Targeting the Stress Response Kinase GCN2 to Restore Immunity in the Tumor Microenvironment

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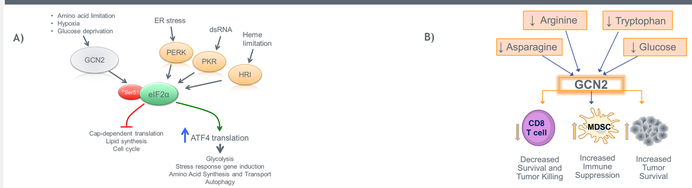
1. Abstract

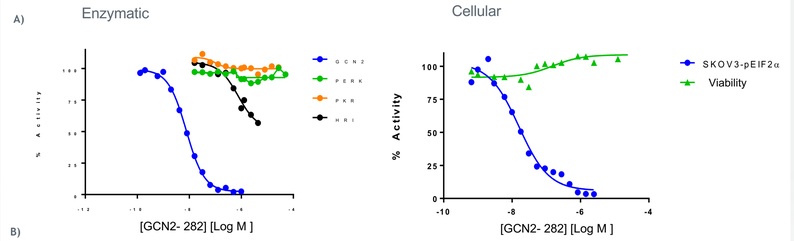
Recent advances in cancer metabolism suggest that targeting amino acid metabolism represents a promising strategy for the development of novel therapeutic agents. Tumor, stromal and myeloid-derived suppressor cells (MDSC) within the tumor microenvironment (TME) create a nutrient-poor environment that inhibit immune function and support tumor growth. GCN2 (**g**eneral **c**ontrol **n**onderepressible **2**), a stress response kinase, plays a key role in maintaining cellular homeostasis under a wide range of stressors. Phosphorylation of GCN2 (pGCN2) in response to stress leads to inhibition of global protein synthesis and subsequently leads to 1) T cell anergy and apoptosis, 2) enhanced MDSC-dependent immune suppression and 3) tumor cell survival. Collectively, these roles suggest that GCN2 inhibition could have both a direct anticancer and an immune-activating effect. Treating nutrient-deprived T cells in vitro with a RAPT GCN2 inhibitor (RPT-GCN2i) rescued $CD4^+$ and $CD8^+$ T cell proliferation and effector functions. The RPT-GCN2i also reversed T cell suppression mediated by MDSCs derived from healthy donors or cancer patients. Using syngeneic mouse tumor models, we demonstrated that translational induction of **a**ctivating **t**ranscription **f**actor 4 (ATF4) is a strong marker of GCN2 pathway activation in vivo. Oral administration of an RPT-GCN2i exhibited notable drug-target occupancy and potently inhibited GCN2 kinase and ATF4 in the TME. RPT-GCN2i as a single agent and in combination with checkpoint blockade or angiogenesis inhibitor (anti-VEGFR) led to delays in tumor growth rate in various syngeneic tumor models. Our results show that inhibition of GCN2 is an attractive approach for enhancing antitumor immune response and therefore GCN2 is a promising therapeutic target for the treatment of cancer.

2. Introduction



A) GCN2 is a stress response kinase detecting amino acid starvation in the tumor microenvironment.[1] B) Activation of GCN2 leads to 1) T cell anergy, apoptosis and enhanced T_{reg} suppression and a decrease overall T cell function 2) increases myeloid-derived suppressor cell function and 3) increased tumor survival. [2]

3. GCN2i Potently Reduces EIF2α Phosphorylation

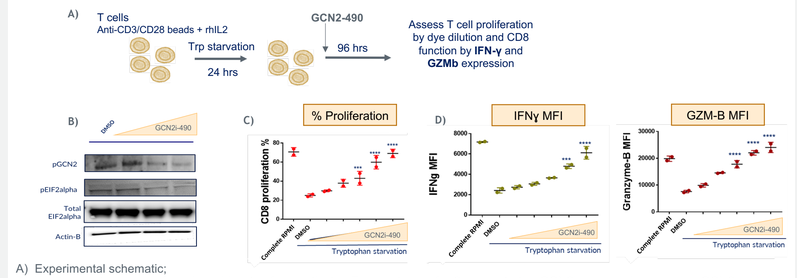


B)

ID	GCN2 Enzyme IC_{50} (nM)	PERK Enzyme IC_{50} (nM)	PKR Enzyme IC_{50} (nM)	HRI Enzyme IC_{50} (nM)	SKOV3 Cellular pEIF2a IC_{50} (nM)	SKOV3 Cellular Toxicity IC_{50} (nM)	Bioavailability (Rodent %F)
GCN2-490	4.5	>50000	390	440	16	>25000	22%
GCN2-282	7.6	>50000	4400	>25000	24	>25000	80%

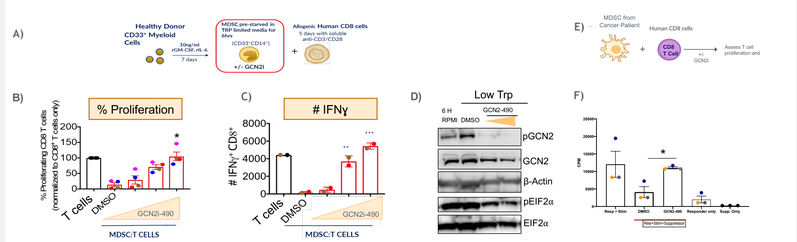
A) Enzymatic and cellular potency for GCN2-282; B) Potency and selectivity parameters for GCN2-490 and GCN2-282. For enzymatic assays, compounds were incubated with recombinant human kinases and EIF2α-GFP substrate. Phosphorylation of EIF2α was measured by TR-FRET and used to calculate inhibition of kinase activity. For cell-based pEIF2α assay, SKOV-3 cells were incubated with compounds and then stimulated with halofuginone (1 hour) to activate GCN2 and then pEIF2α was measured by AlphaLisa. For toxicity assessment, SKOV3 cells were incubated with compounds for 72 hours and viability was assessed with CellTiter-Glo reagent.

4. GCN2i Restores Human T Cell Proliferation And Function In Tryptophan Limited Conditions



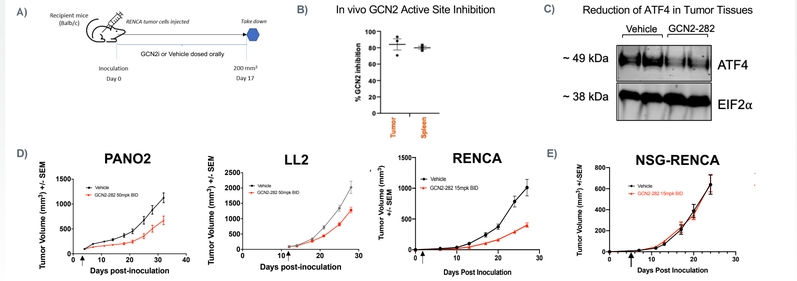
A) Experimental schematic;
B) Western blot analysis of whole cell lysates from activated T cells starved for 24 hrs with the GCN2i showed decrease in pGCN2 and pEIF2alpha
C) $CD8^+$ T cell proliferation was assessed by dye dilution (CD4 proliferation is similarly increased – data not shown)
D) T cell functional markers were analyzed by flow cytometry. *P < 0.05; **P < 0.01, ***P < 0.001, ****P < 0.0001

5. Treatment Of Human $CD33^+$ MDSC With GCN2i Reverses Their Immuno-suppressive Function



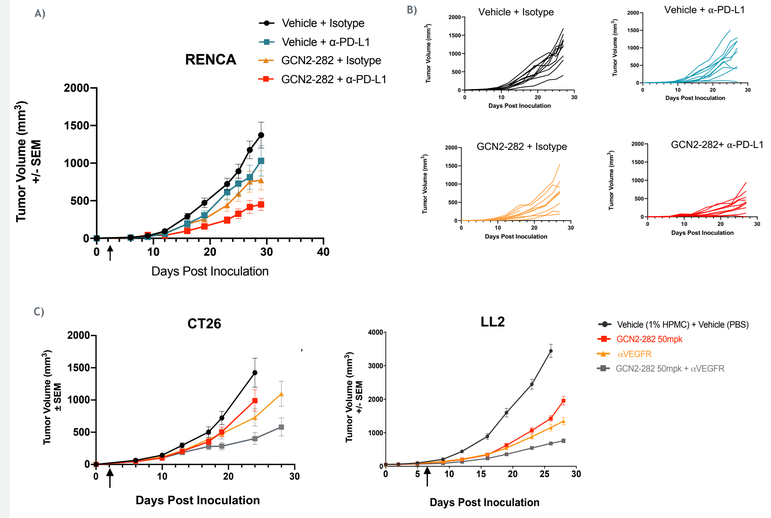
A) $CD33^+$ myeloid cells from healthy donor (isolated and expanded as previously described) were pre-starved in tryptophan-limited media and pretreated with GCN2-490 for 6hrs and then washed. Pre-starved and pretreated MDSC were co-cultured in complete media with activated $CD8^+$ T cells. B) Proliferation (n=4) and C) IFNγ (n=2) were measured by flow cytometry. Similar increases in $CD107a^+CD8^+$ cells were also observed (data not shown). D) Western blot indicating GCN2 pathway at 6 hours under low tryptophan conditioned human MDSCs in the presence or absence of GCN2-490. E) Schematic of cancer patient derived MDSC Suppression Assay. F) MDSCs from cancer patients (n=3) were collected. The Allo-MLR reaction was set up in a 1:2:4 ratio (Stimulator :Responder: Suppressor) and GCN2-490 was added to the respective wells. Cells were cultured for 4-6 days and on D6, 50 µl/well of 20 µCi/ml ^{3}H methyl thymidine media was added to the cells. Cells were harvested and the counts per minute were read using top counter. The data represents the mean ± SEM incorporated thymidine (raw c.p.m. values). *P < 0.05; **P < 0.01, ***P < 0.001, ****P < 0.0001

6. GCN2i Shows In Vivo Target Engagement and Single Agent Antitumor Response Mediated by Immune Cells



A) Schematic of in vivo target engagement with GCN2-282 in RENCA tumor model
B) KiNativ platform was used to examine GCN2 active site occupancy in tumors and spleen (n=3).
C) ATF4 levels in tumor tissues was assessed via Western blot following dosing of mice with GCN2-282 or Vehicle.
D) Mice bearing subcutaneous PANO2, LL2 and RENCA tumors began treatment on days indicated by the arrow. Mice received either vehicle or 15 mpk GCN2-282 dosed orally BID (mean ± SEM, n = 10 per group).
E) NSG Mice bearing subcutaneous RENCA tumor received either vehicle or GCN2-282 dosed orally BID; (mean ± SEM, n = 10 per group). Arrow indicates day of randomization of RENCA tumors at size 40-80 mm^3

7. GCN2i in Combination with Checkpoint and Angiogenesis Blockade Demonstrates Combinatorial Anti-Tumor Activity



A) Mice bearing subcutaneous RENCA tumors began treatment on days indicated by the arrow. Mice received 200 ug of α-PD-L1 or isotype control twice weekly and either vehicle or 15 mpk GCN2-282 dosed orally BID. Tumor volume represented as mean + SEM, n=10 mice/group and significance tested on day 30. C) Mice bearing subcutaneous CT26 and LL2 tumors began treatment on days indicated by the arrow. Mice received 40mg/kg of aVEGFR2 (orange and grey) three times weekly and either vehicle or GCN2-282 dosed orally.

8. Conclusions

- RAPT Therapeutics is developing potent and selective inhibitors of the stress response kinase GCN2
- GCN2i inhibited phosphorylation of GCN2 and EIF2α in human $CD8^+$ T cells and human MDSC cultured under amino-acid starved conditions
- GCN2i reverses immune suppression mediated by human MDSCs
- GCN2i restores proliferation and immune activation of human CD8+ and CD4+ T cells cultured under amino-acid starved conditions
- GCN2i demonstrates in vivo target engagement in RENCA tumors
- GCN2i demonstrates single-agent antitumor activity which is immune dependent
- Our orally bioavailable GCN2i demonstrates antitumor activity alone and in combination with anti-PD-L1 or anti-VEGFR
- Thus, our data collectively demonstrates that GCN2 is a promising therapeutic target for the treatment of cancer

9. References

1. Pakos-Zebrucka, Karolina et al. “The integrated stress response.” *EMBO reports* vol. 17,10 (2016)
2. Halaby, M. J., et al. "GCN2 drives macrophage and MDSC function and immunosuppression in the tumor microenvironment." Science immunology **4**(42) (2019).

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