# A Novel STING Antagonist for Interferonopathies and Autoimmune Diseases

Shenghua Zhou, SreeRupa Challa, Vishal Nair, Lakshmi Bhagat, Diane Schmidt, Leena Suppiah, Seetharamaiyer Padmanabhan, Anjaneyulu Sheri, Dillon Cleary, Rayomand Gimi, Ron Mastrolia, Ireneusz Nowak, Geeta Meher, Nezam Afdhal, Sam Baskaran, and Radhakrishnan Iyer

Spring Bank Pharmaceuticals, Inc., 35 Parkwood Ave, Hopkinton, MA 01748, USA.

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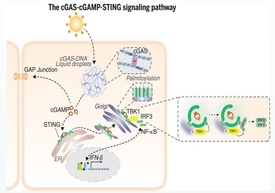
## INTRODUCTION

Cellular immune responses to dsDNA result in the activation of the cGAS-STING pathway for IFN production and play an indispensable role in antimicrobial defense; however, aberrant activation of the STING pathway has been hypothesized to cause autoimmune diseases including SLE, Aicardi–Goutières Syndrome, Sjögren's syndrome, SAVI, etc. Reported here is the discovery of a small molecule STING antagonist, SB 36, that uniquely inhibits aberrant STING-signaling.

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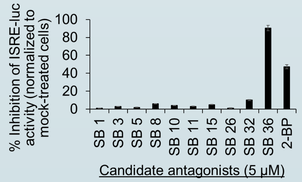
## STING ANTAGONIST DISCOVERY APPROACH

- A focused library of different heterocyclic and cyclic dinucleotide scaffolds were synthesized utilizing the crystal structure of 2',3'-cGAMP complexed with STING.
- Primary screening was performed in HEK293-based SZ14 cells stably expressing ISG54 ISRE-luciferase reporter gene against SBP's synthetic STING agonist (SB 11285).
- Promising compounds were further evaluated for antagonistic activity in THP1-Dual-WT cells against SB 11285 and 2',3'-cGAMP - the natural STING agonist.
- Based on early leads having drug-like properties, detailed SAR studies were performed and SB 36 was identified as a potent STING antagonist.
- SB 36 was further evaluated for antagonist activity against constitutive IFN signaling in TREX1-KO and Gain of Function (GOF) STING mutant cell lines (THP1-KI-hSTING-M155, and THP1-KI-hSTING-S154).
- STING-dependent antagonist activity of SB 36 was demonstrated in THP1-Dual-WT, RAW-ISG, and HEK293-TLR9 cells against various type I IFN inducers - LPS (TLR4), R848 (TLR7), ODN2006 (TLR9) and 3p-hpRNA (RIG-I).
- As an *in vivo* proof-of-concept, the STING-dependent antagonist activity of SB 36 was evaluated in normal C57BL/6 mice against SB 11285- and 2',3'-cGAMP-induced IFN-β production and/or interferon-stimulated gene (ISG) expression.

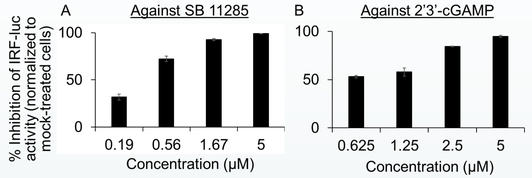
## RESULTS

### Screening of compound library using SZ14 cells stably expressing ISRE-luciferase reporter gene



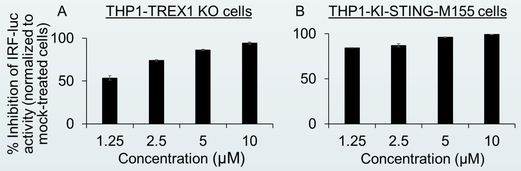

SZ14 cells in 96-well plates were pre-treated with candidate antagonists or control 2-BP for 1 hr, followed by stimulation with SB11285 agonist for 5h. The levels of ISRE-luc activity were determined and the percent inhibition was calculated against ISRE-luc activity in DMSO-treated cells.

### SB 36 inhibits STING-mediated type I IFN expression in THP1-WT cells




THP1-Dual-WT cells were pre-treated with the antagonist compound SB 36 for 2h, followed by stimulation with SB 11285 (A) or 2',3'-cGAMP (B) for 18h. The levels of IRF activity were determined and the percent inhibition was calculated against activity in DMSO-treated cells.

### SB 36 inhibits constitutive type I IFN expression in TREX1 KO cells and GoF-STING mutant cells




THP1-TREX1 KO (**A**) and THP1-KI STING-M155 (GoF) cells (**B**) were treated with SB 36 once daily for 3 days. The levels of IRF activity were determined and the percent inhibition was calculated against activity in DMSO-treated cells.

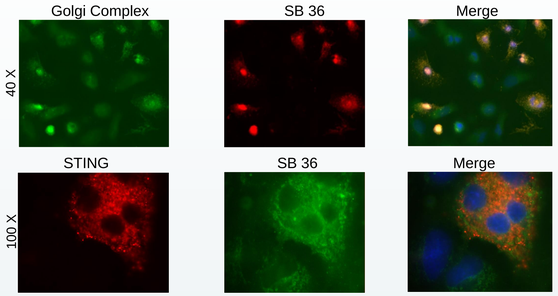
### SB 36 does not antagonize other PRR-induced type I IFN and NF-κB signaling pathways

| IRF activity/IC50 (μM) | | | |
|---|---|---|---|
| U-IFN | Ht-DNA (cGAS) | 3p-hpRNA (RIG-I) | LPS (TLR4) |
| NI | 6.5 | 7.15 | NI |

| NF-κB activity/IC50 (μM) | | | | |
|---|---|---|---|---|
| Ht-DNA (cGAS) | 3p-hpRNA (RIG-I) | LPS (TLR4) | R848 (TLR7) | ODN2006 (TLR9) |
| 7.46 | 9.04 | NI | NI | NI |

THP1-WT, RAW-ISG, and HEK293-TLR9 cells were pre-treated with SB 36 followed by various type I IFN inducers, including U-IFN (recombinant human type I IFN), Ht-DNA/lipo, 3p-hpRNA (RIG-I)/lipo, LPS (TLR4), R848 (TLR7), and ODN2006 (TLR9). Cells were incubated for 18h. The levels of IRF activity or NF-κB activity were determined and normalized against IRF or NF-κB activity in DMSO-treated cells. IC50 values were calculated using Xlfit. NI, no inhibition.

### SB 36 is localized at the Golgi

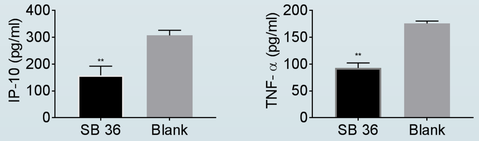



A549 cells transfected with FL-STING were treated with biotinylated compound SB 36 along with 2',3'-cGAMP, fixed, permeabilized and stained for imaging the Golgi compartment, STING and Biotin.

### SB 36 binds to FL-STING


A549 cells were transfected with FL-STING and the cell lysates were incubated with biotin-SB 36 (16h at 4°C), followed by incubation with avidin-magnetic beads (12h at 4°C). Beads were then washed extensively and the cell lysate + biotin-SB 36 complexes were subjected to SDS-PAGE and Western blot, Blots were probed with anti-STING antibody to detect STING. Biotin-TPOT is a non-specific Biotin control.

### SB 36 inhibits 2',3'-cGAMP-induced IP-10 and TNF-α production in human PBMCs




Human PBMCs were treated with SB 36 for 1h, followed by treatment with 2',3'-cGAMP. The cells were then incubated for 20 hours @ 37°C. The production of IP-10 and TNF-α in culture supernatant was measured using ELISA. Statistical significance was calculated by student's t test.

### SB 36 is a potent small molecule STING Antagonist

| | SB 11285 | 2'3'-cGAMP | | M155 | S154 | TREX1 |
|---|---|---|---|---|---|---|
| | THP-1 | THP1 | RAW | [GOF] | [GOF] | KO |
| $IC_{50}$ (μM), IRF-luc | 0.05-0.15 | 0.5 | 0.06 | 0.4 | 2.8 | 1.09 |

### SB 36 inhibits SB 11285-induced IFN-β and ISG expression in mice

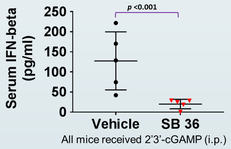

All mice received SB 11285 (i.p)

**Q-PCR analysis of ISG expression in spleen**

| | Vehicle | SB 36 | *p* value (t test) |
|---|---|---|---|
| IFN-β | 2.04±0.5 | 1.23±0.33 | <0.02 |
| IRF7 | 23.66±12.35 | 6.04±4.68 | <0.02 |
| IFIT2 | 98.67±49 | 30.22±20.89 | <0.02 |
| IFIT3 | 19.95±8.16 | 9.04±3.93 | <0.02 |
| RIG-I | 9.56±4.78 | 3.1±1.72 | <0.02 |

Mice were pre-treated with vehicle or SB 36, 10mg/kg via i.p. injection for 1h, followed by treatment with SB 11285, 2mg/kg. Blood, spleen, and liver samples were collected at 4h post-SB 11285 treatment. **Top panels**: Expression of IFN-β in blood, spleen and liver were assessed using ELISA. **Table**: The expression of ISGs in spleen at 4h post-treatment with SB 11285 were analyzed using qRT-PCR. All samples were normalized to GAPDH housekeeping gene expression. Results are shown as fold-increase over untreated mice (n=2).

### SB 36 inhibits 2',3'-cGAMP-induced IFN-β production in C57BL/6 mice




All mice received 2'3'-cGAMP (i.p.)

Mice were pre-treated with vehicle or SB 36, 10 mg/kg via i.p. injection, followed 1hr later with 10 mg/kg, i.p. 2'3'-cGAMP. Blood samples were collected at 4h post-cGAMP treatment. The production of IFN-β was assessed using ELISA.

## CONCLUSIONS

- SB 36 is a novel small molecule STING antagonist that inhibits constitutive and STING agonist-induced type I IFN expression *in vitro* in wild-type, as well as, GOF STING mutant cells and TREX1-KO human, and mouse cells.
- SB 36 co-localizes with STING in the Golgi and inhibits 2',3'-cGAMP-induced cytokine production in human PBMCs
- SB 36 administered by i.p. route in C57BL/6 mice inhibits type I IFN and ISG expression induced by both synthetic and natural STING agonists.
- SB 36 and analogs have the potential for broad therapeutic applications in interferonopathies and autoimmune diseases.

## REFERENCES

1. A Ablasser and ZJ Chen. *cGAS in action: Expanding roles in immunity and inflammation*. *Science*, 363 (**6431**), **2019**. 2. S Zhou, et al., *Characterization of SB 11285 as a novel STING agonist for Immuno-oncology*. 2017 Keystone Symposia Conference-C7: Cancer Immunology and Immunotherapy, March 19, 2018, British Columbia, Canada. 3. S Challa, et al., *Pharmacodynamic studies of SB 11285, a systemically bioavailable STING agonist in orthotopic tumor models*. AACR Tumor Immunology and Immuno-therapy, November 27-30, 2018, Miami, Florida.

**For additional information, please contact:**
**szhou@springbankpharm.com and kiyer@springbankpharm.com**