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

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**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.

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

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**Introduction**

Basal Cell Carcinoma (BCC) is the most diagnosed skin cancer in the US with 4 million diagnosis a year (Citation). 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) (Varjosalo and Taipale, 2007). 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 (Atwood *et al*., 2012). Small molecule drugs that inhibit SMO have been used to modulate the Hedgehog Signaling pathway and treat BCC. It has been found that BCC tumors can acquire resistance to these SMO inhibitor drugs, which motivates investigation of recurrent mutations of the GLI1 transcription factor, located downstream of SMO (Pricl *et al*., 2015, Kasper *et al*., 2006). 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 (Niewiadomski *et al*., 2014).

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 (Homisko *et al*., 2019, Gu *et a*l., 2018). Additionally, MAP2K1 stabilizes GLI and activates Hedgehog Signaling (Liu *et al*., 2014). Cobimetinib is effective as a MAP2K1 inhibitor used to treat melanoma with an IC50 of 4.2 nM (Rowdo *et al*., 2016). Pyruvate Dehydrogenase Kinase (PDHK) regulates glycolysis which is more highly activated in cancer cells than normal cells (Golias *et al*., 2018). A PDHK inhibitor, Dichloroacetic acid (IC50, 81.03 mM), upregulates apoptosis of ovarian cancer cells (Zhou *et al*., 2018). 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 ciliogenesis and is correlated to cancer (Melo-Hanchuk *et al*., 2017, Evangelista *et al*., 2008). 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. The purpose of this project is to investigate the role of these kinases in the activation of 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 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.

**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 before or after 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 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.

**Proliferation Assay**

To confirm that kinase inhibitors are not cytotoxic in general, 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 Zinc 05007751) 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 for 10 minutes. 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**

**MAP2K/MEK1 Inhibition does not impact Activation of Hedgehog Signaling.**

Previous evidence of the role of MAP2K1 in cancer and its ability to activate Hedgehog Signaling suggests that inhibiting this kinase with Cobimetinib would decrease Hedgehog Signaling activity. GLI1 expression, indicative of Hedgehog Signaling, is not affected in the presence of kinase inhibitor at the concentrations tested (**Figure 1**). In Wild Type (3T3) cells, fold change does not vary between cells under serum starved (SS) and serum starved with Hedgehog (HH SS) conditioned media regardless of Cobimetinib concentration (**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**).

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**Figure 1: Inhibition of MAP2K1/MEK1 by Cobimetinib does not impact Hedgehog Signaling.**

**A.** GLI1 expression of Wild Type 3T3 Cells grown in serum starved media (SS) or serum starved with Hedgehog conditioned media (HH SS) in the presence of Cobimetinib at varying concentrations. Expression of GLI1 is normalized based on expression of housekeeping gene GAPDH. Standard error bar corresponds to technical replicates. **B.** GLI1 expression of Basal Cell Carcinma (BCC) cells grown in serum starved media (SS) in the presence of Cobimetinib at varying concentrations. Expression of GLI1 is normalized based on expression of housekeeping gene GAPDH. Standard error bar corresponds to technical replicates.



**Figure 2: Treatment of BCC cells with MAP2K1/MEK1 inhibitor Cobimetinib affects cell proliferation at high concentrations.** Measurement of the absorbance at 570 nM as a means of determining cell viability through an MTT assay. BCC cells were treated with increasing concentrations of inhibitor drug over the course of 6 days.

**PDHK Inhibition by Dichloroacetic Acid does not activate Hedgehog Signaling**

Increased activity of PDHK in cancer cells suggests that inhibition with Dichloroacetic acid could reduce Hedgehog Signaling if PDHK is plays a role in the signaling pathway. Hedgehog signaling activity measured through GLI1 expression is not affected in the presence of Dichloroacetic acid at the concentrations tested (**Figure 3**). Fold change only varies at the highest concentration of serum starved Wild Type cells. In the Hedgehog conditioned serum starved media, there is a large increase of GLI1 expression at concentrations higher than the IC50 of 80 nM Dichloroacetic Acid. In BCC cells, GLI1 expression is within the standard error bars for all concentrations of inhibitor. To assess overall toxicity of the drug, an MTT assay was conducted where cytotoxicity is consistent over a period of 6 days in the BCC cell line (**Figure 4**).

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**Figure 3: Inhibition of PDHK by Dichloroacetic Acid does not impact Hedgehog Signaling.**

**A.** GLI1 expression of Wild Type 3T3 Cells grown in serum starved media (SS) or serum starved with Hedgehog conditioned media (HH SS) in the presence of Dichloroacetic acid at varying concentrations. Expression of GLI1 is normalized based on expression of housekeeping gene GAPDH. Standard error bar corresponds to technical replicates. **B.** GLI1 expression of Basal Cell Carcinma (BCC) cells grown in serum starved media (SS) in the presence of Dichloroacetic acid at varying concentrations. Expression of GLI1 is normalized based on expression of housekeeping gene GAPDH. Standard error bar corresponds to technical replicates.



**Figure 4: Treatment of BCC cells with PDHK inhibitor Dichloroacetic Acid does not affect cell survival.** Measurement of the absorbance at 570 nM as a means of determining cell viability through an MTT assay. BCC cells were treated with increasing concentrations of inhibitor drug over the course of 6 days.

**NEK**

The effect of NEK1 on ciliogenesis and its correlation in cancer make its inhibition by Zinc05007751 likely to affect activation of Hedgehog Signaling. NEED DATA HERE



**Figure 6: Treatment of BCC cells with NEK1 Inhibitor Zinc05007751does not affect cell survival.** Measurement of the absorbance at 570 nM as a means of determining cell viability through an MTT assay. BCC cells were treated with increasing concentrations of inhibitor drug over the course of 6 days.

**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

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