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# Design, synthesis, and biological evaluation of C7-functionalized DMXAA derivatives as potential human-STING agonists

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STING, a central protein in the innate immune response to cytosolic DNA, has emerged as a hot target for the development of vaccine-adjuvants and anticancer drugs. The discovery of potent human-STING (hSTING) agonist is expected to revolutionize the current cancer immunotherapy. Inspired by the X-ray crystal structure of DMXAA (5,6-dimethylxanthenone-4-acetic acid) and hSTING$^{G230I}$ complex, we designed various DMXAA derivatives that contain a hydrogen bonding donor/acceptor or a halide at the C7 position. While 7-bromo- and 7-hydroxyl-DMXAA showed notable binding to mouse-STING (mSTING), our newly synthesized C7-functionalized DMXAA derivatives did not bind to hSTING. Nevertheless, our newly developed synthetic protocol for the C7-functionalization of DMXAA would be applicable to access other C7-substituted DMXAA analogues as potential hSTING agonists.

## 1. Introduction

### 1.1. STING and DMXAA

STING (stimulator of interferon genes) is a key protein of the innate immune system and the body's first line of defense against pathogenic invasions of bacteria and viruses.[1] STING controls the transcription of type 1 interferons (IFNs) and pro-inflammatory cytokines upon binding to cyclic dinucleotides (CDNs).[2] Aberrant localization of DNA in the cytosol by pathogen-derived DNA, by self-DNA that has leaked from the nucleus of the host cell as a consequence of DNA damage, or by mitochondrial DNA (mtDNA) infiltrated due to mitochondrial stress induces the production of cyclic GMP-AMP (cGAMP) by cyclic GMP-AMP synthase (cGAS).[3] Alternatively, gram-negative and gram positive bacteria have also been reported to secrete CDNs. CDN then binds to STING and induces an "open" to "closed" conformational change.[4] This structural change of STING serves as a signal to complex it with TANK-binding kinase 1 (TBK1) and IκB kinase (IKK) and relocate them to perinuclear regions of the cell.[5] These kinases phosphorylate the transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor-κB (NF-κB) for their activation.[6] The incitement of IRF3 and NF-κB triggers the induction of cytokines and proteins, such as the type I interferons (IFNs), which initiate anti-pathogen activity via various pathways including the modulation of T-cells.

STING has emerged as a promising target for the development of novel immunization, autoinflammation, and cancer therapeutics.[7] Especially, the development of STING agonist which can recruit T-cells at the site of tumors is expected to greatly improve the efficacy of antibody-based checkpoint inhibitors such as Keytruda® and Opdivo®. DMXAA (**1**, Vadimezan) was firstly synthesized by Denny and coworkers as a potent antitumor agent.[8] Baguley and Ching showed that the antivascular action of DMXAA results from its immune modulation via the induction of cytokines in mouse models.[9] A cocktail treatment comprising DMXAA, paclitaxel, and carboplatin passed the phase II clinical trial against non-small-cell lung cancer but failed in the subsequent human phase III trials.[10] In 2012, the research team led by Vogel discovered that expression of IFN-β in response to DMXAA in murine macrophages requires STING, indicating that DMXAA targets the mouse-STING (mSTING) pathway.[11] In their contemporaneous studies, the Fitzgerald research team[12] and the Mitchison group[13] reported that DMXAA binds to mSTING but does not bind to human-STING (hSTING) despite their sequence identity (68% amino acid identity and 81% similarity) and structural similarity.

Atomic-level understanding of the interaction between DMXAA and hSTING became available by elegant structural, biophysical, and cellular essay studies reported by Patel and coworkers.[4,14] The Patel research team identified three point substitutions (S162A, G230I, and Q266I) of hSTING which synergistically rendered the mutated hSTING highly sensitive to DMXAA (Figures 1A). Notably, the substituted I266, together with I165, L170, and I235 side chains, formed a nonpolar pocket that maximizes the hydrophobic interaction with the aromatic methyl groups of DMXAA consistent with its highest binding affinity (Figure 1B). The crystal structure of DMXAA

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bound to hSTING$^{G230I}$ revealed hydrogen bonding (salt-bridge) between

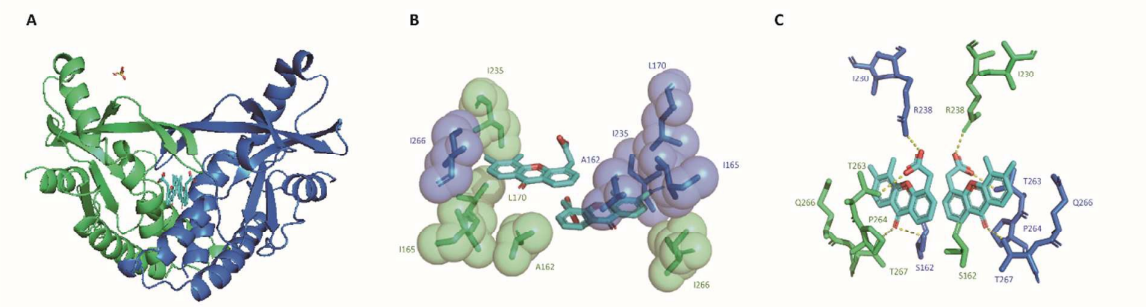



**Figure 1**. Patel's X-ray crystal structures of DMXAA bound to mutated hSTING. (A) The 2.37 Å crystal structure of DMXAA bound to hSTING$^{S162A/G230I/Q266I}$. (B) Crystal structure representation of DMXAA bound to hSTING$^{S162A/G230I/Q266I}$ with an emphasis on hydrophobic interactions around DMXAA. (C) The 2.51 Å crystal structure of DMXAA bound to hSTING$^{G230I}$ with representation of hydrogen bonding as dotted line.

the carboxylic acid moiety of DMXAA and the guanidino group of R238. It also disclosed additional hydrogen bonding between the ketone moiety of DMXAA and the hydroxyl groups of T267 and S162 (Fig. 1C).

### 1.2. Design of C7-functionalized DMXAAs as potential STING agonists

The seminal report by Patel and coworkers[14] provided insight to design novel DMXAA derivatives with potentially higher affinity to hSTING. In fact, the Patel research team concluded their report[14] by suggesting the synthesis of DMXAA analogues with polar groups at C1/C2 and C7 for presumed intermolecular hydrogen bondings with hSTING, respectively. Our group's interest in developing hSTING agonists prompted us to devise DMXAA derivatives with hydrogen bonding donor/acceptor at the C7 position (Figure 2). We envisioned that the hydroxyl group attached at C7 via a proper methylene-based linker would hydrogen bond with the primary amide group of Q266 in hSTING. Notably, our preliminary virtual docking studies indicated that DMXAA derivatives **2**, **3**, and **4** complexed to hSTING are 0.47, 0.14, and 0.84 kcal/mol more stable than DMXAA-hSTING complex, respectively. The docking model of **4** to hSTING predicted hydrogen bondings of the hydroxyl group of the ligand with Q266, R169, and R232 of hSTING (Figure 2).

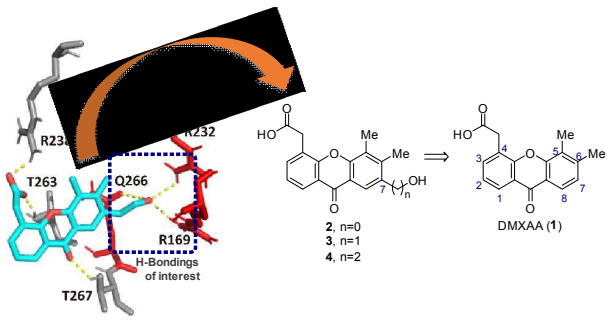
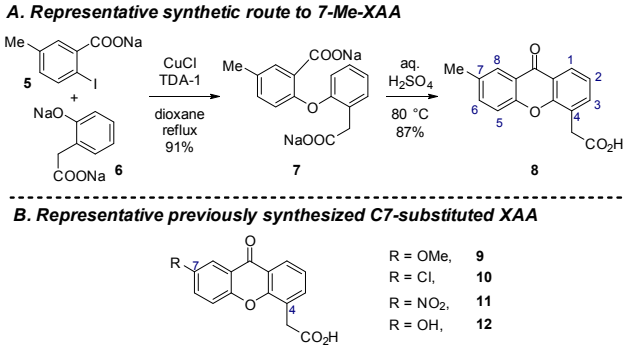



**Figure 2**. Design of DMXAA derivatives with hydrogen bonding donor/acceptor at the C7 position based on the docking model of **4** to hSTING.

### 1.3. Previous synthesis of C7-monosubstituted XAA derivatives

In 1989, Denny and coworkers reported the synthesis and structure-activity relationship studies of monosubstituted xanthenone-4-acetic acids (XAA) against the colon 38 tumor in vivo.[15] They synthesized various monosubstituted XAA derivatives including 7-substituted XAAs. A synthetic route for 7-Me-XAA (**8**) is presented in Scheme 1A. Firstly, Iodide **5** and sodium phenolate **6** were coupled by copper catalyzed cross coupling reaction to produce **7**. The resulting **7** was heated in aqueous sulfuric acid to yield 7-Me-XAA (**8**) by Friedel–Crafts acylation-type reaction. Other 7-substituted-XAAs (**9**–**12**) could be accessed using analogous synthetic procedures (Scheme 1B). Importantly, 7-Me-XAA, along with 8-Me-XAA, was active in stimulating human leukocytes to produce IL-6 and IL-8 and for inhibition of tube formation by ECV304 human endothelial-like cells. On the other hand, 5- and 6-Me-XAAs were the most active compounds in murine cell systems hinting at a substitution site dependent species specificity of XAA.[16,17]




**Scheme 1**. Denny's synthesis of C7-monosubstituted XAA derivatives.

### 1.4. Previous synthesis of C7-substituted DMXAA derivatives

The synthetic protocol developed for the synthesis of DMXAA[8] could be applied to the synthesis of 7-Me-DMXAA by Xie et al (Scheme 2A).[18] Cross-coupling between the sodium salt of 2-iodo-3,4,5-trimethylbenzoic acid (**13**) and sodiumphenolate **6** followed by an acid mediated cyclization produced 7-Me-DMXAA (**15**). As an effort to streamline the synthesis of DMXAA (**1**), Yang and Denny

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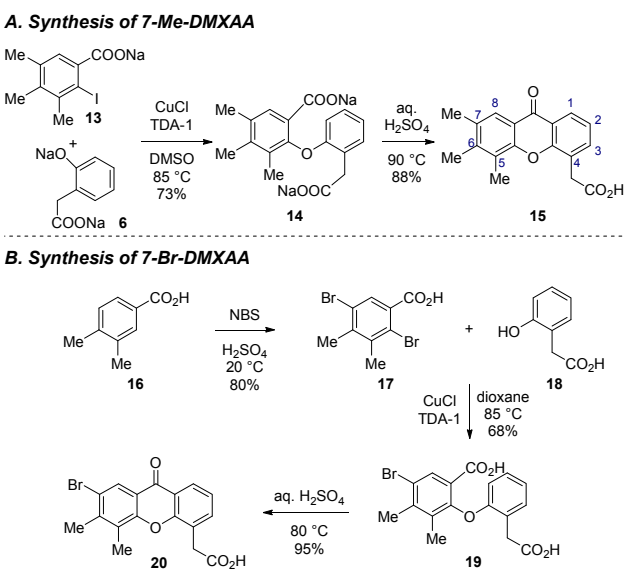
devised a synthetic strategy which utilized 7-bromo-DMXAA (**20**) as a direct precursor to DMXAA (Scheme 2B).[19] 3,4-Dimethylbenzoic acid (**16**) could be converted to dibromo compound **17** upon treatment with NBS under acidic conditions. They found that the bromo group at the meta position of the carboxylic acid moiety in **17** activated the bromo group at the ortho position to allow a regioselective cross-coupling with 2-hydroxyphenylacetic acid (**18**) to yield diacid derivative **19** in 68% yield. Subsequent cyclodehydration of diacid **19** afforded 7-bromo-DMXAA (**20**).




**Scheme 2**. Synthesis of 7-substituted DMXAA

## 2. Results and Discussion

### 2.1 Synthesis of C7-functionalized DMXAA derivatives

Based on our aforementioned rational design, we set out to synthesize C7-functionalized DMXAA analogues **2–4**. Previous syntheses of various C7-substituted XAA were possible due to the commercial availability of the trisubstituted benzene derivative (Scheme 1). However, limited commercial availability of the pentasubstituted benzene derivatives necessary for the synthesis of C7-functionalized DMXAAs by Denny's method prompted us to devise a novel synthetic strategy. For an efficient access to various C7-functionalized target DMXAA derivatives, we devised a divergent synthetic strategy that utilizes a commercially available DMXAA itself. Our synthesis of the designed DMXAA derivatives commenced with the methyl ester formation of the carboxylic acid moiety of DMXAA (**1**) to make the handling of downstream compounds easier (Scheme 3). We subsequently installed the synthetic handle at the C7 position of methylester derivative **21** by an electrophilic aromatic halogenation of the xanthone framework. Treatment of **21** with molecular bromine and aluminium trichloride provided the C7-brominated derivative **22** in 58% yield along with C2,C7-dibrominated compound **23** in 4% yield. Hydrolysis of brominated methylester derivative **22** under basic condition yielded 7-bromo-DMXAA (**20**) in 75% yield. More efficient and selective C7-monohalogenation of xanthone derivative **21** was accomplished in the presence of *N*-iodosuccinimide and trifluoroacetic acid to produce C7-iodinated derivative **24** in 97% yield. Hydrolysis of the methylester moiety of **24** provided 7-iodo-DMXAA (**25**).[20]

With a robust access to 7-iodo-DMXAA derivative **24** in hand, we next investigated the C7 hydroxylation. Initial evaluations of a palladium catalyzed hydroxylation of **24** were not successful.[21] Successful hydroxylation of xanthone-based iodide **24** was achieved when a combination of $Cu(acac)_2$ and *N*,*N*′-bis(4-hydroxyl-2,6-dimethylphenyl)oxalamide (BHMPO), a catalytic system reported by Ma and coworkers,[22] was applied. Under these reaction conditions, the desired 7-hydroxy-DMXAA (**2**) was obtained in 62% yield. It is notable that the methylester moiety underwent a concomitant hydrolysis during this transformation.

We envisioned that a substitution of the C7-iodide to the vinyl group in **24** would enable a synthetic access to both alcohols **3** and **4** with one and two methylene linkers, respectively. This substitution was attained by Suzuki–Miyaura cross coupling reaction. Treatment of aryliodide **24** with Buchwald's third generation XPhos-based palladacycle precatalyst and vinylboronic acid pinacol ester under basic conditions resulted in the formation of vinyl derivative **26**. With vinyl derivative **26** in hand, we firstly explored its transformation to **4**. A hydroboration reaction of **26** with $BH_3$·THF followed by an oxidative work-up afforded primary alcohol derivative **27** in 41% yield. A hydrolysis of the methylester moiety of **27** yielded 7-(2-hydroxyethyl)-DMXAA (**4**) in 90% yield.

Upon testing various oxidative cleavage reactions of vinyl derivative **26**, we discovered that **26** was most efficiently converted to aldehyde **28** in the presence of ruthenium trichloride catalyst and [bis(acetoxy)iodo]benzene oxidant in a $CH_2Cl_2$/water two phase system.[23] Hydrolysis of the methylester moiety of aldehyde derivative **28** produced 7-formyl-DMXAA (**29**). We envisaged that the hydrogen bond accepting ability of the aldehyde functionality in **29** would provide an interesting additional entry with respect to our initial design principle of STING agonists. Finally, reduction of the aldehyde moiety in **28** afforded benzyl alcohol derivative **30**. Hydrolysis of **30** yielded 7-hydroxymethyl-DMXAA (**3**) in 78% yield.

### 2.2 Biological evaluations of our C7-functionalized DMXAA derivatives

Our synthetic campaign provided us not only with the target DMXAA analogues **2–4** but also with various synthetic precursors and their derivatives (Scheme 1). With an access to assorted C7-functionalized DMXAA derivatives, we firstly tested their binding to mSTING by thermal shift assay using differential scanning fluorimetry (DSF).[24] We immediately noticed that the presence of free carboxylic acid moiety in DMXAA derivatives was crucial for the interaction with mSTING. The thermal shift data identified 7-bromo-DMXAA (**20**) and 7-hydroxy-DMXAA (**2**) to show the strongest ligand-mSTING interaction among tested DMXAA derivatives. The observed $K_d$ of 7-bromo-DMXAA (**20**) was 149 μM (Observed $K_d$ of DMXAA from this experiment: 83.4 μM) and that of 7-hydroxy-DMXAA (**2**) was 522 μM. The ligand-mSTING binding affinity strongly depended on the C7 substituent in the order of Br > OH > $CH_2OH$ >> $CH_2CH_2OH$ ~ I ~ CHO. The highest affinity between 7-bromo-DMXAA (**20**) and mSTING might be due to a strong

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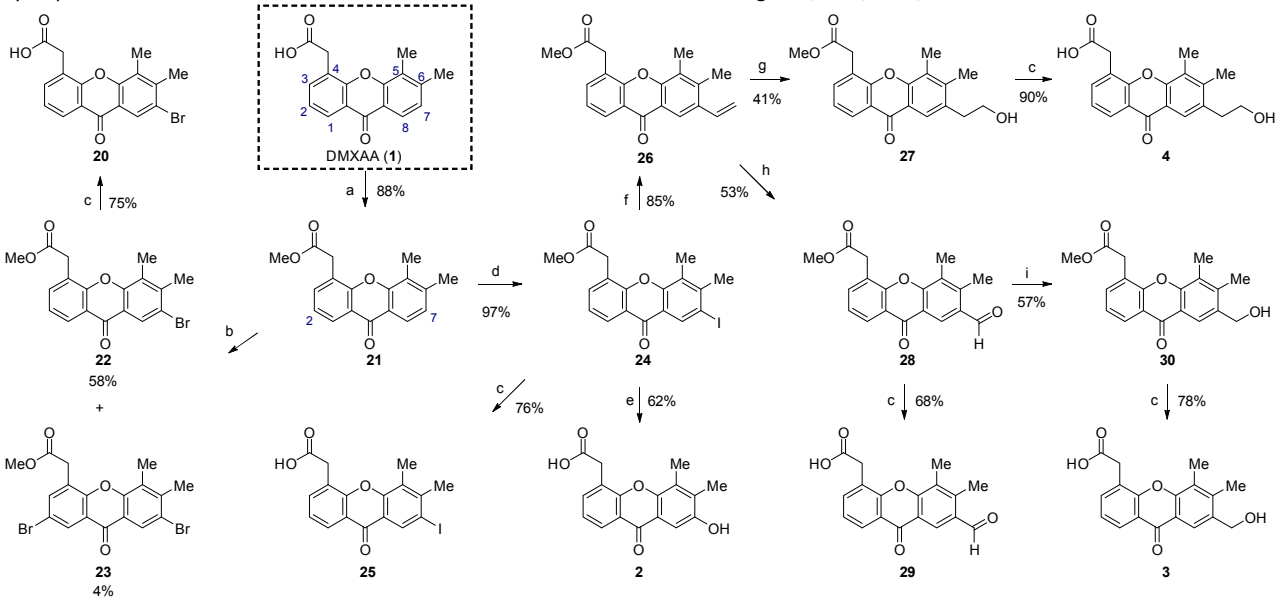
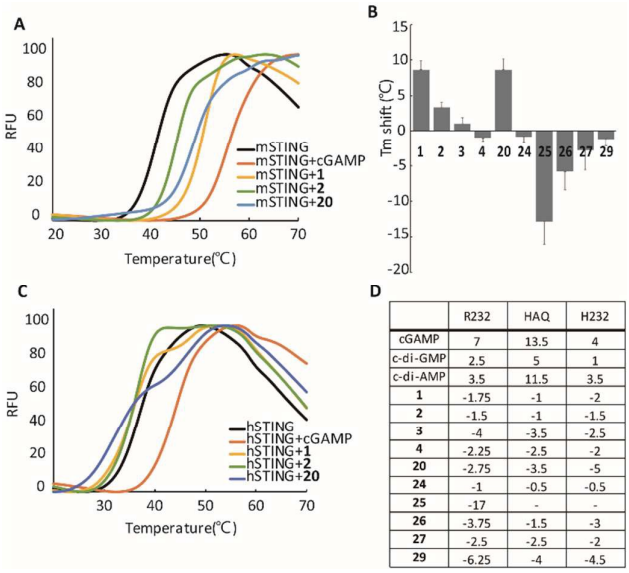
hydrophobic interaction between the bromine atom and the surrounding I165, I266, L170, and I235 residues.




**Scheme 3**. Synthesis of various C7-functionalized DMXAA derivatives. Reagents and conditions: (a) EDC, HOBt, DIPEA, MeOH, DMF, $CH_2Cl_2$, 23 °C. (b) $Br_2$, $AlCl_3$, $CS_2$, 23 °C. (c) NaOH, $H_2O$, MeOH, 50 °C. (d) NIS, TFA, $CH_2Cl_2$, 23 °C. (e) $Cu(acac)_2$, *N*,*N*'-bis(4-hydroxy-2,6-dimethylphenyl)oxalamide, KOH, 1,4-dioxane, $H_2O$, 80 °C. (f) Pd XPhos G3, 4,4,5,5-tetramethyl-2-vinyl-1,3,2-dioxaborolane, $Na_2CO_3$, 1,4-dioxane, $H_2O$, 90 °C. (g) $BH_3$·THF, THF, 23 °C; $H_2O_2$, NaOH, $H_2O$, 23 °C. (h) $RuCl_3$, $PhI(OAc)_2$, $CH_2Cl_2$, $H_2O$, 23 °C. (i) $NaBH_4$, MeOH, 23 °C.

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| | R232 | HAQ | H232 |
|---|---|---|---|
| cGAMP | 7 | 13.5 | 4 |
| c-di-GMP | 2.5 | 5 | 1 |
| c-di-AMP | 3.5 | 11.5 | 3.5 |
| **1** | -1.75 | -1 | -2 |
| **2** | -1.5 | -1 | -1.5 |
| **3** | -4 | -3.5 | -2.5 |
| **4** | -2.25 | -2.5 | -2 |
| **20** | -2.75 | -3.5 | -5 |
| **24** | -1 | -0.5 | -0.5 |
| **25** | -17 | - | - |
| **26** | -3.75 | -1.5 | -3 |
| **27** | -2.5 | -2.5 | -2 |
| **29** | -6.25 | -4 | -4.5 |

**Figure 3**. Thermal shift assay data of STING + C7-functionalized DMXAA derivative complexes. (A) DSF data of mSTING + C7-functionalized DMXAA derivative complexes. (RFU: Relative Fluorescence Unit) (B) $\Delta T_m$ of mSTING + C7-functionalized DMXAA derivative complexes. (C) DSF data of hSTING + C7-functionalized DMXAA derivative complexes. (D) $\Delta T_m$ of hSTING + C7-functionalized DMXAA derivative complexes. (R232: $hSTING^{R232}$, HAQ: $hSTING^{HAQ}$, H232: $hSTING^{H232}$)

We then tested the interaction between our newly synthesized DMXAA derivatives and hSTING using DSF based thermal shift essay. We conducted our studies using three different natural variants of hSTING, namely, $hSTING^{R232}$, $hSTING^{HAQ}$, and $hSTING^{H232}$ (Figure 3D). Notably, we could not observe any interaction between our C7-functionalized DMXAA derivatives and hSTING. We reasoned that the lack of thermal shift might be due to either no binding of the ligand or binding but no change in the conformation of hSTING. We, therefore, conducted an ITC (isothermal titration calorimetry) experiment using select DMXAA derivatives (**2**, **3**, **4** and **20**) and hSTINGs ($hSTING^{HAQ}$ and $hSTING^{H232}$). The ITC experiment did not reveal any specific binding between these ligands and hSTING. Our studies revealed that the introduction of a hydrogen bonding donor/acceptor or a halide at the C7 position of DMXAA did not improve the binding to hSTING. It is important to note that our design of C7-functionalized DMXAA was based on the co-crystal structure of DMXAA and "mutated" hSTING. We reason that the subtle structural discrepancy between "natural" and "mutated" hSTINGs might have contributed to the lack of interactions between our C7-functionalized DMXAA analogues and hSTING.[25]

## 3. Conclusions

We rationally designed, synthesized, and tested the biological activity of various C7-functionalized DMXAA derivatives based on the x-ray crystal structure of DMXAA bound to $hSTING^{G230I}$. While 7-bromo-DMXAA (**20**) and 7-hydroxy-DMXAA (**2**) showed notable binding to mSTING, none of our newly synthesized C7-functionalized DMXAA derivatives showed any affinity to hSTING. Our studies demonstrated that the introduction of a hydroxyl group appended at the C7-

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position of DMXAA by linkers of different length fell short to induce a substantial hydrogen bonding with the side chain of Q266 residue in hSTING to restore the binding of the ligand to the protein. Our data illustrates the challenge in establishing the strongly angle-dependent hydrogen bonding in protein-ligand interactions even with co-crystal structures available.[26] Nevertheless, our findings provide a basis for the selective C7-functionalization of DMXAA and would be applicable to access other C7-substituted DMXAA analogues.

## Conflicts of interest

There are no conflicts to declare.

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