Novel Dinucleotides that Activate cGAS- STING Signaling for Immuno-oncology

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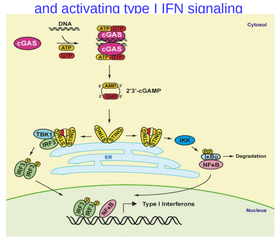
INTRODUCTION

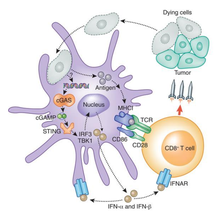
Immunotherapy has recently emerged as a transformative approach for the treatment of cancer; nevertheless, many patients remain unresponsive to treatment. It is being recognized that Induction of type I interferons (IFN) and interferon-stimulated genes (ISGs) in tumor cells and within the tumor microenvironment (TME) is essential for modulating the host immune response and inducing apoptosis of tumor cells. Furthermore, the antigen-presenting cells within TME can cause induction of adaptive immune response, through priming of CD8+ T cells and tumor killing. Importantly, the DNA released from damaged cells and cancer cells can be sensed by cyclic GMP-AMP synthase (cGAS) leading to the synthesis of cyclic GMP-AMP (2`3`-cGAMP), a second messenger that activates of Stimulator of Interferon Genes (STING) pathway resulting in the production of type I IFN and ISGs. The cumulative effects of activation of innate and adaptive immune response result in a powerful anti-cancer strategy. Therefore, therapeutic agents that activate the cGAS-STING signaling pathway in tumor cells and TME are urgently needed. Herein, we describe the discovery of novel potent, first-in-class small molecules for application in immuno-oncology.

cGAS-STING Mediated Cytosolic DNA Sensing and activating type I IFN signaling



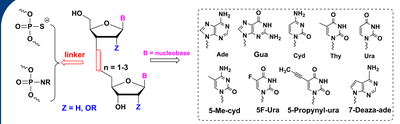
Cai X, Chiu YH & Chen ZJ, Molecular Cell. The cGAS-cGAMP-STING Pathway of Cytosolic DNA Sensing and Signaling. 2014; *54, 289-296*

Role of the cGAS–STING pathway in antitumor immunity



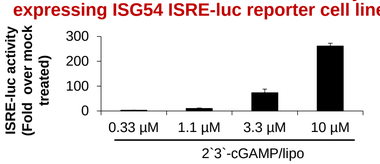
Chen Q, Sun LJ & Chen ZJ. Nature Immunology. Regulation and function of the cGAS–STING pathway of cytosolic DNA sensing. 2016;17 (10):1142–49.

RESULTS



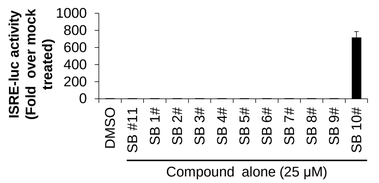
A nucleic acid-based scaffold is a logical template to incorporate diversity attributes for targeting specific protein-nucleic acid interactions including pattern recognition receptors (PRRs) such as RIG-I, cGAS, NOD2 that contain nucleotide-binding domains. The specificity of such nucleotide-protein binding can be optimized using a network of p-stacking, hydrogen bonding, hydrophobic, ionic, and van der Waals interactions. Using structure guided drug design of cGAMP bound to STING, a focused nucleotide library of small molecules was designed to mimic the variety of interactions that exist between cGAMP and STING. Both rigid and flexible scaffolds were used to create variable spatial display of non-covalent interactions within individual members in a library of di-, tri-nucleotides termed SMNH library. The SMNH library contained multiple chemical substitutions including backbone modifications along with different base and sugar substituents and was assembled using well-established solid-phase or solution-phase methods. The library was screened for ISG54 ISRE-luciferase induction in SZ14 cells that express the PRRs, including STING, and lead identification and optimization was carried out.

SZ14 – A novel HEK293-derived stably expressing ISG54 ISRE-luc reporter cell line



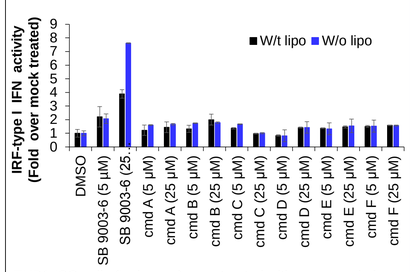
To allow for the screening of molecules that might enhance type I IFN responses through the cGAS-STING pathway, we generated HEK293 based cells stably expressing an ISG54 ISRE-luciferase reporter gene. Clone SZ14 is one of the best representative clones, highly responsive to STING ligands.

Screening of nucleic acid-based library in SZ14 cells



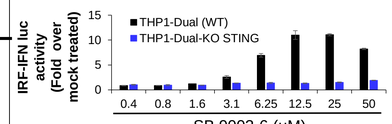
SZ14 were treated with compound/digitonin for 30 min, then were incubated for an additional 5 hrs. ISG54 ISRE-luciferase activity was determined and normalized to DMSO-treated cells (average ± standard deviation of triplicate wells).

SB 9003-6 induces IRF in monocytic cells



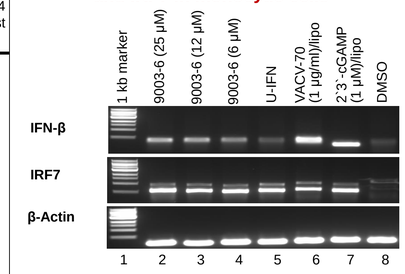
THP1-ISG cells (Invivogen) were treated with compound alone (W/o lipo) or with lipofectamine 2000 (W/t lipo) for 22 hrs. IRF-type I IFN activity was determined and normalized to DMSO-treated cells (mean ± standard deviation of triplicate wells).

SB 9003-6-induced type I IFN response is dependent on STING signaling



THP1 cells (Invivogen) were treated with 9003-6 alone for 22 hrs. IRF-type I IFN luciferase activity in culture supernatants was determined and normalized to DMSO-treated cells (mean ± standard deviation of triplicate wells).

SB 9003-6 induces the expression of IFN-β and IRF7 in monocytic cells



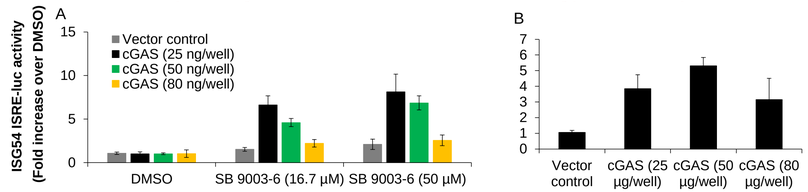
THP1 cells were treated with compound SB 9003-6 or controls for 22hrs. RNA samples were prepared and the expression of IFNβ, IRF7 and β-actin was determined using OneStep RT-PCR kit from Qiagen (semi-quantitative RT-PCR). U-IFN, universal type I IFN.

RESULTS

2`3`-cGAMP induces IFN-β expression within 5 hrs, it takes longer for SB 9003-6 to activate IFN-β gene expression in THP1-WT but not in STING KO cells

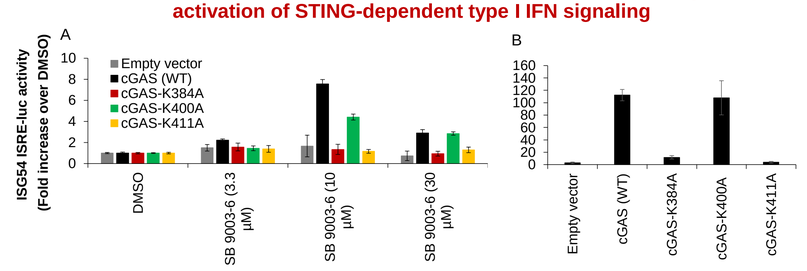
THP1-WT (A) and STING KO cells (B) were treated with compound SB 9003-6, DMSO, or controls U-IFN and 2`3`-cGAMP for 5 and 22 hrs. The expression of IFN-β was determined using OneStep RT-PCR kit from Qiagen (semi-quantitative reverse transcription-PCR, starting with equal amounts of RNA). PCR products were subjected to 1% agarose gel electrophoresis.

The cGAS pathway is essential for 9003-6-induced type I IFN responses



SZ14 cells were transfected with plasmids encoding human cGAS (wild-type) and internal control Renilla-luciferase reporter gene and incubated for 24 hrs, followed by treatment with (A) compound SB 9003-6, (B) DMSO for an additional 21 hrs. The ISRE-luc activity in the total cell lysate of individual wells was measured and normalized to Rellina-luciferase activity (ISRE-luc/pRL-TK fold). Data shown in A panel are ISRE-luc/pRL-TK fold increase over DMSO-treated cells as shown in panel B (average ± standard deviation of triplicate wells). Data shown in panel B are ISRE-luc/pRL-TK fold increase.

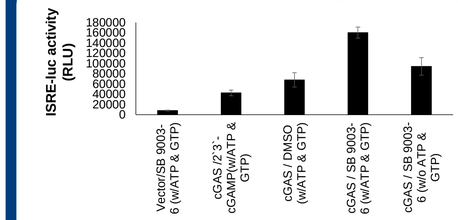
K384 and K411 residues in cGAS play an important role in mediating 9003-6 activation of STING-dependent type I IFN signaling



SZ14 cells were transfected with expression plasmid encoding human cGAS (wild-type) or cGAS mutant (K384A or K411A) together with a plasmid encoding Renilla-luciferase reporter gene and incubated for 24 hrs, followed by treatment with (A) SB 9003-6, (B) DMSO for an additional 22 hrs. The ISRE-luc activity in the total cell lysate of individual wells was measured and normalized to Rellina-luciferase activity (ISRE-luc/pRL-TK fold). Data shown in A panel are ISRE-luc/pRL-TK fold increase over DMSO-treated cells as shown in panel B (average ± standard deviation of triplicate wells). Data shown in panel B are ISRE-luc/pRL-TK fold increase.

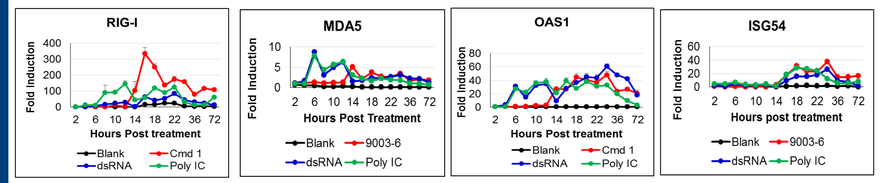
RESULTS

ATP and GTP enhance SB 9003-6-induced type I IFN signaling in SZ14 cells



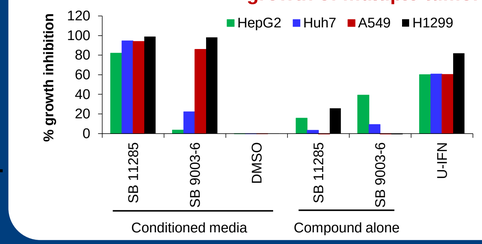
SZ14 were transfected with a cGAS expression plasmid for 24 hrs, followed by compound treatment in the presence of ATP and GTP (w/ATP & GTP) or absence of ATP and GTP (w/o ATP & GTP) for 21 hrs. ISG54 ISRE-luciferase activity was determined and shown as Relative Light Units (RLU) (average ± standard deviation of triplicate wells). (SB 9003-6 final concentration: 20 μM, ATP and GTP: 2 mM, 2`3`-cGAMP: 10 μM)

Kinetics of SB 9003-6-dependent induction of PRRs and ISGs in THP1 cells



THP1 cells were treated with either 20uM SB 9003-6 (Cmd 1) or 1.8ug/ml dsRNA or 18ug/ml Poly IC or control. Samples were collected every 2 hrs for 24hrs and at 36, 48 & 72 hrs after treatment. RNA was extracted and gene expression was evaluated by real time PCR. Fold change was calculated by ΔΔct method comparing with 0 hr sample.

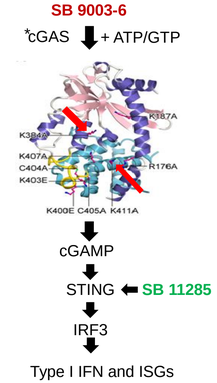
SB 9003-6 and SB 11285-treated THP1-conditioned medium significantly inhibits the growth of multiple tumor-derived cell lines



Tumor cells in 96-well plate were treated once daily with compound-treated THP1 (WT) conditioned media or compound alone for 3 days. Cells were fixed with 4% paraformaldehyde and stained with DAPI. Cells were imaged at 10x magnification (9 fields per well) using the ImageXpress Micro (Molecular Devices) and the total number of survival cells were analyzed using MetaXpress software. Percentage growth inhibition is calculated based on the equation recommended by NCI. U-IFN, universal type I IFN.

CONCLUSIONS

- **The analog SB 9003-6 appears to be an agonist of cGAS whereas SB 11285 is a STING agonist**
- **Residues K384 and K411 in cGAS play critical roles in SB 9003-6-induced activation of type I IFN response.**
- **SB 9003-6 induces IFN-β and ISGs, including multiple pattern recognition receptors in human monocytic cells.**
- **Both SB 9003-6 and SB 11285 suppress tumor cell growth in conditioned media.**



∗Kato K, Ishii R, Goto E, Ishitani R, Tokunaga F, et al. (2013) Structural and Functional Analyses of DNA-Sensing and Immune Activation by Human cGAS. PLoS ONE 8(10): e76983.doi:10.1371/journal.pone.0076983. Figure 4A

ACKNOWLEDGEMENTS

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