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Complete List of Authors:	Caldwell, Steven; University of Pittsburgh, Chemistry Janosko, Chasity; University of Pittsburgh, Chemistry Deiters, Alexander; University of Pittsburgh, Chemistry

Development of a Light-Activated STING Agonist

Steven E. Caldwell‡, Chasity P. Janosko‡, and Alexander Deiters*

Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, United States
Center for Systems Immunology, University of Pittsburgh, Pittsburgh, PA 15260, United States

‡ These authors share co-first authorship.

Abstract

The STING pathway is critical to innate immunity and is being investigated as a potential therapeutic target. Existing agents targeting STING suffer from several undesirable effects, particularly the possibility of systematic activation, which increases the risk of autoimmune disorders. In this proof-of-concept study, we report the development of a light-activated STING agonist, based on the potent compound SR-717. We first screened the activity of the non-caged agonist toward 5 human STING variants to identify the most viable target. A photocaged agonist was designed and synthesized in order to block an essential interaction between the carboxy acid group of the ligand with the R238 residue of the STING protein. We then investigated the selective activation of STING with the photocaged agonist, demonstrating an irradiation-dependent response. The development and characterization of this selective agonist expands the growing toolbox of conditionally controlled STING agonists to avoid systematic immune activation.

Introduction

The Stimulator of Interferon Genes (STING) pathway has been shown in recent years to have an important role in the innate immune response.[1] The STING pathway plays a crucial role in combatting infection in response to the recognition of viral or damaged DNA by cyclic GMP-AMP (cGAMP) synthase (cGAS)[2] or by the secretion of other cyclic dinucleotides (CDNs) from invading bacteria.[3] Upon binding of a cyclic dinucleotide, the signaling protein STING is activated as a protein dimer, initiating the STING signaling cascade (**Fig. 1A**), which ultimately leads to $CD8^+$ T cell priming and elicits an anti-cancer immune response.[4] The mechanism of CDN-STING binding has been extensively investigated, including several protein structures.[5-8] Interestingly, the active STING dimer can adopt either of two conformations. For example, while cGAMP[5] and c-di-AMP[6] bind to human STING in a closed conformation, c-di-GMP interacts with the same protein in an open conformation.[8] Independent of the conformation, stabilization of the STING dimer leads to repositioning of the C-terminal domains of each monomer, leading to polymerization and initiating binding to TBK1.[6] This protein complex is subsequently trafficked to the golgi apparatus, where TBK1 phosphorylates transcription factors, such as nuclear factor κB (NFκB) and interferon regulatory factor 3 (IRF3).[9] Type I interferon (IFN) signaling is widely implicated in antitumor immunity by inhibiting proliferation and promoting apoptosis of tumor cells.[10] While this effect is beneficial for an anti-cancer response, STING is widely expressed across various cell types, including immune, non-immune, and cancerous cells.[9] Broad STING expression poses an issue,

as systemic activation of STING can lead to autoimmune disorders, such as systematic lupus erythematosus.[11] This demonstrates a need for more selective STING agonists which specifically target only cells in the afflicted area of the body, thereby minimizing off-target effects.

Because CDN binding to STING has been well-studied, several CDN derivatives have been synthesized toward the goal of therapeutic STING agonists.[12, 13] CDNs make for successful agonists via injection into solid tumors,[14] but suffer from a lack of stability and cell permeability, limiting their use in other cancer types.[4] While active transporters were recently determined to be required for CDN uptake, several common immunotherapeutics were shown to inhibit CDN uptake by these transporters,[15] complicating use in combinatorial therapies. Several CDN derivatives aim to increase stability through the introduction of phosphorothioate moieties,[16, 17] delivery through encapsulation in tumor-targeting[18, 19] or inhalable nanoparticles[20] or hydrogels,[21] or cell permeability through masking the negatively charged phosphate groups using a prodrug approach.[17] To address both the inherent instability and low cell permeability of CDNs, several non-nucleotide, small molecule STING agonists, such as diABZI3,[22] MSA-2,[23] and SR-717 (see **1** in **Fig. 1A**)[24] have been developed as well. A non-symmetrical dibenzimidazole dimer diABZI3 demonstrated increased activity as compared to cGAMP against mouse and various human variants of STING in cell-based assays, as well as a reduction in tumor volume in mice.[22] More recently, the pH-dependent agonist MSA-2 led to increased selectivity for activation in cancerous cells, and various dimerized derivatives showed drastically improved efficacy over the original compound,[23, 25] as well as in combinatorial treatment with bifunctional antibodies.[26] We found the small-molecule agonist SR-717 of particular interest, because it binds human STING in the closed conformation, similar to cGAMP, with a low micromolar EC_{50} reported in ISG-THP1 cells. This agonist was also shown to have anti-tumorigenic properties in a mouse xenograft model, while showing promise across STING isoforms.[24] The human homolog of STING is known to have several prevalent single point mutations, as shown in **Table S1** in the ESI.[27] Each of these mutations affect substrate binding and allow for preferential activity of certain agonists to certain variants.[14] Several of the human variants have been shown to be less or non-responsive to CDN-treatment, highlighting the importance of non-nucleotide based agonists, such as **1**, in order to eventually target patients expressing these widespread mutations. In cancer treatment in particular, a prodrug approach offers the potential for enhanced tumor specificity, with a caged compound being selectively activated by the chemical makeup of a cancer cell or the tumor microenvironment.[28-30] Here, we are investigating the use of a light-activated caging group as a stepping stone for a prodrug design of the STING agonist **1**. Light provides a distinct advantage over other external triggers as it is bioorthogonal and can be controlled temporally and spatially.[31]

Results and Discussion

The crystal structure of human STING in complex with **1**, along with the data reported during the original structure activity relationship study were utilized to rationally design an appropriate prodrug approach for **1**. Two molecules of **1** are shown to overlap at the dimer interface of the wildtype human STING protein (**Fig. 1B**). The terminal carboxylate of each molecule of **1** forms a hydrogen bond to R238 of each protein monomer, a residue that is conserved across four of the

five common STING variants. By appending a photocaging group to the carboxylic acid group of **1**, we hypothesized that this critical interaction will be disrupted due to removal of a hydrogen bond and the introduction of significant steric bulk, thereby blocking STING dimerization and activation. This hypothesis is further supported by the original structure-activity relationship studies, which showed that the methyl ester derivative of **1** exhibits no binding to recombinant STING, in contrast to the corresponding carboxylic acid derivative.[24]

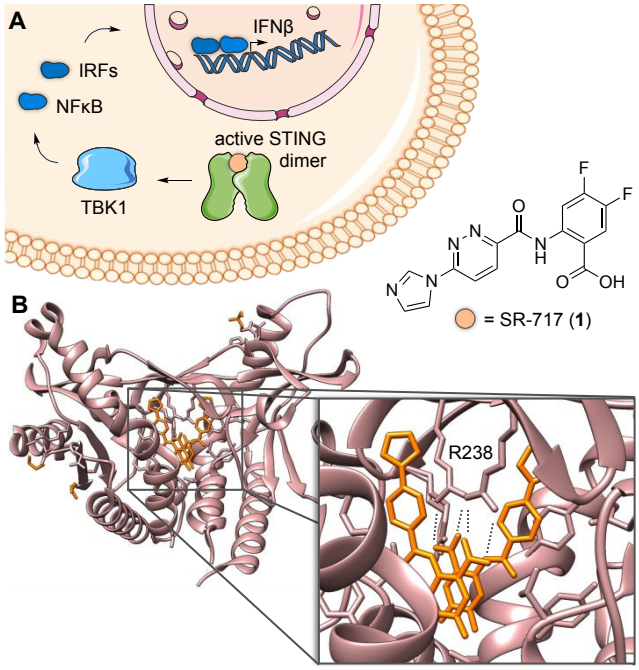

Fig. 1. Design of a photocaged STING agonist. A) Activation of STING leads to the transcription of immune responsive genes. B) Crystal structure of **1** binding human STING (PDB: 6XNP). Binding occurs as a dimer and critical hydrogen bond interactions between each molecule of **1** and the R238 of each dimer are indicated as dashed lines.

To experimentally validate our hypothesis, we first synthesized and tested two ester-containing derivatives of **1**, the benzyl ester **2** and the propylphenyl ester **3** (**Fig. 2**). These ester groups were chosen because they closely resemble nitrophenyl caging groups without being light sensitive. The synthesis of **2** began with the assembly of the imidazolyl-pyridazine acid **6**[24] from the commercially available starting material **5**, and subsequent conversion to the acyl chloride intermediate **7**. Concurrently, the amine **8** was Boc-protected to prevent interference with future substitution reactions. The Boc-protection of **8** is low yielding simply due to the presence of unreacted starting material. While this may be surprising, it is consistent with reported syntheses[32] of similar compounds and could likely be improved with a longer reaction time. Hydrolysis of the methyl ester **9** allows the resulting carboxylic acid **10** to undergo an S_N2 reaction with benzyl bromide to afford **11**. Then a simple Boc deprotection with trifluoroacetic acid (TFA) releases the amine and enables an amidation reaction with the synthesized acyl chloride intermediate **7**, to yield the benzyl ester **2**. The propylphenyl ester **3** was assembled using a similar approach, with

an alternative method of installation of the propyl ester to the Boc-protected amine. Instead of the carboxylic acid acting as a nucleophile in an S_N2 reaction, the propyl ester was added via a HATU-mediated esterification to produce **13**. This route was optimal, because attempts to construct the brominated version of the propyl group were met with difficulty, likely due to the presence of the tertiary carbon center which would favor an elimination product over the substitution product. Unfortunately, the yield of this reaction was quite low, which we attribute to the carboxyl's inherent poor electrophilicity. This is supported by the increased yield when using the carboxyl as nucleophile as in the synthesis of **11** and **12**. Furthermore, multiple other conditions were attempted including the use of different coupling agents and increasing the reaction temperature, but only the reported conditions were able to provide any product.

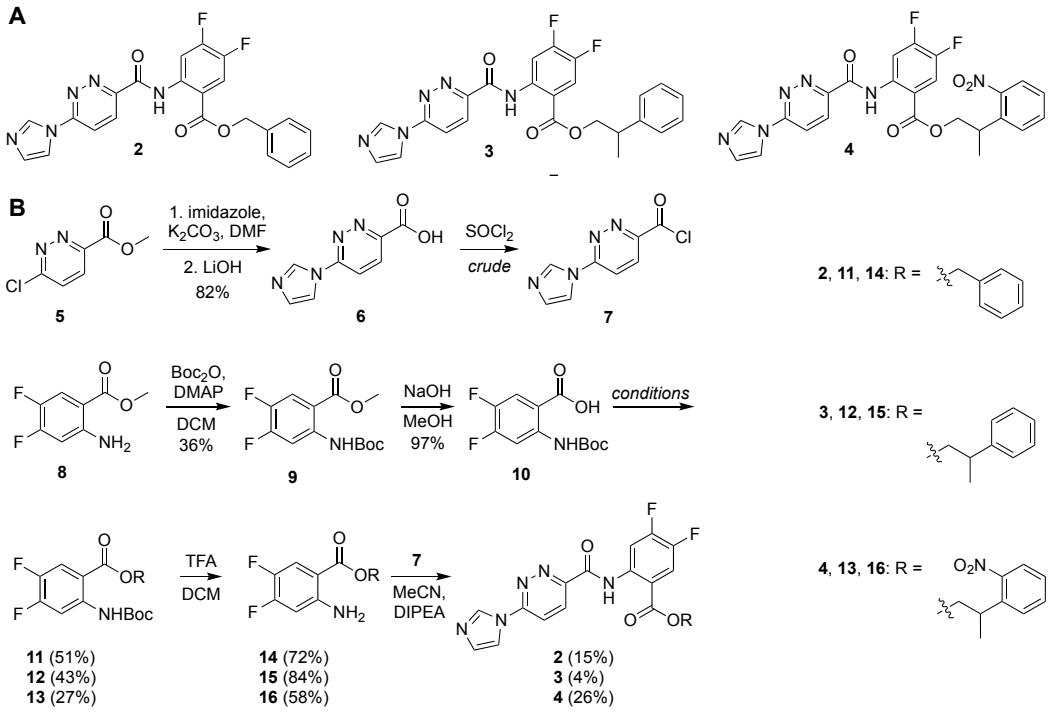

Fig. 2. Synthesis of STING agonist derivatives. Conditions used for esterification of **10** were as follows: **11**, benzyl bromide, DIPEA, MeCN; **12**, 2-phenylpropan-1-ol, HATU, DMAP, TEA, DMF; **13**, 2-(2-nitrophenyl)propan-1-ol, HATU, DMAP, TEA, DMF.

The agonist **1** was previously shown to bind to the mouse and all common human variants of recombinant STING protein in thermal stability assays, suggesting the activity of this ligand might be unaffected by commonly found mutations. Because testing the activity against human STING is more translatable to applications in immunotherapy, and the activity of **1** for the different human STING mutants had not yet been characterized in cells, we first aimed to directly compare the cellular response with compound treatment of each variant. To this end, we introduced the appropriate mutations for $STING^{AQ}$, $STING^{Q}$, and $STING^{REF}$ into the pMSCV-$STING^{WT}$ and pMSCV-$STING^{HAQ}$ plasmids[33] (**Table S1** and **Table S2** in the ESI) in order to obtain expression

vectors for all five human variants. STING activity was then assessed by expressing each STING mutant in HEK293T cells, along with a firefly luciferase reporter under the control of an interferon β (IFNβ) promoter and a constitutively expressed *Renilla* luciferase transfection control (**Fig. 3A**). The IFNβ promoter contains responsive elements for IRF-1, IRF-2, and NFκB, which all act downstream of STING. Thus, in response to STING activation, expression of firefly luciferase would be expected.[34] After transfection, cells were treated with **1** (20 μM) for 2 hours and relative luminescence values were normalized to the corresponding DMSO negative control, as no STING response should be elicited in the absence of STING agonist. STING^{HAQ} and STING^{AQ} expressing cells treated with **1** showed a 7-fold increase over the corresponding negative controls, suggesting successful activation of the pathway, while no significant response over background activation was noted for STING^{WT}, STING^{Q}, or STING^{REF} at this concentration (**Fig. 3B**). Background activation has been previously noted when high levels of STING are expressed, so it is likely that minimizing the transfection levels of STING could ascertain the differences in activation of STING^{WT}, STING^{Q}, or STING^{REF}. However, it was clear from this study that **1** most potently activates STING^{HAQ} and STING^{AQ} when equal levels of each protein are expressed (**Fig. S1** in the ESI).

For all further experiments, we moved forward with the STING^{HAQ} mutant, as it is the most prevalent mutation found in the human population.[27] In order to further characterize the activation of STING^{HAQ} by **1**, a dose-response activity assay was performed by treating cells transfected with the reporter system described above using increasing concentrations of **1** (ranging from 10 nM to 200 μM). A nonlinear fit (log(agonist) vs. response) of the data revealed an EC_{50} of 17 μM (**Fig. 3C**), which is slightly higher than the previously determined EC_{50} of 2.1 μM of **1** in THP-1 cells.[24]

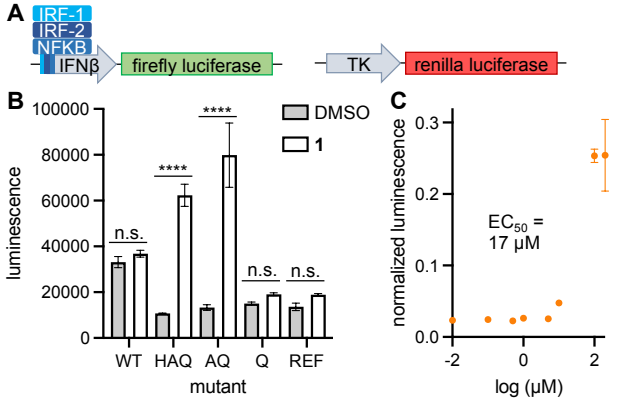

Fig. 3. Activation of human STING mutants with 1. A) Activation of each STING variant was evaluated using a dual luciferase assay. B) Cells transfected with each variant were treated with 100 μM of **1** and STING activation was analyzed. C) Cells expressing STING^{HAQ} were treated with increasing concentrations of **1**. Three biological replicates were averaged and error bars represent standard deviations. Two-way ANOVA tests were performed, n.s. $p > 0.05$, **** $p < 0.0001$.

To test if binding to STING was sufficiently inhibited by blocking the carboxy group of **1**, activation of STING^{HAQ} with the esters **2** or **3** was analyzed using the luciferase reporter. While cells treated with **1** showed a significant response, cells treated with increasing concentrations of **2** (**Fig. 4A**) or **3** (**Fig. 4B**) showed no activation of the IFNβ reporter at any compound concentration. This observation suggests that the presence of the ester functionality quantitatively blocked agonist activity in both cases and either ester scaffold could have been used for the generation of a photocaged STING agonist.

Next, we sought to synthesize a photocaged compound based on the benzyl ester **2**, though a photocaged derivative of **2** was not synthetically accessible, presumably due to the steric hindrance introduced by the addition of an *ortho*-nitro substituent, preventing formation of the amide bond. Thus, we speculated that a photocaged derivative of **3** could be more synthetically accessible, as the *ortho*-nitrophenyl would be placed further from the amide functionality. The photocaged compound **4** was assembled analogously to the negative control compound, propylphenyl ester **3** (**Fig. 2**). The low yields of the final amidation step are likely due to a combination of the low reactivity of the imidazoyl-pyridazine carboxyl and low solubility. Initial attempts at amidation through traditional coupling conditions (HATU, DCC, EDC, etc.) afforded no product and the compounds poor solubility in common chromatography solvents led to difficulties with purification. The potential for background activity from the non-irradiated photocaged agonist **4** was also tested by measuring the STING response to compound treatment in the absence of irradiation (**Fig. 4C**). With the careful exclusion of ambient light, no background activity was observed, similar to the negative control compound **3**.

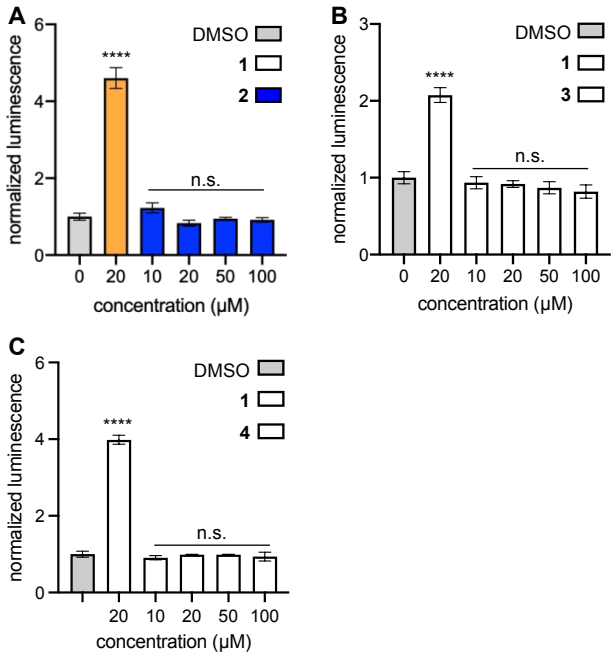

Fig. 4. Background activation of STING using ester derivatives. Cells were treated with DMSO, **1** (20 μM) and increasing concentrations of A) **2**, B) **3**, or C) **4**. While **1** produced a statistically significant response, no response was observed with any concentration of **2**, **3**, or **4**. Three biological replicates were averaged and error bars represent standard deviations. One-way ANOVA tests were performed, n.s. $p > 0.05$, **** $p < 0.0001$.

Prior to analysis of STING activation in cells, the stability of **4** was first assessed by HPLC (**Fig. S2** in the ESI) to ensure the compounds would be stable throughout the course of testing. Each compound was incubated in a solution of phosphate buffered saline (PBS, pH 7.4) with 10% fetal bovine serum (FBS) to simulate assay conditions. Samples were analyzed by HPLC periodically over an 8-hour time period. The area of the peaks corresponding to **1** and **4** were quantified at each timepoint to show complete stability over 8 hours, suggesting stability throughout the course of cell treatment.

Once the stability of the agonists was confirmed, we aimed to validate the light-induced decaging of **4** prior to moving into cell-based assays (**Fig. 5A**). A compound solution prepared at 100 μM in PBS was irradiated with a 365 nm (0, 5 s, 10 s, or 20 s) or 405 nm (0 , 10, 20, 30, 60, or 120 s) LED, then immediately analyzed by HPLC. The peak corresponding to **4** (15.8 min) completely disappeared with increasing light (365 nm) exposure and two new peaks appeared, corresponding to **1** (19.7 min) and the decaging byproduct 1-nitro-2-(prop-1-en-2-yl)benzene at 18.3 min as confirmed by HRMS (**Fig. 5B and C**). While irradiation with 365 nm light produced a complete response with only a 5 s irradiation, even 2 min irradiation at 405 nm was required for complete decaging (**Fig. S3** in the ESI). From this study, it was determined that irradiation with 365 nm should be used in later assays for the most efficient decaging, though 405 nm could be used if UV-exposure was a concern.

After demonstrating this compound can be used in cells without inducing background activation of STING and successful decaging via HPLC, activation of this signaling pathway in response to the decaging of **4** was next analyzed. Cells were treated with **4**, then irradiated for increasing amounts of time using a 365 nm LED. A dose-response with respect to irradiation time was observed (**Fig. 5D**) for cells treated with **4**, with a complete response observed after 120 s of irradiation, matching the positive control and showing successful optical activation of the STING pathway in mammalian cells. To ensure irradiation did not contribute to STING activation, this assay was also performed with the negative control **3**. No STING response was noted with increasing irradiation times (**Fig. S4** in the ESI).

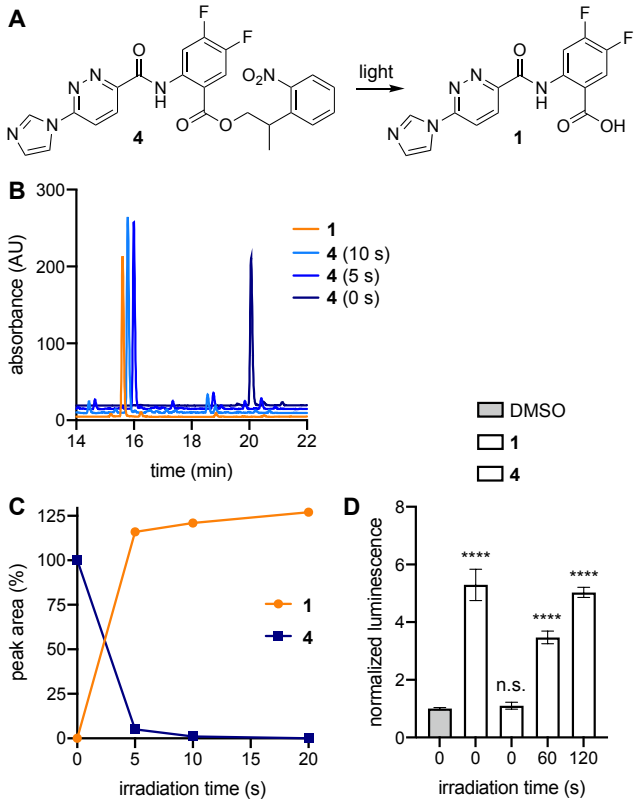

Fig. 5. Photoactivation of STING using 4. A) Irradiation of **4** with 365 nm light leads to decaging and release of **1**. B) Samples (100 μM) were prepared in PBS, irradiated for various lengths of time with a 365 nm LED, and analyzed by HPLC. HPLC chromatograms show complete disappearance of the peak corresponding to **4** (19.7 min) with a 5 s irradiation, suggesting successful decaging of **4** to release the active agonist, **1** (15.8 min). C) The peak area corresponding to **4** and **1** at each irradiation time was normalized to the peak area of the corresponding non-irradiated control. D) Cells were treated with DMSO, **1** (20 μM), or **4** (20 μM), then irradiated. STING activation increases with increasing irradiation times. Three biological replicates were averaged and error bars represent standard deviations. One-way ANOVA tests were performed, n.s. $p > 0.05$,*** $p < 0.001$, **** $p < 0.0001$.

Beside photo-activation, we also investigated the effect of these compounds on cell health and a cell viability assay was performed. HEK293T cells were treated in triplicate with increasing concentrations of **1** or **4** for 72 hours, followed by analysis via XTT assay. Absorbance values were normalized to a DMSO control, showing no decrease in cell viability with compound treatment (**Fig. S5** in the ESI).

Summary

In summary, we developed the photocaged STING agonist **4** in a step toward mitigating systemic overactivation of the STING pathway, which is a common concern in the field as this can lead to dangerous autoimmune disorders. The caging site in **4** was selected based on the analysis of the structure of the ligand-protein complex and the caging group was chosen based on synthetic considerations. We have demonstrated fast light-dependent decaging of our agonist via HPLC studies, with an immediate and almost complete response noted after a minimal light exposure of 5 s. Furthermore, selective activation of **4** was assayed in mammalian cells using an IFNβ-responsive firefly luciferase construct to monitor STING signaling. Importantly, the caged compound displayed no background activation in the absence of light stimulation. We further investigated the selectivity of the parent agonist **1** to reveal preference for $STING^{HAQ}$ and $STING^{AQ}$ over the wildtype protein, demonstrating a path toward targeted treatment for patients expressing these common mutations. The overexpression of each widely expressed STING variant in HEK293T cells allowed for a direct comparison of the variable response to this agonist in different populations, an important yet sometimes overlooked consideration in the development of STING agonists. The new agonist **4** elicited no toxicity in HEK293T cells even at increased concentrations. During our studies, photocaged analogs of another STING agonist, MSA-2, were reported.[36, 37] Together with our results this demonstrates that caging and pro-drug approaches are likely broadly applicable to various small molecule activators of STING signaling. Due to the diverse binding modes of each STING agonist to each STING variant, it is important to gain selective control over a collection of agonists. The results obtained in our study help add to the rapidly developing foundation for conditional activation of the STING pathway by rendering the potent agonist **1** photoactivatable.

Acknowledgements

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Footnote

Electronic supplementary information (ESI) available: Synthesis and characterization of new compounds, experimental details, supplementary data.

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