Role of MEK Mutations in Trametinib Resistant Acute Myeloid Leukemia Cells

by
Sofia Beer

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Human Development and Family Sciences
(Honors Scholar)

Presented May 13, 2021
Commencement June 2021

AN ABSTRACT OF THE THESIS OF

Sofia Beer for the degree of Honors Baccalaureate of Science in Human Development and Family Sciences presented on May 13, 2020. Title: Role of MEK Mutations in Trametinib Resistant Acute Myeloid Leukemia Cells.

Abstract approved: _____
Viktor E. Bovbjerg

Acute myeloid leukemia (AML) is a blood malignancy characterized by a differentiation and proliferation defect of myeloid progenitors leading to accumulation of myeloid blasts. It is one of the most common leukemias in adults. Trametinib (Mekinist) is a small molecule inhibitor that targets MEK1 (MAP2K1) and MEK2 (MAPK2K2). It has been approved by the Food and Drug Administration (FDA) to treat advanced melanoma. The benefit of trametinib is limited by the development of resistance. In AML, a combination of trametinib with tyrosine kinase inhibitors (TKIs) has been shown to improve the response in AML cell lines[1]. Moreover, a phase 1/2 study demonstrated trametinib's benefit in *RAS*-mutated myeloid malignancies including AML[2]. This work aims to study trametinib resistance mechanisms in AML.

MOLM13, MV4;11, and OCI-AML2 AML cell lines were made resistant to trametinib. Exome sequencing as well as Sanger sequencing revealed $MEK1^{K57N}$ and $MEK1^{L215P}$ mutations in these resistant cells. Therefore, site directed mutagenesis as well as gateway cloning were used to generate $MEK1^{K57N}$ and $MEK1^{L215P}$ open reading frame (ORF) harboring lentivirus vectors. Then, MOLM13 AML cells were transduced with a lentivirus containing wild-type and mutant MEK1 to establish AML cell lines expressing wild type or mutant MEK1. A drug sensitivity assay was used to analyze trametinib sensitivity in these cells. Moreover, western blot analysis was used to study alteration of signaling pathways in acquired trametinib resistant AML cell lines (MOLM13 and OCI-AML2).

The drug sensitivity assay demonstrated that MOLM13 cells harboring $MEK1^{K57N}$ has lower sensitivity to trametinib in comparison to MOLM13 cells transduced with wild type MEK1. This indicated that $MEK1^{K57N}$ confers resistance to trametinib in AML cells. The finding confirmed the hypothesis that $MEK1^{K57N}$ plays a role in the drug resistance of trametinib in AML cells. This information can be used to further investigate ways to possible circumvent this mutation and find ways to use trametinib to treat AML. This can potentially be done through using combination therapy, using this information as a treatment selection tool, and finding ways to block the mutated protein from activating.

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Sofia Beer, Author

Acknowledgements

I am extremely grateful to everyone in the Tyner/Druker lab that welcomed me in. I entered this lab right as my sophomore year had ended and I had no prior experience working in a professional lab. Despite this lack of experience, everyone in the lab was very kind and made me feel at home.

I want to especially thank Dr. Alisa Damnernsawad and Dr. Jeffrey Tyner. They worked with me very closely to help me learn and grow in the lab. Without Alisa, I would not know anything about a lab. She not only taught me everything I know but she was always there to answer my questions and never got frustrated with my lack of knowledge. Dr. Jeffrey Tyner showed me a lot of support while in the lab and has continued to show me support since leaving.

I am also very thankful for Dr. Viktor Bovbjerg. He has put in a tremendous amount of time helping me be successful in this thesis. He has worked with me since my sophomore year and has always helped me when I need it. In addition, I want to thank Dr. Philip McFadden for taking the time to be on my thesis committee.

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Chapter 1: Introduction

1.1 Acute Myeloid Leukemia

Leukemia is a cancer that begins in the bone marrow. The bone marrow will start producing abnormal white blood cells, these cells then continue to grow and can then overrun the healthy blood cells (7). Acute myeloid leukemia (AML) is one of the most common leukemias in adults, around 20,000 people are diagnosed annually (7). It starts in the bone marrow and quickly moves to the blood. It develops from myeloid progenitor cells, also called myeloid blasts, which, in a healthy scenario, are the cells that would develop into mature blood cells including red blood cells, some types of white blood cells, and platelets (10). AML is then caused by abnormal myeloid progenitor cells that failed to properly differentiate and are proliferative. This leads to accumulation of myeloid blasts in the hematopoietic system (10). Prognostic factors can include age, coexisting conditions, and poor performance status. Factors including white-cell count, prior myelodysplastic syndrome or cytotoxic therapy for another disorder, and leukemic-cell genetic changes can help to determine whether or not the patient is likely to be resistant towards therapies (4). The current conventional treatment for acute myeloid leukemia is intensive induction chemotherapy. Induction therapies usually include a combination of an anthracycline (such as daunorubicin, doxorubicin, or idarubicin) and cytarabine (also called cytosine arabinoside or ara-C) (18). Other treatments include: Gemtuzumab ozogamicin (CD33 antibody-drug conjugate), Midostaurin (FLT3 inhibitor), Gilteritinib (FLT3 inhibitor) Venetoclax (BCL2 inhibitor) in combination with hypomethylating agents (Azacytidine or Decitabine), Glasdegib (Hedgehog pathway inhibitor), Enasidenib (IDH2 inhibitor) and Ivosidenib (IDH1 inhibitor) (18).

In addition to chemotherapy, bone marrow transplants are commonly used to treat AML. Currently, for people over 60, only about half of the people go into remission after their first round of chemotherapy. Five-year survival rate is around 28 percent (2). Due to this low rate of success, it is imperative that other treatment strategies for AML are created. (4).

1.2 MAPK/RAS Pathway

Dysregulation of signaling pathways are heavily involved in the pathogenesis of cancer. Most cancerous tumors arise from mutations, these can be inherited genetically, or can arise spontaneously due to other environmental factors (1). Oncogenic mutations can dysregulate signaling pathways that normally regulate cell growth, therefore, leading to cancer progression (1). Sometimes oncogenic genes can cause genes to be overexpressed or it can produce dysregulated proteins (1).

There are many signaling pathways that, in the context of mutations, can cause the growth of cancerous tumors. The MAPK/RAS is an example of one. This pathway is involved in transducing signals from the extracellular matrix to the nucleus where specific genes can then be transcribed (12). The MAPK/RAS signaling pathway is involved in a variety of cellular functions such as proliferation, differentiation, transcription regulation, cell growth, and development (12). It is also involved in stimulating angiogenesis, or the development of new blood vessels, all of its roles are very important for a tumor to form. This pathway is commonly dysregulated because RAS is one of the most common oncogenes (12). NRAS mutations have been found in 11% of AML patients, while KRAS mutations have been found in 5% of AML patients (13).

RAS is the beginning of the pathway and can be stimulated through factors such as epidermal growth factors, tumor necrosis factors, protein kinase activators, and SRC members (3). The pathway can be activated through binding of a signal to a protein tyrosine kinase receptor (13). RAS is a GTPase, it serves as a regulator of the signaling cascade (19). Guanine exchange factors cause GDP to get switched with GTP (19). Once RAS is activated, RAF is recruited to bind to the plasma membrane through lipid binding and binding of the switch 1 domain of RAS. RAF then starts the signaling cascade through phosphorylation of MAP2K1/2 (MEK1/2) which then phosphorylates MAPK1/3 (ERK1/2) (13). ERK activation occurs and then ERK 1/2 phosphorylates a transcription factor, FOS (13). FOS then can translocate to the nucleus to activate gene transcription (13). FOS will sometimes work in parallel with mediators such as JNK, SAPK, and NF-KB (13). It can also regulate RAF and ERK, this can allow for cross talk between other signaling pathways (13). Abnormal MAPK signaling can lead to cells proliferating uncontrollably and can cause resistance to apoptosis (6). The MAPK pathway plays

a role in not only leukemia but many other cancers as well (6). In leukemia, when there is hyper-activation of the MAPK pathway, it can play a role in transforming myeloid cells to be cancerous (6).

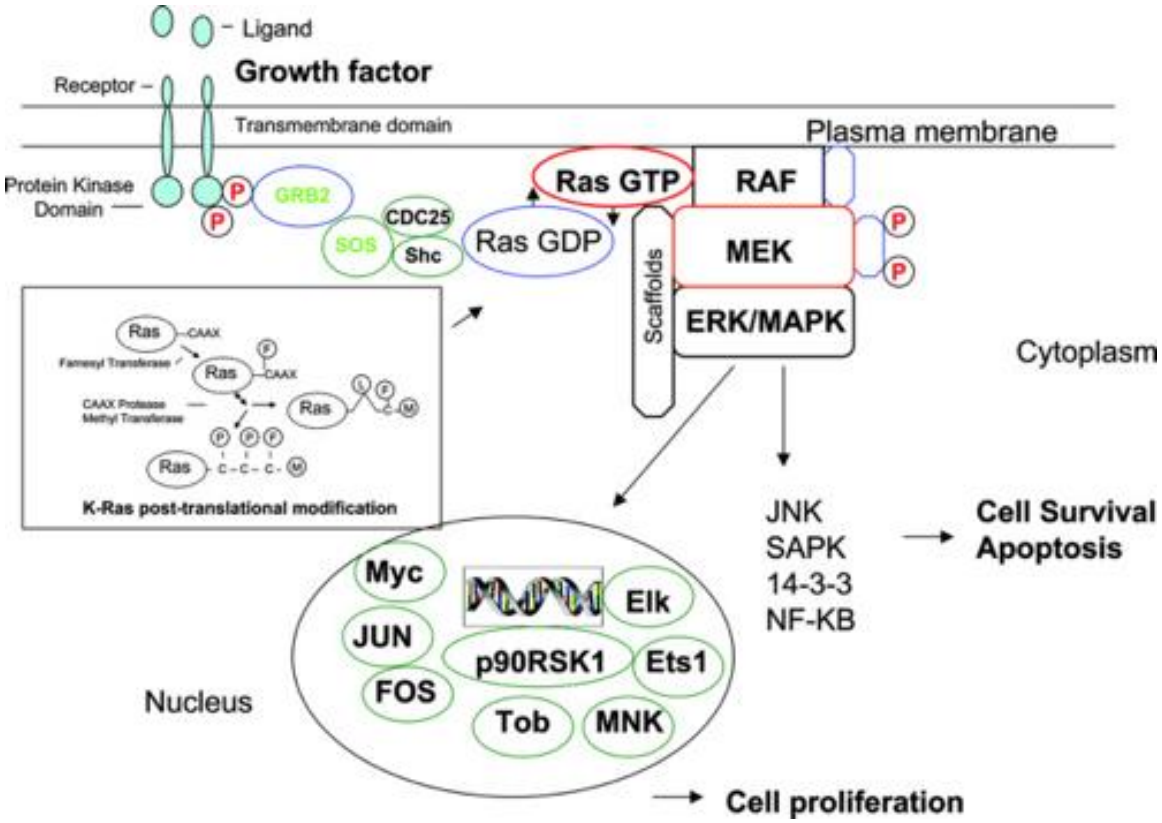


Figure 1. The RAS/RAF/MEK Pathway. This pathway is activated by a ligand binding to a tyrosine kinase receptor. Activation of RAS activates RAF, which activates MEK and eventually ERK. (24).

In acute myeloid leukemia, about 10-15% of patients have mutations that cause dysregulation of the MAPK signaling pathway (14). This involves activating mutations in NRAS, KRAS, PTPN11, and NF1 (20).

1.3 Targeted Drug Therapy and Trametinib

Targeted drug therapy, unlike chemotherapy, focuses on targeting the specific tissue environment, genes, or proteins that are contributing to the growth of the cancer (9).

Chemotherapy targets rapidly proliferating cells and kills them. Therefore, it is also toxic to normal cells that are rapidly proliferating (9). Targeted drug therapy only targets cells with abnormal genes or proteins, having subtle effects on healthy cells (9). Targeted drug therapy can be cytostatic, which means it blocks tumor cell proliferation (9). It can also induce apoptosis in tumor cells. There are many different types of targeted drug therapies. Common ones include hormone therapies, signal transduction inhibitors, immunotherapies, and many more. Hormone therapies work by preventing the body from producing hormones that are required for hormone-sensitive tumors (9). Hormone therapies are currently being used for breast and prostate cancer (9). Signal transduction inhibitors interfere with inappropriate signaling that results from signaling cascades (9). These come from a series of biochemical reactions that produce certain responses (9). Immunotherapies trigger the immune system to destroy cancer cells (9). There are monoclonal antibody immunotherapies that recognize specific molecules on the surface of cancer cells while others bind to other immune cells to help kill cancer cells (9).

Trametinib is a targeted drug that inhibits MEK, also known as mitogen-activated protein kinase (11). Trametinib inhibits both MEK1 and MEK2. MEK1 and MEK2 are a part of the RAS/MAPK signaling pathway (11).

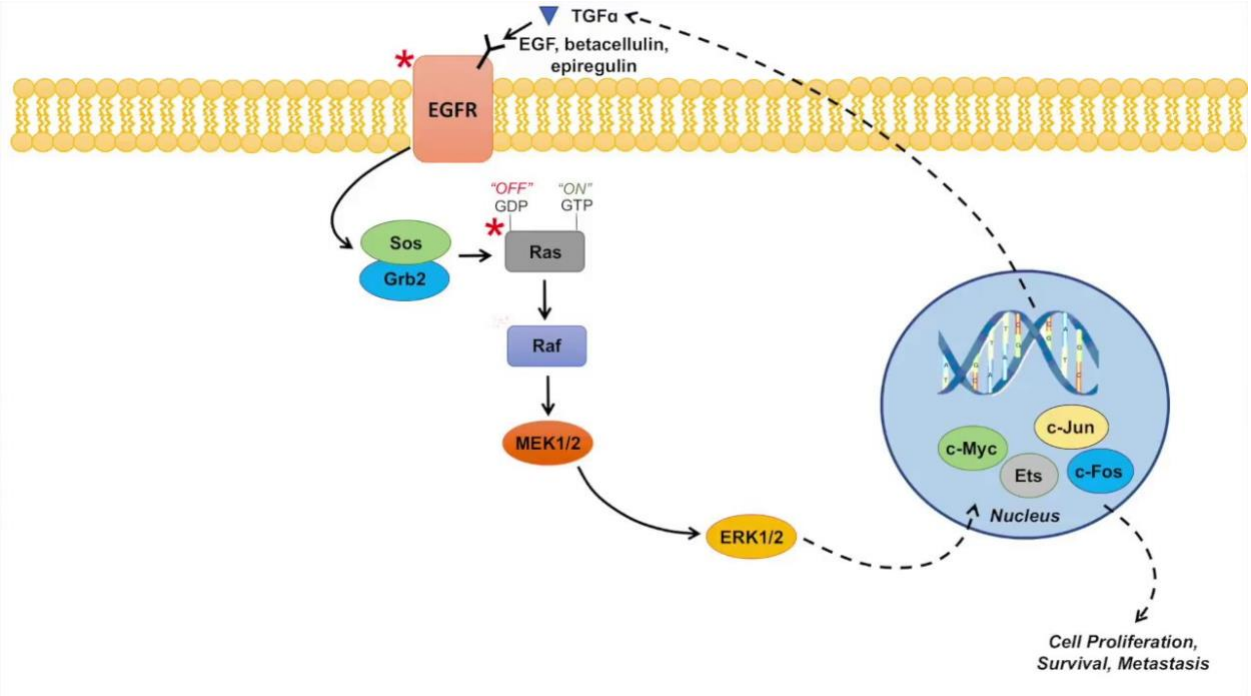


Figure 2. RAS Activation. When GDP is active the pathway is inactive, when GDP is phosphorylated to GTP the signaling cascade continues. (25).

Trametinib is administered orally (11). It was approved by the FDA for use in May 2013 for people with metastatic melanoma with BRAF V600E or V600K mutations (11). 50% of melanoma patients hold a mutation in BRAF, about 95% of those are either V600E or V600K mutations. In the RAS-RAF-MEK- ERK pathway BRAF is immediately upstream of MEK (11). A mutation in BRAF causes MEK to stay continuously active (11). This results in increased proliferation, and therefore increased survival of cancer cells (11).

Although targeted drug therapy is a promising way to treat cancer, there are still some drawbacks, including drug resistance. Drug resistance occurs when cancers become tolerant to certain treatments (5). There are many factors that contribute to drug resistance including: drug inactivation, drug efflux, DNA damage repair, cell death inhibition, epithelial-mesenchymal transition, and drug target alteration (5). Understanding these mechanisms of drug resistance is imperative to improve the manner by which targeted drug therapies are used as well as for the improvement of cancer treatment in general.

1.4 Trametinib Resistance in Melanoma

Currently, there are some known mechanisms of resistance of trametinib when used to treat cancerous melanoma cells. Three main ones are the MEK 1/2 Mutation, BRAF Gene Amplification, and the NRAS mutation.

The MEK 1/2 mutation is de novo and occurs in around 26% of treatment-insensitive tumors (15). MEK contains a helix A/C substructure which acts as a negative regulator (15). The helix is near the region where allosteric inhibitors (such as trametinib) and ATP bind. A mutation in MEK allosterically alters the binding of ATP. This intrinsically increases the kinase activity of MEK, therefore increasing the ERK levels up to 20-fold (15). This increases the proliferation of cells and increases the inhibition of apoptosis resulting in easier tumor growth. This overexpression of MEK results in a decreased sensitivity towards MEK at least by 10-fold,

causing resistance to trametinib (15).

BRAF gene amplification existed in 36% of patients being treated with trametinib (15). When the number of the copies of BRAF increases so does the activity of the BRAF kinase because there is a greater concentration of it (15). This creates an excess of MAPK. Excess of MAPK has two major consequences, one of them being an increase in the basal level of pERK. This causes an upsurge of proliferation, metastasis, and apoptosis resistance (15). The second consequence of an increase in MAPK is that it increases the IC50 of trametinib needed to inhibit pERK (IC50 is the concentration of drug required to inhibit a biological process by half) (15).

An NRAS mutation can also occur. The protein NRAS is responsible for regulating cell division (15). This protein has a very high mutation rate, it is the second highest oncogenic stimulus for cutaneous metastatic melanoma (15). The mutations of the NRAS gene occur most commonly at codon 61 and the rest at codon 12 or 13 (15). This can hyperactive the RAS/RAF/MAPK and P13KT/AKT cascades resulting in an increase in protein expression and therefore an increase in proliferation and cell cycle dysregulation (15). This promotes metastasis and can only be overcome by increasing the dosage of the drug beyond what is safe for humans (15).

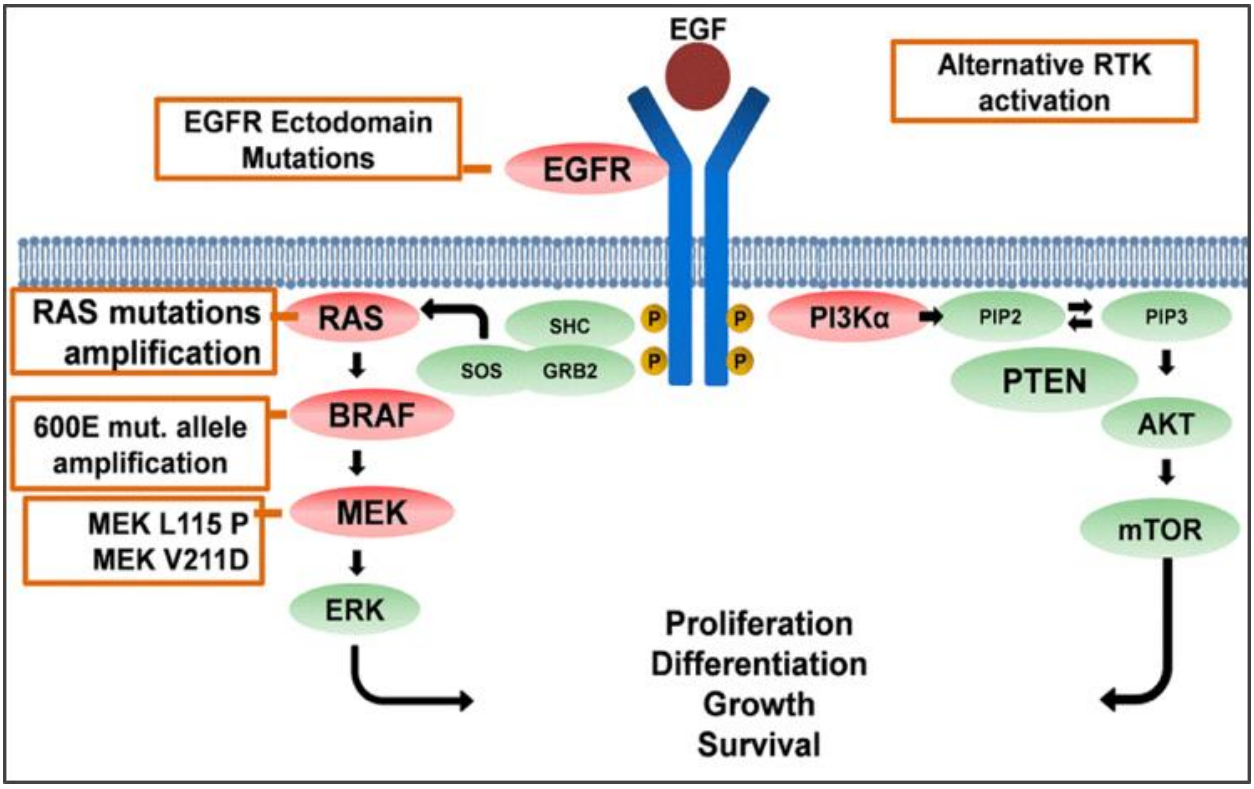


Figure 3. Mutations of the RAS/RAF/MEK Pathway. MEK mutations, BRAF gene amplification, and RAS mutations amplification can all lead to resistance. (26).

1.5 Some Examples of Acute Myeloid Leukemia Resistance with Other Drugs

There are many resistance pathways that have been studied when acute myeloid leukemia has been treated with other drugs. Drug resistance related proteins and enzymes include overexpression of P-gp, MRP1 (ABCC1) and LRP, Glutathione S-transferases, and Protein kinase C (16). Overexpression of P-gp has been shown to be associated with higher white blood cell count, worse chromosomal abnormalities, and shorter overall survival (16). It has been found that drug resistant MOLM13 AML cells had upregulation of P-gp and down regulation of Bcl-2, which is an antiapoptotic protein (16). MRP1 is part of the ABC cassette transporters and can affect how drugs are distributed in cells, it can prevent drugs from entering the nucleus which can be cytotoxic. LRP is associated with resistance to drugs in AML. It can lead to drug resistance by blocking nuclear pores and therefore stopping drugs from entering the nucleus. It can also transport drugs from the nucleus to transport vesicles and release it through exocytosis (16). Glutathione S-transferase is hypothesized to be involved in drug resistance by inactivating

the drug activity directly by catalyzing the synthesis of anticancer drugs with glutathione and intracellular GST inhibiting the effects of anticancer drugs by attacking intracellular DNA (16). Protein kinase C (PKC) can phosphorylate P-gp, a drug resistance pump, on serine residues. It can enhance the activity of P-gp (16).

Another mechanism for resistance in AML is gene alterations in the drug targeting gene (16). This includes: Fms-like tyrosine kinase 3 (FLT3), Wilms tumor (WT1), and the RAS family. A mutation in FLT3 can result in uncontrollable cell proliferation making AML resistant to chemotherapy (16). The expression of WT1 in an AML patient has been directly correlated with the severity of the disease (16). A mutation in WT1 increases the risk of AML relapse (16).

MicroRNAs (miRNAs) can also play a role in drug resistance. The role of miRNAs is to silence RNA and post-transcriptionally regulate gene expression (17). They do this by base pairing with complementary mRNA (17). miRNA has also been shown to play a role in drug resistance by repairing damaged DNA caused by antineoplastic drugs (16). They also have the ability to downregulate proteins, leading to cell cycle arrest and drug resistance (16). Lastly, miRNAs can express in low levels and can result in reducing the activation of apoptosis pathways leading to drug resistance (16).

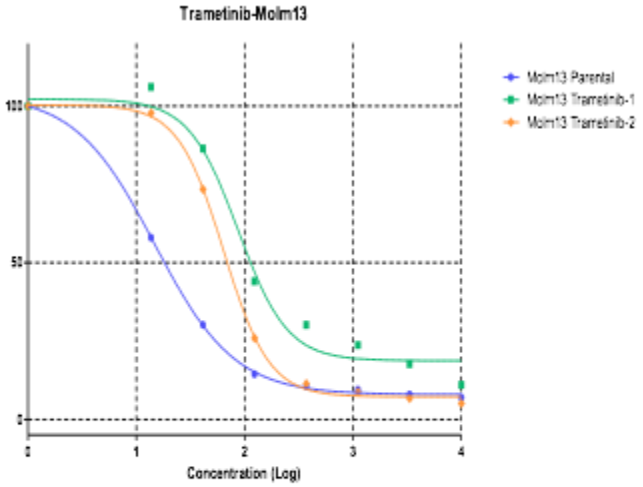
Signaling pathways also play a role in drug resistance. The P13K/AKT signaling pathway and autophagy have been found to result in drug resistance. When the P13K/AKT is activated excessively it can lead to regulating the activity of the JNK-p38 MAPK pathway. This can lead to drug resistance. AKT can also phosphorylate substrates that induce tumor cells to resist drugs directly (16). The P13K/AKT pathway can also regulate the expression of P-gp which is downstream from it (16). Autophagy can be looked at as having positive and negative effects. It initially can suppress tumor formation, but this can also lead to drug resistance (16).

1.6 Previous Work Done by Alisa Damnernsawad

Prior to my joining the Druker/Tyner Lab, Dr. Alisa Damnernsawad performed some experiments that led to the idea of this thesis. Dr. Damnernsawad generated cells that were

resistant to trametinib. Resistant MOLM13 and OCI-AML2 cells were generated. Figures 4 and 5 show the concentration of the cells viable when treated with trametinib. In both the MOLM13 and OCI-AML2 cells that were resistant to trametinib showed higher viability than the parental cells when treated with a dose gradient of trametinib. The stability of the resistant cells was also tested, and the resistant cells showed to be stable even after trametinib withdrawal of two weeks. The MOLM13 cells were also frozen and they still remained resistant to trametinib.

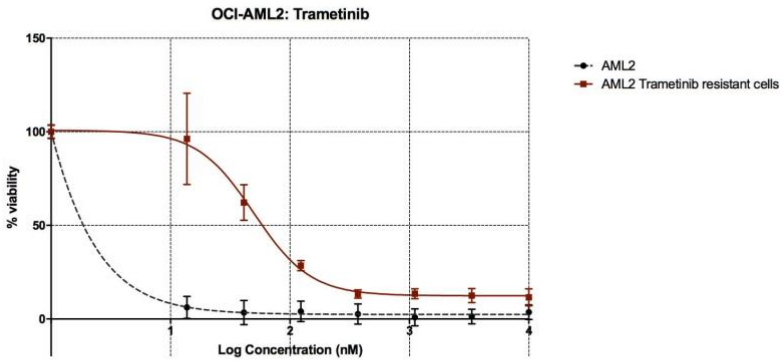
Exome sequencing was performed on the resistant MOLM13 cells and the results are shown in Table 1. The results showed the resistant cells had at least one allele that had the MEK1^{K57N} mutation. The cells were most likely heterozygous as opposed to homozygous. The results also showed that 25% of the resistant MOLM13 cells have the MEK1^{L215P} mutation. This led to the project performed for my thesis. I wanted to further investigate whether these mutations played a role in drug resistance to trametinib.



	Molm13 Parental	Molm13 Trametinib-1	Molm13 Trametinib-2
True IC50	18.24	113.8	69.31

	Molm13 Parental	Molm13 Trametinib-1	Molm13 Trametinib-2
Total Area	140.1	241.7	197.4

Figure 4. Dr. Alisa Damnernsawad. Data showing the concentration of MOLM13 cells treated with trametinib. The cells were shown to be resistant to trametinib due to the higher survival rate.



	AML2	AML2 Trametinib
True IC50	1.862	58.61

	AML2	AML2 Trametinib
Total Area	68.57	199.3

Figure 5: Dr. Alisa Damnernsawad. Data showing the concentration of OCI-AML2 cells treated with trametinib. The cells were shown to be resistant to trametinib due to the higher survival rate.

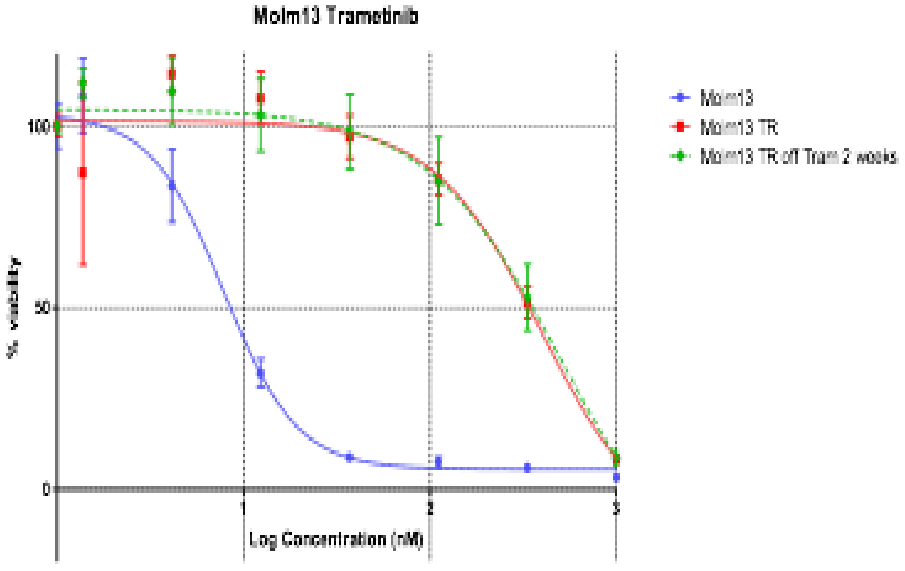


Figure 6: Dr. Alisa Damnernsawad. Data showing that trametinib resistance was maintained after being taken off of it for two weeks. The trametinib resistance was maintained shown by the higher viability.

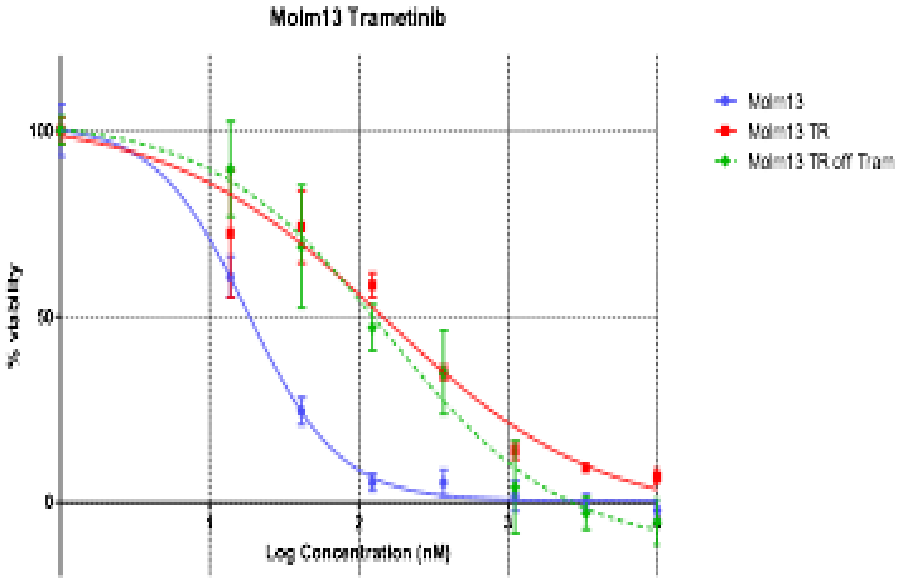


Figure 7. Dr. Alisa Damnernsawad. Data showing that trametinib resistance was maintained after being taken off of it for two weeks and being frozen. The trametinib resistance was maintained shown by the higher viability.

	MEK1^{K57N}	**MEK1^{L215P}**
AML2 Trametinib Resistant	50%	No
MOLM13 Trametinib Resistant 1	No	25%
MOLM13 Trametinib Resistant 2	50%	No
MV-411 Trametinib Resistant 1	No	No
MV-411 Trametinib Resistant 2	50%	No

Table 1. Dr. Alisa Damnernsawad. Table showing the results of exome sequencing, MEK1^{K57N} was found in 50% of AML2, MOLM13, and MV-411 alleles of the trametinib

resistant cells. Presumably, 1 of the 2 copies of alleles is mutated in every resistant cell. $MEK1^{L215P}$ was found in 25% of MOLM13 trametinib resistant cells. Presumably, half of the resistant cells carried the mutation in 1 of the 2 alleles.

1.7 Problem Statement and Overview of Thesis

In this thesis, the potential role of $MEK1^{K57N}$ and $MEK1^{L215P}$ mutations in trametinib resistance of AML cells is investigated. Specifically, OCI-AML2 as well as MOLM13 AML cell lines were examined.

Hypothesis: The mutations of MAP2K1, alternatively called MEK1, cause resistance of trametinib in AML cells due to the previous exome sequencing revealing the five resistant cells contained mutations in the MAP2K1 gene including MAP2K1 $MEK1^{K57N}$ and $MEK1^{L215P}$.

Chapter 2: Methods and Materials

This experiment was conducted through cloning, transfection, transduction, and finally a drug sensitivity assay that measured the survival of cells with or without the MAP2K1 $MEK1^{K57N}$ mutation when both types of cells were treated with trametinib.

2.1 MEK Mutagenesis/LR Clonase Reaction

In this project the MAP2K1 wild type, $MEK1^{K57N}$, and $MEK1^{L215P}$ mutations were inserted into lentiviral expression vectors in order to express the gene variants in AML cell lines, MOLM13, MV4;11, and OCI-AML2.

2.1.1 Site directed mutagenesis

Site directed mutagenesis had previously been done to generate $MEK1^{K57N}$ and $MEK1^{L215P}$. Specifically, a QuikChange II XL Site-Directed Mutagenesis Kit was used. Using this instead of

traditional site directed mutagenesis allowed site specific mutations to occur in not only ssDNA, but also dsDNA. The three-step procedure is outlined below:

1. Mutant Strand Synthesis Reaction (Thermal Cycling)
2. Dpn I Digestion of the Amplification Products
3. Transformation

2.1.2 Gateway Cloning

Gateway Cloning technology was used to transfer target gene variants from donor vector, pDONR223, into lentivirus vector, pInducer. In Gateway Cloning, targeted genes were transferred from the entry vector, pDONR223, to the destination vector, pInducer, via DNA recombination processed by the LR clonase enzyme. pDONR223 holds the wild-type or mutant MAP2K1 between attL1 and attL2 sequences and pInducers have attR1 and attR2 sequences (8). In the LR reaction, LR clonase enzyme will recombine in between attL and attR sequences allowing transfer of the insert from the entry clone to the destination vector. The donor vector holds the ccdB gene which, later, is replaced by the inserted gene (8). Any cell that still expresses ccdB dies, while the ones with the inserted genes propagate. Because of this the ccdB system greatly increases the efficiency of cloning because only the clones with the correctly inserted gene should grow (8).

2.1.3 Sanger Sequencing

Sanger sequencing was then performed to confirm the correct DNA sequences of the expression vector products. This protocol involves:

1. The double-stranded DNA (dsDNA) is denatured into two single-stranded DNA (ssDNA).
2. A primer that corresponds to one end of the sequence is attached.
3. Four polymerase solutions with four types of dNTPs but only one type of ddNTP are added.
4. The DNA synthesis reaction initiates and the chain extends until a termination nucleotide is randomly incorporated.

5. The resulting DNA fragments are denatured into ssDNA.
6. The denatured fragments are separated by gel electrophoresis and the sequence is determined (21).

2.2 Transfection

In order to generate the virus, two packaging plasmids were used as well as the expression vector was transfected into HEK293T cells using lipofectamine (3). This multi-plasmid system helps to increase the safety of using the lentivirus, since no single construct contains all of the genetic material needed to form a functional virus. Lipofectamine forms a lipid bilayer surrounding the vectors into liposomes. These vectors containing liposomes fuse with the plasma membrane of the HEK293T cells and unload the vectors into the cell cytosol (3). HEK293T cells containing packaging and expression vectors will produce the virus. The virus will be harvested and used to transduce AML cell lines (3).

2.3 Transduction

AML cell lines, MOLM13, MV4;11, or OCI-AML2 cells were transduced with the virus using the spinoculation technique. Since the expression vector used had a puromycin resistance gene as well as a green fluorescent protein (GFP) encoding gene, cells that successfully receive the virus were able to withstand puromycin treatment and express GFP (23). Therefore, puromycin selection was used to select cells containing the virus. After this, the cells with the virus were cultured for amplification (23). The cells were confirmed for the presence of the lentiviral vector and MAP2K1 variants through GFP detection as well as DNA sequencing (23).

2.4 Drug Sensitivity Assay

A drug sensitivity assay was used to determine trametinib sensitivity of cells containing wild-type and mutant MAP2K1 (22). Wild type as well as mutants harboring AML cells was treated with variation of trametinib concentrations for 72 hours. Then, the amount of survival cells was determined using MTS assay (22). The half maximal inhibitory concentration (IC50) and area

under the curve (AUC) was determined from a dose-response curve (22). High IC50 as well as AUC means less sensitivity to the drug (22).

2.5 Western Blots

Western blot analysis is a technique used to detect specific proteins in a sample. The general protocol for a western blot is shown below:

1. Make a lysis buffer
2. Measure the protein level
3. Loading the dye
4. Running the gel
5. Transfer the gel
6. Blocking (fill tray with 5% BSA TBST)
7. Add the primary antibodies
8. Add the secondary antibodies
9. Use Image Lab for detection and imaging

2.6 Statistical Analysis

To analyze the data, graphs were made of the data of the resistance in the different genes analyzed. The area under the curve was calculated to see the total survival of cells.

2.7 Results

2.7.1 Western Blots: MOLM13 Cells

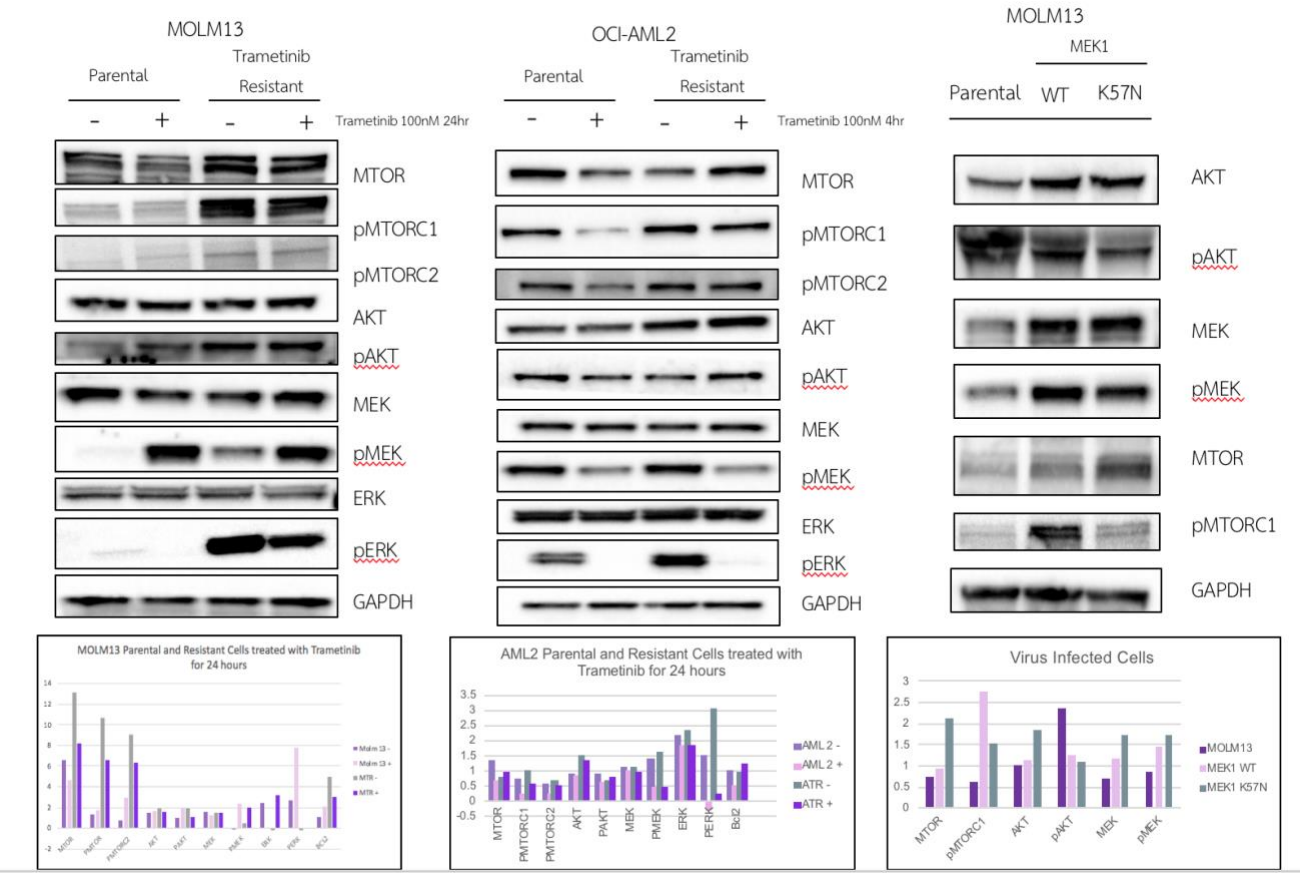


Figure 8: Sofia Beer. Western Blot Results.

Trametinib resistant cell lines demonstrated increased levels of p-MEK, p-ERK as well as p-mTORC1 (p-mTOR complex 1) had enhanced activity of MAPK and mTOR pathways. MOLM13 trametinib resistant cells showed an increase in p-AKT, which can activate mTORC1, suggesting that up-regulation of mTORC1 activity in MOLM13 trametinib resistant cells might be partially enhanced by AKT activity.

2.7.2 Western Blots: OCI-AML2 Cells

Trametinib resistant cell lines demonstrated decreased levels of p-MEK, p-ERK. There were also enhanced levels of p-mTORC1 and p-mTORC2 in the presence and absence of trametinib. There was a slight downregulation of p-AKT in the trametinib resistant cells when not treated with trametinib.

2.7.3 Mutagenesis

The below figure shows the successful mutagenesis of the MOLM13 cells. The MEK1^{L215P} results from replacing a cytosine with a thymine. This mutation results in proline replacing leucine. The MEK1^{K57N} mutation comes from replacing a guanine with a thymine. This results in replacing a lysine with an asparagine. These images show the successful insertion of both mutations.

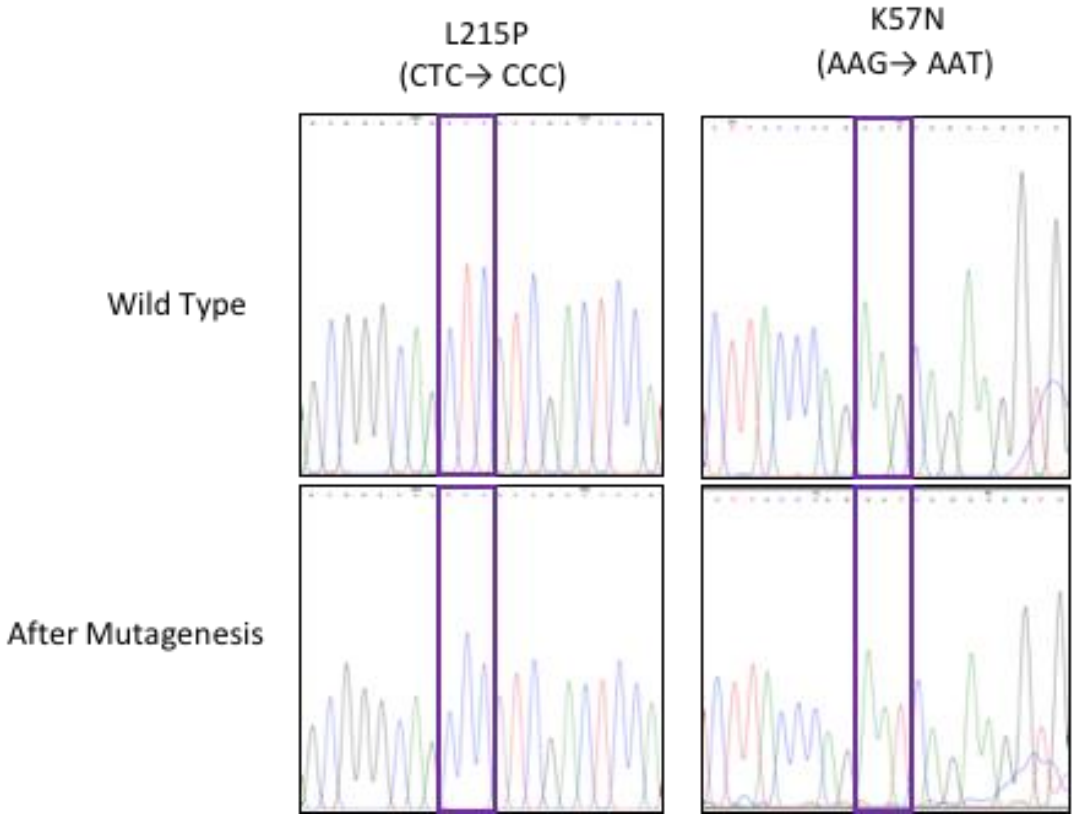


Figure 9. Dr. Alisa Damnernsawad. Confirmation of Mutagenesis through Sanger Sequencing. This figure shows the successful mutagenesis of the MEK1^{L215P} and MEK1^{K57N} genes. The CTC sequence was successfully converted to CCC which creates the MEK1^{L215P} mutation. The MEK1^{K57N} mutation substitutes asparagine for lysine at amino acid 57. This is successfully done by substituting G for T.

2.7.4 GFP+ Cell Sorting

These images show the results of the GFP+ cell sorting. Only the cells with GFP were shown under fluorescence. The wild type had 34.7% viable cells while the cells with the MEK1^{K57N} mutation had 43% of viable cells. Only the cells that successfully had been transduced with the lentiviral vector and the MAP2K1 variants.

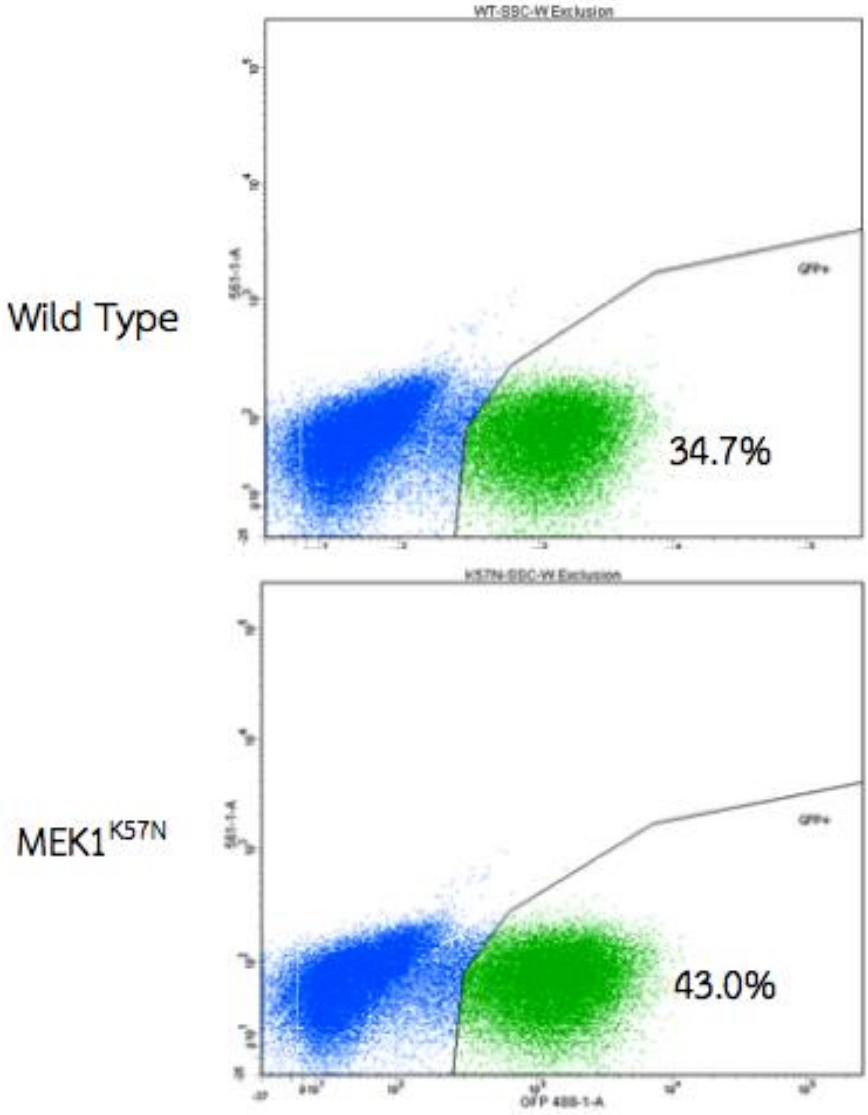
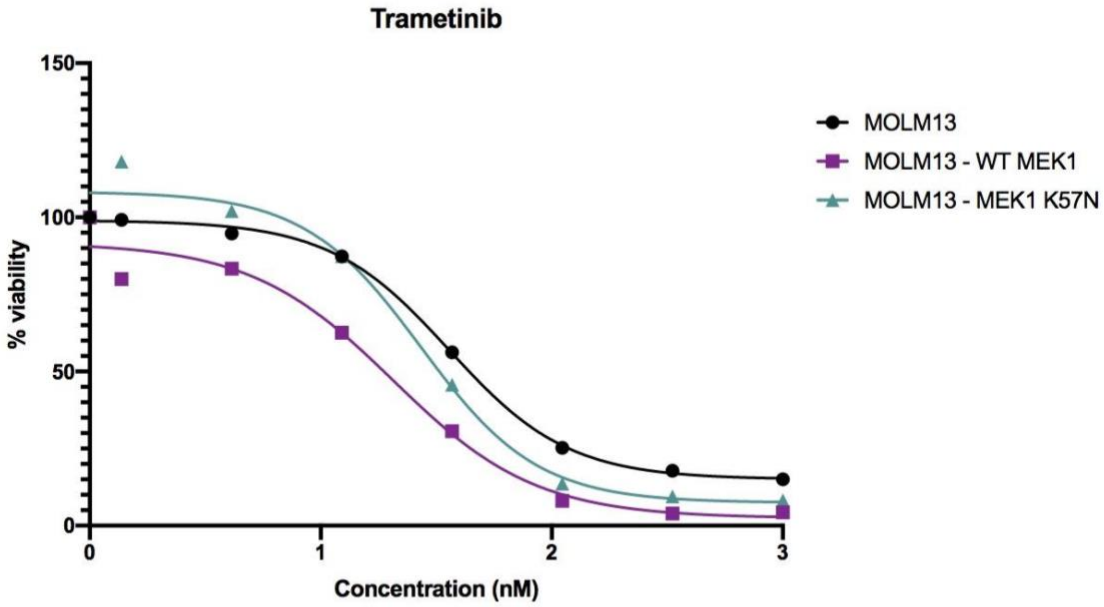


Figure 10: Dr. Alisa Damnernsawad. Viral Transduction Efficiency in MOLM13 Cells: Results of GFP Detection. From this data it was shown that there were enough viable cells successfully transduced.

2.7.5 Drug Sensitivity Assay

The drug sensitivity assay demonstrated that MOLM13 cells harboring MEK1^{K57N} have decreased sensitivity to trametinib in comparison to MOLM13 cells transduced with wild type MEK1. This indicated that MEK1^{K57N} confers resistance to trametinib in AML cells. The results for MOLM13 cells with MEK1^{L215P} were not successfully collected. There was not enough time to finish this portion of the experiment and will hopefully be done in the future.



	MOLM13	MOLM13 - WT MEK1	MOLM13 - MEK1 K57N
Total Area	175.2	122.4	168.3

Figure 11. Dr. Alisa Damnernsawad. Results of drug sensitivity assay, MOLM13 MEK1^{K57N} had higher viability than MOLM13 cells.

Chapter 3: Discussion and Future Directions

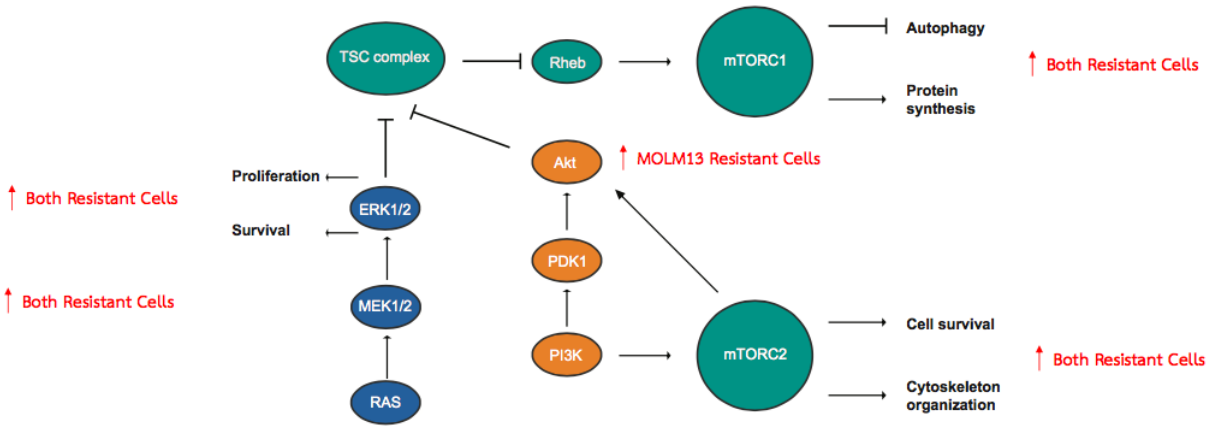


Figure 12. Dr. Alisa Damnersawad. MAPK Pathway. This shows the results of the Western Blot results in comparison to the MAPK, AKT, and mTORC1/2 pathways.

3.1 Western Blots: MAPK Pathway

The western blots revealed expected results in terms of the MAPK pathway. The upregulation of pERK in trametinib resistant cell lines shows that the MAPK pathway is hyperactivated in the presence of trametinib resistant cell lines. This would cause increased cell proliferation and, therefore, increasing cancer growth. In both resistant cell lines, there seemed to be an increase in the activity of pERK and pMEK. Shown in the above figure, MEK 1/2 activates ERK 1/2 through phosphorylation. Knowing this information could lead to knowledge of what to target in the resistant cells in order to re-sensitize them.

3.2 Western Blots: MTOR/AKT Pathway

MOLM13 trametinib resistant cells showed an increase in p-AKT, which can activate mTORC1, suggesting that up-regulation of mTORC1 activity in MOLM13 trametinib resistant cells might be partially enhanced by AKT activity.

3.3 Drug Sensitivity Assay

The results of the drug sensitivity assay showed that MOLM13 with the $MEK1^{K57N}$ showed greater survival than those cells without the mutation. The $MEK1^{K57N}$ cells were less sensitive than the wild type. This indicates that this mutation contributes to the drug resistance of trametinib. The experiment was not able to be finished with the $MEK1^{L215P}$ mutation due to time constraints. In the future this experiment should be completed to see if the $MEK1^{L215P}$ mutation also contributes to trametinib drug resistance in AML cells. Through this information future experiments can test whether the resistant cells exhibit a differential apoptotic rate. Lower apoptotic rates would indicate a contribution to the survival of cells. In addition to assessing the resistant cell rate of apoptosis, studying the effect on the rate of proliferation of cancer cells in MOLM13 and AML2 cells would also help further this investigation.

3.4 Conclusion and Future Directions

The work done in this experiment could lead to the use of trametinib as a targeted drug therapy for patients with acute myeloid leukemia. In order for a clinical trial to be done there would be many steps that would have to be taken before this. The first step would be to complete the experiment with the $MEK1^{L215P}$ variant. This would be able to give information on whether the $MEK1^{L215P}$ variant plays a role in the drug resistance as well as the $MEK1^{K57N}$ variant.

The knowledge of $MEK1^{K57N}$ contributing to the resistance towards trametinib could result in being a treatment selection tool. Physicians could test patients to see if they have this mutation and could only use trametinib on patients without this mutation.

Once it is established that these MEK mutations confer resistance to trametinib, the next goal would be to devise strategies to overcome or reverse mechanism of resistance. If successful, such efforts would enable the use of trametinib in these patients. A possible approach would be to target signaling downstream from the activated MEK or signaling in pathways that crosstalk with activated MEK. Another approach would be to achieve this would be to inhibit the transcription and/or translation of the mutated gene.

This first approach would involve testing Rheb to see if inhibiting Rheb would affect the MTOR

levels. Rheb is connected to the MAPK pathway through the TSC complex. The TSC complex has been found to be a tumor suppressor. It negatively regulates mTORC1 through being a GTPase activating protein, it activates Rheb, which is a small GTPase. The MTOR pathway plays a role in the regulation of cell apoptosis. From the Western Blots performed, both resistant cell lines were found to be upregulated by mTORC1. When activated, mTORC1 inhibits autophagy as well as activates protein synthesis. By finding a way to target and inhibit Rheb, trametinib could still potentially be used to treat AML even after some patients gain resistance.

In addition, testing re-sensitization of resistant cells to trametinib should be done. This could be done by potentially using the information gained from this experiment. If $MEK1^{K57N}$ is contributing to the resistance to Trametinib in AML cells, blocking this protein from activating would reverse resistance. Re-sensitization could be done at the protein level, using small molecule inhibitors or at the DNA and RNA level using shRNA or CRISPR-Cas9. Small molecules are an example of protein-protein interactions, small molecules can either bind allosterically, which is binding not at the active site of the protein or can bind orthosterically (27). These small molecules could block the activity of proteins encoded by the mutated DNA (27). shRNA is another way to re-sensitize the cells. shRNA can silence genes by having a 21-29 basepair strand that has the same sequence as the mRNA that is trying to be silenced (28). It then also has a loop 8 bases long and a complementary strand (28). CRISPR could also potentially be used. This is a system that can be used to alter DNA and therefore modify the gene function (29). This could be used to change the DNA of the trametinib resistant cells to eliminate the mutation that contributes to the resistance.

Methods to evaluate the effectiveness of the proposed interventions include assessment of apoptosis and of cell proliferation. Specifically, once one of these mechanisms is put into action the apoptotic rate could be tested. This would be done by using flow cytometry (30). This instrument will be able to accurately analyze the number of cells that have gone through apoptosis compared to the total number of cells (30). A high apoptotic rate would indicate that the re-sensitization technique used is being successful (30). Cell proliferation would also need to be tested to determine if the re-sensitization is succeeding and fewer cancer cells are being produced. This can be measured through multiple methods. One method is detecting lactate

dehydrogenase, when cells proliferate the amount of lactate dehydrogenase released increases and this can be detected using tetrazolium salts (30). Formazan dye will change color and then the absorbance can be read using a spectrophotometer and a microplate reader (30). This will measure the proliferative activity of the cells. Proliferative cells can also be analyzed by looking at antigens that are only released by cells that are proliferating. Measuring ATP concentration can also be a way to detect proliferation. ATP is not detected in dead cells and is strictly controlled in living cells (30). Bioluminescent luciferase and luciferin can be used, the luciferase will produce light and the intensity is proportional to the concentration of ATP (30). An ERK, MTOR, or AKT inhibitor could be used to test whether this could re-sensitize the resistant cells to trametinib (30). These inhibitors could potentially inhibit the resistance mechanisms. Also, we could test whether resistant cells lower the apoptotic rate, therefore increasing survival of the cells (30). Testing whether the resistant cells increase the proliferation of cancerous cells can also be done.

In order to use trametinib on patients, combination therapy could also be explored. ERK is directly downstream of MEK, a drug that inhibits ERK could be used in combination with trametinib so that is AML cells gain resistance to trametinib the downstream protein would still be inhibited. This would make sure that the cells still would not rapidly proliferate because ERK would be inhibited. The effects of inhibiting MEK as well as ERK would need to be more extensively explored to make sure it is safe. ERK and Akt both directly inhibit the TSC complex so the effect of inhibiting ERK would need to be explored further to make sure there are no other adverse effects on the human body.

Overall, the results of this project have given insight into the future of treating acute myeloid leukemia. It is shown that trametinib could possibly be a treatment of acute myeloid leukemia. It has shown that $MEK1^{K57N}$ plays a role in the trametinib resistance of MOLM13 cells. Further research should be conducted to find a way to circumvent this mutation to be able to use trametinib on acute myeloid leukemia patients.

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