Preclinical studies of SR-8314, a highly selective ENPP1 inhibitor and an activator of STING pathway

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Abstract

Purpose: STING (stimulator of interferon genes) plays an important role in innate immunity by activating type I interferons in response to cytosolic nucleic acid ligands such as cyclic dinucleotides (CDNs). In recent years, STING has become an attractive therapeutic target for cancer immune therapy and hydrolysis resistant CDNs have been developed as a new class of cancer therapeutics. These CDNs have been shown to possess potent preclinical efficacy but early results from phase I trials have been disappointing. Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1 or NPP1) is natural antagonist of STING pathway. ENPP1 constitutively hydrolyzes 2′3′-cGAMP, a CDN that is the natural ligand for STING. Previously, we reported that SR-8291, a highly selective ENPP1 inhibitor, induces STING activity and demonstrates anti-tumor activity in B16F10 melanoma and CT26 colorectal models. In this study, we extend these findings and report the discovery of SR-8314, an analog of SR-8291 that shows improved physiochemical and developability properties.

Methods: With the application of computational techniques (ICM, Maestro), human NPP1 homology model was built by utilizing the crystal structure of mouse NPP1 (PDB: 4GTW). Series of docking simulations on built in ligands were performed within the substrate binding pocket and that led to the identification of lead NPP1 inhibitor SR-8314. Binding of SR-8314 to ENPP1 was evaluated using thermal shift assay. Activity of recombinant human ENPP1 using ATP as a substrate was measured using Cell Titer Glo (Promega) reagent. IRF-Lucia reporter activity, RT-PCR and Western Blotting were performed to evaluate the effect of SR-8314 in 2′3′-cGAMP primed THP1 dual reporter cells (Invivogen). In vivo efficacy studies of intraperitoneally-dosed SR-8314 and SR-8291 were performed in a syngeneic murine tumor model. Tumor T cell infiltration, tumor and plasma pharmacokinetics were assessed by flow cytometry and mass-spectrometry, respectively.

Results: SR-8314 has higher binding affinity towards ENPP1 and it potently inhibits ENPP1 activity with a Ki value of 0.079μM. A significant increase in gene expression of IFNβ, ISG15 and CXCL10 along with an increase in the secretion of IFNβ was observed in SR-8314 treated THP1 cells. We show anti-tumor activity as well as an increase in $CD3^+$, $CD4^+$ and $CD8^+$ T cells in both SR-8314 and SR-8291 treated tumors. In addition, there was a decrease in tumor associated macrophages in SR-8314 treated tumors.

Conclusions: In summary, we show SR-8314 as a potent inhibitor of ENPP1 that promotes STING activity in vitro. SR-8314 displays promising anti-tumor activity and has ideal candidate properties that warrant further evaluation.

Introduction

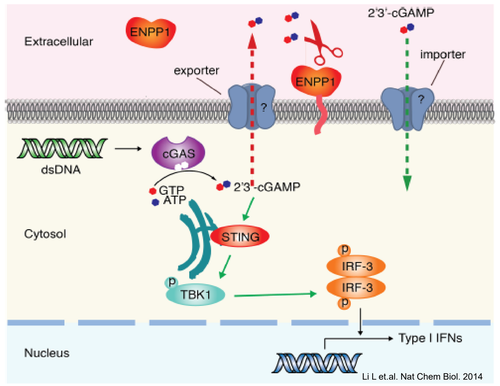


Fig 1: A schematic model for activation of STING pathway. Phosphodiesterase resistant CDN's that showed potent antitumor immunity in pre-clinical studies are currently undergoing clinical trials. As these CDN's are bulky and require intra-tumoral delivery, alternative approaches must be explored to activate STING.

Methods

Computational Methods The 2.7 A° crystal structure of mouse ENPP1 bound AMP (PDB: 4GTW) was used as a structural target to construct 3D model human ENPP1 in complex with AMP (**Fig 2**) and ATP. The 3D model was generated using the ICM-Homology and build functionality within ICM 3.8-5 (Molsoft, San Diego, CA) where the missing loops predicted though PDB-database search and the energy of the system minimized by an ICM simulation through a series of random global moves, and gradient local minimization in the internal coordinates space.

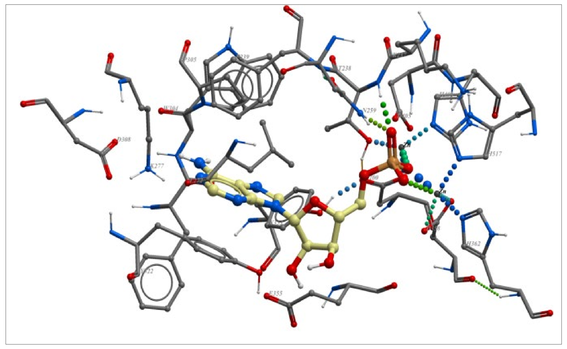


Fig 2: Homology model of hENPP1 in complex with redocked AMP. Catalytic active site clipped and residues labelled. The dotted lines are H-Bonds and black circles are Zn^2 atoms. Key residues; Asp218, Thr256, Asp277, Tyr340, Tyr371 and Asp376 depicted in stick model.

Results: SR-8314 selectively binds and inhibits ENPP1 activity

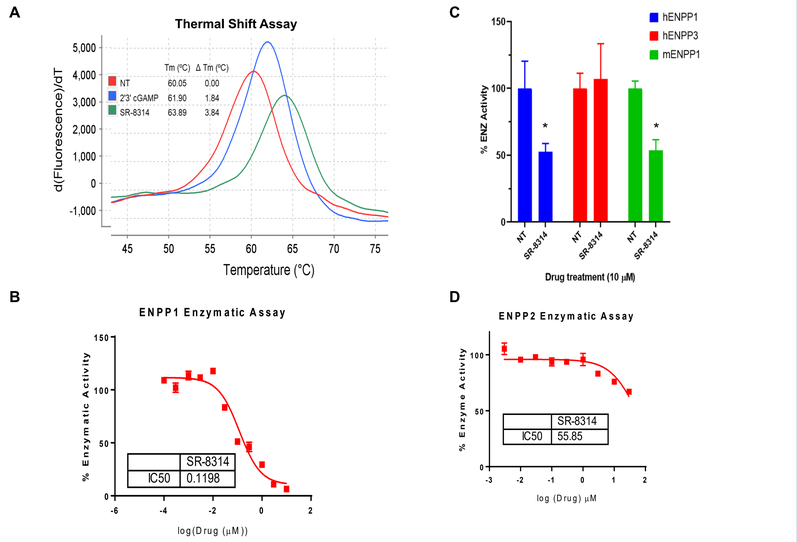


Fig 3: (**A**) Protein melt assays were performed with recombinant ENPP1 protein and sypro orange dye in the presence or absence of indicated compounds at 100 μM. (**B**) Activity of recombinant ENPP1 protein was measured by incubating different concentrations of SR-8314 and thymidine 5'-monophosphate *p*-nitrophenyl ester as a substrate. (**C**) HEK293T cells were transfected with indicated ENPP constructs. After 24 hours, cell lysates were treated with 10 μM of indicated compounds for three hours at 4°C and ENPP activity was assessed as described in B. (**D**) Activity of recombinant ENPP2 protein was measured by incubating different concentrations of indicated compounds and Bis(*p*-Nitrophenyl) phosphate as substrate. *p<0.05

Results: SR-8314 stimulates STING pathway

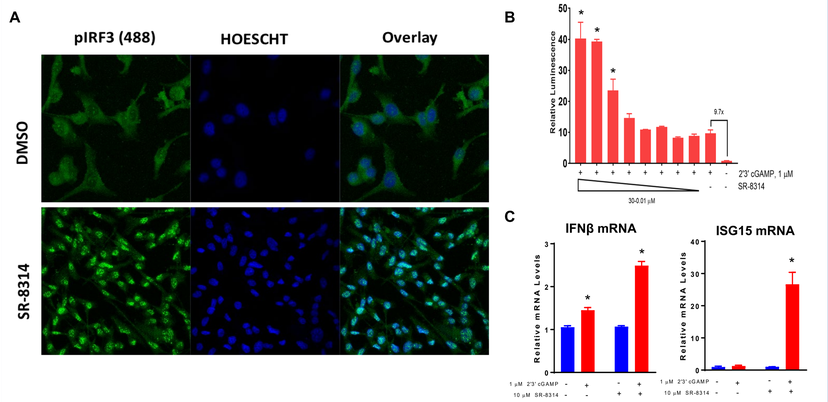


Fig 4: (**A**) Immunofluorescence was used to determine the localization of phospho-IRF3 in MDA-MB-231 cells treated with 10 μM SR-8314 for 48 hours. (**B**) Activity of an interferon stimulated reporter was measured in THP1 dual cells (Invivogen) treated with various doses of SR-8314 for 48 hours (**C**) Expression of IFNβ and ISG15 were measured in THP1 dual cells treated with SR-8314 for 48 hours. *p<0.05

Results: SR-8314 stimulates lymphocyte infiltration

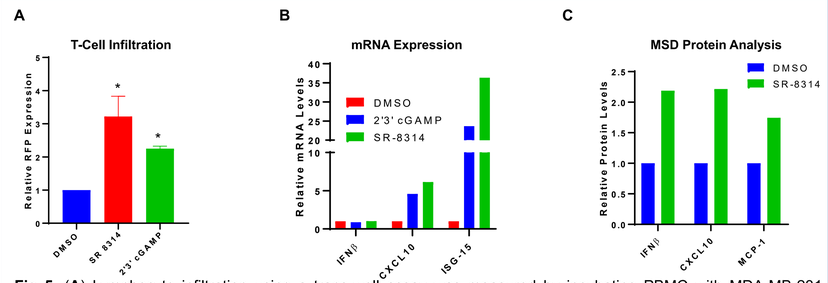


Fig 5: (**A**) Lymphocyte infiltration using a trans-well assay was measured by incubating PBMC with MDA-MB-231 spheroids and conditioned media that was collected after treating 2D cell culture of MDA-MB-231 with 10 μM SR-8314 for 72 hours. (**B**) Expression of IFNβ, CXCL10, and ISG-15 were measured from the 2D cell culture of MDA-MB-231 as described in (A). (**C**) The levels of IFNβ, CXCL10 and MCP-1 were measured from the conditioned media as described in (A) using MSD multiplex assay. *p<0.05

Results: SR-8314 shows anti-tumor activity in a KPC model

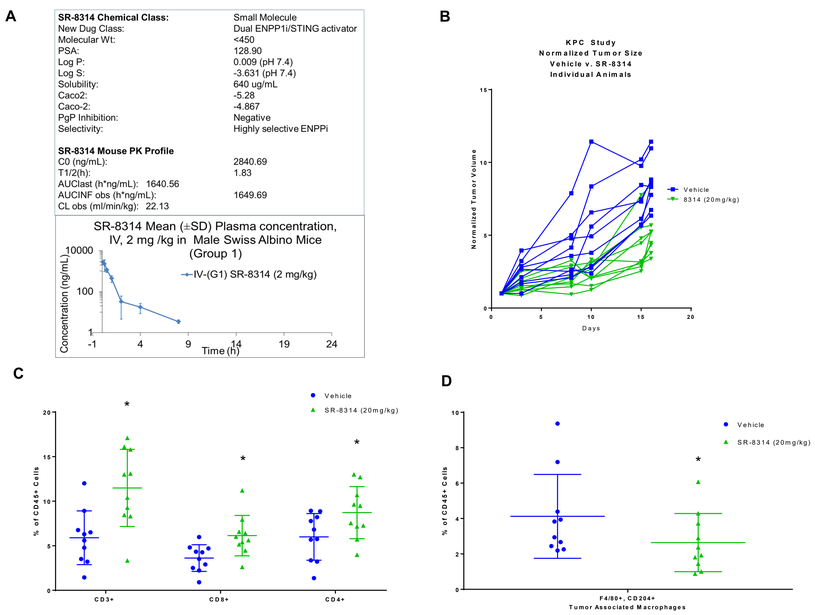
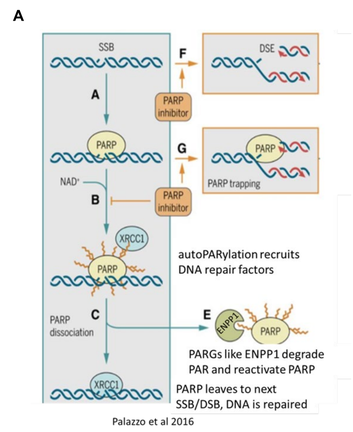


Fig 6: (**A**) Pharmacokinetic parameters of SR-8314. (**B**) SR-8314 inhibits tumor growth of KPC mouse model of PDAC. SR-8314 was administered once daily via IP at a dose of 20 mg/kg. (**C**) & (**D**) At the end of the study, tumors were excised and analyzed for T-cells and tumor-associated macrophages using flow cytometry. *p<0.05

Results: SR-8314 shows synergy with PARP inhibitor



Drug treatment		CI Values	ED50 Drug Concentration		Chou-Talalay
		ED50	ENPP1i (μM)	Olaparib (μM)	
Individual	SR-8314	--	*NC	--	--
	Olaparib	--	--	23.7	--
SR-8314:Olaparib	1:1	0.393	7.585	7.585	Synergy
	10:1	0.609	1.41	14.104	Slight Synergy
	1:10	0.475	34.294	3.429	Synergy

CI Values*	
X > 1	Competitive
X = 1	Additive
X < 1	Synergistic

Fig 7: (**A**) A schematic showing the potential role of ENPP1 in DNA repair mediated by PARP. Image adapted from Pommier Y et.al., 2016 (**B**) SR-8314 and Olaparib were tested at different ratios in MDA-MB-468 cells and the data was analyzed by the Chou-Talalay method to demonstrate synergy. *Not Calculated

Conclusion

- SR-8314 is a potent and selective small molecule inhibitor of ENPP1.
- SR-8314 induced phosphorylation and translocation of IRF3 to the nucleus.
- ENPP1 inhibition by SR-8314 induces expression of immune cell attracting chemokines and cytokines.
- SR-8314 was effective at inhibiting tumor growth and stimulating T-cell infiltration into tumors.
- SR-8314 showed synergy with PARP inhibitor, warranting further investigations using mouse tumor models.

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IACUC Protocol #: 14-531

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

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