Effects of Pak Kinase Inhibition on Emergence of Vemurafenib Resistance in Melanoma

Brooke Jennings

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EFFECTS OF PAK KINASE INHIBITION ON EMERGENCE OF VEMURAFENIB RESISTANCE IN MELANOMA

by

Brooke Jennings

A thesis submitted in partial fulfillment of the requirements for graduation with Honors in the Biology

Christopher Stipp
Thesis Mentor

Spring 2018

All requirements for graduation with Honors in the Biology have been completed.

Lori C. Adams
Biology Honors Advisor

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A thesis submitted in partial fulfillment of the requirements for graduation with Honors in the Department of Biology

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All requirements for graduation with Honors in the Department of Biology have been completed.

Lori C. Adams, PhD.
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Abstract

Melanoma is considered the most dangerous form of skin cancer, and the number of cases of melanoma per year has doubled in 30 years. Mutations in the B-RAF gene account for approximately 50% of genetic driver mutations in skin melanoma. Patients with B-RAF mutant melanoma are typically treated with a chemotherapy drug called vemurafenib. However, patients can stop responding to this treatment in as little as six months. The main purpose of this study was to determine if inhibiting PAK, a kinase-signaling molecule, in combination with vemurafenib could prevent the treatment-resistance in melanoma. PAK signaling plays an important role in the Hippo tumor suppressor pathway, which is important in cell proliferation and growth. In order to evaluate the effects of PAK kinase inhibition, we utilized a drug called FRAX-486, which is a type of PAK kinase inhibitor in combination with vemurafenib to treat melanoma cells in cell growth experiments. The main findings suggest that PAK inhibition with vemurafenib prevents cell proliferation in the cancer cells. Therefore, these results support the conclusion that PAK kinase inhibition in combination with vemurafenib can prevent emergence of treatment resistant melanoma. This new combination treatment could have the potential to prevent or delay the emergence of treatment-resistant melanoma and prolong the patient’s life.
Acknowledgements

First and foremost, I would like to give my sincerest thanks to my thesis mentor, Dr. Christopher Stipp, for his patience during training, immense knowledge, and enthusiasm for my project. His support was essential for the successful completion of my project, and expansion of my knowledge on not only metastasis and cellular pathways, but also in research as a whole. I would also like to thank Afshin Varzavand, for always being patient while I asked him questions for the second, third, or fourth time. I would also like to express my gratitude for him taking the time to train me on the various protocols. I would like to thank all the lab members as a whole for providing me with an extremely welcoming atmosphere in which to grow and learn. Finally, I would like to thank the biology department and biology honors advisor, Dr. Lori Adams, for their support, advice, and encouragement through the entire process.
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Introduction

Melanoma is a type of cancer arising from melanocytes, the cell type that produces the pigment in skin. Melanoma is considered the most dangerous form of skin cancer (MacGill, 2018). The number of cases of melanoma per year has doubled from 1982 to 2011. In 2011, in the United States alone, there were over 65,000 cases of melanoma, and over 9,000 deaths caused by melanoma (Guy et al., 2015). Mutations in the B-RAF gene account for approximately 50% of genetic mutations in melanoma, and have been found in about 7-15% of cancers (Dhomen & Marais, 2007) (Curtin et al., 2005). Inhibition of the B-RAF kinase is a common target for treatment of melanoma because it is a commonly seen mutation found in the oncogenic cells. Vemurafenib is used to treat metastatic melanoma containing the B-RAFV600 mutations.

Vemurafenib is an orally administered drug that inhibits the oncogenic B-RAF kinase. There are 3 primary highly conserved RAF serine-threonine protein kinase genes (A-RAF, B-RAF, C-RAF) that function in the RAS-RAF-MEK-ERK pathway. The RAS-RAF-MEK-ERK pathway is a kinase cascade that is responsible for transmitting signals from extracellular growth factors, which play a role in many processes such as cell cycle progression, differentiation, survival, and migration (Molina Jr., 2006).

This treatment is effective, with a report of a response rate as high as 84% in B-RAFV600 melanoma treated with the B-RAF-targeted therapy (Chapman, 2011). However, the cancer cells can develop resistance to vemurafenib in as short as 6 months (Chapman, 2011). The resistant cells display an increase in actin stress fiber formation, indicating resistance involves actin cytoskeletal remodeling (Kim, et al., 2016). Monomeric GTPases regulate cytoskeletal rearrangements. PAK kinase signaling downstream of Rac1 (GTPase) results in increased actin polymerization. PAK kinase also has the potential to activate YAP and TAZ, proto-oncogenic
transcriptional co-activators. B-RAF inhibitor resistant cells are shown to have an increase in actin stress fiber formation, and increased accumulation of YAP and TAZ in the nucleus. Overexpression of constitutively active YAP has also shown to cause resistance (Kim, et al., 2016). It has been concluded that YAP/TAZ activation in cancer cells is oncogenic and stimulates resistance to anti-cancer drug therapies (Kim et al., 2016). PAK is a possible contributor to the emergence of vemurafenib resistance because of its potential to activate YAP and TAZ.

PAK, also known as p21-activated kinase, is a member of a family of enzymes that serve as targets for small GTP binding proteins. PAK is a possible contributor to vemurafenib resistance because of its potential to activate YAP and TAZ, the proto-oncogenic transcriptional co-activators that are the targets of the Hippo tumor suppressor pathway. Typically, this signaling pathway plays key roles in the biological processes of organ size control, cell proliferation and apoptosis, stem cell homeostasis, and cellular differentiation (Kim et al., 2016).

PAK signaling downstream of Rac1 is a possible activator of YAP and TAZ (Fig 1). Rac1 stimulates the PAK kinase proteins, which in turn activates LIMK. LIMK is an actin-binding kinase that subsequently phosphorylates and inactivates the actin-severing protein cofilin, which in turn favors actin polymerization and F-actin accumulation. Subsequently, F-actin is binding AMOT, dislodging YAP, which then goes to the nucleus to initiation gene expression leading to abnormal cell growth (Feng, 2014). This potential pathway could explain the upregulation of YAP and TAZ as well as actin remodeling seen in resistant cells (Fig 1).

It is known that YAP/TAZ activation of the Hippo pathway and actin remodeling result in emergence of treatment-resistant melanoma, however the role of upstream signaling by PAK kinases in resistance remains largely unexplored (Kim et al., 2016). Successful long term
outcomes of the research presented here may include the ability to shut down this pathway through a PAK kinase inhibitor to prevent the development of vemurafenib-resistant cells. Here we report the effects of PAK kinase inhibition on emergence of vemurafenib resistance and on some of the signaling proteins that lie downstream of PAK that might contribute to drug resistance.

**Figure 1:** Schematic representation of Hippo-dependent and Hippo-independent pathways resulting in YAP activation (Feng, 2014).
In order to evaluate the effects of PAK kinase inhibition, we utilized a drug called FRAX-486, which is a type of PAK (p21-activated kinase 1) kinase inhibitor (Chow et al., 2012). To test the effects of PAK kinase inhibition on vemurafenib resistance, we completed a population doubling versus time experiment. Cells were treated with four different conditions: a negative control, vemurafenib alone, FRAX-486 alone, and a combination of FRAX-486 and vemurafenib. The cell populations’ doubling over time were examined to determine how the conditions affected cell growth and proliferation. Western blots were completed with to examine the effect of FRAX-486 treatment on signaling proteins in the treated cells. Proteins associated with the predicted path of resistance, as well as up and downstream components of PAK kinase signaling were examined. Overall based on the in vitro studies, PAK inhibition seems to be an ideal potential target for preventing treatment-resistance in patients. In future research, the PAK inhibition coupled with vemurafenib should be tested with small animal trials to evaluate results and possible side effects in an in vivo model.
Materials and Methods

Reagents

Vemurafenib was purchased from LC Laboratories, and FRAX-486 was purchased from Selleckchem. Vemurafenib was used at 3 μM and FRAX-486 was used at 50 nM. These were each dissolved in dimethyl sulfoxide.

Cell Culture

A375 cells were purchased from ATCC. Prior to this study, the cells had been transduced with firefly luciferase to enable bioluminescence imaging of viable cells. Growth media was prepared with DMEM 1X media, 50 mL 10% Fetal Bovine Serum, 5 mL Penicillin (100 U/mL) /Streptomycin (100 μg/mL) solution, 5 mL MEM Non-Essential Amino Acids, and 2 mL L-glutamine. Cells were plated at 300,000 cells in a six well plate to begin the experiment. The cells were grown under four primary conditions: a negative control (no drug treatment), vemurafenib alone, FRAX-486 alone, and vemurafenib and FRAX-486 in combination.

Drug Resistance Assay

Growth media was replaced every 4 days. Cells were harvested, counted, and replated as tolerated during the growth experiment. Cell culture was terminated when treated cells either died out or developed resistance to the drug treatment. Cell populations were deemed resistant once they had achieved 10 population doublings under continuous drug treatment. The data was subsequently graphed and analyzed by GraphPad Prism software.
Cell viability assay

For FRAX-486 dose–response analysis, luciferase-expressing cells were treated with variable doses of FRAX-486 in a 96-well plate and incubated for 24 h after plating. For each condition (0 nM, 50 nM, 100 nM, 200 nM, 500 nM, 1000 nM, 5000 nM, 10,000 nM) six wells were plated with 5,000 cells each. A bioluminescence imager quantified viable cells. Data was analyzed by using GraphPad Prism software.

Antibodies

Primary antibodies used for western blot experiments are shown in Table 1 below.

Table 1: Antibodies utilized in Western Blot Experiments

<table>
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<tr>
<th>Name</th>
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<th>Purchasing Company</th>
<th>Catalog number</th>
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<tr>
<td>AKT</td>
<td>1000X</td>
<td>Mouse</td>
<td>BD Transduction</td>
<td>610860</td>
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<tr>
<td>Phospho-AKT</td>
<td>2000X</td>
<td>Rabbit</td>
<td>Cell Signaling Technology</td>
<td>4060S</td>
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<tr>
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<tr>
<td>Phospho-MEK</td>
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<td>Rabbit</td>
<td>Cell Signaling Technology</td>
<td>98105S</td>
</tr>
<tr>
<td>PAK 1/2/3</td>
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<td>Rabbit</td>
<td>Cell Signaling Technology</td>
<td>2604</td>
</tr>
<tr>
<td>Tubulin</td>
<td>1000X</td>
<td>Rabbit</td>
<td>DSHB</td>
<td>12G10</td>
</tr>
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</table>

Secondary antibodies were goat HRP-conjugated anti-mouse IgG and anti-rabbit IgG purchased from Jackson ImmunoResearch.
Western Blot

To prepare protein lysates, cells were seeded in 60 mm dishes and treated and grown for the indicated time points at 37°C in full growth media. Cells were transferred to ice, washed with ice-cold HBSM buffer (20 mM Hepes, pH 7, 150 mM NaCl, 2 mM CaCl2, 5 mM MgCl2 dissolved in H2O), and lysed by Triton X-100 lysis buffer with 2 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration of each lysate was measured using a Red 660 assay kit (G-Biosciences). Lysates were loaded at equal protein amounts of 20 µg per lane into a 9% SDS page gel, and subsequently transferred to a nitrocellulose membrane. After blocking with Aquablock (EastCoast Bio), membranes were incubated with primary antibodies diluted 1:1,000 in Aquablock with 0.1% Tween 20. The primary antibody was stained overnight in at 4°C. The secondary antibody was diluted 1:1,000 in Aquablock with 1% Tween 20, and stained at room temperature for 1 hour. Membranes were scanned on an Odyssey CLx scanner (Licor) with 700 and 800 nm channels set to membrane and normal or low resolution. Protein levels were quantified with the Image Studio software, by utilizing the built-in quantification tool that corrects for background noise.
Results

PAK Inhibition Prevents Resistance in Melanoma

In order to examine the effects of the PAK kinase inhibitor FRAX-486 on cell viability we completed a dose response curve with the following concentrations (in nM): 0, 50, 100, 200, 500, 1000, 5000, 10000 (Fig 2.A). This graph fits the expected logarithmic form. Concentrations towards the right end of the graph indicate highly toxic amounts of the drug that result in cell death. To limit cytotoxicity, 50 nM, a concentration similar to the IC$\textsubscript{20}$ as calculated from the dose-response curve, was chosen for subsequent assays. This concentration was chosen with the expectation that there would be little to no effect alone, but could be successful when used in combination with vemurafenib.

Subsequent cell culture assays were completed in two independent experiments in order to examine the effects of vemurafenib and FRAX-486 in combination, in comparison to a DMSO vehicle control, FRAX-486 alone, and vemurafenib alone. The second experiment included 3 biological replicates run in parallel. The cumulative population doubling is plotted on the y-axis, while time in days is plotted on the x-axis. In both experiments, cells under vemurafenib + FRAX treatment died out around days 55-60 (Fig 2.B & 2.C). The control treatment (DMSO) and FRAX alone reached 10 population doublings in as little as less than 15 days (Fig 2.B). The vemurafenib treated cells reached 10 population doublings at an average of 69 days, while increased growth can be noted on average around 35 days indicating vemurafenib treatment alone did not prevent development of drug-resistance in the cells.

Short-Term Biochemical Assay Shows Inconclusive Results of VEM+FRAX Treatment

In order to assess the potentially involved proteins and pathways for blockage of resistance by PAK kinase inhibition, lysates were generated and immunoblotted for various
Figure 2. PAK Kinase Inhibition Prevents Resistance in Melanoma Cell Line

A  Dose response curve for PAK Kinase Inhibitor (FRAX-486). Bars indicate the standard error of the mean of the six wells per condition. The x-axis indicates the log concentration of FRAX-486, while the y-axis indicates the percentage of viable cells.

B  Population doublings vs. time graph evaluated the effects of treatment. This represents a single trial of a cell assay. Cells were re-fed with corresponding treatment every 4-5 days. DMSO and FRAX alone treatments reached 10 population doublings within 15 days. Vemurafenib treatment reached 10 population doublings around 81 days. The VEM-FRAX combination treatment cells died off by day 60.

C  Population doublings vs. time graph evaluated the effects of treatment. This graph represents three trials run simultaneously. Cells were re-fed with corresponding treatment every 4-5 days. Vemurafenib treatment reached 10 population doublings on average by 65 days. The VEM-FRAX combination treatment cells died off by around day 55.
potential proteins (see Figure 1) involved in vemurafenib resistance. Proteins involved in the MAP-kinase pathway targeted for treatment with vemurafenib were also tested. Tubulin served as the loading control for equal amounts of protein sample loading.

When comparing the cells under vemurafenib (VEM) treatment and the vemurafenib and FRAX-486 combination (VEM+FRAX) treatment we expected to see a down-regulation of TAZ, pERK, and pAKT and/or AKT in the VEM+FRAX cells because these proteins are associated with resistance in melanoma. We also expected to see a down-regulation of phosphorylation of MEK at position 298 in the VEM+FRAX cells because this position on MEK is phosphorylated by activated PAK.

Overall, there were no dramatic changes in any of the signaling protein levels when comparing the vemurafenib treated cells with the VEM+FRAX combination (Fig 3). The protein levels of TAZ, pMEK, pAKT, and pERK are all highest in the DMSO and FRAX treated cells, while they are significantly lower in both the VEM treated and VEM+FRAX treated cells. The protein levels of TAZ and pMEK moderately decrease with time in both the VEM treated and VEM+FRAX treated cells. Interestingly, the protein levels of pERK was significantly diminished by day 2 of treatment and then moderately increased by day 7 in both the VEM treated and VEM+FRAX treated cells. These results indicated either the mechanism by which PAK inhibition blocks drug resistance has not been identified, or that the PAK inhibition mechanism is functioning at sub-detectible levels during the beginning of treatment.

**PAK Inhibitor Concentration Levels in Relation to pMEK S298 Signal Inhibition**

In order to assess the relationship of FRAX 486 concentration and the inhibition of the downstream target of PAK, lysates were generated from cells treated with the following
Figure 3. Protein involvement in resistance prevention via VEM+FRAX is inconclusive
Immunoblotting for the indicated proteins, quantification for relative intensity of the band can be seen underneath each lane. The protein levels of TAZ, pMEK, pAKT, and pERK are all highest in the DMSO and FRAX treated cells, while they are significantly lower in both the VEM treated and VEM+FRAX treated cells. The protein levels of TAZ and pMEK moderately decrease with time in both the VEM treated and VEM+FRAX treated cells. The protein levels of pERK was significantly diminished by day 2 of treatment and then moderately increased by day 7 in both the VEM treated and VEM+FRAX treated cells. Tubulin served as the positive loading control.
concentrations of FRAX 486 (in nM): 0, 50, 100, 200, 500, 1000. They were subsequently blotted for total MEK and pMEK S298, the target site for PAK signaling. Tubulin served as the loading control for equal amounts of protein sample loading. The relative amount of pMEK S298 and total MEK decreased with increasing concentrations of FRAX 486 (Fig 4.A).

A dose response curve was created to visualize the percentage of target inhibition. From this graph, the IC$_{50}$ was calculated to be 540 nM (Fig 4.B). The IC$_{50}$ for target inhibition corresponds to the concentration of FRAX 486 that inhibits 50% of the amount of pMEK S298 in untreated cells.
Figure 4. Dose Response Curve for FRAX 486 Target Inhibition.

A Immunoblotting for the indicated proteins at varying levels of PAK inhibitor concentrations; quantification for relative intensity of the band can be seen underneath each lane. PAK inhibition results in a decreased level of phosphorylated MEK and total MEK. Tubulin served as the loading control.

B Dose response curve for PAK Kinase Inhibitor (FRAX-486). The x-axis indicates the log concentration of FRAX-486, while the y-axis indicates the percentage of the pMEK signal inhibited by FRAX 486.
Discussion

This study aimed to evaluate the effects of PAK kinase inhibition on emergence of vemurafenib resistance and potentially related biochemical signaling pathways. Our results strongly indicate that combining PAK kinase inhibition via FRAX-486 with vemurafenib treatment can prevent development of resistance in melanoma cells. In the cell culture assays, cells under vemurafenib treatment alone began to show signs of resistance by day 35 on average. However, cells under VEM-FRAX combination treatment eventually died out between days 55-60, and resistance was never developed. Treatment with FRAX alone showed no effect on cell growth in comparison to the DMSO control. This provides evidence that the FRAX treatment at the 50 nM dose we chose may not result in cytotoxicity in normal cells; there may be minimal to no effects. Furthermore, these results indicate that PAK kinases are playing an important role in the development of resistance in melanoma cells.

According to the short-term biochemical assay, there were no significant differences in the levels of selected signaling proteins within the first seven days of treatment. However, there are several possible explanations for this observation. Phosphorylation of MEK at the 298 position is associated with activation by PAK. One possible way the treatment of PAK kinase inhibition is effective is through deactivation of PAK and subsequent effects on cell proliferation occurring at sub-detectible levels at the beginning of treatment. The protein levels of pERK are highest in the DMSO and FRAX treated cells. The protein levels of pERK are extremely diminished in the VEM and VEM+FRAX treated cells. There was a two fold increase from day 2 to day 7 in pERK in the VEM and VEM+FRAX treated cells. It is possible the cells begin to combat the vemurafenib treatment by attempting to reactivate the ERK protein by
phosphorylation. Although not tested in this study, PAK kinase inhibition may work to prevent resistance by having a slow build up over time of cumulative inhibition of ERK phosphorylation.

The inconclusive results of the short-term biochemical assay are further rationalized by the results of the FRAX 486 concentration vs. pMEK S298. These concentration lysates were used to determine the signaling IC$_{50}$ of 540 nM for the FRAX 486 drug. For the drug resistance experiments we chose a FRAX 486 concentration close to the IC$_{20}$, 50 nM. This value is significantly lower, which leads us to believe that the blockage of resistance is taking place at a sub-detectible level in the short term.

This study faced constraints mostly due to time. The results from the short-term biochemical assays were difficult to draw conclusions from. Moving forward, biochemical assays on long-term lysates need to be assessed. In the drug-resistance assays differences between cell treatment growths weren’t seen until around days 20-30. Ideally, when making the new lysates, they should be from cells grown under the treatment conditions until at least day 30.

Previous studies have shown increased PAK-1 expression in cancers of the bladder, breast, lung, and T cell lymphomas (Ong et al., 2011)(Field and Ye, 2015)(Pandolfi et al., 2015)(Gan et al., 2016). However, the role of PAK in metastasis with relation to the Hippo pathway was not explored. As discussed in previous literature, PAK seems to play an essential role cancer metastasis. Developing a potential treatment to target PAK would be beneficial in a large number of cancers.

Previous research has also linked PAK signaling directly to resistance in $B$-$RAF$ mutant melanomas like studied here (Lu et al., 2017). However, the major focus in Lu et al. was on the MAP Kinase pathway. The potential role of the Hippo pathway was not explored despite the evidence of cytoskeletal rearrangement in drug-resistant melanoma. Furthermore, the effects of
PAK inhibition with the common B-RAF inhibitor was only studied after the cells developed resistance to the B-RAF inhibitor treatment. The avenue of potential preventative treatment was not investigated (Lu et al., 2017).

Another future avenue for this project involves evaluating the role of Src/PAK signaling in vemurafenib resistant melanoma. It has been shown that oncogenic Src results in cancerous cellular transformation through activation of Rac1 (Servitja, et al., 2003). It is possible that vemurafenib resistance is developed through a pathway involving Src signaling through Rac1, and subsequent downstream signaling through PAK.

In conclusion, PAK appears to play an important role of development of resistance in vemurafenib treated B-RAF melanoma. This means PAK kinase inhibition could be an extremely important therapeutic target moving forward. Further investigation into the mechanism will provide insight to the cancer community, as it is possible PAK kinase is an important factor in a variety of cancers. Subsequent animal trials will allow evaluation of the potential treatment in an in vivo setting.
Literature Cited


