GENETIC SILENCING OF AKT INDUCES MELANOMA CELL DEATH

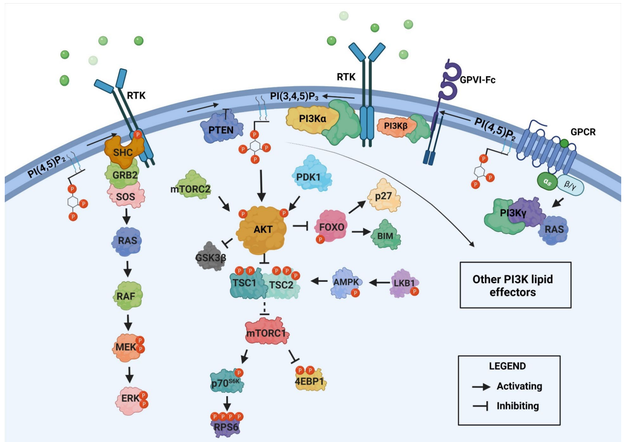
Gennie L. Parkman[1], Tursun Turapov[1], David A. Kircher[1], William J. Burnett[1], Christopher M. Stehn[1], Kayla O'Toole[1], Ryan Flaherty[1], Riley C. Elmer[1], Katie M. Culver[1], Ashley T. Chadwick[1], Karly Stanley[1], Mona Foth[1], Robert H. I. Andtbacka[1], David H. Lum[1], Robert Judson-Torres[1], Matthew W. VanBrocklin[1], John E. Friend[2], Martin McMahon[1] and Sheri L. Holmen[1]

Huntsman Cancer Institute at the University of Utah[1], Kazia Therapeutics Limited[2]

BRAF-mutant melanoma: A therapeutically challenging malignancy

- Melanoma is the 5th most common cancer for men & women in the U.S. (Cancer Facts & Figures 2022).
- Approximately 50% of all melanomas harbor an activating BRAF mutation.
- Targeted therapy options for BRAF-mutant melanoma exist, but most patients will experience primary or secondary resistance.
- The five-year survival rate of stage IV melanoma remains at 30%, highlighting the need for new therapeutics to treat this disease.

MAPK and PI3K pathway alterations co-occur and play a significant role in melanoma



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SiRNA-mediated knockdown of AKT1,2,3 leads to cell lethality

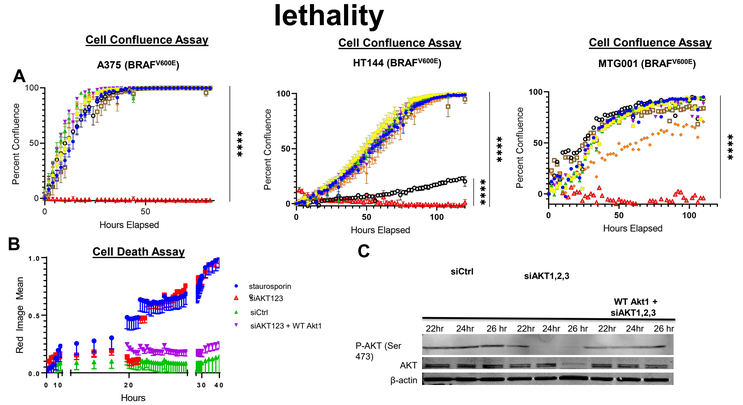

Figure 1: A, Cell confluence assay under genetic inhibition. A375, HT144, and MTG001 showed little sensitivity to individual siRNAs against AKT1, 2, and 3 and AKT1+2 or AKT2+3, while siAKT123 led to complete cell lethality in all cell lines ($p<0.0001$ in all cell lines). **B**, Cell death assay comparing cells treated with staurosporine, siCtrl, siAKT123, and siAKT123 + WT Akt1. **C**, Immunoblotting of A375 cells treated with siCtrl, siAKT123, and siAKT123 + overexpression of wildtype mouse Akt1 exhibited complete knockdown of phospho-AKT (Ser473) at 24 and 26 hours in siAKT123 vs siCtrl which was rescued by addition of mouse Akt1.

Rescue of melanoma cells from siRNA-mediated knockdown of AKT123 is dependent on Akt kinase activity and T308 phosphorylation

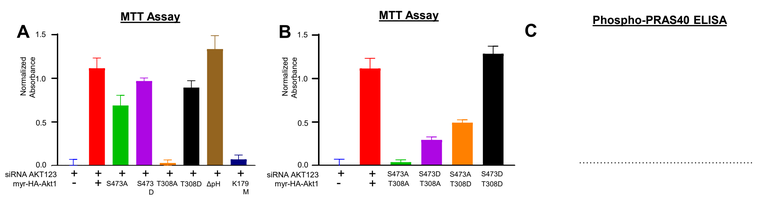

Figure 2: A-B, MTT assay of stable A375 cell lines expressing phospho and kinase mutants evaluating cell viability post 48 hours siAKT123 transfection. Cell lines expressing T308A or K179M mutations were unable to significantly rescue siAKT123 knockdown while all other cell lines expressing phospho mutations were able to rescue this phenotype. **C**, Phospho-PRAS40 kinase ELISA of A375 myrAkt1 stable cell lines expressing phospho and kinase mutants demonstrate undetectable levels of phospho-PRAS40 in T308A; S473A, T308A; S473D, T308A; and K179M cells.

SiAKT123 significantly decreases mTOR activity

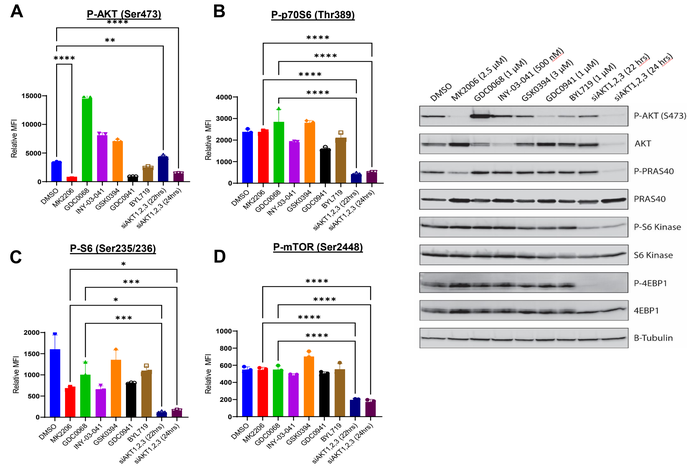

Figure 3: A-D, Luminex quantitative immunoassay of phosphorylation of AKT (Ser473), P70S6 (Thr389), P-S6 (Ser235/236A, MTG001 cells treated with DMSO control, MK2206 (2.5μM), GDC0068 (1μM), INY-03-041 (500nM), GDC0941 (1μM), BYL719 (1μM), siAKT123 at 22 hours (50nM), or siAKT123 at 24 hours (50nM). **E**, Immunoblotting of cell lysates treated with DMSO control, MK2206 (2.5μM), GDC0068 (1μM), INY-03-041 (500nM), GSK0394 (3μM), GDC0941 (1μM), BYL719 (1μM), siAKT123 (50nM) at 22 hours, and siAKT123 (50nM) at 24 hours reveals complete knockdown of pAKT (Ser473) and total AKT by siAKT123 at 24 hours, as well as knockdown of p-PRAS40, p-S6 Kinase, and p-4EBP1.

Activated SGK can rescue the lethal effect of siRNA-mediated knockdown of AKT1,2,3

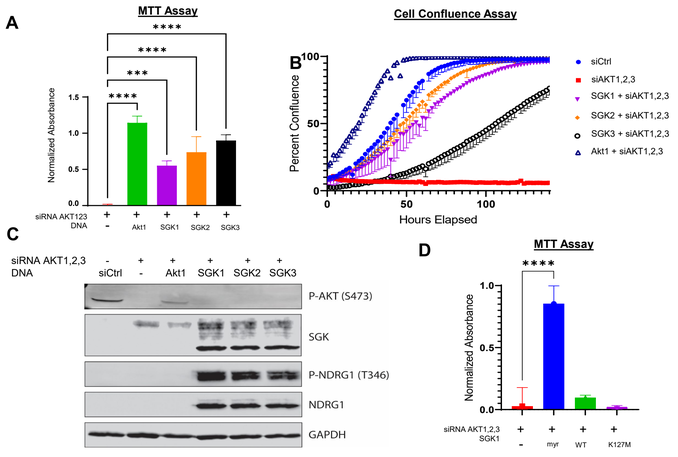

Figure 4: A, MTT assay of stable A375 cell lines expressing myrAkt1, myrSGK1, myrSGK2, or myrSGK3 evaluating cell viability post 48 hours siAKT123 transfection compared to siAKT123 alone**. B**, Cell confluence assay evaluating cell proliferation in A375, A375 myrAkt1, A375 myrSGK1, A375 myrSGK2, and A375 myrSGK3 cell lines treated with siCtrl or siAKT123. **C**, Immunoblotting of A375, A375 myrAkt1, A375 myrSGK1, A375 myrSGK2, and A375 myrSGK3 treated with siCtrl or siAKT123 demonstrating knockdown of p-AKT except for in A375 myrAkt1 cell line. **D**, MTT assay of A375 myrSGK1, A375 wt SGK1, and A375 myrSGK1-K127M cell lines evaluating cell viability post 48 hours siAKT123 transfection.

Combined inhibition of AKT and SGK decreases melanoma cell proliferation and increases overall survival

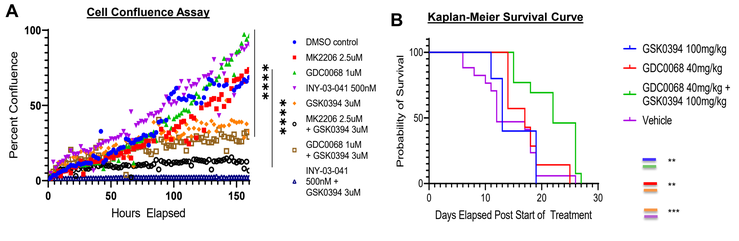

Figure 5: A, Cell confluence assay under pharmacological inhibition. HT144, MT001, and MTG004 cells were treated with DMSO control, MK2206 (2.5μM), GDC0068 (1μM), INY-03-041 (500nM), GSK0394 (3μM), MK2206 (2.5μM) + GSK0394 (3μM), GDC0068 (1μM) + GSK0394 (3μM), and INY-03-041 (500nM) + GSK0394 (3μM). **B**, Kaplan-meier survival curve of immunocompetent mice tolerized to luciferase and GFP were injected subcutaneously with YUMM 3.2 $BRAF^{V600E}$;*Cdkn2a*$^{-/-}$;*Pten*$^{-/-}$ mouse melanoma cells treated q.d. with vehicle, 100 mg/kg GSK0394, 40 mg/kg GDC0068, or the combination of GSK0394 plus GDC0068. Statistical analysis was performed using a log rank Mantel-Cox test, where **p<0.01 and ***p<0.001.

Combined inhibition of AKT and SGK suppresses mTOR signaling

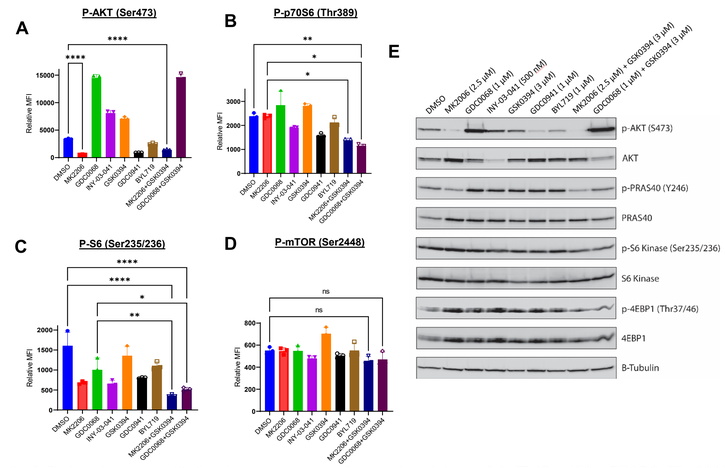

Figure 6: A-D, Luminex quantitative immunoassay of phosphorylation of AKT (Ser473), P70S6 (Thr389), P-S6 (Ser235/236A, MTG001 cells treated with DMSO control, MK2206 (2.5μM), GDC0068 (1μM), INY-03-041 (500nM), GDC0941 (1μM), BYL719 (1μM), MK2206 (2.5μM) + GSK0394 (3μM), or GDC0068 (1μM) + GSK0394 (3μM). **E**, Immunoblotting analysis of lysates treated with pharmacological inhibition and used for Luminex analysis (A-D). Decreased levels of p-PRAS40, p-S6, and p-4EBP1 with combination treatments versus single agent alone were observed.

Dual PI3K/mTOR inhibitors and third generation mTOR inhibitors block proliferation *in vitro* and increase overall survival of BRAF-mutant melanoma *in vivo*

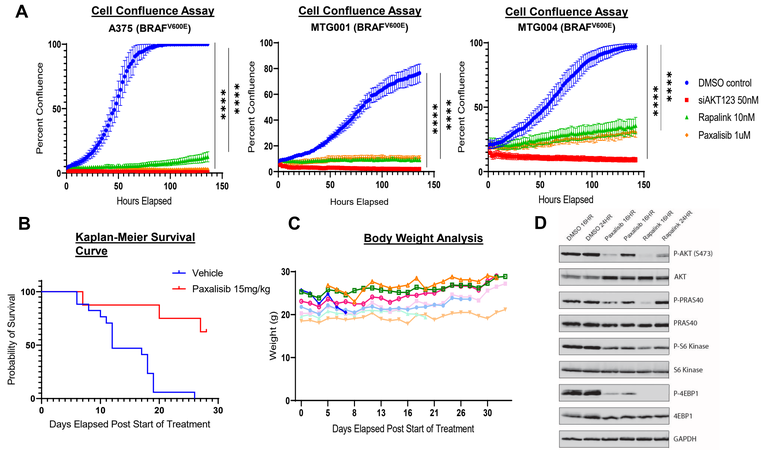

Figure 7: A, Cell confluence assay under genetic and pharmacological inhibition. A375, MT001, and MTG004 cells were treated with DMSO control, siAKT123 (50nM), Rapalink (10nM), and Paxalisib (1μM). **B**, Kaplan-meier survival curve of immunocompetent mice tolerized to luciferase and GFP injected subcutaneously with YUMM 3.2 $BRAF^{V600E}$;*Cdkn2a*$^{-/-}$;*Pten*$^{-/-}$ mouse melanoma cells and treated q.d. with vehicle or 15 mg/kg paxalisib for 28 days or until experimental endpoint. Statistical analysis was performed using a log rank Mantel-Cox test. **C**, Body weight analysis of individual mice represented by varying colors in the paxalisib *in vivo* cohort showing tolerability of drug treatment. **D**, Immunoblotting analysis of lysates treated with pharmacological inhibition and used for MTT analysis in A.

Conclusions and Future Directions

- Genetic silencing of AKT induces melanoma cell death through suppression of downstream mTOR signaling and is dependent on functional kinase activity.
- Genetic silencing is superior to pharmacological inhibition as it prevents reactivation of the PI3K>AKT pathway following relief of negative feedback.
- Activated SGK1 can rescue lethal effects of siAKT123 knockdown.
- Combination of AKT and SGK inhibition decreases melanoma cell proliferation and leads to increased overall survival in a BRAF-mutant melanoma mouse model but tumors still grow through treatment.
- Second and third generation mTOR inhibition more effectively diminishes melanoma cell viability and leads to substantially increased overall survival.
- Dual PI3K/mTOR inhibition may represent an effective therapeutic strategy in this refractory disease.
- Next steps will be to test combination of PI3K/mTOR inhibitors with standard of care BRAF/MEK targeted inhibitors.

Acknowledgements

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