CHARACTERIZATION OF SB 11285 AS A NOVEL STING AGONIST FOR IMMUNO-ONCOLOGY

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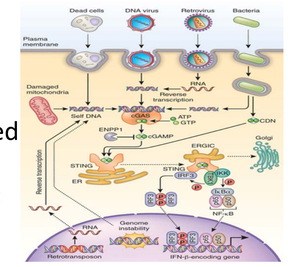
BACKGROUND AND INTRODUCTION

Immunotherapy has emerged as a trans-formative approach for the treatment of cancer. It is believed that the induction of IFNs and ISGs within the tumor microenvironment (TME) is essential for efficient immune-mediated anti-tumoral response.

Indeed, the cGAS-STING-TBK1/IKKε-IRF3/NF-κB pathway provides a potential target within TME for immuno-modulators to enhance the production of IFNs and ISGs [1,2].

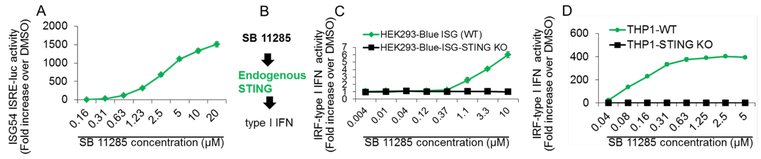


Chen Q, et al., Nature Immun. 2016, 17: 1142-1149

We recently disclosed that **SB 11285** is a highly potent STING agonist that induces the production of IFNs and ISGs in human monocytic cells [3,4]. Reported here are studies elucidating the mechanism of action (MOA) of **SB 11285** and its potential for activating innate immune cells in the TME to elicit potent anti-tumoral activity. **SB 11285** was discovered by screening and SAR-based optimization of a proprietary focused library of dinucleotides for their ability to activate type I IFN signaling in conjunction with proprietary SZ14 cells that stably express ISG54 ISRE-luciferase reporter gene. We further characterized the immune-modulatory properties of **SB 11285**. The results are presented here.

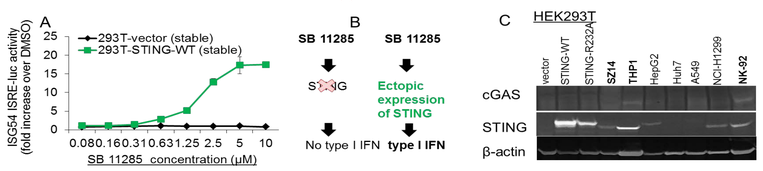
RESULTS

SB 11285 induces dose-dependent type I IFN response in SZ14 cells which is STING-dependent



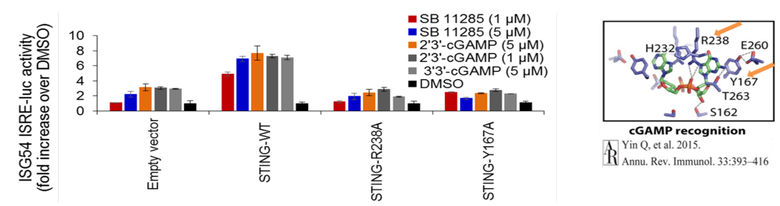
(**A**) In-house developed SZ14 cells were treated with SB 11285/digitonin for 30 min, then were incubated for an additional 5 hrs. ISG54 ISRE-luciferase activity was determined using Steady-glo luciferase reagent (Promega) and normalized to DMSO-treated cells (mean ± standard deviation of triplicate wells). (**B**) Cartoon shows the potential mechanism of action of SB 11285. (**C**) HEK-Blue WT vs. STING KO cells were similarly treated with SB 11285/digitonin and incubated for 20 hrs. Levels of SEAP (under the control of IRF-inducible promoter) in the supernatant were determined with Quanti-blue and normalized to DMSO-treated cells. (**D**) THP1-Dual WT vs. STING KO cells were stimulated with **SB 11285** alone for 20 hrs. Levels of IRF-type I IFN activity were determined using Quanti-luc and normalized to DMSO-treated cells. Results are shown as fold induction over DMSO-treated cells (mean ± standard deviation of triplicate wells per stimulant).

SB 11285 induces type I IFN response in HEK293T cells ectopically expressing wild-type STING



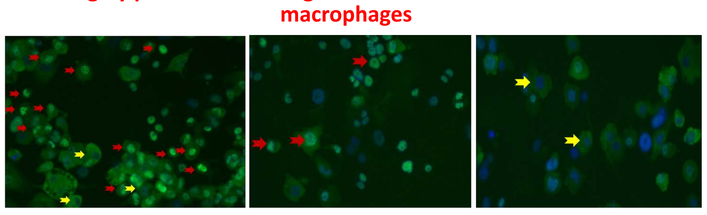
(**A**) HEK293T cells stably expressing wild-type STING or vector were transfected with plasmids encoding ISRE-luciferase reporter gene and Renilla-luciferase reporter gene and incubated for 18 hrs, followed by treatment with SB 11285 in presence of digitonin for an additional 5 hrs. ISRE-luciferase activity was determined using Dual-glo luciferase assay system (Promega) and normalized to DMSO-treated cells (average ± standard deviation of triplicate wells). (**B**) Cartoon shows the potential role of STING in SB 11285-induced activation of type I IFN signaling. (**C**) Western blot analysis showing the expression of STING and cGAS in various cell lines.

Residues R238 and Y167 are critical for the activation of STING-dependent type I IFN response by SB 11285



HEK293T cells were transfected with expression plasmid encoding human wild-type STING (WT) or STING mutant (R232 or Y167A) together with plasmids encoding ISRE-luc reporter gene and Renilla-luc reporter gene and incubated for 18 hrs, followed by treatment with SB 11285 or cGAMP in presence of digitonin for an additional 5 hrs. The ISRE-luc activity was measured using Dual-glo luciferase assay system and normalized to Rellina-luciferase activity. Data shown are ISRE-luc/pRL-TK fold increase over DMSO-treated cells (mean ± standard deviation of triplicate wells).

SB 11285 is highly potent in inducing IRF3 nuclear translocation in THP1-derived macrophages



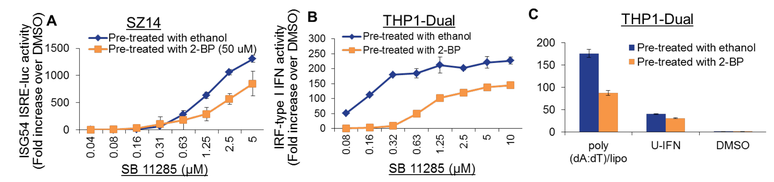
THP1 (WT)-derived macrophages were treated with SB 11285 for 2, 4, and 6 hrs. Cells were fixed with 4% paraformaldehyde and treated with 0.5% Triton X-100, stained with rabbit anti-IRF3 and Alex Fluor 488 conjugated anti-rabbit IgG antibody (green). Nuclei were identified using DAPI (blue) staining. Cells were imaged on ImageXpress Micro (40x). Images were analyzed using ImageJ. IRF3 and DAPI images were overlapped and nuclear translocated **p-IRF3 (green)** is indicated by **the red arrow**. The yellow arrow indicates cells without IRF3 nuclear translocation. **IRF3 nuclear translocation is a strong indicator that type I IFN signaling is activated. More red arrows mean more cells responded to compound treatment. Magnification *(40x)*.**

MATERIALS AND METHODS

To investigate the MOA of SB 11285, we utilized various cell lines expressing type I IFN-inducible reporter genes, including SZ14 cells, and cells deficient in STING, as well as, TLR adaptor MyD88 (InvivoGen). Primary human innate immune cells were obtained from ZenBio and STING-deficient (goldenticket <*Tmem173*^{gt}>) mouse cells from the Jackson Laboratory. Palmitoylation inhibitor, 2-BP, was obtained from Wako, and TBK1/IKKε inhibitor, BX795, from InvivoGen. Analysis of type I IFN-inducible reporter activities, as well as, the production of type I IFN and other cytokines and chemokines were done using luciferase assays, ELISA and multiplexed ELISA (Quansys). IRF3 nuclear translocation and *in vitro* tumor cell growth inhibition were assessed using the ImageXpress Micro (Molecular Devices).

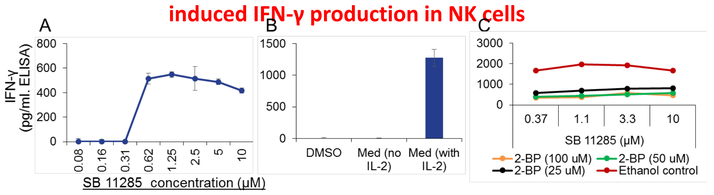
RESULTS

Palmitoylation inhibitor, 2-BP, inhibits SB 11285-induced type I IFN response in SZ14 and THP1 cells



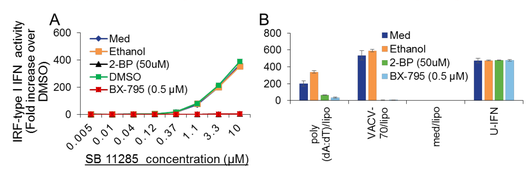
SZ14 (**A**) or THP1-Dual cells (WT) (**B** and **C**) were pre-treated with palmitoylation inhibitor (2-BP. 50 μM) or control ethanol for 1 hr, followed by treatment with SB 11285 (**A and B**) or controls (**C**) for 5 hrs (SZ14) or 20 hrs (THP1). (**A**) The ISRE-luc activity in the cell lysate was measured and normalized to DMSO-treated cells. Data shown in **A** are ISRE-luc fold increase over DMSO-treated cells (mean ± standard deviation of triplicate wells). (**B** and **C**) Levels of IRF-dependent luciferase were measured and shown as fold increase over DMSO-treated cells (mean ± standard deviation of triplicate wells).

SB 11285 activates NK cells; palmitoylation inhibitor, 2-BP blocks SB 11285-induced IFN-γ production in NK cells



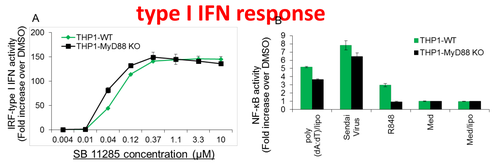
NK92 cells were treated with SB 11285 alone (**A**), DMSO, medium (with or without IL-2) (**B**) for 24 hrs. (**C**) NK92 cells (without IL-2) were pre-treated with 2-BP or control ethanol at indicated concentrations for 1 hr, followed by treatment with SB 11285 for 24 hrs. Levels of IFN-γ in culture supernatants were measured using ELSIA and shown as pg/ml.

The palmitoylation and TBK1/IKKε inhibitors completely blocked SB 11285-induced IRF-type I IFN signaling in mouse macrophage cell line



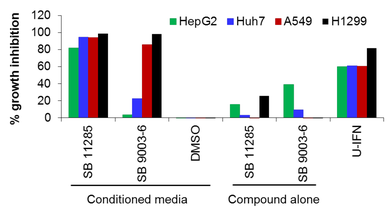
RAW-Lucia-ISG cells were pre-treated with palmitoylation inhibitor (2-BP. 50 μM) or control ethanol, TBK1/IKKε inhibitor BX-795 (0.5 μM) or control DMSO for 1 hr, followed by treatment with SB 11285 alone (**A**), or controls (**B**) for 18 hrs. Levels of IRF-dependent secreted luciferase were measured and shown as fold induction over DMSO treated cells (mean ± standard deviation of five wells per stimulant).

TLR-MyD88 signaling pathways are not involved in SB 11285-induced activation of type I IFN response



THP1-Dual (WT) and THP1-Dual-MyD88 KO cells were stimulated with SB 11285 alone (**A**) or controls (**B**) for 19 hrs. The levels of IRF-type I IFN luciferase activity were determined by measuring the relative light units using Quanti-luc (**A**) or the levels of NF-κB-induced SEAP(**B**) in cell culture supernatant were measured using Quanti-blue and normalized to DMSO-treated cells. The results are shown as fold induction over DMSO-treated cells (mean ± standard deviation of triplicate wells per stimulant). **Note:** Since TLR7 agonist, R848, predominately induces NF-κB activity in THP1 cells, NF-κB activity was measured.

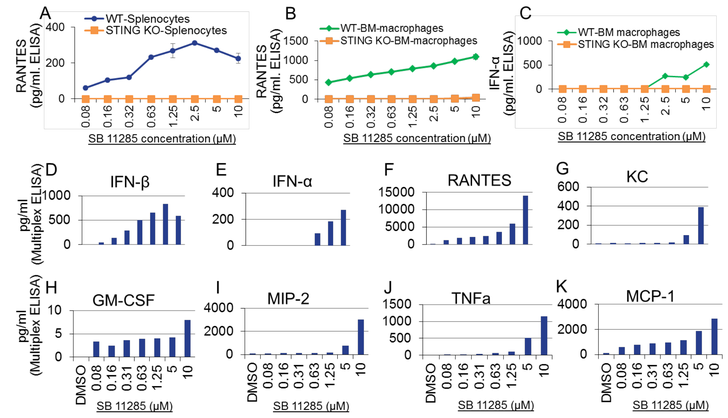
SB 11285 significantly inhibits the growth of multiple tumor-derived cell lines



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 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.

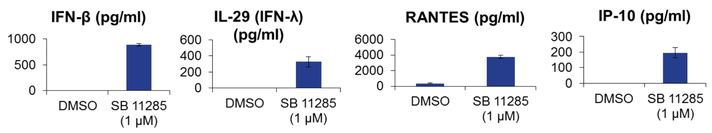
RESULTS

SB 11285 induces STING-dependent type I IFN, other cytokines and chemokines in mouse splenocytes and macrophages



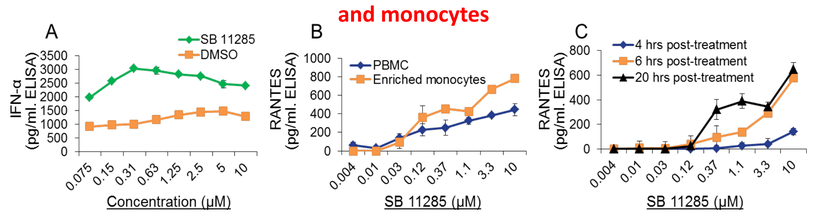
Fresh splenocytes (**A**) and bone marrow-derived macrophages (**B-K**) from C57BL/6 (wild-type) and STING knockout mice were treated with SB 11285 alone or controls (data not shown) in triplicate wells for 24 hrs. The production of mouse cytokines and chemokines in culture supernatants were measured using regular ELISA (**A-C**) or multiplexed ELISA (**D-K**) and the results are shown as pg/ml (average ± standard deviation of duplicate wells per concentration).

SB 11285 induces type I/III IFN and other cytokines and chemokines in THP1 cells



THP1 (WT) cells were treated with SB 11285 alone or controls in triplicate wells for 20 hrs. The production of cytokines and chemokines in culture supernatants were measured using ELISA and the results are shown as pg/ml (average ± standard deviation of duplicate wells per concentration).

SB 11285 induces type I IFNs, other cytokines and chemokines in human PBMCs and monocytes



PBMCs isolated from healthy donor (**A**) and enriched monocytes (**B and C**) were stimulated with SB 11285 alone (**A and B**) or controls (data not shown) for 20 hrs. The production of IFN-α (**A**) or RANTES (**B and C**) in cell culture supernatants was quantified using regular ELISA and the results are shown as pg/ml (average ± standard deviation of duplicate wells per concentration).

CONCLUSIONS

- **SB 11285** is a potent STING agonist:
  - SB 11285-induced STING signaling is blocked by palmitoylation inhibitor and TBK1/IKKε inhibitor.
  - TLR-MyD88 signaling pathways are not involved in SB 11285-induced type I IFN response.
  - SB 11285 activates NK cells in STING-dependent manner.
- **SB 11285** induces type I/II/III IFNs, as well as, cytokines and chemokines in primary human PBMC and monocytes, as well as, mouse bone marrow-derived macrophages.
- Pharmacodynamic studies in syngeneic mouse models have shown that **SB 11285** has potent anti-tumor activity (see **Poster # 1036**).
- **SB 11285** is being advanced to further preclinical development.

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