**Cell Reports**

**Binding-Pocket and Lid-Region Substitutions Render Human STING Sensitive to the Species-Specific Drug DMXAA**

**Graphical Abstract**

**Highlights**

- Residues critical for species-selective STING sensitivity to DMXAA are uncovered
- S162A/Q266I substitutions endow hSTING with the same DMXAA sensitivity as mSTING

In Brief

The anticancer drug DMXAA specifically activates the STING pathway in a species-dependent manner. Gao et al. combine calorimetric, structural, and cellular studies to investigate the mechanism underlying DMXAA species selectivity. They find a critical role for a lid residue at position 230 and unveil the structural basis for the mouse specificity of DMXAA. This work also suggests ways to render human STING responsive to DMXAA.

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**Authors**

Pu Gao, Thomas Zillinger, ..., Winfried Barchet, Dinshaw J. Patel

**Correspondence**

winfried.barchet@ukb.uni-bonn.de (W.B.), pateld@mskcc.org (D.J.P.)

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Binding-Pocket and Lid-Region Substitutions Render Human STING Sensitive to the Species-Specific Drug DMXAA

Pu Gao,1,6 Thomas Zillinger,3,5 Weiji Wang,2 Manuel Ascano,4 Peihong Dai,2 Gunther Hartmann,3 Thomas Tuschi,4 Liang Deng,2 Winfried Barchet,3,* and Dinshaw J. Patel1,*

1Structural Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
2Dermatology Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
3Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn, University of Bonn, Bonn 53127, Germany
4Laboratory of RNA Molecular Biology, Howard Hughes Medical Institute, Rockefeller University, New York, NY 10065, USA
5Co-first author
*Correspondence: winfried.barchet@ukb.uni-bonn.de (W.B.), pateld@mskcc.org (D.J.P.)

SUMMARY

The drug DMXAA (5,6-dimethylxanthenone-4-acetic acid) showed therapeutic promise against solid tumors in mouse models but subsequently failed in human clinical trials. DMXAA was later discovered to activate mouse, but not human, STING, an adaptor protein in the cyclic dinucleotide cGAMP-mediated signaling pathway, inducing type I interferon expression. To facilitate the development of compounds that target human STING, we combined structural, biophysical, and cellular assays to study mouse and human chimeric proteins and their interaction with DMXAA. We identified a single substitution (G230I) that enables a DMXAA-induced conformational transition of hSTING from an inactive “open” to an active “closed” state. We also identified a substitution within the binding pocket (Q266I) that cooperates with G230I and the previously identified S162A binding-pocket point substitution, rendering hSTING highly sensitive to DMXAA. These findings should facilitate the reciprocal engineering of DMXAA analogs that bind and stimulate wild-type hSTING and their exploitation for vaccine-adjuvant and anticancer drug development.

INTRODUCTION

The endoplasmic reticulum transmembrane protein STING (stimulator of interferon genes) (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Jin et al., 2008; Sun et al., 2009; Zhong et al., 2008) is a central player in the innate immune response to cytosolic double-stranded DNA (Burgett and Vance, 2013). STING, which responds to various forms of pathogen-derived DNA, as well as to self-DNA, functions as an adaptor protein that recruits and activates TANK binding kinase (TBK1) and IκB kinase (IKK), which, following their phosphorylation, activate nuclear transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor kappa B (NF-κB), respectively. STING was shown to be a direct sensor of bacterial cyclic dinucleotides (CDNs) (Burgett et al., 2011), although it was subsequently demonstrated that the host-encoded cytosolic DNA-sensor cyclic GMP-AMP synthase (cGAS) (Sun et al., 2013) produces the second messenger cyclic GMP-AMP (cGAMP) (Wu et al., 2013), which then binds and activates STING. Independent studies by several groups demonstrated that a noncanonical cGAMP linkage isomer, c[G(2',5')pA(3',5')p], is produced by cGAS upon DNA binding (Ablasser et al., 2013; Diner et al., 2013; Gao et al., 2013a; Zhang et al., 2013). Follow-up structure-function studies showed that human and mouse STING (hSTING and mSTING, respectively) undergoes an “open” to “closed” conformational transition upon binding c[G(2',5')pA(3',5')p] (Gao et al., 2013b; Zhang et al., 2013). Our studies have primarily focused on the R71/G230/R232/R293 variant of hSTING (hSTINGR232).

The xanthenone derivative compound DMXAA (Vadimezan, 5,6-dimethylxanthenone-4-acetic acid; Figure 1A) was initially identified as a small molecule that exhibits immune modulatory activities through the induction of cytokines and disrupts tumor vascularization in multiple mouse models (Baguley and Ching, 2002). DMXAA in combination with paclitaxel and carboplatin was evaluated in a phase II clinical trial against non-small-cell lung cancer, but ultimately failed in human phase III trials (Lara et al., 2011). Recently, it was demonstrated that DMXAA-induced interferon-β (IFN-β) production by murine macrophages is dependent on STING, suggesting that mSTING is the protein target of DMXAA (Prantner et al., 2012). Despite the high sequence identity between mSTING and hSTING (68% amino acid identity and 81% similarity) (Diner et al., 2013), DMXAA activates mSTING but has no effect on hSTING (Conlon et al., 2013; Kim et al., 2013), which hampers DMXAA’s therapeutic potential in humans.

Our earlier structure-function studies revealed that mSTING binds to DMXAA using the same pocket as the natural cyclic dinucleotide c[G(2',5')pA(3',5')p] and induces a similar “open” to “closed” conformational transition (Gao et al., 2013b). Given that identical residues line the DMXAA binding pocket of both mSTING and hSTING, it is unclear why DMXAA only activates mSTING. Following our initial observation that a point substitution...
Gluc, RLU

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rendered it partially sensitive to DMXAA (Gao et al., 2013b), we (S162A) of hSTING placed within the CDNs/DMXAA binding site and vaccine adjuvants. which are needed for the development of anticancer therapies b variants with potential IFN- provides a critical guide for future rational drug design of DMXAA rendered hSTING highly sensitive to DMXAA. These findings with the previously identified binding-pocket S162A substitution, that Q266I binding-pocket and G230I lid substitutions, together teric, biophysical, and cellular techniques. Our studies establish tion on STING-ligand complexes (Gao et al., 2013b), we subdi- (Conlon et al., 2013; Kim et al., 2013). Therefore, the noncon- (F) Intermolecular contacts in the complex. The bound DMXAA is shown in biscuit color, with individual monomers colored in yellow and magenta. (G) Two expanded views of the hydrophobic interac- tions of the G230I substitution (in green) in the complex (blue box region in E). Other residues lining the hydrophobic pocket are shown in yellow. See also Figures S1 and S2.

RESULTS

The Lid Region of the Ligand Binding Pocket Is Important for DMXAA Recognition

Within STING, DMXAA (Figure 1A) and c [G(2,5)pA(3,5)p] share the same ligand binding pocket (Gao et al., 2013b), which in human and mouse proteins is composed of identical amino acids. Despite the fact that the hSTING and mSTING C-terminal domains (CTD, aa 140–379) exhibit ~76% amino acid identity (Figure S1), DMXAA only binds and activates mSTING, and has no effect on hSTING (Conlon et al., 2013; Kim et al., 2013). Therefore, the noncon- served residues between the two species that are located outside the DMXAA binding pocket must play a role in distinct DMXAA recognition. Guided by the available structural information on STING-ligand complexes (Gao et al., 2013b), we subdivided the nonconserved residues located in the STING CTD into four groups (groups 1–4). We then substituted hSTING residues with their mSTING counterparts for each of the four groups (Figure S1). These residues are located either along the dimer interface or within the regions that undergo large conformational changes during the “open” to “closed” transition.

(S162A) of hSTING placed within the CDNs/DMXAA binding site rendered it partially sensitive to DMXAA (Gao et al., 2013b), we reasoned that either smaller substituents or slightly modified DMXAA variants could be promising candidates for the activation of hSTING and have potential for development as anticancer drugs or vaccine adjuvants.

Here, we describe our detailed investigation of the mechanism of DMXAA species selectivity through a combination of structural, biophysical, and cellular techniques. Our studies establish that Q266I binding-pocket and G230I lid substitutions, together with the previously identified binding-pocket S162A substitution, rendered hSTING highly sensitive to DMXAA. These findings provide a critical guide for future rational drug design of DMXAA variants with potential IFN-β-stimulating activity in humans, which are needed for the development of anticancer therapies and vaccine adjuvants.
associated with complex formation. We also generated a construct containing the combined substitution in all four groups (hSTINGgroup1234).

We performed isothermal titration calorimetry (ITC) experiments to measure the DMXAA binding affinity of hSTING CTD (aa 140–379) containing various group substitutions. hSTINGgroup1234 showed a comparable exothermic binding curve and binding affinity (K_D: 0.69 μM) (Figure 1B) to mSTING (K_D: 0.49 μM) (Gao et al., 2013b). Similar to what was found for wild-type (WT) hSTING protein, no detectable binding to DMXAA was observed for the isolated group1, group3, or group4 substitutions of hSTING (Figure S2A). Only group2 substitutions of hSTING exhibited detectable endothermic binding with DMXAA (K_D: 3.12 μM; Figure 1C).

To validate the binding results, we used an IFN-β luciferase reporter assay to further test the responsiveness of hSTING group substitutions to DMXAA stimulation in human 293T cells, which lack endogenous STING expression. For this cellular assay, we used full-length hSTING (WT and substitutions) and mSTING (WT) constructs, which were expressed at moderate levels to allow ligand-dependent activation of the IFN-β promoter. We confirmed that hSTING-transfected 293T cells responded to DMXAA, whereas hSTING-transfected cells did not (Figure 1D), left panel). Consistent with the ITC results, among the individual group substitutions, only the hSTINGgroup2 substitutions showed responsiveness to DMXAA (Figure 1D, middle panel). Inversely, removing the group2 substitutions from the combined group1234 substitutions (hSTINGgroup1234) strongly diminished DMXAA activation, whereas loss of any of the other groups was tolerated (Figure 1D, right panel). These results indicate that group2 residues from mSTING, which are located within the lig region of the binding pocket, play an important role in DMXAA recognition.

Crystal Structure of DMXAA Bound to hSTINGgroup2
We proceeded to solve the crystal structure of DMXAA bound to hSTINGgroup2 (aa 155–341) at 1.88 Å resolution (for X-ray statistics, see Table S1) with the complex containing two molecules of DMXAA per hSTINGgroup2 dimer (Figure 1E). The results were similar to what we had previously observed for the complex of mSTING and DMXAA (Gao et al., 2013b). The four-stranded, antiparallel, β-pleated sheet formed a lid covering the binding pocket, indicative of the formation of a “closed” conformation of STING upon complex formation. The aromatic rings of the two DMXAA moieties were aligned in parallel, with complex formation mediated by both intermolecular van der Waals contacts and hydrogen-bond interactions (Figure 1F). We observed excellent superposition of hSTINGgroup2 and mSTING in their complexes with DMXAA, as shown in Figure S2B (root-mean-square deviation [rmsd]: 0.95 Å).

To elucidate the molecular basis underlying DMXAA species selectivity, we compared the structure of the hSTINGgroup2, DMXAA complex with that of the mSTING-DMXAA complex (Gao et al., 2013b). We found that in the hSTINGgroup2-DMXAA structure, the side chain of the substituted residue I230 (G230 in WT protein) is located in a hydrophobic pocket composed of residues from both the four-stranded, antiparallel β-sheet region (R232, I235, R238, and Y240) and the adjacent long α-helix (L170 and I171) (Figure 1G). The amino acids that form the hydrophobic pocket are identical between human (Figure 1G) and mouse (Figure S2C) proteins. This isoleucine-mediated hydrophobic interaction may help stabilize the β sheet and other parts of the protein, facilitating DMXAA-mediated formation of the “closed” conformation by mSTING or hSTINGgroup2, thereby explaining the absence of complex formation by WT hSTING with a glycine at this position.

G230 of hSTING and I229 of mSTING Are Critical Contributors to Differential DMXAA Recognition
To support our conclusions based on our structural findings described above, we generated the G230I single substitution in hSTING and tested its IFN-β induction activity using the luciferase assay. Indeed, hSTINGG230I alone was sufficient to mimic the effects observed for hSTINGgroup2, resulting in an induction of IFN-β almost identical to that found for hSTINGgroup2 (Figure 2A). Using the same method, we also generated and tested reverse substitutions on mSTING (I229G or I229A). As expected, mSTINGG230G and mSTINGG230A showed a significant decrease in DMXAA-mediated IFN-β induction (Figure 2B).

We also solved the crystal structure of DMXAA bound to hSTINGG230I (aa 155–341) at 2.51 Å resolution (for X-ray statistics in Table S1), with hSTINGG230I in the complex forming a “closed” conformation (Figure 2C). The detailed intermolecular contacts in the complex (Figure S3A) are similar to those observed for the hSTINGgroup2-DMXAA structure (Figure 1F). We observed excellent superposition of hSTINGG230I and hSTINGgroup2 in their complexes with DMXAA, as shown in Figure S3B (rmsd: 0.61 Å). The I230 residue, which is positioned within a hydrophobic pocket (Figure 2D), forms the same intramolecular contacts as observed in the structures of the hSTINGgroup2-DMXAA (Figure 1G) and mSTING-DMXAA (Figure S2C) complexes. Taken together, our structural and functional data strongly demonstrate that the substitution of Gly with Ile at position 230 results in the gain of function of hSTING for DMXAA recognition.

hSTINGQ266I Is Activated by DMXAA
Guided by the structures of complexes of hSTING substitutions with DMXAA, we next tested additional substitutions within the ligand binding pocket to identify more constraints that would help in the design of future modifications on DMXAA. We generated five substitutions (G166S, I235L, Q266I, Q266L, and Q266V) in hSTING (Figure S1) to either enhance the hydrophobic interaction or introduce additional hydrogen bonds with DMXAA. The initial IFN-β induction results showed that only the Q266I substitution in hSTING conferred DMXAA sensitivity at a level similar to that previously observed for the S162A substitution (Gao et al., 2013b; Figure 3A). Q266L resulted in a less pronounced gain of DMXAA-mediated IFN-β induction, whereas G166S, I235L, and Q266V showed no effects (Figure 3A). We next tested whether the S162A/Q266I double substitution would augment DMXAA recognition, and indeed observed an enhanced DMXAA-induced IFN-β induction similar to that found for mSTING (Figure 3B). These results were confirmed by ITC studies, which showed that hSTINGS162A/Q266I binds to DMXAA with higher affinity (K_D: 1.99 μM; Figure 4C) than either hSTINGS162A (Figure S3C) or hSTINGQ266I (Figure S3D).
Besides the prevalent allelic hSTING variant (R71/G230/R232/R93, hSTING\(^{R232}\)) four major nonsynonymous variants are found with high frequencies in the human population: R71H/G230A/R293Q (hSTING\(^{HAG}\)), 20.4%; R232H (hSTING\(^{H232}\)), 13.7%; G230A/R293Q (hSTING\(^{GAQ}\)), 5.2%; and R293Q (hSTING\(^{Q293}\)), 1.5% (Yi et al., 2013). To determine whether the substitutions for all major hSTING alleles (listed in Figure 3D) and S162A and Q266I substitutions were effective in all natural hSTING alleles, which showed a variable maximal IFN-\(\beta\) induction (Figure 3B for hSTING\(^{R232}\) and Figures S4A and S4B for other hSTING alleles), which showed a variable maximal IFN-\(\beta\) induction for different alleles but clear sigmoidal dose responses that diverged by less than one order of magnitude in their EC\(_{50}\). Taken together, these results indicate that the Q266I substitution renders hSTING responsive to DMXAA. Further, hSTING containing Q266I and S162A substitutions lead to a DMXAA-dependent IFN-\(\beta\) reporter response close to that observed for mSTING. (Figure 3F). The crystal structures of hSTING\(^{S162A/Q266I}\) and hSTING\(^{G230I}\) in their bound complexes with DMXAA superimpose with an rmsd of 0.70 Å (Figure S4C). The details of the intermolecular contacts in the complex are shown in Figure S4D, with the same intermolecular hydrogen-bonding interaction network as observed in the hSTING\(^{R232}\)-DMXAA (Figure 1F) and hSTING\(^{G230I}\)-DMXAA (Figure S3A) complexes.

Crystal Structure of DMXAA Bound to hSTING\(^{S162A/Q266I}\)

To better understand how S162A and Q266I substitutions facilitate the IFN induction of hSTING by DMXAA, we solved the cocrystal complex of DMXAA with hSTING\(^{S162A/Q266I}\) (aa 155–341) at 2.42 Å resolution (X-ray statistics in Table S1). The complex adopts the “closed” conformation, as reflected by the positioning of two DMXAA in the binding pocket and the formation of the four-stranded, antiparallel \(\beta\) sheet lid over the bound ligands (Figure 3F). The crystal structures of hSTING\(^{S162A/Q266I}\) and hSTING\(^{G230I}\) in their bound complexes with DMXAA superimpose with an rmsd of 0.70 Å (Figure S4C). The details of the intermolecular contacts in the complex are shown in Figure S4D, with the same intermolecular hydrogen-bonding interaction network as observed in the hSTING\(^{R232}\)-DMXAA (Figure 1F) and hSTING\(^{G230I}\)-DMXAA (Figure S3A) complexes.

hSTING\(^{S162A/G230I/Q266I}\) is More Sensitive to DMXAA than mSTING in IFN-\(\beta\) Induction

We next tested whether combining the G230I lid substitution with the binding-pocket substitutions S162A/Q266I would further enhance hSTING sensitivity to DMXAA. We generated the triple mutant of hSTING and tested its binding to DMXAA by ITC, as well as IFN induction by DMXAA in transfected cells. The ITC titration for hSTING\(^{S162A/G230I/Q266I}\) with added DMXAA is plotted in Figure 4A and shows a higher binding affinity (K\(_D\): 0.99 μM) than that observed for hSTING\(^{R232}\) (K\(_D\): 3.12 μM; Figure 1C) or hSTING\(^{G230I}\) (K\(_D\): 1.99 μM; Figure 3C), indicating that all three substitutions individually contribute to an increased DMXAA sensitivity. This increase in affinity translates to synergistic functional effects, based on our luciferase reporter assays in which hSTING\(^{S162A/G230I/Q266I}\) showed approximately two
orders of magnitude higher sensitivity than hSTING^{G230I} as well as an order of magnitude higher sensitivity than either hSTING^{S162A/Q266I} or mSTING for IFN-β induction by DMXAA (Figure 4B).

We also solved the crystal structure of DMXAA bound to hSTING^{S162A/Q266I} (aa 155–341) at 2.37 Å resolution (X-ray statistics in Table S1) in the “closed” conformation (Figure 4C). As expected, we observed both the hydrophobic pocket
surrounding I230 (Figure 4D), which was the same as in the hSTINGG230I-DMXAA complex (Figure 2D), and the hydrophobic interactions within the DMXAA binding pocket (Figure 4E), which were the same as in the hSTINGS162A/Q266I-DMXAA complex (Figure 3G).

**DMXAA Activates Type I IFN and Proinflammatory Cytokine and Chemokine Production in mSTING-Deficient BMDCs Reconstituted with hSTING Substitutions**

We previously showed that c[G(2,5)pA(3,5)p] and its linkage analogs induce type I IFN and proinflammatory cytokine/chemokine production in a STING-dependent manner in bone-marrow-derived macrophages (Gao et al., 2013b). To test whether various hSTING substitutions can rescue the deficiency of type I IFN and proinflammatory cytokine/chemokine production in response to DMXAA in mSTING-deficient bone-marrow-derived dendritic cells (BMDCs), we generated BMDCs from homozygous functional null STING mice (Goldenticket, STINGGt/Gt) (Sauer et al., 2011). Retroviruses carrying WT hSTING or hSTING mutants (hSTINGG230I, hSTINGS162A/Q266I, hSTINGG230I/Q266I, and hSTINGS162A) were used to transduce these BMDCs. Although WT hSTING did not induce the upregulation of IFN-β mRNA after DMXAA treatment, we observed 2.6-, 3.1-, 4.2-, and 2.2-fold increases in IFN-β mRNA levels in BMDCs expressing hSTINGG230I, hSTINGS162A/Q266I, hSTINGG230I/Q266I, and hSTINGS162A.
hSTINGS162A, respectively. Similar to the results obtained from the luciferase reporter assays, we found that hSTINGG230I/Q266I in BMDCs expressing hSTINGG230I/Q266I had the highest IFN-β mRNA induction after DMXAA treatment, corroborating that G230I substitution and the pocket substitutions S162A/Q226I lead to synergistic effects on hSTING sensitivity to DMXAA. We also observed upregulation of CXCL10, CCL5, and IL-6 mRNAs in BMDCs expressing various hSTING mutants (Figure 4F), with hSTINGS162A/G230I/Q266I eliciting the strongest induction among the four mutants after DMXAA treatment. We also collected supernatants at 18 hr after DMXAA treatment. At this time point, hSTINGS162A/G230I/Q266I induced the highest level of CXCL10 production compared with the other hSTING mutants (Figure S4E). We confirmed hSTING protein expression in transduced cells by western blot analysis (Figure 4G).

**DISCUSSION**

Functional studies have demonstrated that DMXAA activates mSTING, but not hSTING (Conlon et al., 2013; Kim et al., 2013). DMXAA showed great promise in mouse cancer models, underscoring its potential for human application, notwithstanding the outcome of a phase III clinical trial for non-small-cell lung carcinoma (Lara et al., 2011). Hence, it is important to recognize that although DMXAA itself is no longer a viable drug, pharmacological modulation of STING remains an ideal therapeutic strategy to pursue. For this purpose, we sought to define the molecular basis underlying DMXAA species selectivity.

Given that DMXAA binding involves interactions with identical amino acids in both mSTING and hSTING (Gao et al., 2013b), nonconserved residues that do not participate in direct interaction with DMXAA must contribute to species-specific response to DMXAA.

We identified a hydrophobic interaction between the substituted I230 and the residues from both the lid region and other parts of the protein in the hSTINGG230I, DMXAA complex (Figure 1G), a distinctive feature that was also found in the structure of the mSTING–DMXAA complex (Figure S2C). All residues that form the hydrophobic pocket that contains I230 are conserved in both hSTING and mSTING proteins. The gain of function of hSTINGG230I and, inversely, the loss of function of mSTINGQ229G and mSTINGQ266A in their ability to induce IFN gene expression in response to DMXAA further confirmed the critical role of this residue in species-specific recognition of DMXAA (Figures 2A and 2B). Our crystal structure of the hSTINGG230I–DMXAA complex also exhibited the active “closed” conformation (Figures 2C and 2D), further supporting our conclusion that this single point substitution outside of the binding pocket of hSTING critically modulates sensitivity to the otherwise mouse-selective DMXAA ligand. Hydrophobic interactions could help facilitate formation of the lid region and other parts of the protein, allowing mSTING to form the “closed” conformation more readily than hSTING in response to DMXAA.

In general, our structural studies indicate that mSTING is induced more readily to assume the “closed” conformation than hSTING in response to CDNs and their analogs. To overcome this intrinsic disadvantage of hSTING, we need to design better-fitting DMXAA analogs to allow hSTING to overcome the energy barrier when transitioning from an “open” to a “closed” state. To eventually enable the rational design of suitable DMXAA modifications, we systematically introduced hSTING substitutions within the binding pocket and tested their influence on DMXAA-induced IFN-β production. Following this strategy and guided by our cocrystal structures of STING substituents with DMXAA, we identified two point substitutions within the ligand binding pocket: S162A (reported previously; Gao et al., 2013b) and Q266I, each of which strongly promotes DMXAA recognition (Figure 3A). Our data suggest that modestly altered DMXAA derivatives might be sufficient to bind and activate hSTING. By introducing the above substitutions into the predominant hSTING alleles, we were able to restore a dose-dependent response to DMXAA in all cases (Figures S4A and S4B).

Strikingly, the S162A/G230I/Q266I triple substitution of hSTING showed an order of magnitude higher activity than mSTING (Figure 4B), indicating that all three substitutions are required to confer a synergistic effect to DMXAA recognition. hSTINGS162A/G230I/Q266I might therefore be used as a benchmark hSTING synthetic allele in future drug development studies using humanized mouse models.

In summary, we have provided a comprehensive structural, biophysical, and functional analysis of DMXAA’s association with select substitutions within hSTING. Our results highlight the critical role of the lid residue at position 230 (229 in mSTING) and unveil the structural basis for the mSTING selectivity of DMXAA. Our structural and functional results also shed light on strategies to restore an efficient hSTING response to DMXAA based on the binding-pocket S162A and Q266I substitutions. Toward this goal, our current efforts to generate reciprocal DMXAA derivatives with synthetic chemist collaborators are directed toward the synthesis of analogs containing polar groups (OH, OCH3, F, Cl, and NO2) at the C1/C2 (S162A substitution) and C7 (Q266I substitution) positions within the DMXAA ring so as to form additional intermolecular hydrogen bonds, as well as to replace the six-membered aromatic rings with their five-membered counterparts. Given that two molecules of DMXAA bind to the STING dimer, ongoing efforts are also being directed toward the generation and evaluation of covalently linked DMXAA dimers. In this context, it should be noted that 7-methyl-XAA and 8-methyl-XAA, which involve the redistribution of CH3 substitutions on the DMXAA scaffold, have been identified as weak yet human-active and possibly also human-selective DMXAA analogs (Tijono et al., 2013). Finally, we are conducting an ongoing program to screen small-molecule libraries using high-throughput approaches and mass-spectroscopy-based detection assays to identify potential candidates with scaffolds distinct from DMXAA that target hSTING. Imitating the effects of these amino acid substitutions by rational design of reciprocal DMXAA derivatives should lead to the development of human-active STING agonists for antitumor, antiviral, and vaccine adjuvant applications.

**EXPERIMENTAL PROCEDURES**

**Crystallization and Structure Determination**

Crystals were grown using the sitting-drop vapor diffusion method, and diffraction data were collected at synchrotron beamlines. All structures were solved using the PHASER, COOT, and Phenix programs.
**Isothermal Titration Calorimetry**

The thermodynamic parameters of the binding reactions of STING with cGAMP isomers and DMXAA were measured by ITC using a MicroCal ITC200 calorimeter at 25°C.

**Reconstitution of STING-Deficient Murine BMDCs with hSTING**

BMDCs were generated by culturing bone marrow cells from STING<sup>−/−</sup> mice in complete medium in the presence of GM-CSF for 10 days. BMDCs (1 x 10<sup>6</sup> cells/well) were infected with retroviruses expressing hSTING (WT and various substitution mutants). At 48 hr after retroviral infection, cells were stimulated with DMXAA.

**Luciferase Assay**

HEK293T cells were reverse transfected with STING expression plasmids and reporter constructs as described previously (Gao et al., 2013b). DMXAA was added by culture medium replacement 12 hr later. Luciferase expression was determined after another 12 hr.

For further details regarding the materials and methods used in this work, see the Supplemental Experimental Procedures.

**ACCESSION NUMBERS**

The following coordinates have been deposited to the Protein Data Bank: DMXAA-hSTING<sub>pA(3′,5′)p(5′,5′)pA(3′)</sub> (4QXO), DMXAA-hSTING<sub>pA(3′)p(5′)pA(3′)p(5′)pA(3′)</sub> (4QXP), DMXAA-hSTING<sub>pA(3′)p(5′)pA(3′)p(5′)pA(3′)</sub> (4QXR).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.010.

**AUTHOR CONTRIBUTIONS**

The structural and in vitro binding assays were performed by P.G. under the supervision of D.J.P. The luciferase assays monitoring interferon induction were performed by T.Z. under the supervision of W.B. and G.H. The assays describing DMXAA stimulation of BMDCs were performed by W.W. and P.D. under the supervision of L.D. All authors participated in the writing of the paper and agree with the contents.

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**REFERENCES**


