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

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Figure S1. Sequence Alignment and the Design of Group Substituents. Related to Figure 1 and 3.

Sequence alignment of STING from mouse and human. The transmembrane domain (1-139 of hSTING; 1-138 of mSTING) and C-terminal domain (140-379 of hSTING; 139-378 of mSTING) are shown in gray and black, respectively. The group substituents are indicated in colored boxes: group1 in blue, group2 in red, group3 in green, and group4 in yellow. Red arrows indicate the mutated residues within the ligand-binding pocket.
Figure S2. ITC Binding Assays for DMXAA with hSTING group1, group3, and group4 Mutants and Structural Comparison of DMXAA Bound to hSTING\textsuperscript{group2} and mSTING. Related to Figure 1.

(A) ITC binding curves for complex formation between DMXAA bound to hSTING\textsuperscript{group1} (aa 140-379) (left), hSTING\textsuperscript{group3} (middle), and hSTING\textsuperscript{group4} (right).

(B) Superposed structures of the complex of DMXAA bound with mSTING (PDB: 4LOL) (silver) and hSTING\textsuperscript{group2} (aa 155-341) (green).

(C) The hydrophobic interactions of I229 with surrounding residues in the mSTING-DMXAA complex, with the same presentations and color codes as used in Figure 1G.
Figure S3. Structural Comparison of DMXAA Bound to hSTING\(^{G230I}\) and hSTING\(^{group2}\), as well as ITC-based DMXAA-Binding Assays for hSTING S162A and Q266I Mutants. Related to Figure 2 and 3.

(A) Intermolecular contacts in the complex of DMXAA bound to hSTING\(^{G230I}\) (aa 155-341). The bound DMXAA is shown in biscuit color, with individual STING subunits in the symmetrical dimer shown in yellow and magenta.

(B) Superposed structures of the complex of DMXAA bound with hSTING\(^{G230I}\) (aa 155-341, yellow) and hSTING\(^{group2}\) (aa 155-341, green).

(C, D) ITC binding curves for complex formation between DMXAA bound to hSTING\(^{S162A}\) (aa 140-379) (panel C) and hSTING\(^{Q266I}\) (panel D).
Figure S4. Dose-Responses of hSTING Variants and their Respective S162A, Q266I Mutants to DMXAA and the Structural Comparison of DMXAA Bound to hSTING\textsuperscript{G230I} and hSTING\textsuperscript{S162A/Q266I}, as well as DMXAA Stimulation of Cytokines/chemokines in Mouse cells. Related to Figure 3 and 4.

293T cells were transfected with reporter constructs and indicated STING variants. After 12 hours, cells were stimulated with ascending concentration of DMXAA (A and B) directly added to medium. Luciferase assay was performed after another 12 hours. Dose-responses shown are representative of 2 independent experiments.

(A) DMXAA dose-response of hSTING variants with S162A, Q266I double substituent. For improved clarity, only hSTING\textsuperscript{H232} is shown as a representative for the corresponding wt variants. Circle refers to magnified view in (B).

(B) DMXAA dose-responses of hSTING\textsuperscript{HAQ} and hSTING\textsuperscript{HAQ} S162A, Q266I on a magnified scale (circle denotes corresponding hSTING\textsuperscript{HAQ} S162A, Q266I curve in (A).

(C) Superposed structures of the complex of DMXAA bound with hSTING\textsuperscript{G230I} (aa 155-341, yellow) and hSTING\textsuperscript{S162A/Q266I} (aa 155-341, cyan).

(D) Intermolecular contacts in the complex of DMXAA bound to hSTING\textsuperscript{S162A/Q266I} (aa 155-341). The bound DMXAA is shown in biscuit color, with individual STING subunits in the symmetrical dimer shown in yellow and magenta.

(E) BMDCs were infected with retroviruses (same as in Figure 4F). Cells were incubated with 50 µg/ml of DMXAA and supernatants were collected 18 hours after treatment. CXCL10 protein levels were determined by ELISA. Data shown are means ± SEM (n=3), representative of two independent experiments.
**Table S1. X-ray Statistics for DMXAA with hSTING Mutants**

| Crystal | hSTING\(^{\text{group2}}\) + DMXAA | hSTING\(^{G230I}\) + DMXAA | hSTING\(^{S162A/Q}\) + DMXAA | hSTING\(^{S162A/G}\) + DMXAA |
|---------|-----------------------------------|------------------------------|-------------------------------|-------------------------------|
| Beam line | APS-ID24C | APS-ID24C | APS-ID24C | APS-ID24C |
| Wavelength (Å) | 1.550 | 1.550 | 1.550 | 1.550 |
| Space group | \(P6_1\) | \(P2\) | \(P6\) | \(P6\) |
| Unit cell | | | | |
| \(a, b, c (\text{Å})\) | 62.9, 62.9, 196.1 | 36.6, 77.9, 79.6 | 148.0, 148.0, 36.1 | 148.6, 148.6, 36.2 |
| \(\alpha, \beta, \gamma (\text{°})\) | 90.0, 90.0, 90.0 | 90.0, 99.0, 90.0 | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 |
| Resolution (Å) | 50-1.88 (1.92-1.88)\(^a\) | 50-2.51 (2.61-2.51)\(^a\) | 50-2.42 (2.55-2.42)\(^a\) | 50-2.37 (2.50-2.37)\(^a\) |
| \(R\text{merge}\) | 0.077 (0.785) | 0.102 (0.643) | 0.118 (1.017) | 0.115 (1.104) |
| \(I/\sigma (I)\) | 15.2 (2.4) | 12.1 (2.3) | 15.4 (2.3) | 25.9 (3.1) |
| Completeness (%) | 97.6 (93.7) | 94.6 (89.5) | 100.0 (100.0) | 100.0 (100.0) |
| Redundancy | 5.8 (6.0) | 3.8 (3.7) | 9.4 (9.0) | 9.1 (8.7) |
| Number of unique reflections | 19022 | 14320 | 17687 | 19048 |
| \(R\text{work}/R\text{free} (%)\) | 17.4/21.8 | 19.7/23.9 | 18.2/22.8 | 18.7/23.1 |
| Number of non-H atoms | | | | |
| Protein | 1499 | 2932 | 2920 | 2928 |
| Water | 137 | 55 | 71 | 58 |
| Ligands(molecule) | 1 | 2 | 2 | 2 |
| Average B factors (Å\(^2\)) | | | | |
| Protein | 38.68 | 68.70 | 56.51 | 68.13 |
| Water | 45.73 | 44.55 | 51.55 | 63.79 |
| ligands | 21.53 | 49.91 | 28.21 | 38.75 |
| R.m.s. deviations | | | | |
| Bond lengths (Å) | 0.013 | 0.007 | 0.014 | 0.007 |
| Bond angles (°) | 1.580 | 1.262 | 1.613 | 1.203 |
| Ramachandran plot | | | | |
| Preferred (%) | 95.4 | 95.3 | 95.8 | 95.3 |
| Allowed (%) | 4.6 | 4.7 | 4.2 | 4.7 |
| Disallowed (%) | 0 | 0 | 0 | 0 |

\(^a\) Highest resolution shell (in Å) shown in parentheses.
**SUPPLEMENTAL EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**
The sequences corresponding to residues aa 140-379 and 155-341 of hSTING were inserted into a modified pRSFduet-1 vector (Novagen), in which the target protein was separated from the preceding His$_6$-SUMO tag by an ubiquitin-like protease (ULP1) cleavage site. The gene sequences were subsequently confirmed by sequencing. The fusion proteins were expressed in BL21 (DE3) RIL cell strain. The cells were grown at 37 °C until OD600 reached approx. 0.6. The temperature was then shifted to 18 °C and the cells were induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture medium at a final concentration of 0.3 mM. After induction, the cells were grown overnight. The fusion proteins were purified over a Ni-NTA affinity column. The His$_6$-SUMO tag was removed by ULP1 cleavage during dialysis against buffer containing 40 mM Tris-HCl, 300 mM NaCl, pH 7.5. After dialysis, the His$_6$-SUMO tag was removed by Ni-NTA affinity column and the sample was further fractionated over a gel filtration 16/60 G200 Superdex column. The final sample of hSTING contains about 16 mg/ml protein, 20 mM Tris-HCl, 150 mM NaCl, pH 7.5. All the mutants were cloned and purified using the same protocol as used for preparation of the wt protein.

**Crystallization**
For crystallization of DMXAA with hSTING mutants (aa 155-341), the protein samples were mixed and incubated with DMXAA (1:2 molar ratio) for 0.5 h at room temperature before setting up crystals. The crystals were generated by sitting drop vapor diffusion method at 20°C, by mixing equal volume reservoir solution with the sample. The detailed conditions are listed below: DMXAA-hSTING$^{group2}$: 2 M LiCl, 10% PEG6000, 0.01 M CaCl$_2$, 0.1 M Tris, pH 7.8; DMXAA-hSTING$^{G230I}$: 0.2 M Li$_2$SO$_4$, 20% PEG 3350, 0.015 mM CYMAL®-7, 0.1 M Tris, pH 8.1; DMXAA-hSTING$^{S162A/Q266I}$: 0.2 M Ca(Ac)$_2$, 15% PEG3000, 0.01 M L-Proline, 0.1 M NaAc, pH 5.1; DMXAA-hSTING$^{S162A/G230I/Q266I}$: 0.2 M Ca(Ac)$_2$, 15% PEG3000, 0.1 M LiCl, 0.1 M NaAc, pH 5.1;

**Structure Determination**
All the diffraction data sets were collected at the Advanced Photon Source, Argonne National laboratories, and were indexed, integrated and scaled using the RAPD online server. All the structures were solved using molecular replacement method in PHASER (McCoy et al., 2007) using the structure of hSTING$^{1232}$ complex bound to c[G(2′,5′)pA(3′,5′)p] (PDB: 4LOH) as the search model. The model building was conducted using the program COOT (Emsley et al., 2010) and structural refinement was conducted using the program PHENIX (Adams et al., 2010). The statistics of data collection and refinement are shown in Table S1.

**Isothermal Titration Calorimetry Binding Assay**

The dissociation constants (Kd) and thermodynamic parameters of binding reactions of hSTING wt and mutants (aa 140-379) with DMXAA were measured by isothermal titration calorimetry using a MicroCal ITC200 calorimeter at 25°C. First, wt and mutant protein samples were dialyzed overnight against working buffer (100 mM NaCl, 30 mM HEPES, pH 7.5) at 4 °C. Then, the protein samples were diluted with working buffer and the lyophilized DMXAA were dissolved in working buffer. The titration was carried out with 16 successive injections of 2.4 µL DMXAA, spaced 180s apart, into the sample cell containing the protein solution. The data were fit using the program Origin 7.0 software.

**STING-Plasmids for Eukaryotic Expression and Generation of Point Mutants**

pMAX-Flag-hSTING and pMAX-Flag-mSTING were described previously (Gao et al, 2013b). Point mutants were generated by site-directed PCR mutagenesis via the Quikchange Primer Design method (Agilent, Santa Clara, CA) using PfuUltra HotStart DNA Polymerase (Agilent) and suitable primers. For combination of multiple distant substitutions and subcloning of constructs, STING fragments were PCR-amplified using Q5 DNA Polymerase (NEB, Ipswich, MA) and full-length constructs were assembled using Gibson Assembly (NEB). Constructs were verified by restriction digestion and Sanger sequencing (Seqlab, Göttingen, Germany). Reporter constructs were described previously (Gao et al, 2013a).
| Name                        | DNA Sequence                                                                 |
|-----------------------------|------------------------------------------------------------------------------|
| hSTINGfw-39bpFlag-GibA   | GCCACCATGGGATTACAAGGATGACGACGATAAGGTCGACT                                      |
|                            | GCCCCACTCCAGCCTGCA                                                             |
| hSTINGrev458-GibA         | AAATCTCCCTTTTTTCACACACTGCG                                                  |
| hSTINGfw420-GibA          | CCCAGCTGAGATCTCCTGCA                                                         |
| hSTINGrev-40bp-GibA       | TGTGGTTTGTCCAAACTCATCGAGCCTCGGATGCGGACCACCGCGG                                |
|                            | CAAGAGAAATCCCTGTCGCGGGA                                                    |
| revbefgroup1               | TGGCAGGATCAGCCCGCAG                                                          |
| fwbefgroup1                | CTGGCGGCTGATCCTGCGCA                                                        |
| revbefgroup2               | ACAGTCCATGGGAGGAGGAGATATACGAG                                                 |
| fwbefgroup2                | CTGATATTTCTCCTCCTCGGGACTG                                                   |
| revbefgroup3               | TGGCGTACTCCAGGACAGCAG                                                        |
| fwbefgroup3                | CTGATGTCCTGAGACGACGCA                                                       |
| revbefgroup4               | ACAGCAGAAGAGCTGCTGCTCAT                                                      |
| fwbefgroup4                | ATGACAGCAGTCTCCTGCTG                                                       |
| revbeforore266             | TGGCGTACTCCAGGACAGC                                                         |
| fwbeforere266              | AGCTTTCTGGAGAACGGGCA                                                        |
| fwafter266                 | TGCCATGTCACAATACTAGTCAAGCT                                                   |
| revafter266                | GGCCCTGCTCAAGCCTATCC                                                       |
| G230R232fw                 | CTGCCCCAGCAGACCGGGTACCGTGCTGCAAGGAT                                                  |
| G230R232rev                | GATCCTTGATGCCAGACCGGTCACCCGGTCAGCTGCCAGACG                                  |
| A230R232fw                 | CTGCCCCAGCAGACCGGTCACCCGGTCAGCTGCCAGACG                                   |
| A230R232rev                | GATCCTTGATGCCAGACCGGTCACCCGGTCAGCTGCCAGACG                                |
| G230H232fw                 | CTGCCCCAGCAGACCGGTCACCCGGTCAGCTGCCAGACG                                   |
| G230H232rev                | GATCCTTGATGCCAGACCGGTCACCCGGTCAGCTGCCAGACG                                |
| group2toGfw                | CTGCCCCAGCAGACCGGTCACCCGGTCAGCTGCCAGACG                                   |
| group2toGrev                | GATCCTTGATGCCAGACCGGTCACCCGGTCAGCTGCCAGACG                                 |
| Q266fw                     | GTGACATGGGCAAAACAAAGTTATCAAGGGGTTGCGGTACTCA                                   |
| Q266Irev                   | GGAGTACGCGCCACCCCCCTTGATAACTTTGTTGCCATGTCAC                                 |
| S162Afw                    | CCCATGGGCTGGCATGGGCTATTACATCGGATATC                                        |
| S162Arev                   | GATATCCGATGTAATGTAATGCCATGCGACGCCCATGGA                                    |
| G230I 232H_fw              | CTGCCCCAGCAGACCGGTCACCCGGTCAGCTGCCAG                                       |
| G230I 232H_rev             | ATGCCAGCAGTGCTGCTGCTGCCAGGCAG                                               |
Cell Culture

293T cells (Life Technologies, Carlsbad, CA) were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 1x MEM non-essential amino acids and 100 U/ml Penicillin/Streptomycin (Life Technologies).

Luciferase Assay

Luciferase assays were performed as described previously (Gao et al, 2013b). Briefly, 3×10^4 293T cells were reverse-transfected with STING constructs (5 ng per 96-well) and reporter constructs (50 ng pIFNβ-Gluc, 10 ng pLenti-EF1-Fluc for normalization, 35 ng pMAX-empty as stuffer, delivered with TransIT-LT1, MirusBio, Madison WI). 12 hours after transfection, medium was replaced with fresh Medium containing DMXAA. 12 hours after stimulation with STING-ligands, cells were resuspended in passive lysis buffer and luciferase activity was determined using the respective substrates Coelenterazine and D-Luciferin (PJK, Kleinblittersdorf, Germany). Gaussia Luciferase values were then normalized to constitutive Firefly Luciferase values (resulting in Relative Light Units, RLU).
Mice
Mice were maintained in the animal facility at the Sloan-Kettering Cancer Institute. All procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animal of the National Institute of Health. \( STING^{Gt/Gt} \) mice were generated in the laboratory of Dr. Russell Vance (University of California, Berkeley).

Generation of Bone Marrow-derived Dendritic Cells
The protocol for generation of bone marrow-derived dendritic cells was described before (Dai et al., 2014). Bone marrow-derived dendritic cells were generated by culturing bone marrow cells from the tibia and femur of \( STING^{Gt/Gt} \) mice in complete medium (CM) in the presence of GM-CSF (30 ng/ml, produced by the Monoclonal Antibody Core facility at the Sloan Kettering Institute) for 10 days. CM is RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM essential and nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES buffer. Cells were fed every 2-3 days by replacing 50% of the old medium with fresh medium. Cells were plated into 6-well plate (1 x 10^6 cells per well) at day 10, the day before retroviral infection.

Retroviruses Transduction and DMXAA Treatment
hSTING cDNAs were cloned into pQCXIP-HA vector. In vitro packaging system was used to generate retroviruses according to protocol (Clontech). BMDCs (1x10^6 cells/well) were seeded into 6-well plates and infected with retroviruses. 48 hours after retroviral infection, cells were stimulated with DMXAA at a final concentration of 50 µg/ml (Sigma). For real-time PCR analysis, cells were collected at 3 hours post DMXAA treatment. Supernatants were collected at 18 hours post DMXAA treatment. CXCL10 protein level was determined by ELISA.

RNA Isolation and Real-time Quantitative PCR
RNA was extracted from cell pellets with an RNeasy Mini kit (Qiagen) and was reverse transcribed with a First Strand cDNA synthesis kit (Fermentas). Quantitative real-time
PCR was performed in triplicate with the Applied Biosystem 7500 Real-Time PCR Detection System (Life Technologies) using Fast SYBR Green Master Mix (Life Technologies) and gene-specific primers. Relative expression was normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GADPH). The following primers were used for PCR: IFN-b (5'-TGGAGATGACGGAGAAGATG-3', 5'-TTGGATGGCAAGGCAAGT-3'), CXCL10 (5'-GTCAGGTTGCCCTGTCTCA-3', 5'-TCAGGGAAGAGTCTGAAAG-3'), CCL5 (5'-GCCACGCTCAAGGAGTATTCTA-3', 5'-ACACACTTGGCGGTTCCCTC-3'), IL-6 (5'-AGGCATAACGCACCTAGGTTT-3', 5'-AGCTGGAGTCACAGAAGGAG-3'), and GAPDH (5'-ATCAAGAAGGTGTGGAAGCA-3', 5'-AGACAACCTGGCTTCATGTG-3').

**Western Blot Analysis**

BMDCs (1 x 10^6 cells) were infected with retroviruses carrying WT hSTING and various hSTING mutants. Cells were collected at 2 days post retroviral infection. Whole-cell lysates were prepared by lysing cells in RIPA buffer. Equal amounts of proteins were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The levels of HA-hSTING in transduced cells were determined by using a mouse monoclonal antibody specific for HA tag (Covance). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control, and was detected using a polyclonal antibody against GAPDH (Cell Signaling).
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