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Discovery of IACS-8803 and IACS-8779, potent agonists of stimulator of interferon genes (STING) with robust systemic antitumor efficacy

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Abstract

Activation of the stimulator of interferon genes (STING) pathway by both exogenous and endogenous cytosolic DNA results in the production of interferon beta (IFN-β) and is required for the generation of cytotoxic T-cell priming against tumor antigens. In the clinical setting, pharmacological stimulation of the STING pathway has the potential to synergize with immunotherapy antibodies by boosting anti-tumor immune responses. We report the discovery of two highly potent cyclic dinucleotide STING agonists, IACS-8803 and IACS-8779, which show robust activation of the STING pathway *in vitro* and a superior systemic anti-tumor response in the B16 murine model of melanoma when compared to one of the clinical benchmark compounds.

Graphical Abstract

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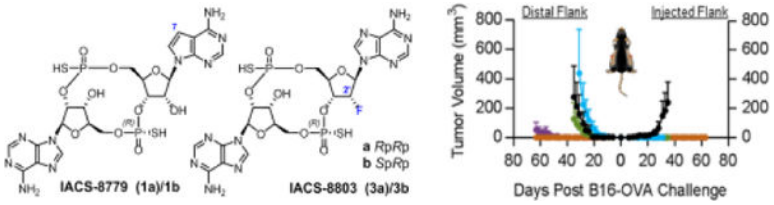
Supplementary material

Experimental procedures and characterization data for synthetic intermediates A1, A3, A4, B1-B2, and final compounds IACS-8779 (1a) / 1b, and IACS-8803 (3a) / 3b are available at the link *https "add link"*. Procedure and data for the additional final compounds are available through reference n. 28.



Keywords

STING agonists; Cyclic dinucleotide; Immune response; T-cell priming; Phosphorothioate esters

Stimulator of interferon genes (STING) is an innate pattern recognition receptor natively localized to the endoplasmic reticulum which plays a key role in host defense through innate detection of pathogenic or damage-associated nucleic acids.[1–4] STING has recently been shown to be required for generation of optimal adaptive anti-tumor immune responses, thus deliberate pharmacological stimulation of the STING pathway is an exciting approach to boost anti-cancer immunity with the potential to synergize with FDA-approved immunotherapy antibodies.[3,4] The STING pathway is activated upon direct binding to STING of cyclic dinucleotides (CDNs),[5,6] second messenger molecules that are either of exogenous bacterial origin (such as 3’,3’-cyclic guanosine-adenosine monophosphate, cGAMP, Figure 1), or produced by a host cyclic GMP-AMP synthase (cGAS).[7] cGAS acts as a sensor of double stranded cytosolic DNA of viral, malignant, or endogenous origin, generating the structurally distinct CDN ligand 2’,3’-cyclic guanosine-adenosine monophosphate (2’,3’-cGAMP, Figure 1), which features a non-canonical 2’,3’-phosphodiester linkage.[8,9] Binding of CDN ligands to STING results in its activation and initiates a signaling cascade culminating in robust engagement of the interferon regulatory factor 3 (IFR3) and nuclear factor kappa-B (NF-kB) pathways, ultimately stimulating production of interferon beta (IFN-β) and other pro-inflammatory cytokines.[10] Beyond its canonical role in immune defense against viral or bacterial pathogens, activation of the STING pathway and resulting IFN-β production have been recognized as critical components of the innate immune sensing of tumors, which is a critical upstream event required for optimal cytotoxic T-cell priming against tumor antigens.[11] Recent reports demonstrated that pharmacological activation of the STING pathway *via* intratumoral administration of synthetic CDN agonists results in significant antitumor responses in a variety of preclinical models, achieves robust tumor regression at both injected and distal uninjected lesions, and is reported to facilitate generation of long-lived immunologic memory.[3,4,12] Clinical evaluation of synthetic STING agonists is underway, with **ADU-S100/MIW815**,[12] a 2’,3’-cyclic diadenosine monophosphate analogue featuring a thiophosphatediester bond (Figure 1) and **MK-1454**, a second agent of undisclosed structure, escalating in Phase I.[13]

Given the key role proposed for the STING pathway in modulating the innate antitumor immune response, we set out to generate high-potency STING agonists and to investigate the role of key nucleobase and ribose modifications with the goal of identifying STING agonists with improved translational potential as cancer therapeutics and ultimately expand the number of available clinical tools. Herein we report the rational design and the discovery of

IACS-8779 (**1a**) and **IACS-8803** (**3a**, Figure 2), two highly potent 2',3'- thiophosphate CDN analogs that show robust activation of the STING pathway *in vitro* and a superior systemic anti-tumor response when compared to the clinical benchmark **ADU-S100** in the B16 melanoma murine model.

At the beginning of our exploration we decided to focus our efforts on 2',3'-linked phosphorothioate analogs bearing a diadenosine monophosphate backbone (2',3'-S2-CDA), due to the excellent feature of this class of synthetic CDNs and the compelling results reported for **ADU-S100**. The 2',3'-phosphodiester linkage offers improved affinity for STING compared to the canonical 3',3'-form.[14,15] The introduction of sulfur atoms within the two thiophophodiester bonds confers improved resistance to phophodiesterase mediated degradation, resulting in enhanced activation of the STING pathway *in vitro* and significantly more robust antitumor responses *in vivo*.[12,16] These advantages proved especially significant for one of the possible stereoisomers of the bis-thiophosphate analogs, the *R*p,*R*p-series in which both the stereogenic thiophosphate groups are in the *R* configuration. The 2,'3'- *R*p,*R*p-S2 analogs also proved to be potent agonists against both mouse STING as well as a number of human STING allelic isoforms, which is significant because these common polymorphic STING alleles are present in a large portion of the human population yet may possess differential responsivity to CDN agonists.[12] At the onset of this project, the 2,'3'-*R*p,*R*p-S2-CDA analog was reported to have the best overall features, and we decided therefore to start our exploration from this lead molecule, targeting nucleobase and ribose modifications specifically designed to have a high probability of success in improving its antitumor efficacy and overall profile.

Amongst the several potential modifications evaluated for the nucleobase portion, we selected to start our exploration by substituting either of the two adenines within the 2',3'-CDA structure with the 7-deaza-adenine core (Figure 2, compounds **1a/b** and **2a/b**). Despite being a rather conservative modification, the 7-deaza substitution has been reported within the context of antiviral nucleotide projects to have significant effects on boosting intrinsic potency against nucleotide recognizing molecular targets, and to offer improved stability against metabolic degradation by adenosine deaminase and phosphorylase.[17]

As for modifications within the ribose portion, we targeted the specific replacement of the 2'-hydroxyl within the 2',3'-CDA structure with either a fluorine or a chlorine atom (Figure 2, compounds **3a/b** and **4a/b**). Both these substitutions were selected in view of their successful replacements of the 2'-OH moiety reported in the context of antiviral nucleotide projects that targeted the inhibition of RNA-dependent-RNA-polymerases, where recognition through the ribose 2'-OH motif is known to play a crucial role.[18,19]

All the above 2,'3- S2-CDA analogs were evaluated *in vitro* for their ability to stimulate the human and mouse STING pathway head to head with two benchmark CDN agonists, 3',3'-cGMP and 2',3'-RR-S2-CDA (**ADU-S100**). The compounds were tested at 1μg/mL in human THP-1-Dual™ and mouse J774-Dual™ cells (Invivogen), featuring stable integration of two inducible reporter constructs enabling simultaneous study of the two main signaling pathways activated by STING, the NF-kB pathway, and the interferon regulatory factor 3 (IRF3) pathway. Data for the activation of the IRF3 pathway are reported in Figure 3a, as

assessed by activity of the luciferase reporter gene. Several of these newly synthetized CDN analogues proved able to act as human and mouse STING agonists, with a selection of them showing equivalent or superior activity relative to the clinical benchmark 2',3'-RR-S2-CDA. A noticeable difference was observed in the effect of 7-deaza-adenine substitution, with regioisomers **1a/b** significantly more active than regiosiomers **2a/b**, and **1a** (**IACS-8779**) showing equivalent activity relative to the clinical benchmark 2',3'-RR-S2-CDA. Within the ribose modifications evaluated in the 2'-position, substitution of the 2'-OH with a 2'-F in **3a/b** proved to be superior to the 2'-Cl analogs **4a/b**, and showed a significant advantage compared to the clinical benchmark 2',3'-RR-S2-CDA. The most promising analogs were then tested in a dose response manner over a range of concentrations (0.5 – 50 ug/mL, Figure 3b), where '8779 was found to be comparable, and the two 2'-F analogs 8802/8803 showed superior activity to the 2',3'-RR-S2-CDA benchmark. In light of these compelling data, compounds **IACS-8779** (**1a**) and **IACS-8803** (**3a**) were selected for evaluation of antitumor activity in mice bearing bilateral B16-OVA melanomas, in a head to head comparison with the 2',3'-cGAMP and 2',3'-RR-S2-CDA benchmarks (Figure 4a).

Mice were implanted bilaterally with 1×10^5 B16-Ova, and all the compounds were administered at the same dose (10 μg) with three intra-tumoral injections, on one flank only, on day 6, 9, 12 post implantation. While all the compounds showed comparable antitumor activity on the injected flank, **IACS-8779** and **IACS-8803** achieved superior regression on the untreated tumor in the contralateral flank, suggesting a more significant systemic immune response compared to the benchmark analogs. Treatment with '8779 and '8803 also resulted in a higher number of mice cured of both tumors compared to benchmarks (Figure 4b). Follow up studies in additional tumor models and dose down experiments are planned with IACS-8779 and IACS-8803, and will be reported in a forthcoming article.[20]

To enable the *in vitro* and *in vivo* studies above described, we required a robust synthetic approach that would allow a modular and efficient assembly of the targeted 2',3'-S2-CDA analogs. We first secured multigram amounts of the necessary building blocks 3'-H-phosphonate intermediates $\mathbf{A_{1–4}}$ and the 2'-phosphoramidite intermediates $\mathbf{B_{1–2}}$, depicted in Scheme 1.

We then employed the protocol developed by Gaffney *et al.* and its subsequent modifications to progress the above intermediates through an amidite-H-phophonate coupling and a first sulfurization step, employing 3-((dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione (DDTT).[21–23,14] Intermediates **5–8** were often carried through to the next step without need of chromatographic purification, as a mixture of stereoisomers at the newly formed phopshorothioate stereogenic center.[24] Cyclization was then achieved with 2-chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane 2-oxide (DMOCP) in pyridine, and followed by a second sulfurization step with Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide)25 in a two-step process reported to be selective for the Rp stereochemistry, [21,24] and hence resulting in the formation of two cyclized diatereosiomer products with *R*p*R*p and *S*p*R*p configuration respectively.[¥]

Removal of the cyanoethyl protecting group was achieved by treatment with base, and the two CDN disatereoisomers were typically separated at this stage by reverse phase HPLC. A

desilylation step afforded the final 2',3'-S2-CDN products as single stereoisomers, that were isolated as sodium salts after a second reverse phase HPLC followed by passage through ion exchange resin.§

The synthesis of the required building blocks intermediates was achieved either according to literature precedents (intermediates $\mathbf{A_2}$ and $\mathbf{B_1}$),[22] or following the route described in Scheme 2 and Scheme 3 for intermediates $\mathbf{A_1}$-$\mathbf{B_2}$ and $\mathbf{A_3}$-$\mathbf{A_4}$ respectively. The 7-deaza-adenine intermediates $\mathbf{A_1}$ and $\mathbf{B_2}$ were both obtained from the same 4-chloro-7-deazaadenine ribose derivative **13**. Following chlorine displacement with ammonia in MeOH, the resulting 4-amino group was selectively benzoylated *via* an *in situ* multi-step sequence, involving transient TMS-protection of the ribose hydroxyls.[26] Selective tritylation of the 5'-hydroxyl, treament with TBSCl and chromatographic separation gave the regioisomeric mono-TBS protected alcohols **14** and **15**, which were progressed to the 3'-H-phosphonate $\mathbf{A_2}$ and to the 2'-phosphoramidite $\mathbf{B_2}$ respectively.

The synthesis of intermediate $\mathbf{A_3}$ started with chloride displacement of triflate **16**,[27] followed by protecting group manipulations to obtain the 2'-deoxy-2'-chlorine-adenosine derivative **17a**. The 5'hydroxyl was temporary protected with dimethoxytrityl to install the 3'-H-phosphonate moiety, and then liberated to yield intermediate $\mathbf{A_3}$. The same synthetic sequence was employed to convert **17b** in the 2'-deoxy-2'-fluorine building block $\mathbf{A_4}$.

In summary, we set out to generate high-potency CDN STING agonists by introducing a focused set of rationally selected modifications within the nucleobase and ribose portions of the 2',3'-CDA structure. This focused approach led to the discovery of **IACS-8779** and **IACS-8803**, two 2',3'-phophothioate-CDA analogs that show robust activation of the STING pathway *in vitro* and a superior systemic anti-tumor response when compared to the clinical benchmark **ADU-S100** in the B16 murine model of melanoma. Additional studies are ongoing towards positioning these molecules for clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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¥In the case of the chlorine analogue **4**, two additional stereoisomers were isolated as final products, suggesting that the cyclization-sulfurization process might have resulted in both Rs and Rp stereochemistry. These additional analogs were found inactive, data not presented.

§Stereochemical assignent of the final products as depicted in Scheme 1 was based on comparison of ^{1}H and ^{31}P-NMR data with authentic samples of 2',3'-RpRp-S2-CDA and 2',3'-SpRp-S2-CDA prepared according to published procedures.[12]

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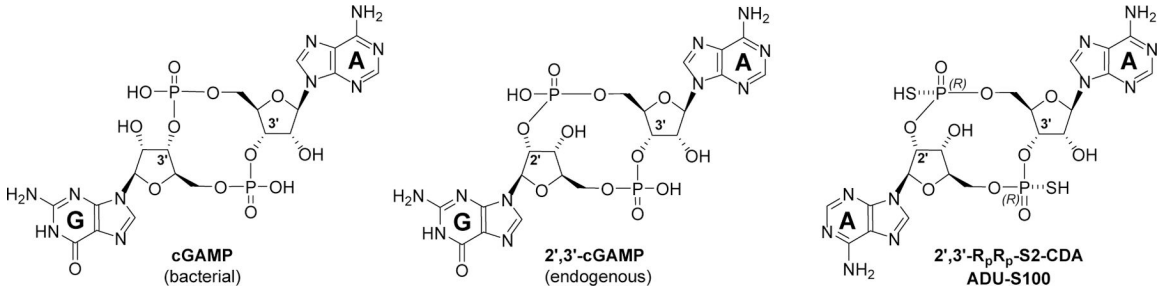


Figure 1.
Cyclic dinucleotide STING agonists.

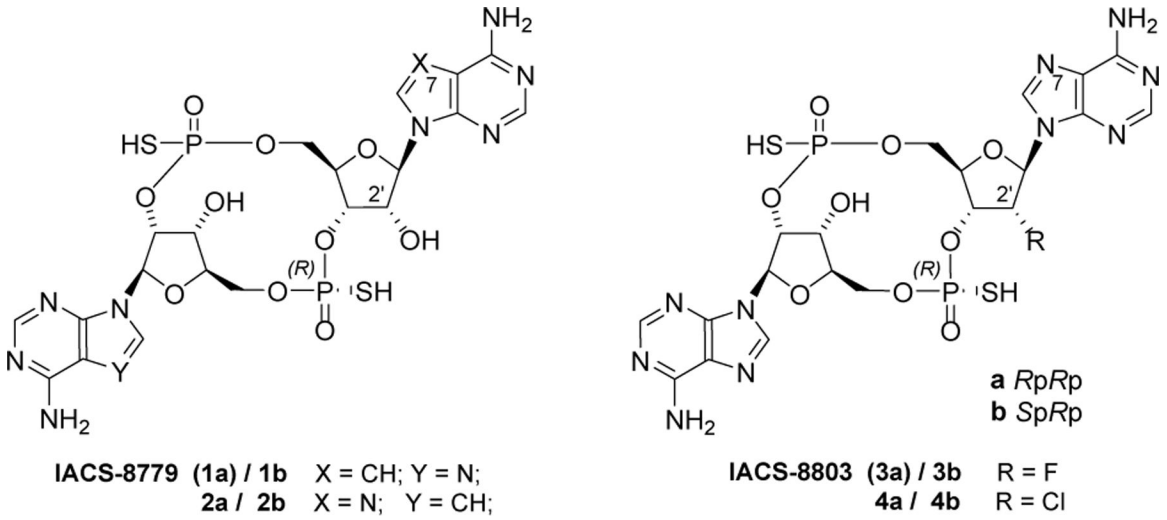


Figure 2.

The 2',3'-linked phosphorothioate diadenosine monophosphate (2',3'-S2-CDA) STING agonists herein described.

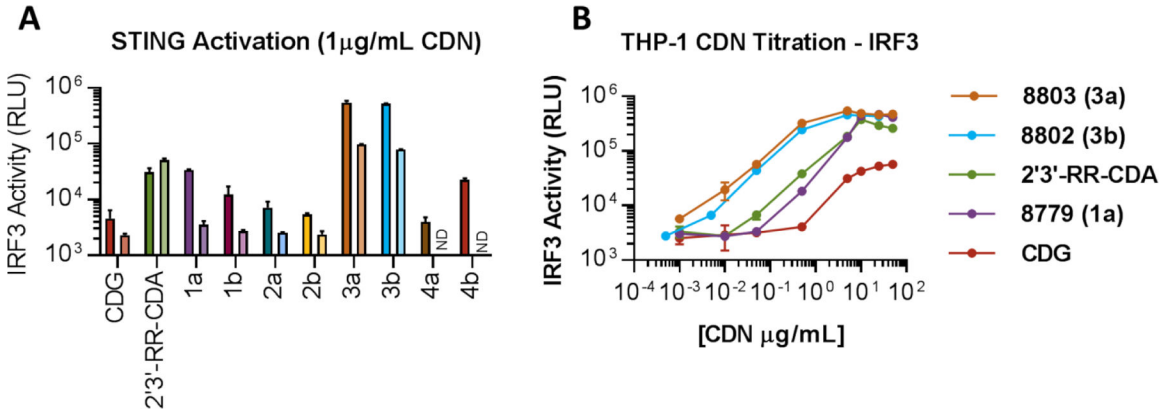


Figure 3.

Evaluation of CDN potency in THP-1 (human) and J774 (mouse) reporter cells; a) CDNs were added at 1 μg/mL to 1×10^5 THP-1 Dual or 5×10^4 J774-dual reporter cells for 20 hours and IF3 activity was measured by luciferase assay. b) CDNs were added at the indicated concentrations in the 0.5 – 50 μg/mL dose range to 1×10^5 THP-1 Dual reporter cells for 20 hours and IF3 activity was measured by luciferase assay.

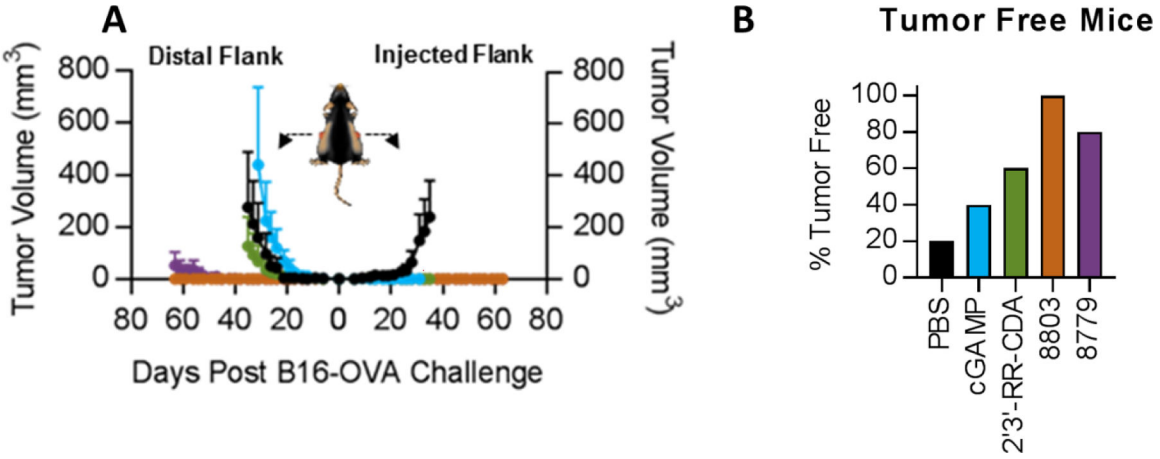
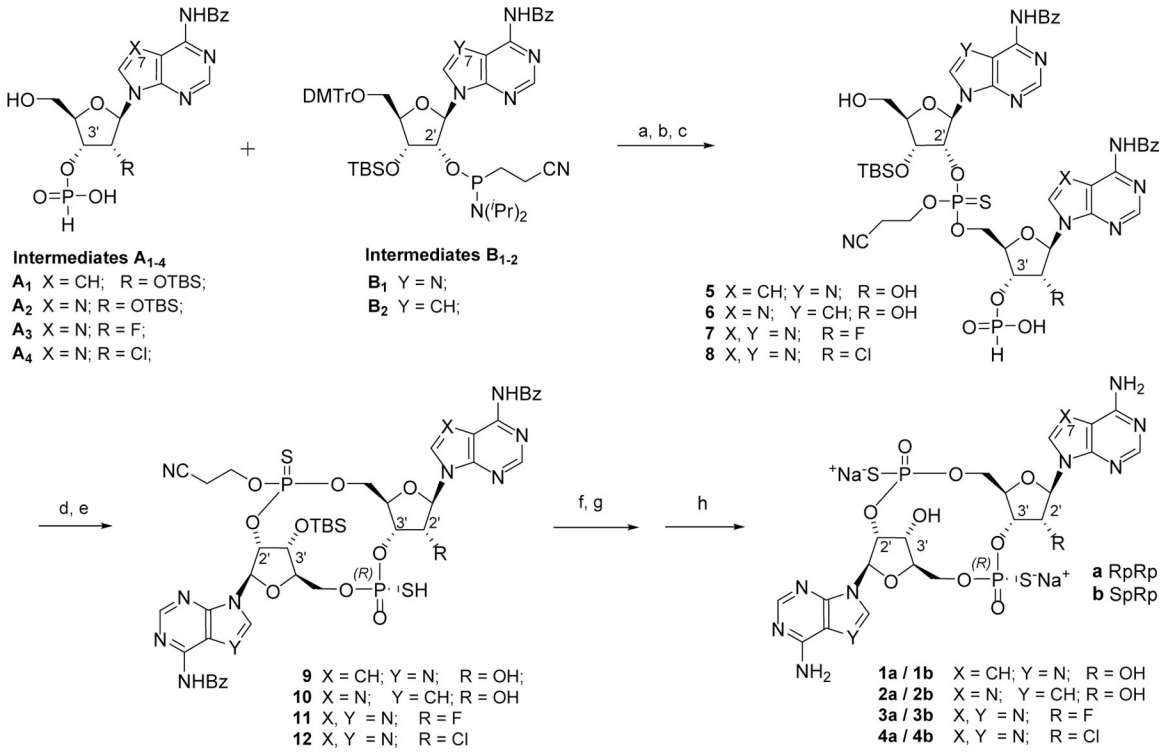


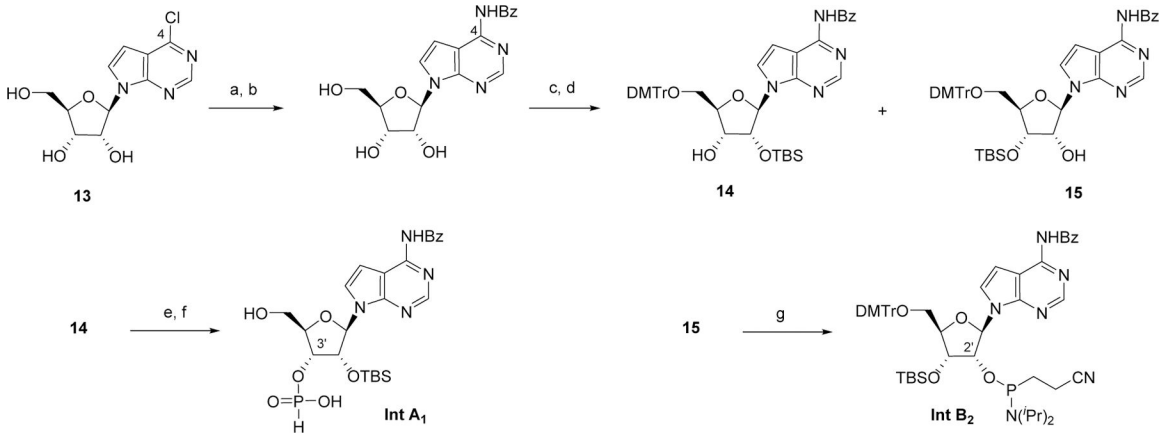
Figure 4.

Antitumor activity of selected CDNs in the B16-Ova melanoma model. Mice (n=5 per group) were implanted bilaterally with 1×10^5 B16-Ova and received 10 ug of each CDN on day 6, 9, 12 post implantation. CDNs were administered by intratumoral injection on one flank only.



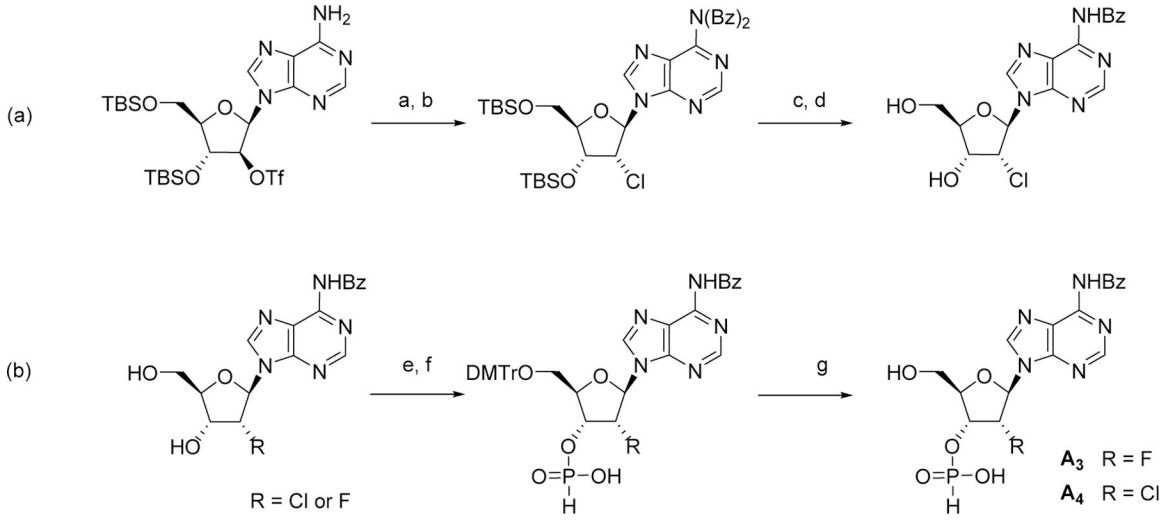
Scheme 1.

Reaction conditions: a) Pyridine, TFA; b) DDTT, acetonitrile; c) aqueous Cl_2HCCO_2H; d) DMOC, pyridine; e) Beaucage reagent, pyridine; f) NH_4OH, MeOH; g) NH_4F, MeOH; h) Dowex®–50WX8 resin (Na^+ form). Beaucage reagent = (3H-1,2-Benzodithiol-3-one 1,1-dioxide); Bz = benzoate; DDTT = 3-((dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione; DMOCP = 2-chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane 2-oxide; TBS = tert-Butyldimethylsilyl.



Scheme 2.

Reaction conditions: a) NH_3, MeOH, 110 °C; b) i. TMSCl, pyridine, 0 °C; ii. BzCl, 0 °C to RT; iii. H_2O, then aq. NH_3 0 °C; c) DMTrCl, pyridine; d) TBSCl, pyridine, $AgNO_3$; e) PivCl, pyridine, H_3PO_3; f) Cl_2CHCO_2H, H_2O, DCM; g) 2-cyanoethyl diisopropylchlorophosphoramidite, DCI, DCM. Bz = benzoate; DMTr = 4,4-Dimethoxytrityl; TBS = tert-Butyldimethylsilyl.



Scheme 3.

Reaction conditions: a) LiCl, DMF, 50 °C; b) BzCl, pyridine, 0–25 °C; c) TBAF, THF; d) aq. NH_3, THF; e) DMTrCl, pyridine; f) PivCl, H_3PO_3, pyridine, 0–30 °C; g) Cl_2HCCO_2H, H_2O, DCM. Bz = benzoate; DMTr = 4,4- Dimethoxytrityl; Tf = trifluoromethansulfonate; TBS = tert-Butyldimethylsilyl.