A Small Molecule IRF3 Agonist Targeting the RIG-I Pathway Modulates Innate Immune Responses and Induces In Vitro Markers of Immunogenic Cell Death in a Murine Colon Carcinoma Tumor Model

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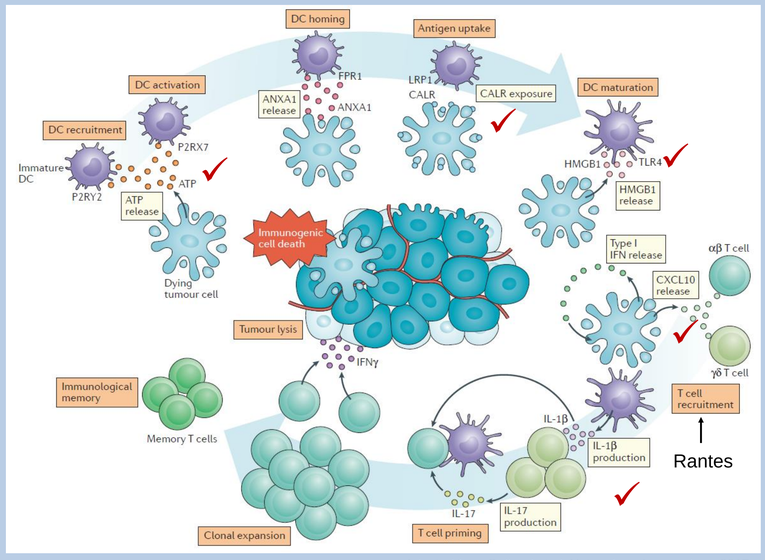
Abstract

The RIG-I innate immune signaling pathway is a key modulator of antiviral immune defense, and nucleic acid agonists of RIG-I have been shown to elicit immunogenic cell death (ICD) in a murine pancreatic tumor model. Kineta has identified small molecule IRF3 immune modulators that activate the RIG-I pathway and induce in vitro signs of ICD in tumor cell lines. The proof of concept compound, KIN131A, activates RIG-I-dependent signaling in reporter cell lines, stimulates chemokine/cytokine production by human PBMCs, and induces activation of dendritic cells. In colon carcinoma CT26 cells, KIN131A treatment causes the release of the danger-associated molecular pattern molecules HMGB1 and ATP, stimulates the translocation of calreticulin from the endoplasmic reticulum to the cell surface, and induces cell death. Together, these data suggest that KIN131A triggers ICD in CT26 cells. Ongoing experiments using the CT26 mouse tumor model are characterizing KIN131A induced ICD in vivo. Preliminary data show that KIN131A inhibits tumor growth, enhances survival and protects a subset of mice from tumor cell re-challenge. These data suggest that small molecule RIG-I agonists can induce ICD in vivo and provide immune mediated anti-tumor properties. The induction of ICD by small molecule RIG-I agonists may provide a unique immunotherapeutic opportunity to induce or boost the antitumor immune response in patients and enhance the efficacy of checkpoint inhibitor-mediated immunotherapy.

Background

- Immune responses against viruses are initiated by pattern-recognition receptors (PRRs), such as the RIG-I-like receptors (RLRs) that are activated by pathogen-associated molecular patterns (PAMPs). The activation of RLRs, and other intracellular PRRs, results in the activation of antigen presenting cells (APC) followed by the induction of an antiviral T cell response.
- Immunogenic cell death is a form of programmed cell death of tumor cells that elicits an anti-tumor T cell response to tumor neo-antigens by facilitating antigen presentation and T cell activation (Figure 1). Several in vitro correlates of ICD have been identified including:
  - Release of the damage-associated molecular pattern (DAMP) molecules ATP and HMGB1
  - Translocation of calreticulin to the cell surface
  - Secretion of CXCL10 and IL-1β
- We have identified a panel of small molecule immunomodulators that activate IRF3 via RLR pathways and induce distinct innate immune signaling by myeloid reporter cell lines, human PBMC and dendritic cells (DC) (Table 1)
- The IRF3 agonists were evaluated for the induction of in vitro markers of ICD in tumor cell lines. KIN131A was selected as the proof of concept compound to demonstrate ICD in the CT26 colon carcinoma tumor model in vivo.



Adapted from Galluzzi et al. Nat reviews Immunology, 2017

Figure 1: Potential Mechanism of KIN131A-induced Immunogenic Cell Death

	PBMC				MoDC			
	Poly I:C	KIN131A	KIN126X	KIN150X	Poly I:C	KIN131A	KIN126X	KIN150X
IFN-α	+++	-	-	-	+	-	-	-
IFN-β	+	-	-	-	-	-	-	-
IFN-λ	++++	-	-	-	+++	-	-	-
IFN-γ	++++	-	-	+++	*n.d.*	*n.d.*	*n.d.*	*n.d.*
IL-1β	-	+	++	+++	-	-	-	-
IL-6	++++	++	+++	+++++	++++	-	-	-
TNF-a	+++	++	++	++++	++++	+	++	-
IL-10	+	-	-	-	++	-	-	-
IL-12p70	++	-	-	-	++	-	-	-
IL-23	++	++	++	++	+++++	-	-	-
Mip-1β	++	++	++	++	+	++++	++++	-
Rantes	+++	++	++	++	++++	++	++	-

-, 0 – 49; +, 50-100; ++,100-499 ; +++, 500-999 ; ++++, 1000-4999 ; +++++, ≥ 5000 pg/ml

Table 1: KIN IRF3 agonists mediate distinct inflammatory cytokine/chemokine profiles by human PBMC and DC. Cytokines, chemokines and interferons were quantified in supernatants 18 h post stimulation.

The IRF3 Agonist KIN131A Activates the RIG-I Pathway

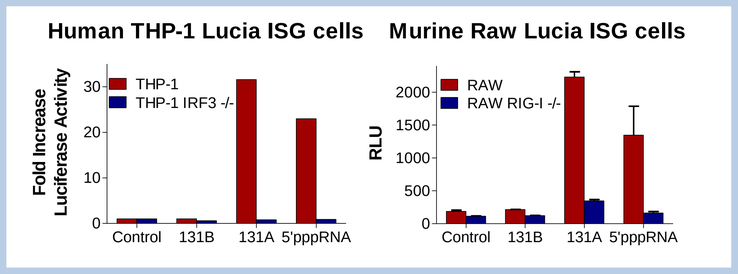


Figure 2: KIN131A stimulates IRF3-dependent luciferase expression in the human and murine myeloid reporter cell lines. Wild type and IRF3 deficient THP-1 cells were differentiated with PMA. Luciferase activity was determined 18 h after stimulation. KIN131B, a KIN131A analog, does not activate the IRF3 pathway.

KIN131A Induces Apoptosis in CT26 Colon Carcinoma Cells

KIN131A treatment elicited dose-dependent apoptosis in murine CT26 cells. Annexin V staining indicated that up to 65% of cells were apoptotic after 16 h of KIN131A treatment (**Fig. 3**).

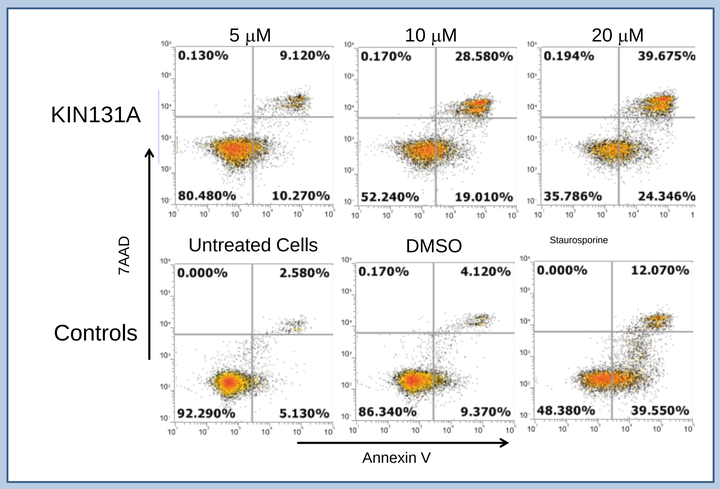


Figure 3: KIN131A induces apoptosis in murine CT26 colon carcinoma cells. Cells were treated for 16 h with the KIN131A at the indicated concentrations. Annexin V and 7AAD staining was used to identify apoptotic and dead cells, respectively

KIN131A Treatment Mediates the Translocation of Calreticulin to the Cell-surface of CT26 Cells

KIN131A induces in vitro markers of ICD including the translocation of calreticulin to the cell surface (Figure 4), the release of ATP and HMGB1 (Figure 5) and the induction of CXCL10 (Table 2).

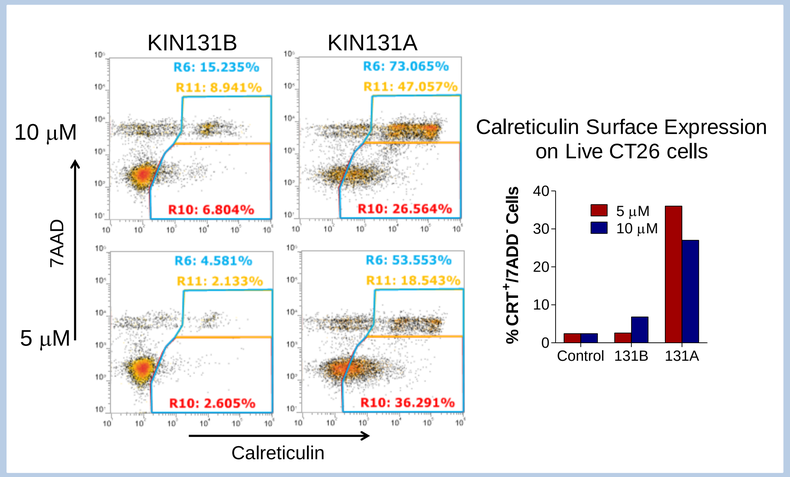


Figure 4: KIN131A mediates the translocation of calreticulin to the cell surface of murine CT26 colon carcinoma cells. Cells were treated for 16 h with KIN131A at the indicated concentrations. 7AAD staining was used to identify live ($7AAD^-$) and dead cells ($CAAD^+$). CRT expression was determined by staining with an anti-CRT antibody against the center region.

CT26 Colon Carcinoma Cells Release DAMPs in Response to KIN131A Treatment

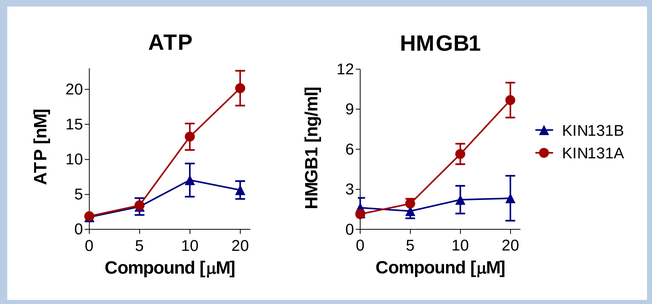


Figure 5: KIN131A induces the release of ATP and HMGB1 by CT26 cells. Cells were stimulated with KIN131A or KIN131B at the indicate concentrations. ATP and HMGB1 levels were determined in supernatants taken 18 h after stimulation.

The RLR agonists KIN131A, and its SAR derivative KIN131A-14, are potent inducers of in vitro markers of ICD by CT26 cells. Cytokine-inducing activity of IRF3 agonists KIN126X and KIN150X (Table 1) does not correlate with apoptosis and CRT expression, suggesting that additional mechanisms are required (Table 2).

KIN	HMGB1	ATP	Annexin V	CRT	CXCL10
131A	+++	+++	+++	+++	+
131A-14	+++++	n.d.	+++++	+++	+++
126X	++	++	++	-	n.d.
150X	-	-	+	-	n.d.

Table 2 : KIN131A, and its derivative KIN13A-14, induce the release of in vitro markers of ICD by CT26 cells.

Intra-tumoral Injection with KIN131A Results in Tumor Regression, Induction of ICD and Anti-tumor Immunity

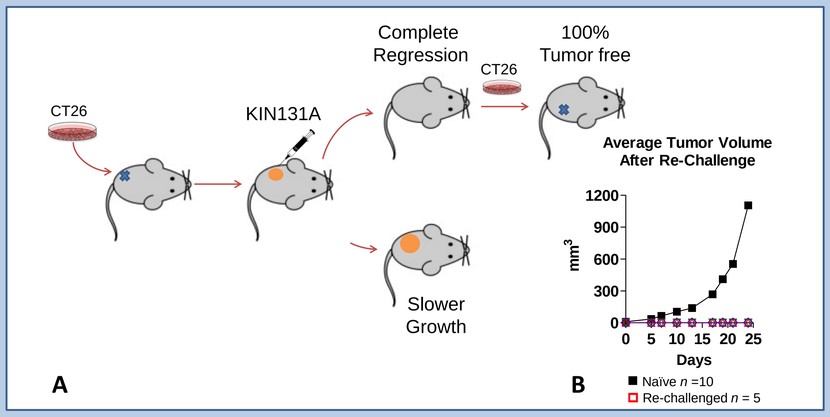


Figure 6: Intra-tumoral injection with KIN131A results in tumor regression and immunity to re-challenge. (**A**) Tumor therapy model. Tumors were induced by injection of 5×10^5 live CT26 cells into the left flank. Established tumors were then injected with KIN131A (n = 40) or DMSO (n = 10). Mice exhibiting complete tumor regression were re-challenged with live CT26 cells into the right flank. (**B**) Tumor immunity after re-challenge with live CT26 tumor cells. Average tumor volume after re-challenge is shown. Black: naïve animals; red: re-challenged animals.

Summary and Conclusions

KIN131A is a RLR agonist with at least two immunomodulatory activities:

1. KIN131A stimulates the RIG-I and IRF3 pathway in myeloid cells and induces the secretion of inflammatory chemokines/cytokines by human PBMC and DC
2. KIN131A induces ICD in the CT26 colon carcinoma model
   - Release of DAMPs (ATP and HMGB1)
   - Induction of programmed cell death (Annexin V^+ and Caspase 3)
   - Translocation of calreticulin to the cell surface
   - Secretion of CXCL10 by CT26 cells
   - Proof of concept in vivo efficacy in the CT26 tumor challenge model by inducing a protective anti-tumor immune response