**Role of kinases MAP2K1, PDHK and NEK1 on Hedgehog Signaling Activation**

**Paige Halas**

**Graduate Program in Cellular & Molecular Biosciences**

**University of California, Irvine**

**Fall 2019 Rotation Report**

This report was written by the student, has a distinct and reachable goal, and represents a reasonable amount of work for one rotation project earning 4 units of academic credit. The student will learn new techniques and approaches consistent with the goals of the rotation program.

Rotation Supervisor’s Name: Dr. Scott Atwood

 Signed\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_  Date \_\_\_\_\_\_\_\_\_\_

Faculty Advisor’s Name: Dr. Eric Pearlman

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**Introduction**

Basal Cell Carcinoma (BCC) is the most abundant skin cancer in the US with 4 million diagnoses a year 1. Evidence supports that this cancer depends on uncontrolled activation of the Hedgehog (Hh) Signaling Pathway. The Hedgehog Signaling Pathway becomes active when a Hedgehog Signaling ligand binds to the Patched1 (PTCH1) receptor on the primary cilia of a cell, thus activating the signal transducer Smoothened (SMO). SMO then moves to the cilium where it binds and inhibits Suppressor of Fused (SUFU), resulting in transcription factor activation of Glioma-Associated Oncogene (GLI) 2. Mutations that occur in regulators of the Hh Signaling Pathway such as PTCH1, SMO and GLI cause uncontrolled activation of the Hh pathway leading to the development of cancers such as BCC 3. Small molecule drugs that inhibit SMO, such as Vismodegib, have been used to modulate the Hedgehog Signaling pathway by preventing release of GLI transcription factors to treat BCC. It has been found that BCC tumors can acquire resistance to SMO inhibitors, thus causing a loss of SMO inhibitor Sufu or the increase of transcription factors in the system 4,5. Additionally, mutations in SMO have caused the drug to be ineffective in suppressing SMO and thus allowing the pathway to proceed as normal 5. BCC resistance and mutations of SMO have directed attention to GLI transcription factors downstream of SMO as targets for BCC treatment. It has been previously found that multi-site phosphorylation regulates the conversion of GLI proteins into transcriptional activators and kinases activate the response in Hedgehog Signaling activity and tumor growth 6. The purpose of this project is to assess Hedgehog Signaling Pathway activation by inhibiting kinases predicted to be active at recurrent GLI mutation sites.

Mitogen-Activated Protein Kinase 2 (MAP2K1) is active at re-current GLI mutations sites determined experimentally to activate Hh Signaling. Mutations in MAP2K1 are correlated with melanoma and inhibiting this kinase reduces metastasis in pancreatic cancer 7–9. Additionally, MAP2K1 stabilizes GLI and activates Hedgehog Signaling 9. Cobimetinib is effective as a MAP2K1 inhibitor used to treat melanoma with an IC50 of 4.2 nM 10. Pyruvate Dehydrogenase Kinase (PDHK) regulates glycolysis which is more highly activated in cancer cells than normal cells 11. A PDHK inhibitor, Dichloroacetic acid (IC50, 81.03 mM), upregulates apoptosis of ovarian cancer cells 12. The prediction of PDHK activity on re-current GLI mutation sites, makes it of interest in assessing its inhibition in Basal Cell Carcinoma. NIMA Related Kinase (NEK1) affects Hh signaling through its role in primary cilia formation and has demonstrated activity in cancer 13,14. Zinc05007751 is a novel selective NEK1 inhibitor with an IC50 of 3.4 μM. Evidence suggests that inhibition of NEK1 would reduce GLI activation and Hedgehog Signaling. As MAP2K1, PDHK and NEK1 kinases are predicted to be active at recurrent mutation sites in GLI, their inhibition is predicted to influence transcriptional activity and activate Hedgehog Signaling.

**Hypothesis**

We hypothesize that MAP2K1, PDHK and NEK1 kinases increase Hedgehog Signaling activity due to their predicted activity at recurrent GLI mutation sites and insight to their role in Hedgehog Signaling or cancer.

**Materials and Methods**

**Kinase Prediction**

A compilation of GLI1 and GLI2 mutations that occur three or more times in cancer cells in the Catalogue of Somatic Mutations in Cancer (COSMIC) database have been generated and previously screened for activation of the Hedgehog Signaling Pathway in the Atwood Lab. Using Group-Based Prediction Software (GPS) and PhosphoNet Kinase Predictor, kinases which are predicted to be active at phosphosites four positions up or downstream from a recurring mutation side are compiled. Kinase candidates having previous evidence of mediating phosphorylation and activating Hedgehog Signaling at a mutation site were eliminated from the compilation. Previous insight into their activity in Hedgehog Signaling or cancer allowed for the selection of MAP2K1, PDHK and NEK1 to evaluate Hedgehog Signaling effects due to kinase activity experimentally.

**Cell Dosing, RNA Extraction, RT-qPCR**

Cobimetinib, Dichloroacetic Acid, and Zinc05007751 are selected as inhibitors for MAP2K1, PDHK and NEK1 due to their availability, specificity and IC50 range. To promote the generation of primary cilia and Hedgehog Pathway activation, 3T3 (Wild Type) cells are serum starved and serum starved with Hedgehog conditioned media immediately before treatment with these inhibitors. Inhibitors are added at their IC50s and serially increased on a logarithmic scale to three higher concentrations. Similarly, Basal Cell Carcinoma Cells are serum starved and treated with inhibitors as in Wild Type cells. These cell types and serum conditions are all treated with diluent only (DMSO) as a control. After 24 hours of incubation, RNA is extracted and quantified from both diluent and drug treated cells using the Zymo Research Direct-Zol RNA Miniprep Plus kit. Extracted RNA is amplified using RT-qPCR with primers specific for the GLI1 gene as a means of quantifying Hedgehog Signaling activation, as well as a GAPDH experimental control to quantify GLI expression.

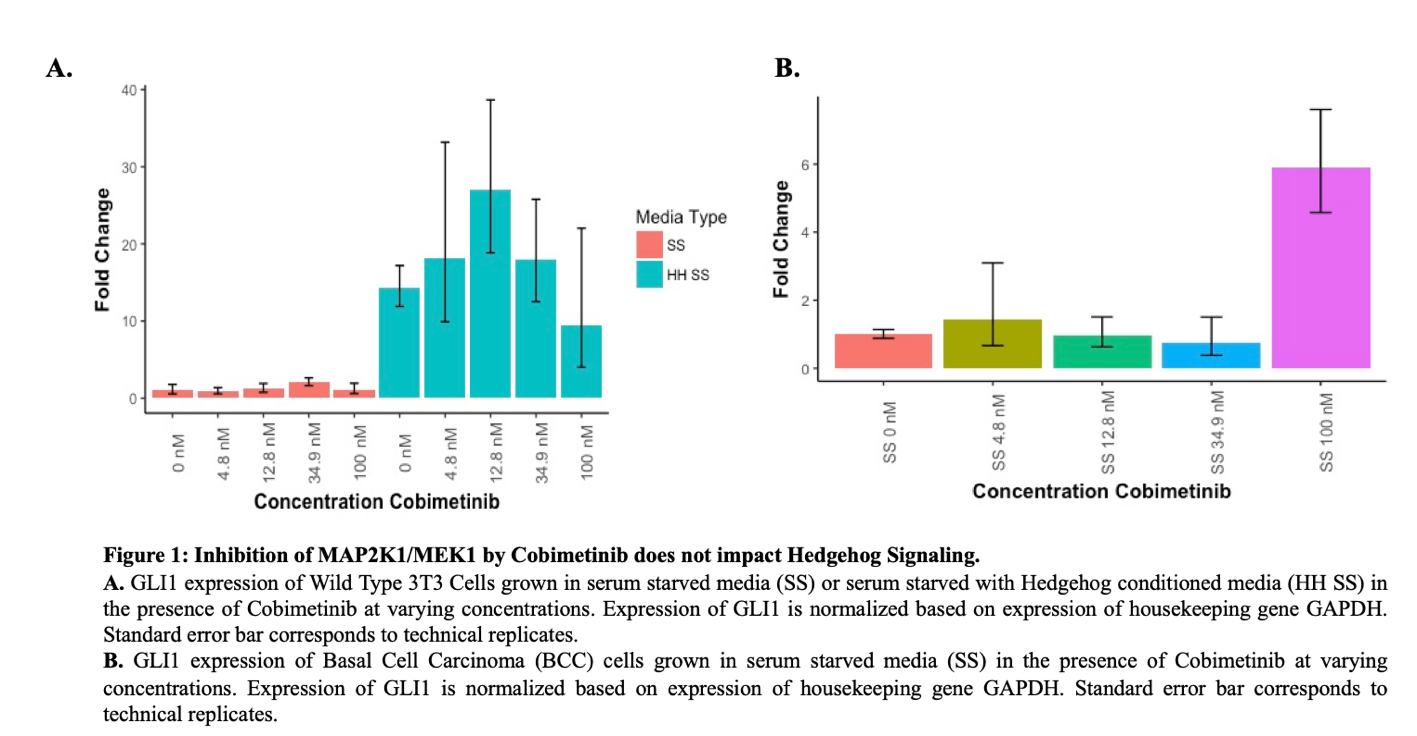
**Metabolic Activity (MTT) Assay**

To confirm that kinase inhibitors would not cause cell death *in vivo*, an MTT Assay is conducted on diluent (DMSO) and drug treated BCC cells. Cells are seeded at 2,000 cells/well and grown to 50% confluence. Once desired confluence is achieved, kinase inhibitors (Cobimetinib, Dichloroacetic Acid, and Zinc05007751) are added at their IC50 and three logarithmically increased concentrations to the cells in replicates of six to allow for variability. When performing the assay, MTT reagent in PBS is added to the each well and allowed to incubate at 36°C for two hours protected from light. After incubation, the MTT reagent is removed and MTT solvent (DMSO) is added to the wells and allowed to shake. Cell proliferation is quantified by measuring the absorbance at 570 nm for BCC cells at 0, 2, 4 and 6 days post drug addition.

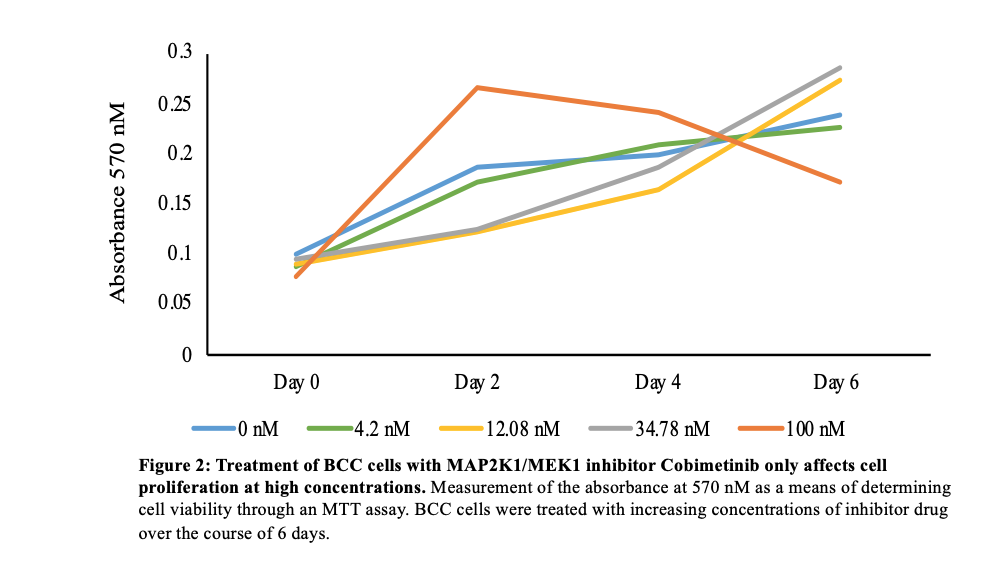
**Results**

**Inhibition of MAP2K1/MEK1 does not impact Activation of Hedgehog Signaling**

Previous evidence of the role of MAP2K1 in cancer and its ability to activate Hedgehog Signaling brought the hypothesis that inhibiting this kinase with Cobimetinib would decrease Hedgehog Signaling activity. GLI1 expression, indicative of Hedgehog Signaling, is not affected in the presence of MAP2K1/MEK1 Cobimetinib kinase inhibitor at the concentrations tested (**Figure 1**). Increased expression of GLI1 is observed for Wild Type (3T3) cells under serum starved with Hedgehog conditioned media (HH SS) in comparison to serum starved media as expected. However, fold change does not vary between Cobimetinib concentrations of SS or HH SS treated 3T3 cells (**Figure 1A**). This observation is consistent in the Basal Cell Carcinoma (BCC) cell line with the exception of the 100 nM concentration of Cobimetinib (**Figure 1B**).

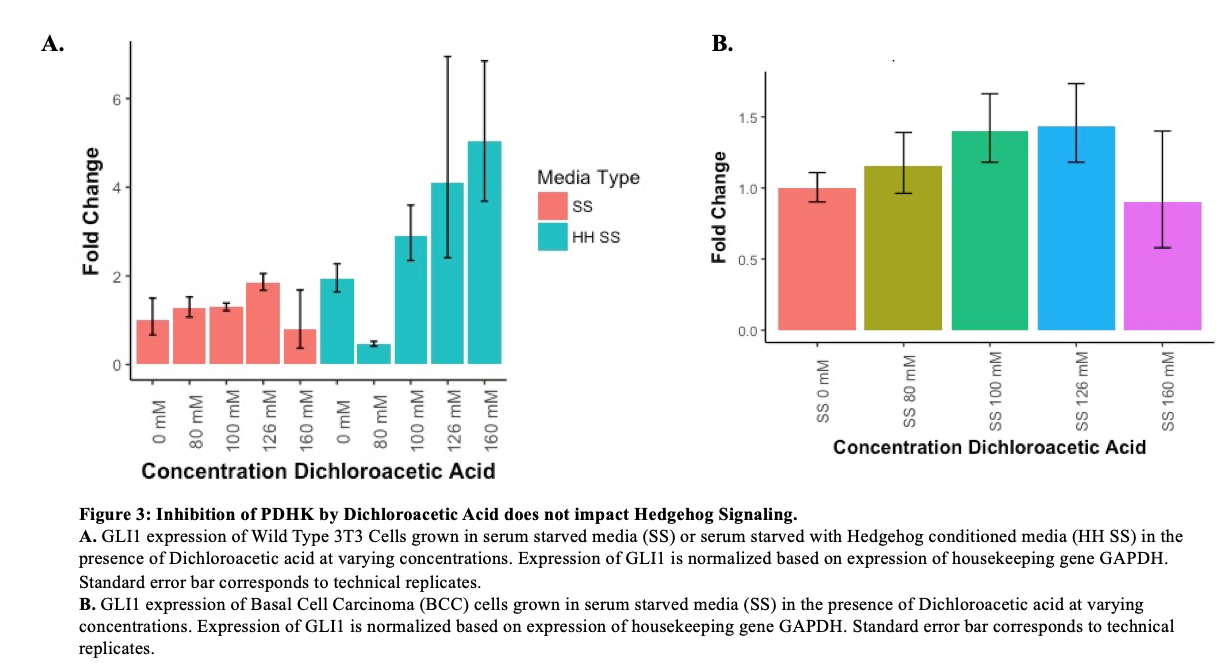


In assessing the cell viability in the BCC cell line, as the concentration of Cobimetinib increases, the drug cytotoxicity is consistent over a period of 6 days with the exception of the highest concentration 100 nM (**Figure 2**).

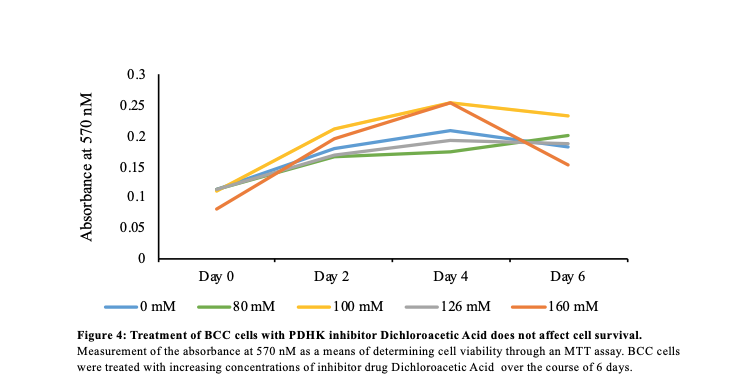


**Hedgehog Signaling activity is not impacted by PDHK Inhibition with Dichloroacetic Acid**

Increased activity of PDHK in cancer cells suggests that inhibition with Dichloroacetic acid could reduce Hedgehog Signaling. Hedgehog signaling activity measured through GLI1 expression is not affected in the presence of Dichloroacetic acid at the concentrations tested (**Figure 3**). Overall, fold change of GLI in the HH SS Wild Type cells is higher than in the SS Wild Type cells as expected. Between concentrations of Dichloroacetic Acid, GLI expression levels are within the standard deviation in the SS condition. In the case of the HH SS treated Wild Type cells, GLI expression is only lower at the IC50 of 80 nM Dichloroacetic Acid (**Figure 3A**). In BCC cells treated with Serum Starved (SS) Media, GLI1 expression is within the standard error bars for all concentrations of inhibitor (**Figure 3B**).

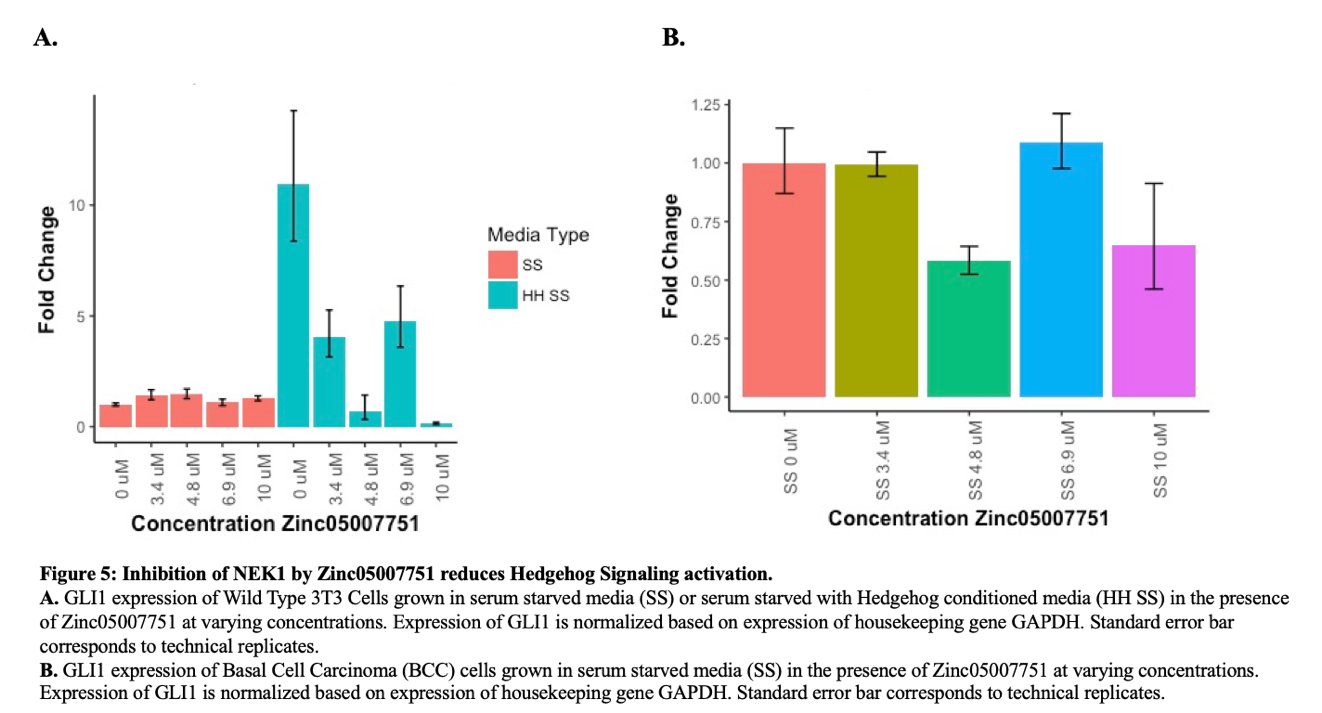


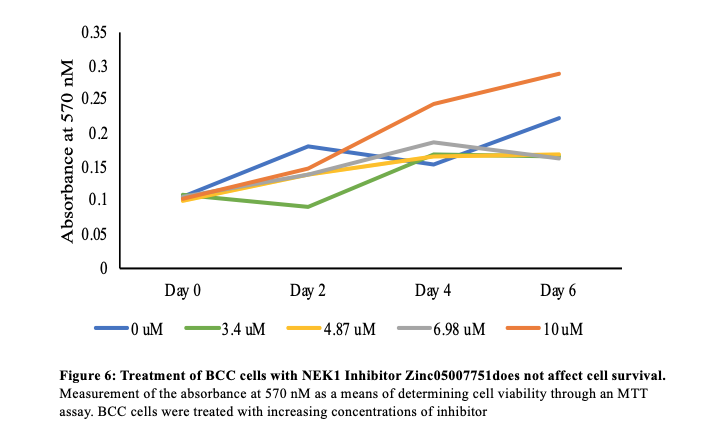
To assess *in vivo* toxicity of the drug, an MTT assay was conducted where absorbance is consistent for all concentrations of Dichloroacetic acid inhibitor over a period of 6 days in the (**Figure 4**).



**NEK1 Demonstrates Activation of Hedgehog Signaling**

The effect of NEK1 on cilia regulation and its correlation in cancer suggests inhibition of NEK1 with Zinc05007751 likely affects activation of Hedgehog Signaling.





**Discussion**

qPCR Prediction

If the presence of selected kinases are responsible for the activation of Hedgehog Signaling, decreased mRNA levels for GLI1 in BCC cell lines are expected in a dose response manner in the presence of kinase inhibitor as compared to diluent treated BCC cells.

MTT prediction

Using a plate reader to measure absorbance, we would expect that if the kinase inhibitor drug is affecting the Hedgehog Signaling Pathway, BCC cell proliferation will be reduced over time as compared to diluent treated BCC cells. An observation of a dose dependent reduction of GLI1 mRNA when treated with kinase inhibitor and a reduction of cell proliferation between diluent and drug treated BCC cells will provide evidence that the predicted kinases could be responsible for activating the Hedgehog Signaling Pathway

**Works Cited**