# Inhibition of ENPP1 using small molecule, SR-8541A, enhances the effect of checkpoint inhibition in cancer models

**Alexis Weston**[1,2], Trason Thode[1,2], Serina Ng[1], David Savitsky[3], Tithi Ghosh Halder[1], Shelby Rheinschmidt[1], Sydney Adamson[1], Katelyn Gutowsky[1], Brian Durbin[1], Raffaella Soldi[1], Srinivas Kasibhatla[1,2], Mohan Kaadige[1,2], and Sunil Sharma[1,2]

[1] Applied Cancer Research and Drug Discovery, Translational Genomics Research Institute (TGen) of City of Hope, 445 N. 5th Street, Phoenix, AZ 85004, [2] Stingray Therapeutics, Texas Medical Center Accelerator (TMCx), 2450 Holcombe Blvd, Suite X, Houston TX, 77021, [3]Agenus Inc., 3 Forbes Road, Lexington MA 02421

## Abstract

**Purpose**: It has become increasingly clear that the activation of both the innate and adaptive immune systems is vital to provide the best outcomes with immunotherapies. As part of the adaptive immune response, checkpoint inhibitors (CIs) have shown promise in the clinic, but seem to only work in a small subset of cancers with response rates below 20%. It is anticipated that activation of the innate immune response may help sensitize multiple cancer types to adaptive immune therapies. cGAS-STING pathway, which is activated in response to cytosolic DNA, has emerged as a key mechanism to activate innate immunity, primarily through type I interferon (IFN) signaling. Several direct STING agonists have been developed but their performance in the clinic has been dissatisfactory. A key limitation with direct STING agonists is the widespread expression of STING in normal tissues, whereby the hyperactivation of STING can lead to a systemic cytokine storm. Thus, there is a need to identify alternative approaches to activate STING in a controlled manner. ENPP1 is the only known direct negative regulator of the STING pathway that hydrolyzes 2′3′ cGAMP, the direct ligand of STING. Highest levels of 2′3′ cGAMP are found in tumors and recent evidence suggests that 2′3′ cGAMP acts locally, as a paracrine immune transmitter. Therefore, inhibition of ENPP1 may produce superior outcomes by activating STING in the tumor microenvironment. Previously, we reported the development of SR-8541A, a highly selective and potent inhibitor of ENPP1 that activates the STING pathway. Here, we show that the inhibition of ENPP1 with SR-8541A enhances the effect of CIs in breast and colon cancer models.

**Methods**: Immune infiltration assays were conducted using human breast cancer cell line derived organoids (MDA-MB-231 and MDA-MB-468). Co-cultures of cancer organoids and immune cells (PBMCs) were exposed to SR-8541A +/- CIs (CTLA-4 and/or PD-1) for 48 hours. Confocal Z-Stack imaging, RT-PCR, and MSD cytokine assays were performed to evaluate the effects. In vivo studies were conducted using syngeneic mouse models (CT-26 and EMT-6), which were engrafted subcutaneously and treated with SR-8541A +/- CIs. Tumor growth was monitored over the course of the study. IHC and RT-PCR were conducted on the tumors.

**Results**: Combination of SR-8541A with CIs showed a significant increase in immune infiltration in both the MDA-MB-231 and MDA-MB-468 organoid models. Corresponding RT-PCR analysis showed activation of IFN signaling (IFN-ß, CXCL10, ISG15). In vivo combination with CIs also exhibited a significant increase in overall efficacy, along with increased levels of CD3+ and CD8+ T-cell infiltration into the tumors, and increased levels of IFN response.

**Conclusion**: In summary, we show that combination of ENPP1 inhibition with checkpoint inhibition promotes a robust antitumor activity by stimulating both innate and adaptive immune response.

## Introduction

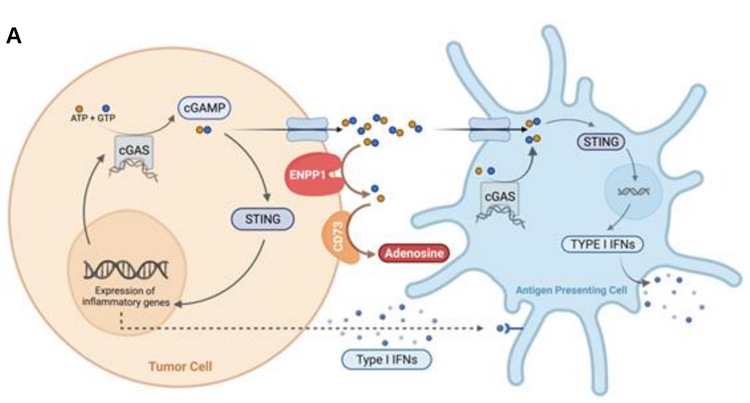


**Figure 1**. **A)** The cGAS-STING signaling pathway and its inhibition by ENPP1 through hydrolysis of 2′3′-cGAMP. **B)** Biological and pathophysiological processes of ENPP1. **C)** TGCA analysis of 33 different tumor types showing ENPP1 and cGAS expression. BRCA, COAD, and PAAD are highlighted as tumor types of interest with a reported <15% response rate to checkpoint inhibition in the clinic.

## Combination of SR-8541A with immune checkpoint inhibitors potentiates the infiltration of immune cells in a breast cancer spheroid model

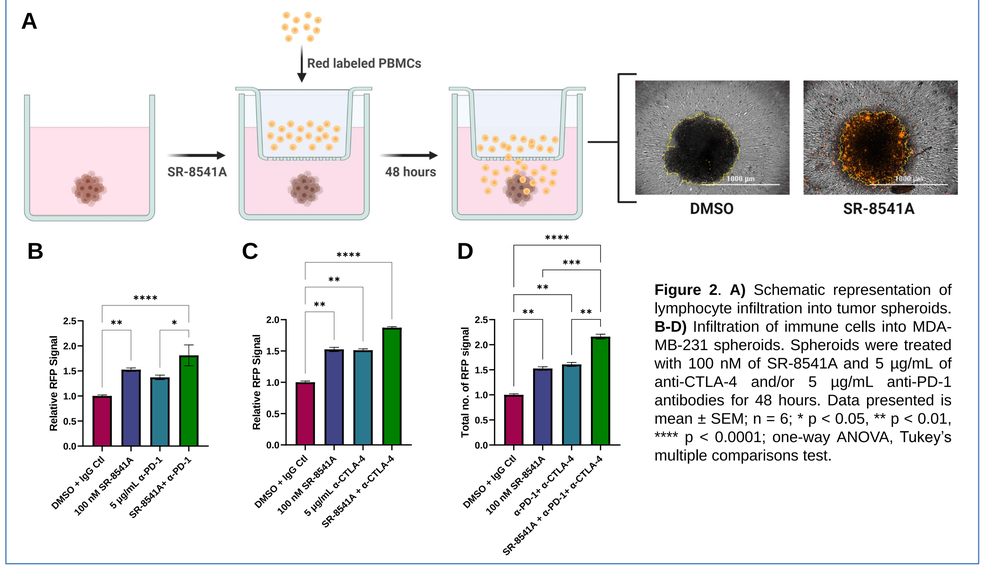


**Figure 2**. **A)** Schematic representation of lymphocyte infiltration into tumor spheroids. **B-D)** Infiltration of immune cells into MDA-MB-231 spheroids. Spheroids were treated with 100 nM of SR-8541A and 5 µg/mL of anti-CTLA-4 and/or 5 µg/mL anti-PD-1 antibodies for 48 hours. Data presented is mean ± SEM; n = 6; * p < 0.05, ** p < 0.01, **** p < 0.0001; one-way ANOVA, Tukey's multiple comparisons test.

## SR-8541A inhibits growth of tumor cells in syngeneic mouse models of breast and colon cancer

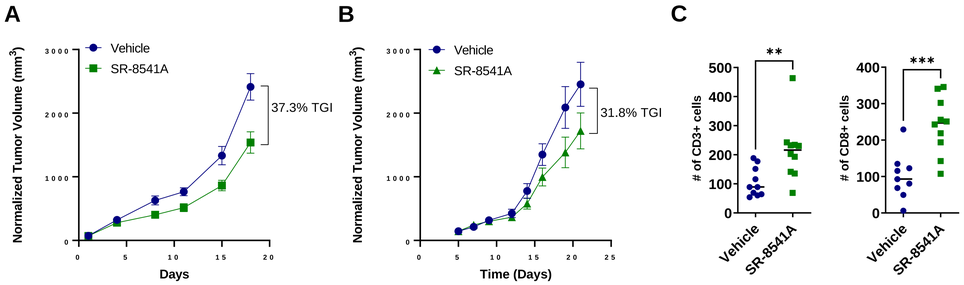


**Figure 3**. **A)** Breast (EMT-6) **or B)** colorectal (CT-26) cells were implanted subcutaneously into the right hind flank of female BALB/cJ mice and treated with 0.2 mg/kg SR-8541A PO, BID. **C)** IHC analysis was performed on colorectal tumors treated with 0.2 mg/kg SR-8541A to identify CD3+ and CD8+ T-cell populations. Data presented is mean ± SEM; n = 10; * p < 0.05, ** p < 0.01, **** p < 0.0001; one-way ANOVA, Tukey's multiple comparisons test.

## Treatment with SR-8541A enhances the effect of double checkpoint inhibition in a CT-26 colon cancer model

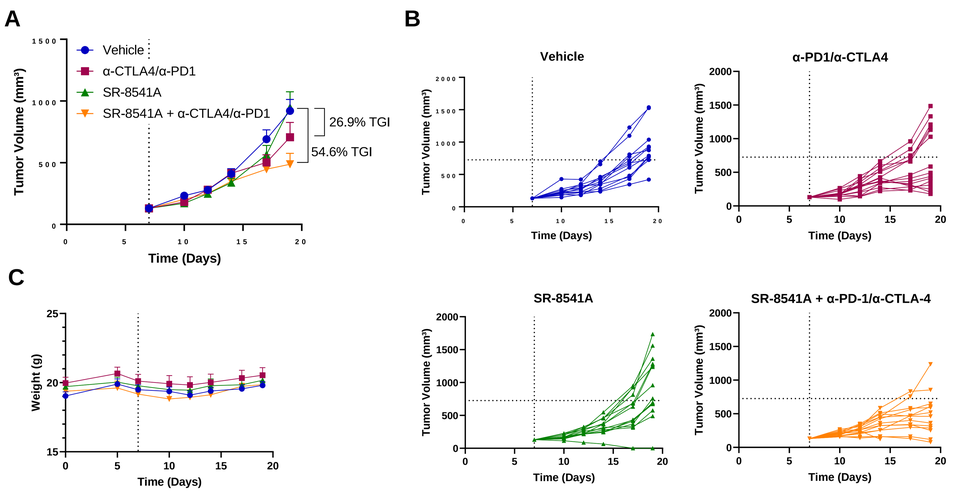


**Figure 4.** CT-26 cells were implanted subcutaneously into the right hind flank of female Balb/cJ mice and treated with 0.2 mg/kg SR-8541A PO, BID +/- 400 µg each of α-CTLA4 [9H10] and α-PD-1 [RMP1-14] IP, 1xW. **A-B)** Tumor growth and **C)** body weight were monitored over 19 days. TGI based on comparison to vehicle group.

## Treatment of SR-8541A along with Botensilimab$^{ms}$ robustly suppresses tumor growth and shows increased immune response

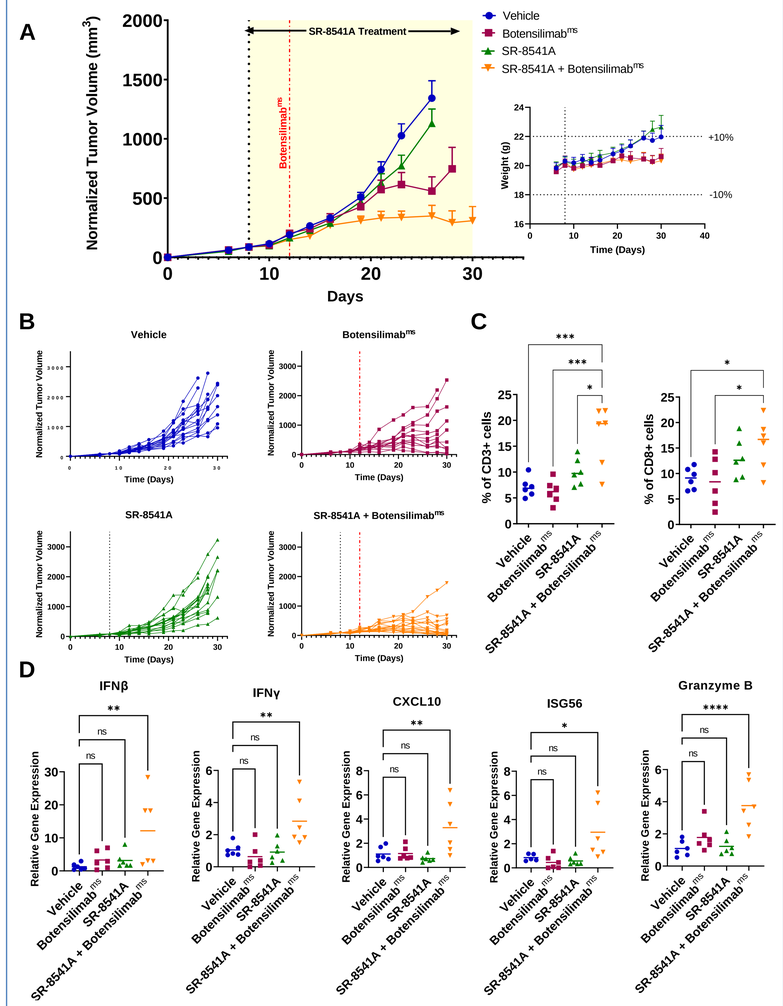


**Figure 5.** CT-26 cells were implanted subcutaneously into the right hind flank of female BALB/cJ mice and treated with 0.2 mg/kg SR-8541A PO, BID +/- 100 µg Botensilimab$^{ms}$ once, day 12. **A-B)** Tumor growth and body weight were monitored over 30 days. TGI calculations were made on day 23 prior to the termination of any mice at the tumor endpoint of 2000 mm$^3$. Tumor samples were collected at endpoint and analyzed via **C)** IHC analysis to identify CD3+ and CD8+ T-cell populations and **D)** RT-PCR for gene expression markers of immune activation. Data presented is mean ± SEM; * p < 0.05, ** p < 0.01, **** p < 0.0001; one-way ANOVA, Tukey's multiple comparisons test.

## Conclusions and Future Studies

- High levels of ENPP1 expression in multiple cancer types promotes resistance to immuno-therapies, such as checkpoint inhibitors in the clinic.
- Inhibition of ENPP1 enhances the effect of checkpoint inhibitors in immune spheroid models.
- Treatment with low dose SR-8541A induces a moderate response as a single agent in murine cancer models and shows increased levels of T-cell infiltration in treated tumors.
- ENPP1 inhibition using SR-8541A enhances the effect of checkpoint inhibitors in murine cancer models, showing both a greater effect on tumor growth inhibition and an increase in immune biomarkers.
- In separate but comparable experiments, the Fc-enhanced α-CTLA-4 (Botensilimab$^{ms}$) showed greater activity compared to the combined first generation α-PD-1/α-CTLA-4 alone and in combination with SR-8541A.
- The IND for SR-8541A was submitted in March 2023, with the plan to start first in patient trials Summer 2023

**Contact Information:**
Alexis Weston
Alexis@StingrayTx.com

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**COI:** AW, TT, MK, SK, and SS own equity in Stingray Therapeutics