.23 (m, 2H), 5.45 (m, 2H), 5.39 (m, 1H), 4.95 (m, 2H), 4.50 (m, 2H), 4.06 (m, 2H). ^{31}P NMR (D_2O, 202 MHz) δ (ppm) -1.88 and -1.93. Exact MS (ESI-TOF for $C_{20}H_{20}F_2N_9O_{11}P_2$ $[M-H]^-$ calculated 662.0726; observed mass: 662.0718.

c-[2'FdAMP(S)-2'FdIMP(S)] (53). Compound **47** was subjected to a similar procedure to that described for preparation of **51**, to give **53** (10 mg; 18% yield). LC-MS: Rt = 3.41 min, m/z =

696 $[M+H]^+$, m/z = 694 $[M-H]^-$. ^1H NMR (D_2O, 300 MHz) δ (ppm) 8.55 (s, 1H), 8.38 (s, 1H), 8.20 (s, 1H), 7.99 (s, 1H), 6.63 (dd, 1H), 6.15 (dd, 1H), 5.16-4.95 (m, 4H), 4.52 (m, 4H), 4.07 (m, 2H). ^{31}P NMR (D_2O, 202 MHz) δ (ppm) 54.80, 54.71, 54.63, 54.57, 54.52, 54.44, 54.33. Exact MS (ESI-TOF for $C_{20}H_{20}F_2N_9O_9P_2S_2$ $[M-H]^-$ calculated 694.0269; observed mass: 694.0261.

c-(2'FdAMP-dIMP) (54). Compound **48** was subjected to a similar procedure to that described for preparation of **51**, to give **54** (5.65 g; 73% yield). LC-MS: Rt = 0.48 min, m/z = 646 $[M+H]^+$, m/z = 644 $[M-H]^-$. ^1H NMR (D_2O, 300 MHz) δ (ppm) 8.20 (s, 1H), 8.11 (s, 1H), 7.94 (s, 1H), 7.99 (s, 1H), 7.85 (s, 1H), 6.25 (m, 2H), 5.62 (m, 1H), 5.46 (m, 1H), 5.08 (m, 2H), 4.48 (m, 1H), 4.36 (m, 1H), 4.23 (m, 2H), 4.08 (m, 2H), 4.09 (m, 2H), 3.02 (m, 1H), 2.69 (m, 1H). ^{31}P NMR (D_2O, 202 MHz) δ (ppm) -1.05 and -1.68. Exact MS (ESI-TOF for $C_{20}H_{21}FN_9O_{11}P_2$ $[M-H]^-$ calculated 644.0820; observed mass: 644.0810.

c-(dAMP-2'FdIMP) (55). Compound **49** was subjected to a similar procedure to that described for preparation of **51**, to give **55** (0.75 g; 48% yield). LC-MS: Rt = 0.56 min, m/z = 646 $[M+H]^+$, m/z = 644 $[M-H]^-$. ^1H NMR (D_2O, 300 MHz) δ (ppm) 8.28 (s, 1H), 8.20 (s, 1H), 7.98 (s, 1H), 7.95 (s, 1H), 7.85 (s, 1H), 6.32 (m, 2H), 5.83 (m, 1H), 5.66 (m, 1H), 5.29 (m, 2H), 4.38 (m, 1H), 4.16 (m, 2H), 4.21 (m, 2H), 3.13 (m, 1H), 2.79 (m, 1H). ^{31}P NMR (D_2O, 202 MHz) δ (ppm) -0.98 and -1.62. Exact MS (ESI-TOF for $C_{20}H_{21}FN_9O_{11}P_2$ $[M-H]^-$ calculated 644.0820; observed mass: 644.0817.

c-[2'FdAM(PS)-dIM(PS)] (56). Compound **50** was subjected to a similar procedure to that described for preparation of **51**, to give **56** (16 mg; 42% yield). LC-MS: Rt = 0.844, 0.946, 2.28, 2.39, 2.72, 2.68 min, m/z = 678 $[M+H]^+$, m/z = 676 $[M-H]^-$. ^1H NMR (D_2O, 300 MHz) δ (ppm) 8.48 (s, 1H), 8.24 (s, 1H), 8.21 (s, 1H), 8.04 (s, 1H), 7.96 (s, 1H), 7.90 (s, 1H), 6.30 (m, 2H),

5.70 (m, 1H), 5.53 (m, 1H), 5.02 (m, 2H), 4.45 (m, 1H), 4.21 (m, 1H), 4.09 (m, 2H), 4.02 (m, 2H), 3.94 (m, 2H), 3.10 (m, 1H), 2.90 (m, 1H). ^{31}P NMR (D_2O, 202 MHz) δ (ppm) 55.95, 55.71 and 54.76, 54.29. Exact MS (ESI-TOF for $C_{20}H_{21}FN_9O_9P_2S_2$ $[M-H]^-$ calculated 690.0355; observed mass: 690.0351.

Biology materials and general procedures. The following reporter cell lines, detection reagents and ligands were obtained from InvivoGen: RAW-Lucia™ ISG Cells (rawl-isg); RAW-Lucia™-ISG-KO STING Cells (rawl-kostg); THP1-Dual™ Cells (thpd-nfis); HEK-Blue™ IFN-α/β Cells (hkb-ifnab); HEK-Blue™ IL-1R Cells (hkb-il1r); HEK-Blue™ IL-6 Cells (hkb-hil6); Quanti-Blue™ (QBA-38-05); Quanti-Luc™ (QLC-38-081); 2',3'-cGAMP (tlrl-nacga23-5); and DMXAA (tlrl-dmx). The HEK-Blue™ IL-28 cell line was obtained through manipulation of HEK-Blue™ ISG cells (InvivoGen catalog code: hkb-isg) to provide a reporter cell line that enables detection of bioactive type III IFNs (IL-28A [IFN-λ2], IL-28B [IFN-λ3] and IL-29 [IFN-λ1]) based on monitoring of ISG54 pathway activation. The following cell culture regents, buffers and screening solvents were purchased elsewhere: PBS (Gibco; 25030-123); DMEM (Gibco; 21969-035); glutamine (Gibco; 25030-24); penicillin/streptomycin (Gibco; 15140-122); Bovine Fetal Serum (Hyclone; SV30160.03); 0.9% saline (OTEC; 600502); and DMSO (Fluka; 41641). The enzymes nuclease P1 (N8630) and snake-venom phosphodiesterase (P3134) were obtained from Sigma Aldrich.

General procedure for *in vitro* screening of CDNs for cytokine induction activity. 180 μL of RAW-Lucia ISG™ cells, RAW-Lucia™ ISG-KO-STING cells or THP1-Dual™ cells (*ca.* 100,000 cells per well) were stimulated for 48 h at 37 ºC in a 5% CO_2 incubator with 20 μL of either saline, or a saline solution of a test compound (CDN or DMXAA) at one of seven different

concentrations (0.08 µg/mL, 0.2 µg/mL, 0.7 µg/mL, 2 µg/mL, 6 µg/mL, 20 µg/mL or 60 µg/mL [or 100 µg/mL, for DMXAA]). The IRF pathway activity was indirectly quantified using QUANTI-Luc™, which was prepared and used according to the manufacturer's instructions. The NF-κB pathway activity (THP1-Dual™ only) was indirectly quantified using QUANTI-Blue™, which was prepared and used according to the manufacturer's instructions.

General procedure for *ex vivo* screening of CDNs for cytokine induction activity. Human blood samples were acquired from healthy donors at the *Etablissement Français du Sang* (EFS Pyrénées-Méditerranée, Toulouse, France), per agreement #21/PLER/TOU/CAYLA01/2013-0071. Briefly, the samples were collected by venipuncture into sodium heparin tubes at the time of donation. The samples were analyzed for rhesus (Rh), blood group, hematocrit and serological status (AgHBS, HIV, HCV, HTLV, HBC, CMV and Syph). The tubes were acquired on the day of collection and subsequently tested (blood analysis, and treatment with test items) on the same day. Each blood sample was diluted (1:2 [v/v]) in RPMI medium and sodium pyruvate and aliquoted into 96-well plates (180-µL wells) containing a test compound at one of seven different concentrations (0.03 µg/mL, 0.1 µg/mL, 0.3 µg/mL, 1 µg/mL, 3 µg/mL, 10 µg/mL or 30 µg/mL) in sterile saline. The plates were incubated at 37 °C in a CO_2 incubator for 18 to 20 h. Then, the supernatants were collected, transferred into the corresponding wells of round-bottom 96-well plates, and either stored at -20 °C, or immediately tested in the reporter cell line. The experiment was performed three times with five different donors per experiment. Five 190-µL aliquots (*ca.* 50,000 cells/well) of the desired reporter cells were plated to a new 96-well plate and incubated with 10 µL of blood supernatant for approximately 20 h at 37 ºC in a 5% CO_2 incubator. The desired cytokine induction activity was determined using QUANTI-Blue™, according to the manufacturer's instructions.

EC_{50} and E_{max} calculations. EC_{50} values were calculated from log(agonist) dose-response curve fitting in GraphPad Prism, using the following equation: Y = Bottom + (Top-Bottom)/(1+10^((LogEC_{50}-X))), where X is expressed as a log of dose, top and bottom are the plateaus (Top represents the E_{max} and is expressed in the same unit as Y). The results are expressed as the mean ± SD from three independent experiments. E_{max} values were normalized to the response induced by 2',3'-cGAMP in each assay and expressed as a mean percentage ± SD from three independent experiments.

General procedure for *in vitro* enzymatic digestion assays. Each CDN (7 µg) was separately incubated with either a solution (21 µL) of an enzyme (either 160 µg SVPD in PBS buffer containing 0.6 mM $MgCl_2$; or 2.5 mU NP1 in 30 mM acetate buffer containing 2 mM $ZnCl_2$ [pH 5.3]), or water (as control), in a water bath at 37 ºC. Aliquots of the reaction mixture were collected at various time points from 0 to 2 hours, heated at 100 ºC for 2 min, and then frozen at 0 °C. Finally, 10 µL of each aliquot was injected directly into the HPLC (Agilent 1290 Infinity UHPLC equipped with a UV-detector; column: Waters Acquity UPLC CSH C18 1.7 µm [2.1 mm x 50 mm; flow rate: 0.3 mL/min]; detection at 254 nm; auto-sampler temperature: 25 °C) for analysis. The following gradient was used: 100% A (10 mM aq. ammonium formate) for 1 minute; then 100% A to 100% B (ACN) over 5 minutes. The remaining CDN was quantified based on HPLC peak area.

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Author contributions

TL and GT designed the cAIMP analogs. TL, MAM, AL and NB synthesized, purified and characterized the cAIMP analogs and intermediates. FV, CB and GT designed the biological assays. FV, ASB, CB and JLV performed the biological assays. MH performed the enzymatic stability assay. All authors contributed to interpreting the results. TL and GQ wrote the manuscript.

Notes

The authors report the following competing financial interests: all authors are currently employees of InvivoGen, several InvivoGen products (reporter cell lines, detection reagents and ligands) were used in this study, and the work described herein was funded entirely by InvivoGen.

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ABBREVIATIONS USED

ACN, acetonitrile; cAIMP, cyclic adenosine-inosine monophosphate; c-diAMP, cyclic di-adenosine monophosphate; c-diGMP, cyclic di-guanosine monophosphate; cGAMP, cyclic adenosine-guanosine monophosphate; cGAS, cyclic GMP-AMP synthase; CDN, cyclic dinucleotide; DCA, dichloroacetic acid; ESI, electrospray ionization; EtOH, ethanol; IFN,

interferon; IL-1, interleukin 1; IL-6, interleukin 6; IL-28, interleukin 28; IRF, interferon regulatory factor; KO, knockout; MeOH, methanol; MS, molecular sieves; NF-κB, nuclear factor-kappa-light-chain-enhancer of activated B cells; NP-1, nuclease P1; PADS, phenacetyl disulfide; RT, room temperature; STING, stimulator of interferon genes; SVPD, snake-venom phosphodiesterase; *t*-$BuNH_2$, *tert*-butylamine; TNF-α, tumor necrosis factor alpha.

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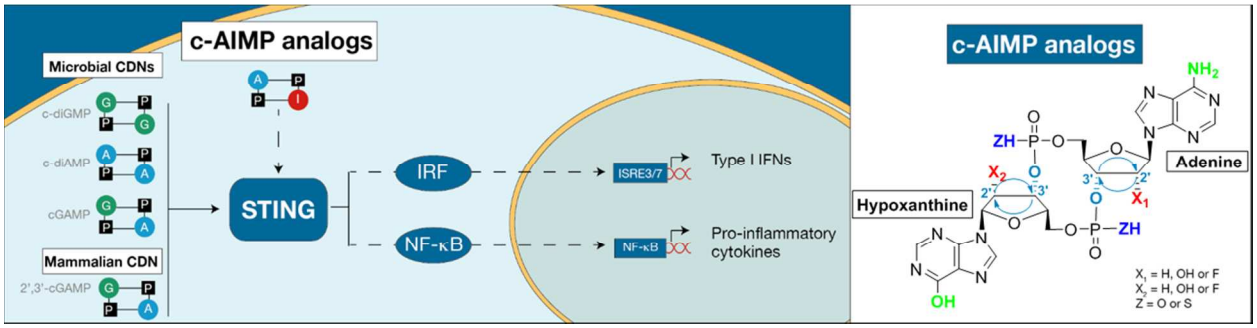
TOC Graphic


c-AIMP analogs
Microbial CDNs
c-diGMP
c-diAMP
cGAMP
Mammalian CDN
2',3'-cGAMP
STING
IRF
NF-κB
ISRE3/7
NF-κB
Type I IFNs
Pro-inflammatory cytokines
c-AIMP analogs
Hypoxanthine
Adenine
X1 = H, OH or F
X2 = H, OH or F
Z = O or S