Investigating Molecules That Confer Sensitivity to AS1411 in Lung Adenocarcinoma Cells

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Abstract

Current approaches in lung cancer treatments are often unsuccessful at curing patients, and can also result in life-altering side effects as a result of their non-specific mechanisms. AS1411, a G-rich DNA oligonucleotide previously developed by this lab, exhibits antiproliferative activity specifically against cancer cells and has shown some success in clinical trials. Previous mechanistic studies have suggested that (1) knockdown of tumor suppressor Ras association domain family 1 isoform A (RASSF1A) in lung adenocarcinoma NCI-H1792 cells increases sensitivity to AS1411, and that (2) AS1411 hinders both complexes of the mechanistic target of rapamycin (mTOR), a serine/threonine kinase, in A549 cells. Through CRISPR-Cas9 knockout, this project examines RASSF1A in NCI-H1792 as a potential predictive biomarker of AS1411 susceptibility. To better understand the manner in which AS1411 mediates cell death, this project also investigates its effect on several other mTOR downstream substrates (SOD1, 4EBP1, TFEB, SGK1, PKCα, and xCT). While the project's preliminary data shows the successful knockout of RASSF1A, the absence of the gene did not improve NCI-H1792 sensitivity to AS1411. Overall, Western blots of previously-prepared A549 whole cell lysates reveal no major difference in phosphorylation of 4EBP1, TFEB, SGK1, and PKCα or in SOD1 levels with AS1411 treatment. However, treatment with AS1411 reduced xCT. This result could potentially lead to the use of xCT inhibition, in combination with AS1411 treatment, as a more effective treatment of lung cancer than current therapies.

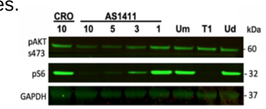


Figure 1: AS1411's Inhibition of mTOR Activity—Preliminary Data. Preliminary Western blots (from Natalia Bilchuk) reveal that AS1411 inhibits mTORC1 (pS6) and mTORC2 (pAKT) in a dose-dependent manner.

Methods

CRISPR-Cas9 Transfection by Electroporation. NCI-H1792 cells were electroporated with the Neon Transfection System. gRNAs RASSF1+503332125, RASSF1-503332148, Negative Control Scrambled #1, and Negative Control Scrambled #2 were used. Vector cells received no gRNA, and untreated cells were electroporated without Cas9 nuclease or sgRNA. Cells were plated following transfection.

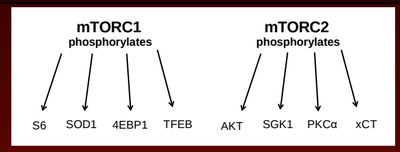
Pierce BCA Protein Assay and Western Blotting. Total protein concentrations of NCI-H1792 CRISPR lysates and A549 whole cell lysates from previous experiments were determined using the Pierce BCA Protein Assay. Samples (35 µg protein/well) were run on NuPage 4-12% Bis-Tris gels and transferred to PVDF membranes. NCI-H1792 membranes were probed for RASSF1A and GAPDH. A549 membranes were probed for SOD1, p4EBP1, pTFEB, pSGK1, pPKCα, and xCT. All blots were developed using the LI-COR infrared fluorescence system. Analysis of signal strength was performed using the LI-COR software.

MTT Cytotoxicity Assay. NCI-H1792 cells were plated 3.0x10^3/well in a 96-well plate and were treated the following day with 10, 5, 2.5, and 1.25 µM of AS1411 or CRO (negative control). Cells were then incubated for 96 hours, after which MTT was added. Cells were lysed 4 hours later. Absorbance was read at 570 nm the following day.

Objectives

Answer the following questions:

1) Does RASSF1A mediate cancer cell sensitivity to AS1411?
2) What other mTORC1/2 downstream pathways does AS1411 affect?



Results

1) Knocking out RASSF1A in NCI-H1792 did not alter AS1411 sensitivity

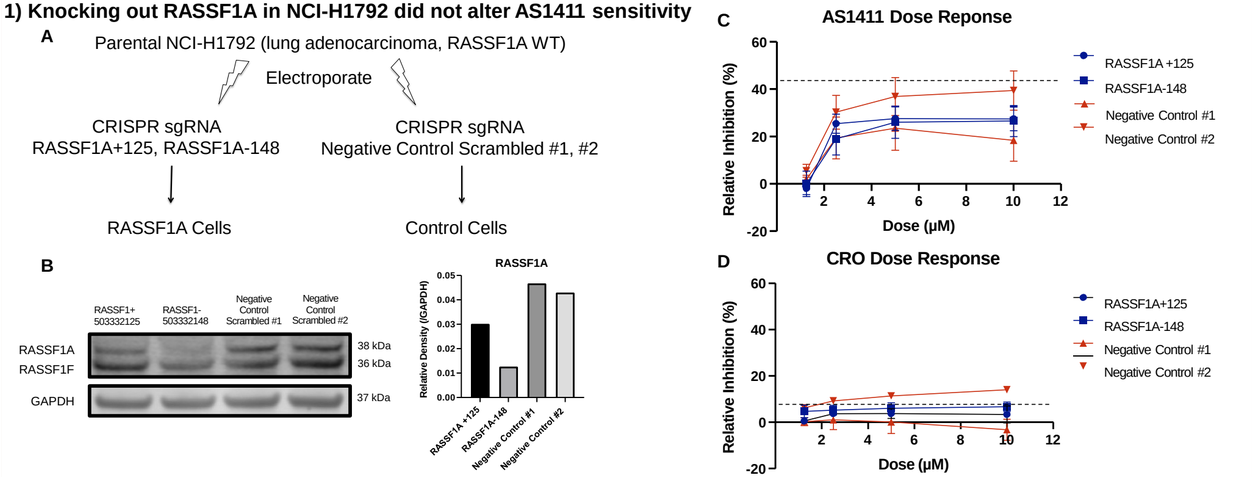


Figure 2: RASSF1A Knockout Confirmation and MTT Cytotoxicity Assay. (**A**) CRISPR cells (RASSF1A+125, RASSF1A-148, Negative Controls #1/#2, Vector, Untreated) were obtained through electroporation with Cas9 and the appropriate gRNA. (**B**) A preliminary Western blot with densitometry reveals a knockout of RASSF1A in CRISPR NCI-H1792 cells treated with RASSF1-148 and decreased RASSF1A in cells treated with RASSF1+125 compared to two negative controls. MTT analysis after treatment with 10, 5, 2.5, and 1.25 µM of AS1411 and CRO revealed that compared to negative controls, (**C**) Cells treated with RASSF1A and control gRNA respond similarly to AS1411, although compared to parental NCI-H1792s (dotted line marks maximum inhibition in parentals), are less inhibited. (**D**) There appears to be no major difference in response to CRO between CRISPR and parental NCI-H1792s cells (dotted line).

2) Not all downstream mTOR targets were affected by AS1411 treatment

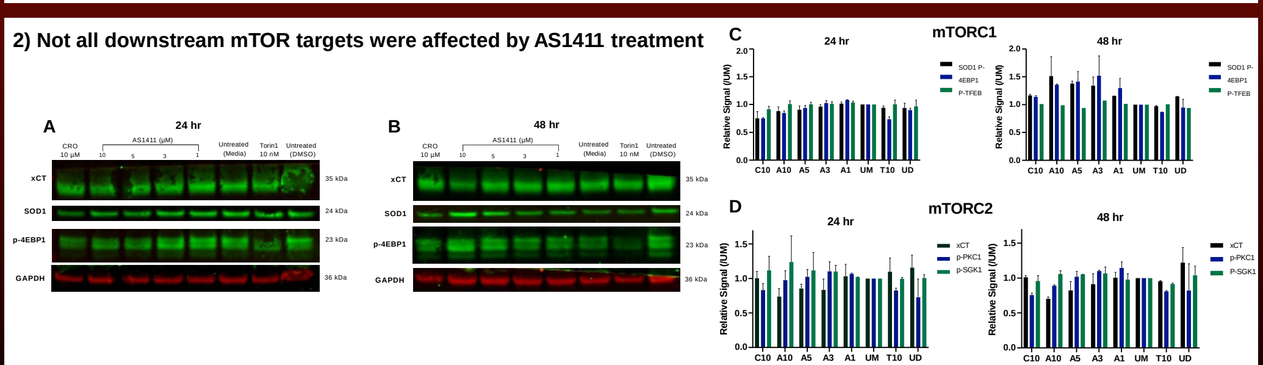


Figure 3: Western blot, A549 24 and 48-hour lysates. Lysates from a previous experiment, in which A549s were lysed 24 hours (**A**) and 48 hours (**B**) after treatment, illustrate a change in xCT, SOD1, and p4EBP1 signal according to AS1411 dose. Densitometry analysis of the examined pathways of mTORC1 (SOD1, p4EBP1, pTFEB) and mTORC2 (xCT, pPKCα, pSGK1) illustrates (**C**) no change in pTFEB over time and increased p4EBP1 and SOD1 signal with AS1411 treatment at 48 hours. (**D**) Analysis of mTORC2 pathways shows an overall decrease in xCT with AS1411 treatment at both 24 and 48 hours, and a minor increase in pSGK1 at 24 hours after AS1411 treatment. There are no major changes in pSGK1, or pPKCα levels across all treatments at 48 hours.

Conclusions

- Role of RASSF1A in Sensitivity to AS1411
  - Successful knockout of RASSF1A
  - **RASSF1A does not determine NCI-H1792 sensitivity to AS1411**
- Effect of AS1411 on mTORC1/2 Downstream Targets
  - 6 downstream effectors were analyzed
  - Some inconclusive and/or inconsistent results
  - pPKCα/βII and pTFEB do not appear to be affected by treatment with AS1411
  - **xCT levels: consistently decreased by AS1411**

Future Directions

- Confirm KO of RASSF1A with additional Western blots
- Assess impact of RASSF1A KO in additional cell lines
- Further assess dose-dependent effects of AS1411 on A549 cells through activity assays of xCT and SOD1, and quantification assay of 4EBP1
- Examine additional downstream targets of mTORC1/2

References

1. Bates PJ, Reyes-Reyes EM, Malik MT, Murphy EM, O'Toole MG, Trent JO. G-quadruplex oligonucleotide AS1411 as a cancer-targeting agent: uses and mechanisms. Biochim Biophys Acta. 2017;1861(5 Pt B):1414-1428.
2. Burbee DG, Forgacs E, Zöchbauer-Müller S, Shivakumar L, Fong K, Gao B, Randle D, Kondo M, Virmani A, Bader S, et al. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. 2001;93(9):691-699.
3. Di Cristofano A. SGK1: the dark side of PI3K signaling. Curr Top Dev Biol. 2017;123:49-71.
4. Gu Y, Albuquerque CP, Braas D, Zhang W, Villa GR, Bi J, Ikegami S, Masui K, Gini B, Yang H, et al. mTORC2 regulates amino acid metabolism in cancer by phosphorylation of the cystine-glutamate antiporter xCT. Mol Cell. 2017;67(1):128-138.
5. Hesson LB, Cooper WN, Latif F. Evaluation of the 3p21.3 tumour-suppressor gene cluster. Oncogene. 2007;26(52):7283-301.
6. Klacz J, Wierzbicki PM, Wronska A, Rybarczyk A, Stanislawowski M, Slebioda T, Olejniczak A, Matuszewki M, Kmiec Z. Decreased expression of RASSF1A tumor suppressor gene is associated with worse prognosis in clear cell renal cell carcinoma. Int J Oncol. 2016;48:55-66.
7. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell. 2012;149(2):274-293.
8. Li W, Yue F, Dai Y, Shi B, Xu G, Jiang X, Zhou X, Pfeifer GP, Liu L. Suppressor of hepatocellular carcinoma RASSF1A activates autophagy initiation and maturation. Cell Death Differ. 2019;26(8):1379-1395.
9. Napolitano G, Esposito A, Choi H, Matarese M, Benedetti V, Di Malta C, Monfregola J, Medina DL, Lippincott-Schwartz J, Ballabio A. mTOR-dependent phosphorylation controls TFEB nuclear export. Nat Commun. 2018;9:3312.
10. Rosenberg JE, Bambury RM, Van Allen EM, Drabkin HA, Lara Jr. PN, Harzstark AL, Wagle N, Figlin RA, Smith GW, Garraway LA, et al. A phase II trial of AS1411 (a novel nucleolin-targeted DNA aptamer) in metastatic renal cell carcinoma. 2014;32:178-187.
11. Wu CC, Hou S, Orr B, Kuo BR, Youn YH, Ong T, Roth F, Eberhart CG, Robinson GW, Solecki DJ, et al. mTORC1-mediated inhibition of 4EBP1 is essential for hedgehog signaling-driven translation and medulloblastoma. Dev Cell. 2017;43(6):673-688.
12. Zhitomirsky B, Yunaev A, Kreiserman R, Kaplan A, Stark M, Assaraf YG. Lysosomotropic drugs activate TFEB via lysosomal membrane fluidization and consequent inhibition of mTORC1 activity. Cell Death Dis. 2018;9(12):1191.

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