The Effects of FLT3 Inhibition on FLT3-Mutated Acute Myeloid Leukemia Cells as a Potential Improved Treatment

By
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April 2024



ALLEGHENY COLLEGE

Allegheny College
Department of Biochemistry

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

_____
Pledge

Approval by the Senior Thesis Committee:

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Dr. Mahita Kadmiel

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Dr. Shaun Murphree

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Abstract

Acute Myeloid Leukemia (AML) affects nearly 500,000 individuals annually, making it a prominent avenue of study. About 30% of patients express a genetic insertion mutation within the FLT3 receptor (known as FLT3-ITD) responsible for regulating the hematopoietic process of these cells, worsening patient prognosis. Current treatments include tyrosine kinase inhibitors to eliminate the negative effects of this mutation, but they lack specificity for FLT3 and FLT3-ITD. A specific FLT3-ITD inhibitor (known as FLT3-IN-3) has been newly developed and would be an improved treatment option with less broad-spectrum activity. To begin examining FLT3-IN-3 as a treatment option, its half-maximal inhibitory concentration value (IC_{50}) and its ability to cause differentiation and apoptosis in FLT3-ITD-positive AML cancer cells need to be investigated. Three AML cell lines were utilized that ranged in their amount of FLT3-ITD expression: MV4-11 (homozygous for ITD), PL-21 (heterozygous for ITD), and NB-4 (no ITD, control). A hemocytometer was used to assess cell viability at FLT3-IN-3 concentrations between 1 nM and 10 uM. Results revealed an IC_{50} value of 300 nM. Cells were then treated at 300 nM for five days before being stained with markers to examine FLT3 expression (CD135), differentiation (CD11b), and apoptosis (phosphatidylserine). Stained cells were imaged with a fluorescent microscope on the Cytation 7 machine and analyzed with ImageJ software. Results revealed an increase in FLT3 expression, differentiation, and apoptosis in the MV4-11 cell line, an increase in apoptosis in the PL-21 cell line, and no change in the NB-4 cell line. These results suggest that FLT3-IN-3 could act as an improved treatment option for patients by providing an effective, specific treatment for FLT3-mutated receptors.

Graphical Abstract

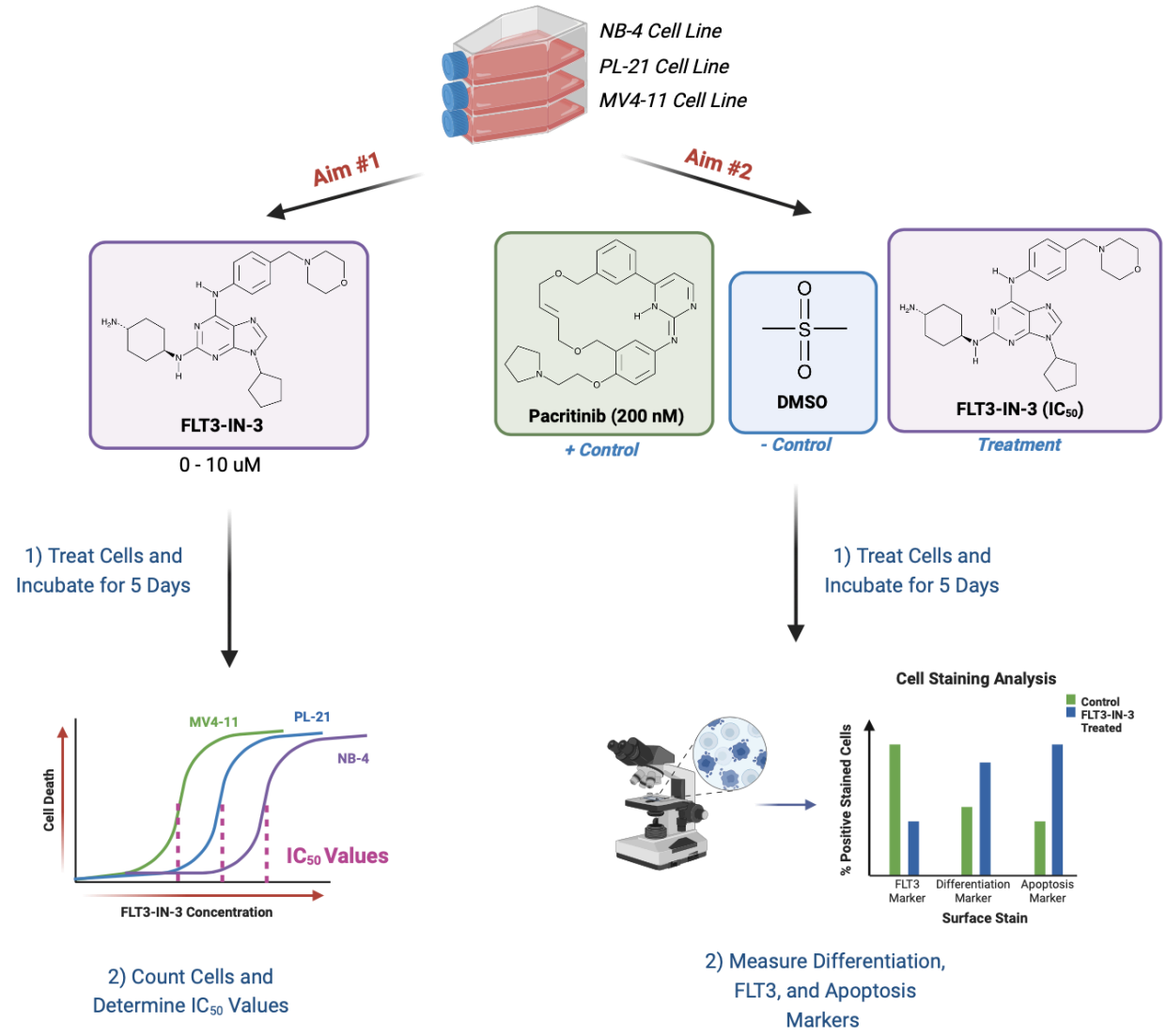


Figure 1. *Overview of COMP research project*. This project determined the IC50 concentration of the potent FLT3-ITD inhibitor FLT3-IN-3 (Aim 1) and examined its effects on FLT3 expression, cellular differentiation, and apoptosis in FLT3-ITD-containing cell lines (Aim 2).

Introduction

Acute Myeloid Leukemia

Cancer pertains to the abnormal growth of tissue within the body that leads to many side effects by preventing space for healthy tissue growth. These effects range in severity but could lead to the death of affected individuals and are, thus, an important avenue of study to determine both treatment and prevention options. Leukemia is a type of cancer that originates within the bone marrow and affects the hematopoietic development process.[1] Even more specific is Acute Myeloid Leukemia (AML), which makes up approximately 80% of all leukemia cases.[2] Acute Myeloid Leukemia is an accumulation of over 20% premature myeloid stem cells and is clinically characterized by three criteria: 1) uncontrolled cellular proliferation, 2) lack of differentiation of myeloid stem cells into mature immune cells, and 3) impaired production of healthy red and white blood cells.[3] The myeloid stem cells are unable to differentiate into mature immune cells through a process known as hematopoiesis.

AML, like almost all cancers, is currently treated through regimens of chemotherapeutic drugs that range in intensity based on the age, health, and strength of the patient.[4] Although the mean age of patients diagnosed with AML is 65 years old, treatment in younger and healthier patients (>60 years old) has an estimated 5-year survival rate of 30-35% which depletes with the increasing age of the patient.[5,4] However, due to the disease being characterized by the lack of differentiation, any mutations within the hematopoiesis process can severely worsen the prognosis of patients. Small-molecule inhibitors for those enzymes involved, such as fms-like tyrosine kinase 3 (FLT3) and isocitrate dehydrogenase (IDH), have proven to be effective. Inhibiting the B-cell leukemia protein 2 (BCL2) through the drug Venetoclax has also shown to be useful in treatment.[4] However, there is still more research to determine whether differentiating

agents used in the treatment of other leukemias such as Vitamin A in Acute Promyelocytic Leukemia (APL) can also be used to treat AML effectively.[4]

Vitamin A can come into the body and be metabolized in multiple different forms, two of which are important for this study: all-trans retinoic acid (atRA) and holo-retinol-binding protein (Holo-RBP). The active metabolite atRA is one of the most common forms and it has the capability of passing directly through the cell membrane to activate gene transcription.[15] However, when the body does not need Vitamin A, it begins to store it in different tissues such as adipose or liver tissue until it is needed.[15] When needed, the Vitamin A then gets secreted into retinol where it becomes bound to a retinol-binding protein and becomes holo-RBP.[15] Holo-RBP can then enter the cell through the STRA6 receptor to start a signaling cascade to ultimately activate the JAK2/STAT5 pathway and induce cell differentiation, as it does in APL.[15]

Hematopoiesis

Hematopoiesis describes the natural process of stem cells differentiating into healthy, mature blood cells through many signaling cascades and transcription factors. Myeloid stem cells, for example, undergo hematopoiesis to develop into different monocytes, megakaryocytes, neutrophils, red blood cells, etc., which are all important for maintaining physical health.[6] If this process is stalled the body will lack the mature immune cells that defend the body from illness and circulate nutrients. There will also be a much higher concentration of stem cells present that take over the bone marrow and prevent space for differentiated cells to thrive (**Figure 2**).

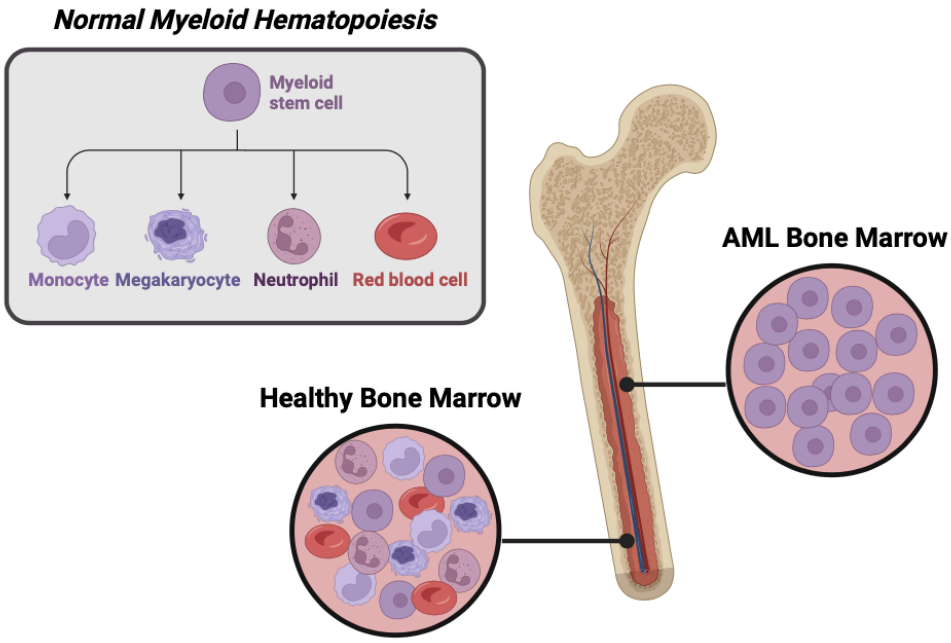


Figure 2. Representation of bone marrow in that of a healthy patient compared to a patient with AML.

Type III Tyrosine Receptor Kinases

An important piece to the hematopoietic process is the presence and activation of type III tyrosine receptor kinases, which mediate signaling pathways to activate differentiation.[7] These receptors are typically two dimers that undergo homodimerization when their ligand binds to their receptor. From there, the kinase phosphorylates the tyrosine residues on the substrate enzymes, allowing more enzymes to bind and a signaling cascade to begin until transcription factors are ultimately sent into the nucleus to start transcription (**Figure 3**). Through this activation, there are subsequent changes in the growth, differentiation, and apoptotic state of the cell.[8]

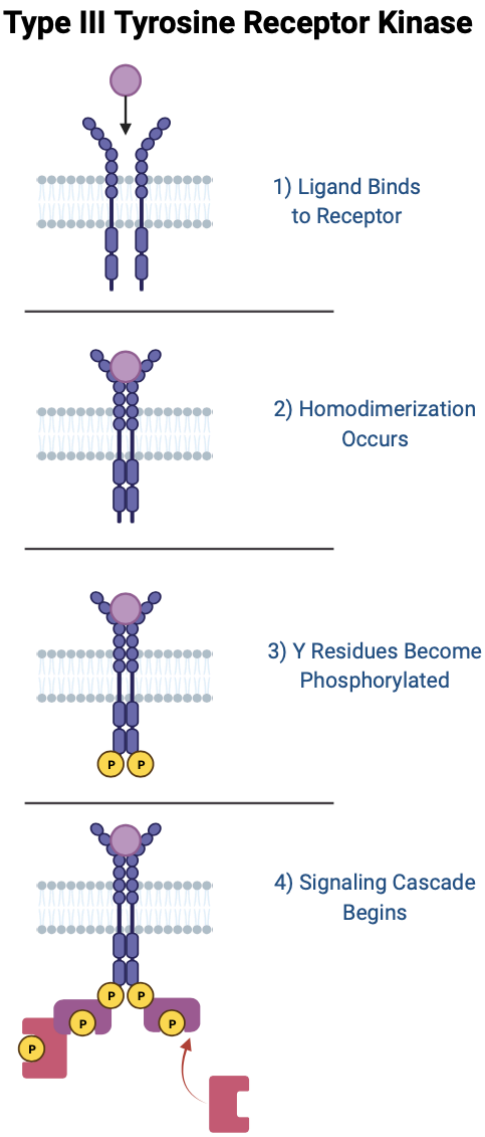


Figure 3. Mechanism of action of a typical Type III Tyrosine Receptor Kinase that acts through phosphorylating tyrosine residues and activating a signaling cascade.

FMS-like tyrosine kinase 3 (FLT3) is an example of a type III tyrosine receptor kinase and is only expressed in stem cells of the hematopoietic system.[7,6] FLT3 is responsible for promoting cell survival, proliferation, and differentiation through the PI3K, RAS, and JAK2/STAT5 signaling processes.[9] Since they control cell growth, they are heavily regulated and monitored by the cell.[10] However, FLT3 expression is limited to early progenitor cells.[11] Once

differentiated into mature erythrocytes and leukocytes, the receptor is no longer expressed on the surface of the cell.[10,11]

JAK2/STAT5 Pathway

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway acts as a control center for cells by leading to the activation of genes that produce several types of cytokines and growth factors.[12] There are four versions of JAK that all act as non-receptor tyrosine protein kinases, but are expressed in different areas of the body.[12] JAK2 is the JAK responsible for mediating STAT recruitment within the hematopoietic pathway. There are also several different STAT molecules that differ in structure and function, but STAT5 is the molecule responsible for regulating cell growth, differentiation, and apoptosis.[12] By putting the two together, the JAK2/STAT5 pathway is an essential component for the proliferation and differentiation of a cell since activation of this pathway results in the production of different cytokines, receptors, and hormones that all stimulate the growth and development of the cell in preparation for cell division.[12] Once the JAK2 protein is recruited by an activated receptor (such as FLT3), it then recruits a STAT5 molecule that will eventually become phosphorylated, dimerize with another STAT5 molecule, and migrate into the nucleus to activate transcription (**Figure 4**).

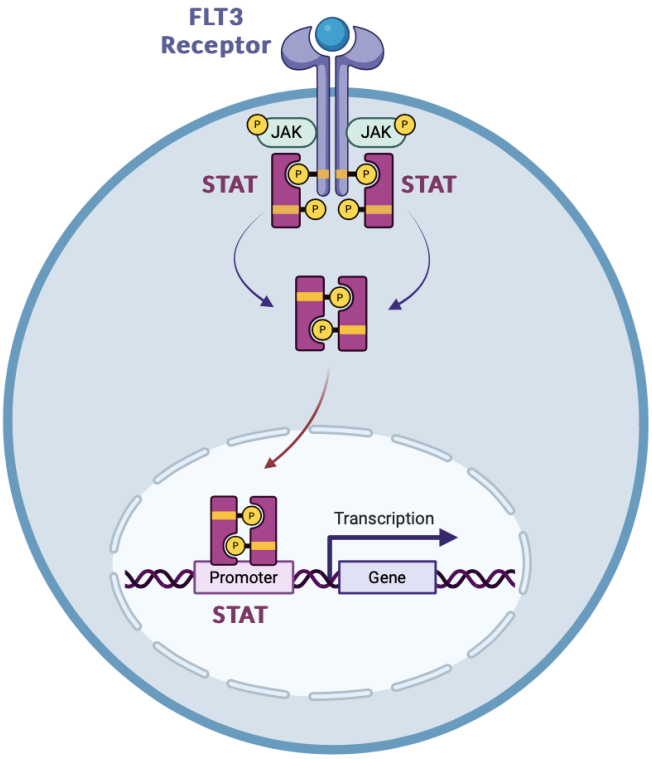


Figure 4. Representative mechanism of the JAK/STAT pathway activated through the ligand-dependent FLT3 receptor activating gene transcription for cell proliferation.

FLT3-Mutated Acute Myeloid Leukemia

FLT3 is of special interest in patients with AML due to the repercussions it has when mutated. In approximately 30% of all AML cases, patients have a mutated FLT3 receptor leading to a much-worsened prognosis.[9,13] There are two types of mutations patients can have: a point mutation or an insertion mutation.[10] The point mutation, commonly known as FLT3-TKD, is a missense mutation within the tyrosine kinase domain of the receptor and typically involves D835 and I836.[10] The insertion mutation is an internal tandem duplication mutation, commonly known as FLT3-ITD, and is the most common type of mutation making up approximately 80% of all FLT3-mutated AML cases.[10] This mutation, the focus of this project, ranges from 3-400 base pairs long and occurs within the juxtamembrane domain of the receptor.[6] When this insertion of

base pairs occurs, there is a shift in the polarity of the amino acid chains that causes a conformational change of the dimer. The new conformation allows for the two dimers to be held together through intermolecular interactions, which allows the receptor to be constitutively activated - meaning it is functional despite the binding of a ligand (**Figure 5**). There is no longer any ligand-dependent regulation of the receptor, allowing the JAK2/STAT5 and other pathways responsible for cell proliferation to run rampant and cause the cell to proliferate faster than it can differentiate.[14]

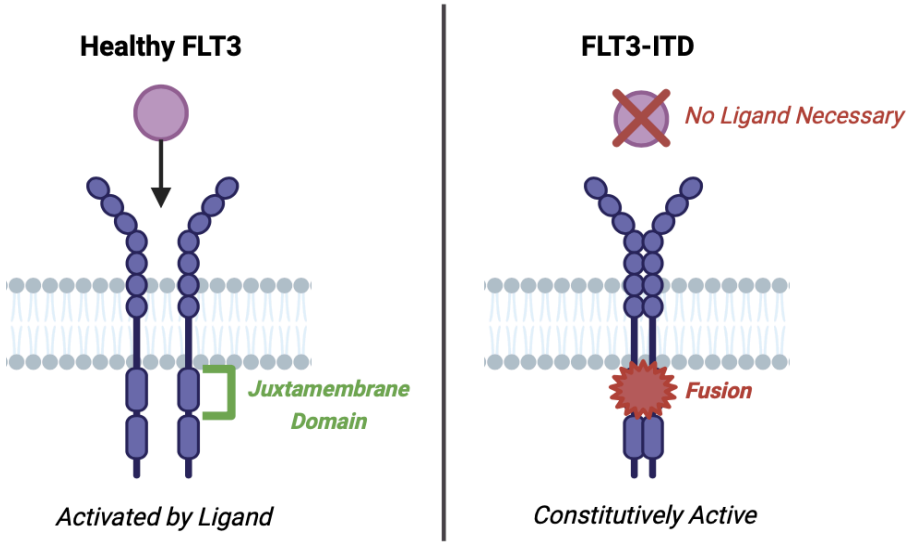


Figure 5. Representation of a healthy, non-mutated FLT3 receptor compared to one presenting with the internal tandem duplication (ITD) mutation within the juxtamembrane domain of the receptor.

A few current treatments are available to AML patients expressing FLT3-ITD to inhibit the FLT3 and aid in lessening the severity of the disease. They are commonly taken in conjunction with traditional chemotherapy drugs, but they range in specificity and potency. First synthesized were the first-generation inhibitors which are broad-spectrum multikinase inhibitors

that are not specific to tyrosine receptor kinases.[9] Later, second-generation inhibitors were found that are more specific to type III tyrosine receptor kinases, but not necessarily specific to FLT3.[9] There are also two different types of FLT3 inhibitors on the market: Type I inhibitors (i.e. Midostaurin and Gilteritinib) that work on both types of FLT3 mutations (point and insertion mutations) and Type II inhibitors (i.e. Quizartinib and Sorafenib) that work solely on FLT3-ITD.[9] However, more research is needed to increase specificity to the FLT3-ITD mutated receptors instead of all FLT3 receptors.

With the current drugs on the market, both wild-type and mutated FLT3 are affected causing the death of healthy cells and mutated cells, but there is another approach that deserves attention. If the mutated cells could instead be forced into differentiation rather than apoptosis they would lose the overactive FLT3 receptor, improving patient prognosis.

The main focus of this study was to test a new, highly specific FLT3 inhibitor that is claimed to be specific to FLT3-ITD at lower concentrations. We were optimistic that the removal of this overactive receptor would lead to either cell differentiation or apoptosis. These results would give us baseline data to investigate further the pathway by which an overactive FLT3 receptor harms the cell. This will be done by comparing the cellular response of cells treated with an FLT3 inhibitor to a JAK2 inhibitor while using a FLT3/JAK2 inhibitor (Pacritinib) as a control. By getting a better understanding of this mechanism, we can investigate in the future which aspect is more important to inhibit. It is known that the JAK2/STAT5 pathway is responsible for cell proliferation.[12] Thus, if inhibiting the FLT3 receptor that also inhibits the JAK2/STAT5 pathway results in more cell death than differentiation, we can investigate new approaches to supplement the cell with sufficient STAT5 to induce differentiation over apoptosis. Vitamin A is a potential avenue for study to complete this task since it activates the JAK2/STAT5

pathway, producing a source of STAT5 that does not stem from the faulty FLT3 receptor (**Figure 6**). Thus, it is hypothesized that if the cells are treated with both an FLT3 inhibitor and concentrated Vitamin A, we can induce differentiation and save the cells from undergoing apoptosis.

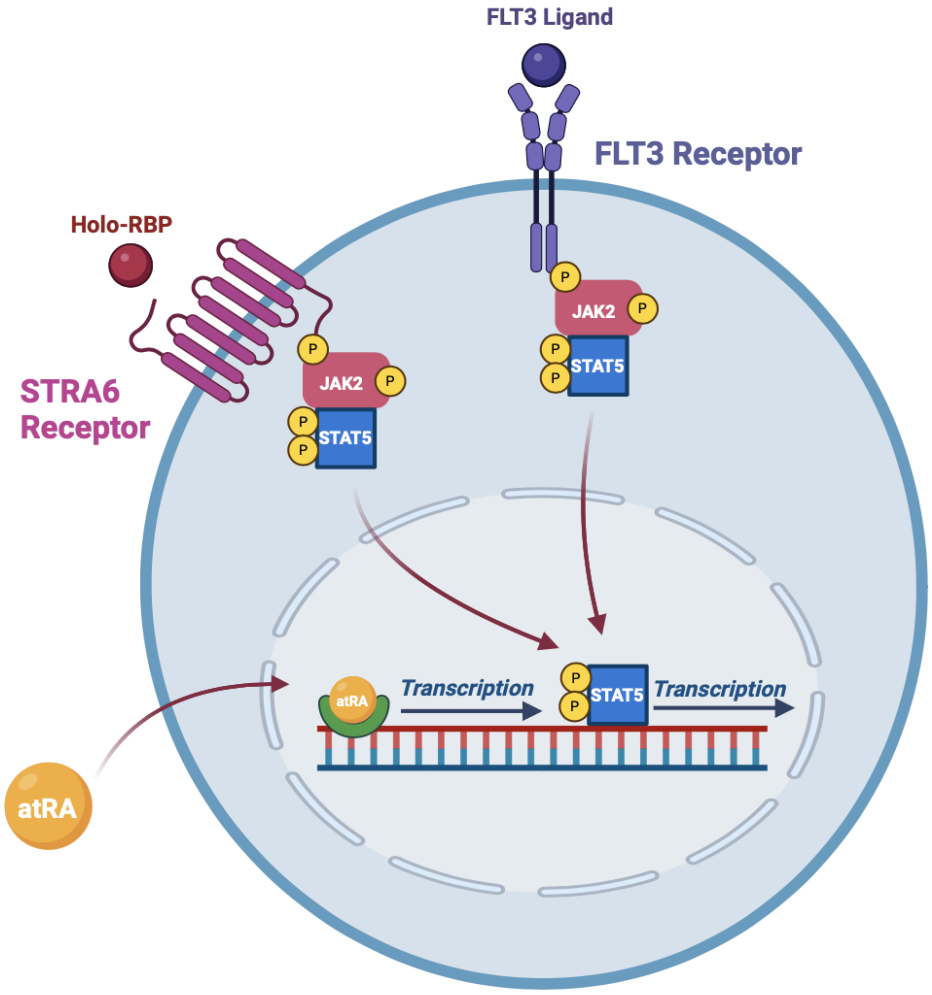


Figure 6. Representative FLT3 and STRA6 receptor mechanisms activating cellular transcription through the JAK/STAT pathway. Two types of vitamin A are present: Holo-RBP and atRA. Holo-RBP activates JAK/STAT through the STRA6 receptor and atRA directly enters the cell to bind to a transcription element within the nucleus, starting transcription.

Study Objectives

In this study, three different cell lines were utilized: NB-4, PL-21, and MV4-11.[16, 17,18] All three cell lines vary in their amount of FLT3-ITD ranging from only mutated to only non-mutated FLT3 (**Table 1)**.[19] Between these three cell lines, we can make comparisons between the true specificity of FLT3-IN-3 to FLT3-ITD in AML cell lines. However, it is important to note that all three cell lines were obtained from different patients with genetically different backgrounds. There may be other genetic factors contributing to any results we find.

Table 1. List of Acute Myeloid Leukemia cell lines with varying levels of the FLT3-ITD mutated gene used in the current study.

Cell Line	Zygosity of FLT3-ITD Gene Mutation
MV4-11 *(Lange et al. 1987)*	Homozygous
PL-21 *(Kubonishi et al. 1984)*	Heterozygous
NB-4 *(Lanotte et al. 1991)*	Not Present

To effectively inhibit FLT3, a highly specific inhibitor developed in the last few years named FLT3-IN-3 was used to treat all three cell lines. A dosage to accurately inhibit approximately 50% of the cells was found by measuring cell viability via a hemocytometer. According to a study performed by Gucky et al.[20] when the inhibitor was developed from a 2,6,9-trisubstituted purine scaffold, they found that concentrations under 1 nM were effective for only inhibiting FLT3-ITD and not the wild-type FLT3. Thus, 1 nM was the starting point of our experiment, but a range of up to 10 uM was examined. On the fifth day of treatment at the determined concentration, cells were stained to be able to determine changes in FLT3 expression,

differentiation, and apoptosis. A fluorescent microscope was used to image the stain cells and images were analyzed to make comparisons between treatments across all three cell lines. For comparison with a similar project being completed with a JAK2/STAT5 inhibitor, a FLT3/JAK2/STAT5 inhibitor known as Pacritinib was also used to treat cells at a concentration of 200 nM as suggested by Hart et al.[21]

It is logical that a mutation such as FLT3-ITD that causes even more frequent proliferation in a disease characterized by uncontrollable growth is problematic. Finding viable treatment options is a concern to researchers, physicians, and patients.

Materials and Methods

Cell Culturing

Three underived, immortalized Acute Myeloid Leukemia cell lines were obtained from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH and utilized in this study: MV4-11, PL-21, and NB-4. Cell lines were grown in a 37℃ incubator under the presence of 5% CO_2 and routinely cultured in T-75 flasks with complete RPMI medium (10% heat-inactivated fetal bovine serum (#16140071, ThermoFisher), 5% penicillin-streptomycin (10,000U/mL, #15140122, Thermo Fisher), and 5% sodium pyruvate (100mM, #11360070, ThermoFisher). Cells were passed every 3-4 days when confluence was reached.

Inhibitor and Control Preparation

FLT3-IN-3 inhibitor (10 mg, #36106, Cayman Chemicals) was dissolved in 1 mL of DMSO to result in a 20.3 mM FLT3-IN-3 solution. A 1 mM stock solution was made by adding 20.3 mM FLT3-IN-3 to DMSO, but this led to the treatment effects of DMSO to

overpower the effects of the inhibitor. Thus, a 1 mM stock solution was made by adding 20.3 mM FLT3-IN-3 to cell culture-grade sterile water. A 1 uM stock was also made from the 1 mM stock at the start of each experiment. DMSO controls were created by adding the same amount of DMSO present in the corresponding FLT3-IN-3 treatment to RPMI media. Pacritinib (1 mg, #16709, Cayman Chemicals) was dissolved in 1 mL of DMSO to result in a 1.058 mM Pacritinib stock solution.

Cell Viability

Cells were treated with FLT3-IN-3 for five days at various concentrations. Cells were seeded at 150,000 cells per well in approximately 1 mL of complete RPMI media in either 12 or 24-well plates. Originally, cells were mixed thoroughly on days two through five, and samples were collected from each well for counting. This process resulted in cell death. Following that information, wells were only disturbed on day five for sample collection. Once samples were collected, cells were stained with 0.4% Trypan blue dye (# 15250061, ThermoFisher). Live, dead, and total cell counts were recorded from an automated hemocytometer (Invitrogen Countess™ 3, #AMQAX2000, ThermoFisher) (**Figure 7**). Cell counts were analyzed to determine the half-maximal inhibitory concentration (IC_{50}) of FLT3-IN-3 with a focus on its effects on the MV4-11 cell line.

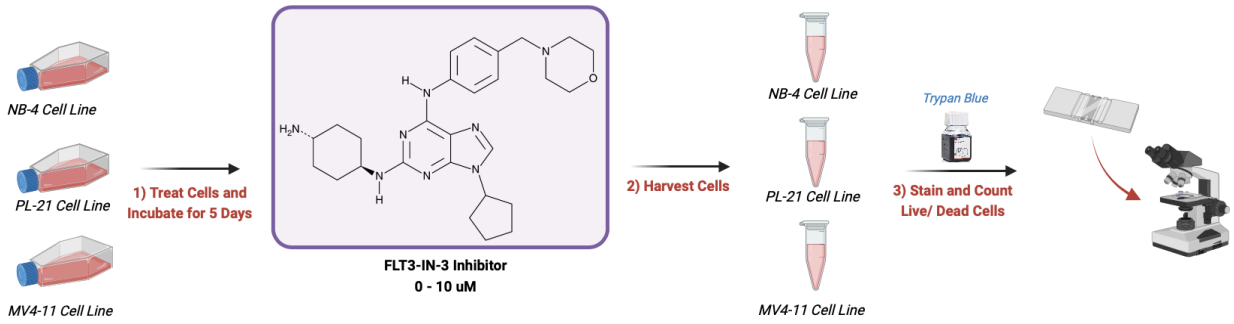


Figure 7. *Experimental Design for Aim 1.* All three cell lines were treated with FLT3-IN-3 inhibitor dissolved in DMSO and sterile grade H_2O at various concentrations between 0 and 10uM. Cells were then incubated for five days before determining viability using a hemocytometer until an IC_{50} value was found.

Cell Surface Staining

Cells were treated at the IC_{50} FLT3-IN-3 concentration determined through the cell viability procedure. After five days, cells were harvested to be stained for FLT3, differentiation, apoptosis, and cell death markers for fluorescent imaging (**Figure 8**). For FLT3 expression: cells were stained for the FLT3 marker, CD135, using 3% PE dye (#12-1351-82, ThermoFisher). For differentiation: cells were stained for a neutrophil marker, CD11b, using 0.5% PE-ef610 dye (#61-0112-82, ThermoFisher). The typical staining procedure for surface stains was followed for the PE and PE-ef610 dyes with an incubation period of 30 minutes on ice (protected from light) followed by two washes with RPMI media. For apoptosis: cells were stained for phosphatidylserine using 1% Pacific Blue Annexin V dye (#640926, BioLegend). For cell death: cells were stained for double-stranded DNA using 2% propidium iodide dye (PI, #P1304MP, ThermoFisher). The typical staining procedure for Annexin V and PI from Biolegend's Pacific Blue Annexin V Detection Kit was followed.

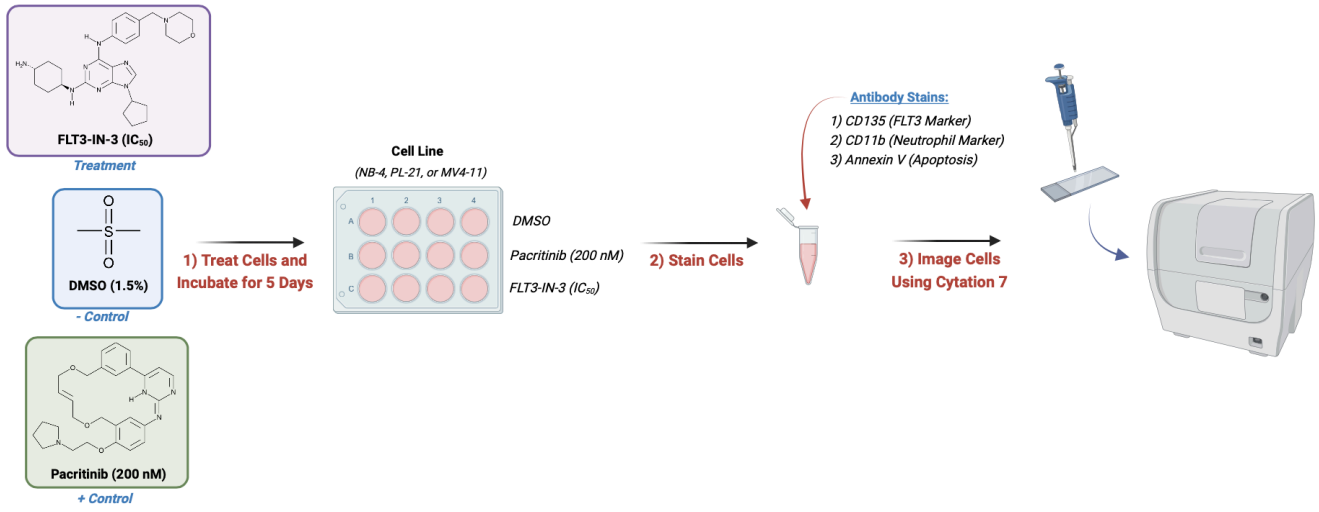


Figure 8. *Experimental Design for Aim 2.* All three cell lines were treated with the FLT3-IN-3 inhibitor dissolved in FLT3-IN-3 (IC_{50}), DMSO (0.3%), or Pacritinib (200 nM), allowed to incubate for five days, stained for FLT3, differentiation, and apoptotic markers, and imaged via a Cytation 7.

Imaging and Analysis

For image preparation, samples were centrifuged at 1500 RPM for 5 minutes at 4℃. The supernatant was poured off and pellets were resuspended in residual media for increased cell concentration. For each sample, 50 uL was placed on a positively charged glass microscope slide and inserted into the BioTek Cytation 7 Cell Imaging Multi-mode Reader by Aligent. Images of the brightfield and fluorescence were taken of each sample. Appropriate lasers were used to obtain fluorescent images based on the stain (**Table 2**).

Table 2. List of each cellular surface stain used, the wavelength of the laser used to obtain fluorescence, and the color of the laser on the Cytation 7 machine.

Cellular Stain	Laser Wavelength (nm)	Laser Color
PE (CD135)	488	Green
PE-ef610 (CD11b)	488	Green
Annexin V	405	Blue
PI	594	Red

Brightfield and fluorescent images of cells were analyzed and compared through the ImageJ software utilizing the Macro and Batch tools. ImageJ allowed for a count of cells to be taken on both the brightfield and the stained image to be able to calculate the percentage of the total amount of cells that were positive for the stain used.

Results

Half-Maximal Inhibitory Concentration of FLT3-IN-3

Cell viability at various concentrations of FLT3-IN-3 was examined to determine the half-maximal inhibitory concentration of the drug. Cells were cultured in either 12 or 24 well plates in 1 mL of media. Concentrations between 0 and 4 nM were prepared and wells were mixed thoroughly to take samples for counting on days 2-4. This procedure resulted in cell death and gave what appeared to be the IC_{50} around 1 nM at day 5 (**Figure S1**). A similar experiment with collecting samples on days 4 and 5 resulted in a new proposed IC_{50} value of around 5 nM (**Figure S2**). However, when cells were undisturbed until day 5, an analysis of cell viability revealed that cells were only 10% dead at 5 nM leading to the conclusion that disturbance of these cells resulted in cell death.

Given this information, cells were treated with concentrations between 1 nM and 35 nM FLT3-IN-3 and left untouched until collection and counting on day 5 (**Figure 9**). Observation of this data led to a proposed IC_{50} value between 13-15 nM. However, control samples of only DMSO that lacked FLT3-IN-3 inhibitor resulted in similar amounts of cell death. This finding led to the hypothesis that any effects seen in the treatment group were caused by the DMSO that FLT3-IN-3 was dissolved within. Further dilution of the solvent was needed.

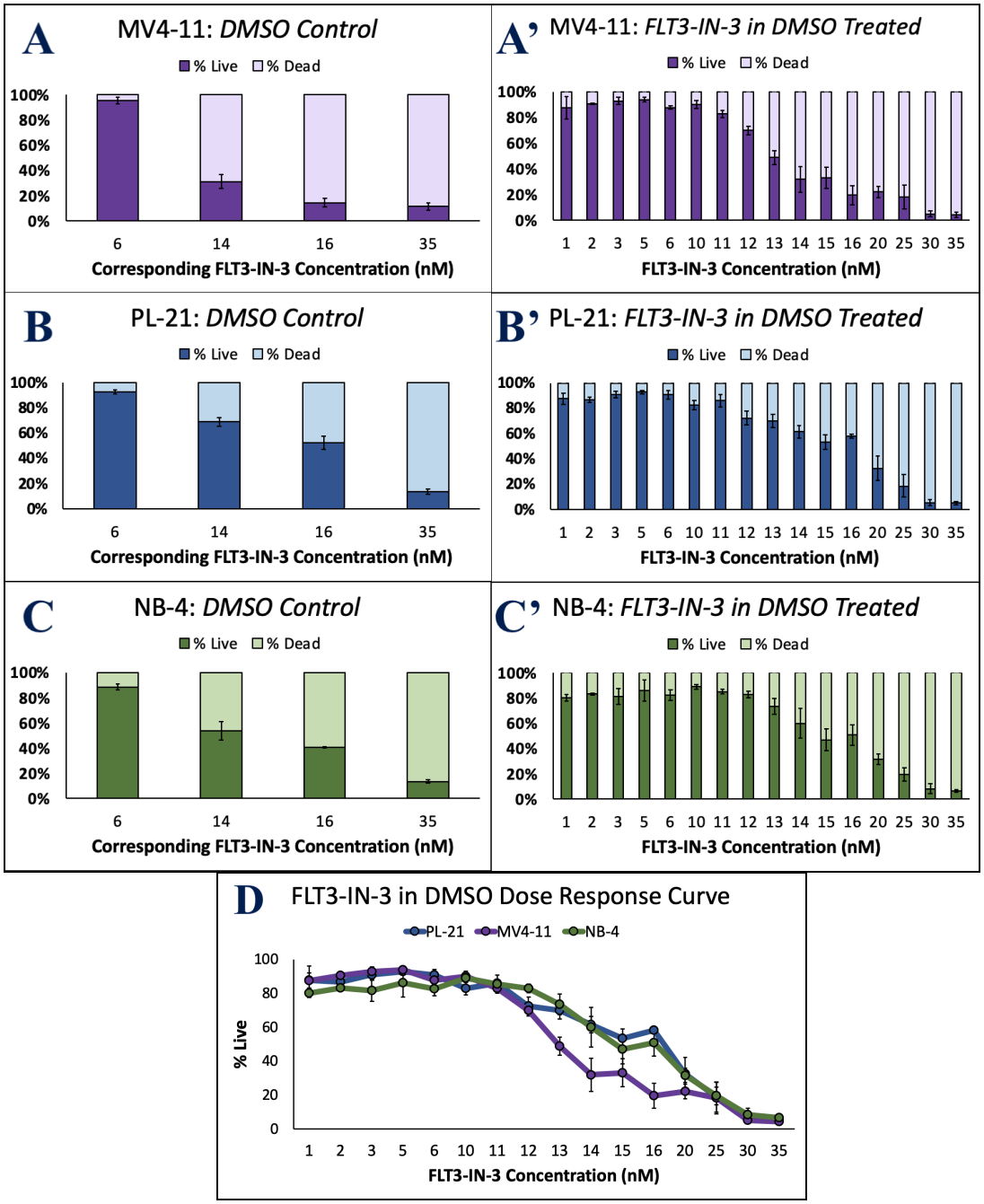


Figure 9. *Summary of Experimental Data Collected Utilizing FLT3-IN-3 in DMSO.* All three cell lines were treated with various concentrations of FLT3-IN-3 ranging from 1 nM to 35 nM throughout four different experiments. Each treatment had between 2 and 4 replicates. Error bars represent the standard deviation between samples. Control samples were run with DMSO at four concentrations that corresponded to the 6, 14, 16, and 35 nM FLT3-IN-3 treatments, and graphs are shown on the left side **[A, B, C]**. **The y-axis of the left-hand graphs shows the FLT3 concentrations that correspond to the treatment conditions for ease of the eye, but actual DMSO concentrations were 0.6%, 1.4%, 1.6%, and 3.5%**. Treatment graphs are shown on the right side **[A', B', C']**. A dose-response curve **[D]** is shown at the bottom of the figure for easier visualization of the IC_{50} around 12-13 nM. Each cell line is color-coated as follows: **[A, A']** MV4-11 = purple, **[B, B']** PL-21 = blue, and **[C, C']** NB-4 = green, and a darker shade represents live cells whereas the lighter shade represents dead cells.

1 mM and 1 uM stock solutions of FLT3-IN-3 were recreated from the original 20.3 mM stock bottle using cell culture-grade water instead of DMSO as a dilutent. FLT3-IN-3 concentrations of 1 nM to 10 uM were investigated in an attempt to determine the range of the IC_{50} value for FLT3-IN-3 (**Figure 10**). Observation of this data revealed that the value must be around 300 nM for both the MV4-11 and PL-21 cell lines and between 400 and 500 nM for the NB-4 cell line. Since priority was given to the MV4-11 cell line for its homozygosity in the ITD mutation, an IC_{50} value of 300 nM was chosen to investigate the ability of the drug to induce differentiation and apoptosis.

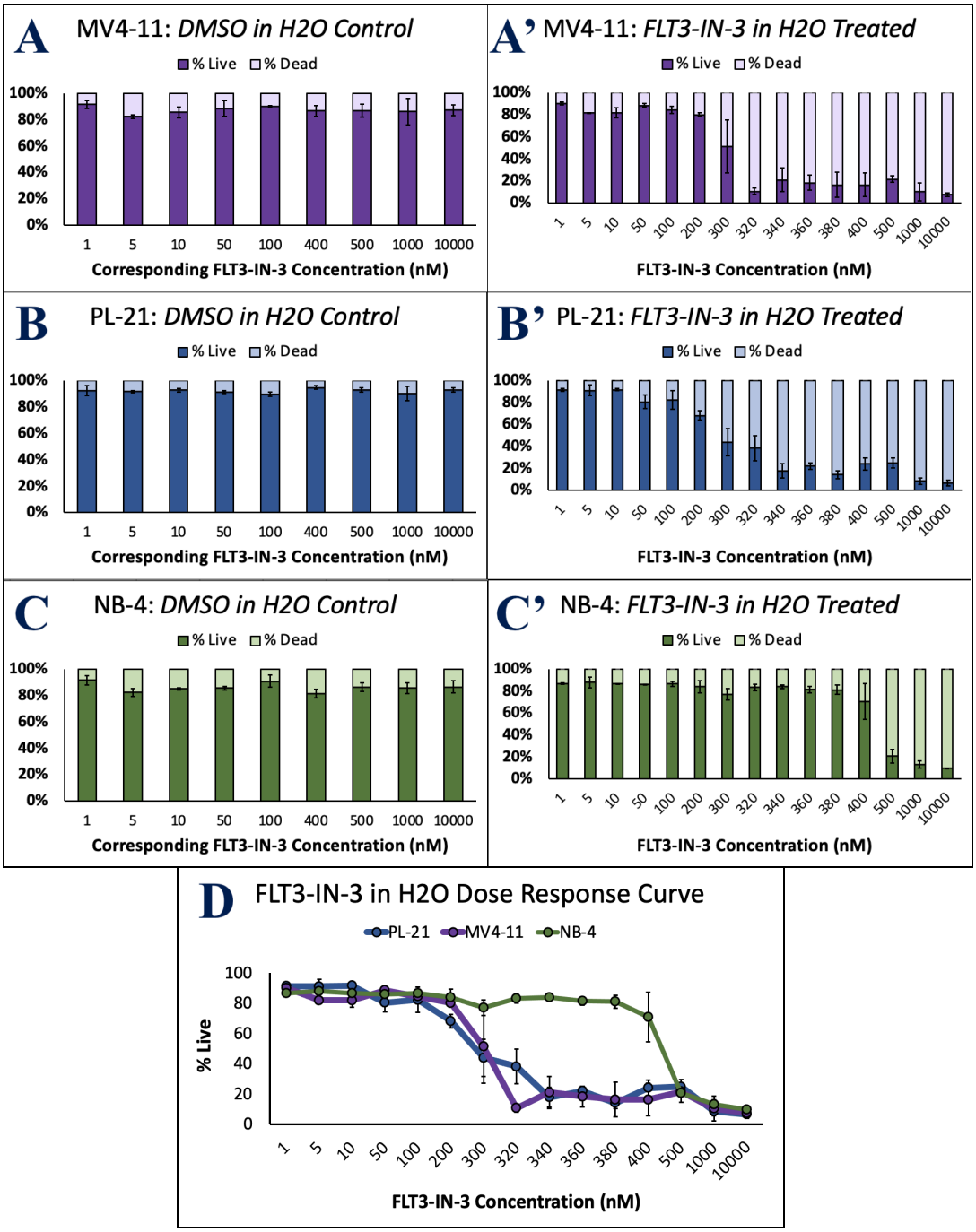


Figure 10. *Summary of Experimental Data Collected Utilizing FLT3-IN-3 in H_2O.* All three cell lines were treated with various concentrations of FLT3-IN-3 ranging from 1 nM to 10 uM. Each treatment was performed in duplicates. Error bars represent the standard deviation between samples. Control samples were run with DMSO in H_2O at each corresponding concentration of FLT3-IN-3 treatments and graphs are shown on the left side **[A, B, C]**. **The y-axis of the left-hand graphs shows the FLT3 concentrations that correspond to the treatment conditions for ease of the eye, but actual DMSO concentrations were 0.0001%, 0.0005%, 0.001%, 0.005%, 0.01%, 0.04%, 0.05%, 0.10%, and 1.0%.** Treatment graphs are shown on the right side **[A', B', C']**. A dose-response curve **[D]** is shown at the bottom of the figure for easier visualization of the IC_{50} around 12-13 nM. Each cell line is color-coated as follows: **[A, A']** MV4-11 = purple, **[B, B']** PL-21 = blue, and **[C, C']** NB-4 = green, and a darker shade represents live cells whereas the lighter shade represents dead cells.

FLT3-IN-3 Effects on FLT3 Expression, Cellular Differentiation, and Apoptosis

To determine the ability of FLT3-IN-3 to induce cellular differentiation and apoptosis, all three cell lines were treated with the drug at the experimentally determined IC_{50} concentration of 300 nM and allowed to incubate for 5 days. Cells were then harvested and stained with markers of either FLT3 expression, differentiation, apoptosis, or cell death, and imaged using a fluorescent microscope (**Figure 11**). Cell lines were also treated with an equivalent amount of DMSO used in the dissolution of the FLT3-IN-3 inhibitor (0.3% DMSO) to act as a control since literature suggests that DMSO could cause cellular differentiation in certain cell lines (Wang et al 2020). Cells were also treated with Pacritinib at a concentration of 200 nM to act as a positive control since literature shows it causes differentiation and apoptosis in acute myeloid leukemia cells (Hart et al 2011).

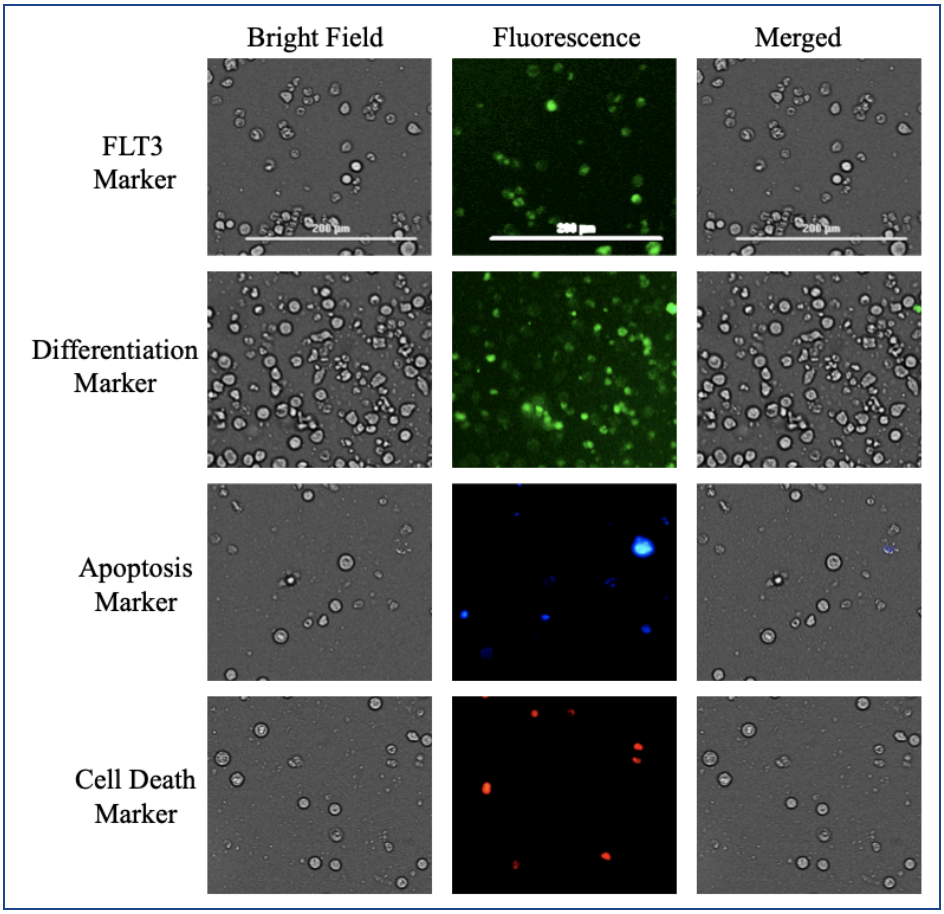


Figure 11. *Representative Images of Stained PL-21 Cells in Negative DMSO Control.* PL-21 cells were treated with 0.3% DMSO (negative control). Cells were stained with either PE surface-stain dye that bound to the CD135 marker present on cells that possess the FLT3 receptor, PE-ef610 surface-stain dye that bound to the CD11b marker present on cells that have differentiated into neutrophils, Pacific Blue Annexin V surface-stain dye**,** or Propidium Iodide, a double-stranded DNA stain.

In terms of FLT3 expression, the MV4-11 cell line showed a potential increase in the FLT3 marker with both FLT3-IN-3 and Pacritinib treatment compared to the DMSO control (**Figure 12A).** For the PL-21 cell line, FLT3 expression remained relatively the same when treated with FLT3-IN-3 and increased in the Pacritinib treatment. The NB-4 data showed that there was no change in FLT3 expression across any treatments.

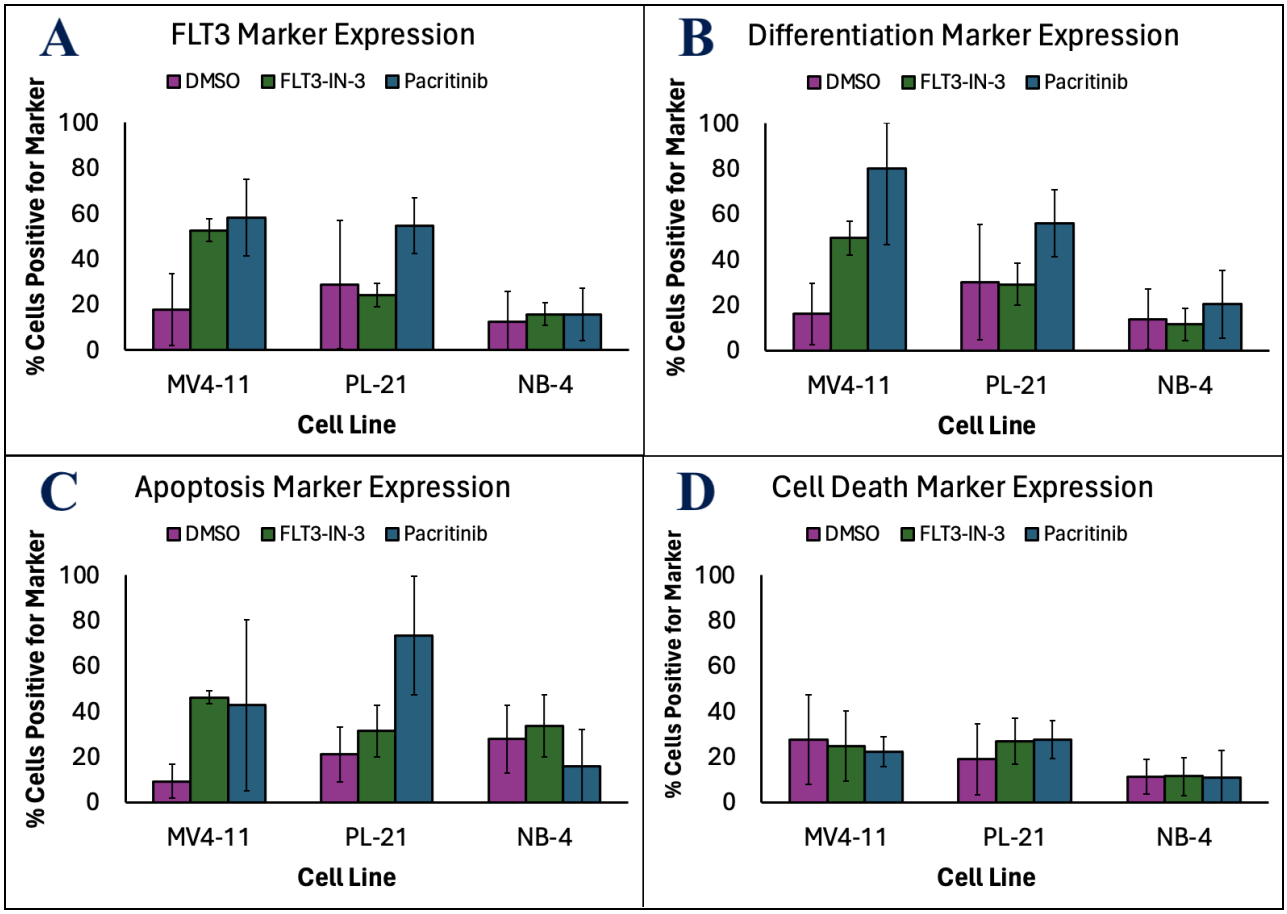


Figure 12. *Summary of FLT3 Marker Expression in MV4-11, PL-21, and NB-4 Cell Lines Treated with FLT3-IN-3.* All three cell lines were treated with 300 nM FLT3-IN-3, 0.3% DMSO (negative control), and 200 nM Pacritinib (positive control). Cells were stained with either **[A]** PE surface-stain dye that bound to the CD135 marker present on cells that possess the FLT3 receptor, **[B]** PE-ef610 surface-stain dye that bound to the CD11b marker present on cells that have differentiated into neutrophils, **[C]** Pacific Blue Annexin V surface-stain dye**,** or **[D]** Propidium Iodide, a double-stranded DNA stain. The percentage of positive cells was calculated through the number of positive cells in the 488 nm (green) laser divided by the total cells in the brightfield image determined by the ImageJ software. The data shown is pooled from 3 experiments, each performed in duplicates. Error bars represent the standard deviation between all six samples. Each treatment is color-coated as follows: DMSO = purple, FLT3-IN-3 = green, and Pacritinib = blue.

The results of the differentiation marker expression for the MV4-11 cell line showed an increase in differentiation in comparison with the DMSO control when treated with both FLT3-IN-3 and Pacritinib (**Figure 12B**). Pacritinib treatment, however, seemed

to increase differentiation roughly ⅓ more than FLT3-IN-3 treatment. For the PL-21 cell line, results were similar to those from the FLT3 expression: FLT3-IN-3 treatment did not cause a change in differentiation whereas Pacritinib produced about a 2-fold increase in differentiation. The NB-4 cell line also produced similar results to those of the FLT3 expression where differentiation was the same throughout all treatments.

Regarding apoptosis, the MV4-11 cell line showed approximately a 3-fold increase in both the FLT3-IN-3 and Pacritinib treatments (**Figure 12C**). The PL-21 cell line resulted in a small increase with FLT3-IN-3 treatment and a 2-fold increase with Pacritinib treatment. The NB-4 cell line remained similar through all treatments and showed no distinctive changes. To control for any cell death that occurred apart from apoptosis, cells were stained with propidium iodide to detect any cell with a destroyed membrane to act as a control against the apoptosis marker expression. These results showed that there was consistent cell death across treatments in all three cell lines (**Figure 12D**).

Discussion

Treatment of FLT3-IN-3 for five days reached its half-maximal inhibitory concentration value at 300 nM on ITD-mutated AML cell lines. Treatment at this dose resulted in a potential increase in FLT3 expression, cellular differentiation, and apoptosis. The increase in FLT3 expression was unexpected but may provide relevant information on the importance of the FLT3 receptor or the necessity of STAT5 for cell survival. Differences seen in cell lines lend support that the inhibitor successfully inhibits only FLT3-ITD at low concentrations and loses specificity as concentration increases. It was also seen through experimentation that these AML cell lines may undergo cell death from physical disturbances.

When originally performing the IC_{50} experiments, there was 50% cell death from a 1 nM concentration of FLT3-IN-3 when wells were vortexed daily to collect samples, just as seen by Gucky et al.[20] However, when wells were only disturbed on days four and five post-treatment, 50% cell death was seen from a 5 nM concentration of FLT3-IN-3. When wells were then left undisturbed until day five of treatment, it took a concentration of 300 nM to result in 50% cell death in the MV4-11 cell line. Thus, it is likely that disrupting the cells caused their death. This finding makes sense, however, when you consider the natural communication and clustering effects of differentiating neutrophils from hematopoietic stem cells.

These stem cells require influxes of molecular signals such as cytokines and hormones to be able to both proliferate and differentiate into their mature residential immune cell state.[22,23] These signals can be within the medium and act from a long distance, or they can be given through cell-cell contact.[23] As the stem cells begin to proliferate and differentiate into more mature hematocytes they begin to form cellular clusters that aid in the process known as feedback control - where the differentiated cells can communicate to their precursors to ensure they all differentiate into the proper tissues.[23,24,25] A study performed by Dao et al.[26] noted the presence of these clusters and identified stem cells, neutrophils, macrophages, and eosinophils within them in a culture of human bone marrow cells providing evidence that this feedback control is an important aspect of cellular differentiation and proliferation.[26] Thus with how important these clusters would be for both proliferation and differentiation, it would be a logical explanation to assume that breaking up these communication clusters regularly would have significant implications for the cell's survival.

Given the differences within the IC50 value and the staining results of the MV4-11 cell line compared to those of the PL-21 and NB-4 cell lines, we can conclude that the FLT3-IN-3

inhibitor is specific to FLT3-ITD receptors at low concentrations just as claimed by Gucky et al.[20] By looking at the IC50 curve in **Fig. 10**, it's evident that MV4-11 and PL-21 reach 50% cell death around 300 nM whereas the NB-4 cell line, which contains only non-mutated FLT3 receptors, doesn't have any cell death until around 450 nM. Within the staining data to examine changes in FLT3 expression, differentiation, and apoptosis, there is also a noticeable difference. The homozygous MV4-11 cell line has the most changes from the DMSO negative control compared to FLT3-IN-3 treatment with clear increases in all three of those factors. The heterozygous PL-21 cell line, however, has only a potential increase in the number of cells undergoing apoptosis. As for the NB-4 cell line, there are practically no changes with FLT3-IN-3 treatment. With the differences within both the IC50 data and the staining data, it's plausible that the inhibitor only acts on FLT3-ITD. However, no other research to date has examined this observed specificity besides Gucky et al in their development of the drug.[20]

The increase in FLT3 expression seen within the MV4-11 cell line was an unexpected yet interesting finding. It appeared that treatment with the FLT3-IN-3 inhibitor caused an increase in the amount of positive-stained cells compared to the negative DMSO control. It is not immediately logical for an inhibitor of a protein to increase that protein's expression, but this result may speak to the importance of either the FLT3 receptor and the other pathways it regulates (PI3K and RAS pathways), or of the STAT5 molecule, especially since the same result was seen in the positive control treatment of Pacritinib, a FLT3/JAK2/STAT5 inhibitor.

As mentioned previously, the FLT3-ITD receptor signals through three main pathways: the JAK2/STAT5 pathway, the PI3K pathway, and the RAS pathway.[27] The PI3K pathway is mainly responsible for regulating cell survival through apoptosis and growth signaling.[28,29] The RAS pathway is mainly responsible for regulating the growth and proliferation of the cell by

regulating steps along the cell cycle.[30,31] However, these pathways get altered when FLT3 activation is constitutive. Literature suggests that the PI3K and RAS pathways have a heavy influence on the progression of cancer by producing anti-apoptotic factors that inhibit apoptosis and push the cell into proliferative cycles.[27] Chen et al.[32] hypothesize that constant activation of these pathways results in the production of reactive oxygen species that proceed to oxidize different tumor suppressor genes that aid in leading the cell to proliferation instead of apoptosis. If these pathways are as important for the survival of the cell as the literature states, it would make sense for the cell to try to increase the number of FLT3 receptors to increase the anti-apoptotic and proliferative signals it gains from the constitutive activation of the PI3K and RAS pathways.

The importance of STAT5 has been expanded on previously for its ability to activate transcription of different cytokines and growth factors leading to the growth, proliferation, and differentiation of the cell.[12] In hematopoiesis especially, STAT5 is necessary for the cell to interpret whether it should keep proliferating or proceed to terminal differentiation.[33] However, within the FLT3-ITD signaling pathway, this influx of STAT5 leaves the cancer cell unable to differentiate appropriately nor undergo apoptosis to obtain healthy numbers of each mature blood cell.[33,34] For similar reasons to the PI3K and RAS pathways, the cell could be increasing in FLT3 receptor expression to be able to produce more STAT5 since it is such an essential piece to the survival and growth of the cell. This theory can be examined further when FLT3-IN-3 is part of a co-treament with Vitamin A where the cell will be supplemented with healthy amounts of STAT5. If the additional STAT5 also increases FLT3 expression, the cell signal from the PI3K and RAS pathways is likely too important for cell survival and an approach to supplement their signals will need to be investigated.

Although several experiments for the IC_{50} determination of FLT3-IN-3 over a five-day time course were performed, time was limited when able to start assessing the differentiating and apoptotic effects of the drug. Barriers in experimental set-up and faulty lab equipment also led to inefficient methods that may have introduced a lot of error within the data from the surface-staining. Populations of different cells from each treatment had to be stained for each variable (FLT3, differentiation, or apoptosis) separately due to the presence of only one laser on the fluorescent microscope, making it impossible to obtain data for all three variables on the same cell. Cells also needed to be placed on a microscope slide to be imaged, but the AML cell lines used in this study are suspension cells that do not adhere to surfaces. This caused them to move around slightly underneath the microscope slide and although they would slow down over a few minutes, it was rare they stopped moving long enough to get images of the brightfield and the laser in the same position. For improved accuracy and confidence of results, the staining experiments should be repeated with a working flow cytometer machine to fix both of the mentioned sources of error.

Conclusions and Future Directions

The results of this study revealed that the FLT3-IN-3 inhibitor provides the potential to be used as a novel treatment option for FLT3-ITD mutated AML patients. The aim to examine FLT3-IN-3 for potential use in treatment was successful with experimental data showing that FLT3-IN-3 treatment of 300 nM for five days likely increases cellular differentiation and apoptosis with high specificity for the FLT3-ITD receptor at this concentration. This result provides a baseline for future treatments that involve combinations of FLT3-IN-3 and Vitamin A to test the polypharmaceutical approach of treating patients with mutated AML and, overall, lead

to better patient prognosis. Results also revealed further insight into the importance of the FLT3 receptor and its mechanism. Future research should examine the drug effects with more advanced techniques, determine the binding pocket for the mutated and wild-type FLT3 receptor, inhibit the RAS and PI3K pathways to determine its effects on FLT3 expression, and attempt polypharmaceutical treatment of FLT3-IN-3 and Holo-RBP to further reveal this inhibitor's full potential in aiding patients.

As mentioned previously, the methods for the second aim of this project were not very efficient. Separate samples of cells were stained for the different markers for differentiation, FLT3 expression, and apoptosis. The cells then needed to be imaged, but they moved a lot under the slide given they are suspension cells. Thus, the data should be recollected with the use of a flow cytometer to remove these errors and inconsistencies. Results from the flow cytometer would have smaller error bars and more validity.

Although it is known that FLT3-IN-3 affects only the mutated FLT3 receptor at low concentrations and loses specificity as concentration increases, the mechanism is unknown. To better understand this concept, the binding pocket should be investigated using software such as SwissDock and PyMol. Upon this investigation, any modifications can also be considered to increase the specificity of the drug to FLT3-ITD.

Similar to the way the FLT3 inhibitor was used in this project, it would be insightful to see the effects of FLT3 expression when using either a PI3K inhibitor or a RAS inhibitor. The importance of these two pathways that stem from the FLT3 receptor can be investigated by seeing if FLT3 expression increases, decreases, or remains the same when the inhibitor is added. If expression increases, similar to the FLT3 inhibitor, it can be assumed that the pathway must have been important enough for cell survival that it started producing more FLT3 receptors. If it

does not change, we can deduce that it might not be an important part of the FLT3 signaling cascade.

Finally, the hypothesized polypharmaceutical approach of treating the cells with both FLT3-IN-3 and Holo-RBP should be tested. This could be done similarly to the methods used within this study, but would be better executed utilizing a flow cytometer. The changes in differentiation and apoptosis can then be compared with those found with solely FLT3-IN-3 treatment. Ideally, there would be less apopsis and more differentiation in the polypharmaceutical treatment. This scenario would mean more cells are undergoing differentiation and losing the overactive receptor than undergoing apoptosis. It is more beneficial in patients to cure the mutation than it is to kill all of their cells.

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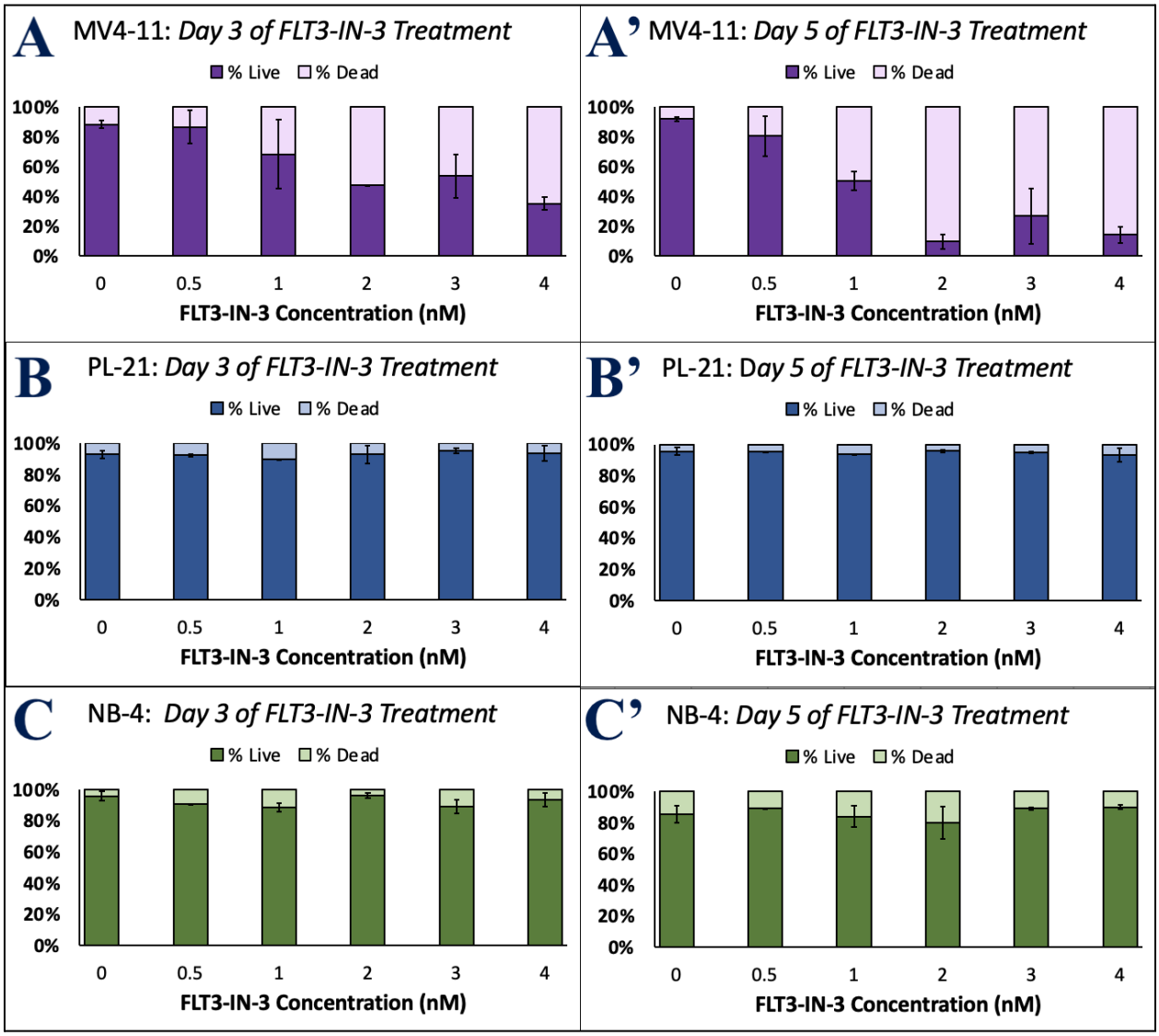
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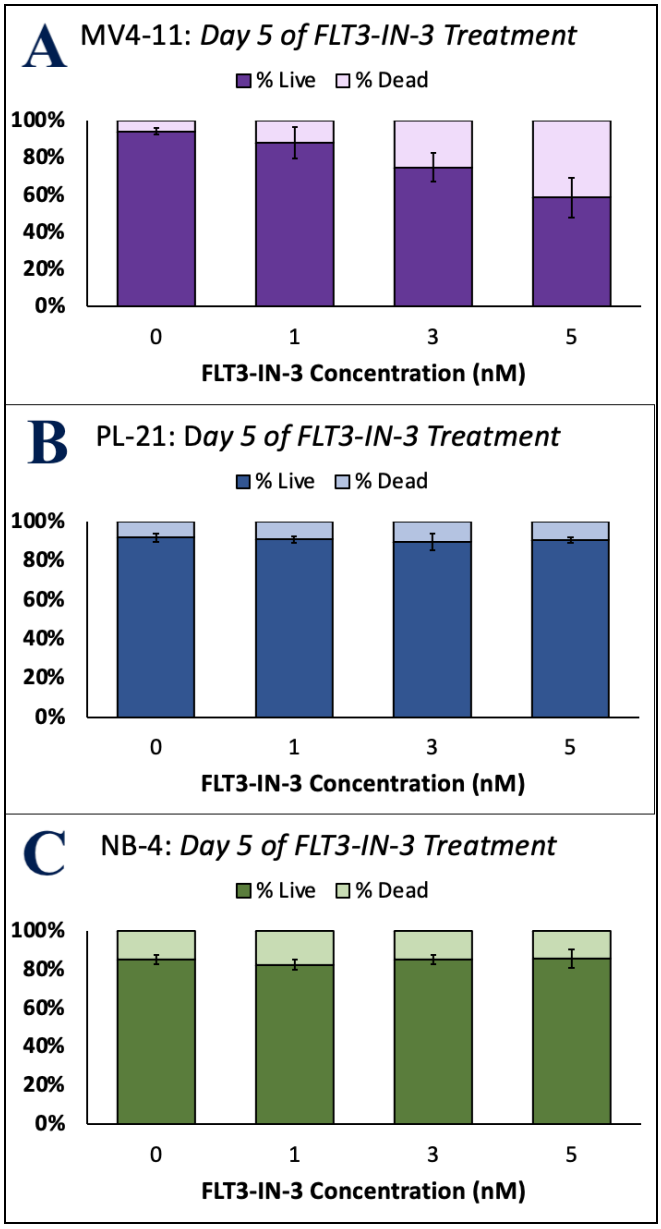
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Supplemental



Supplemental Figure 1. *Cell Viability Data on Days 3 and 5 of FLT3-IN-3 Treatment After Disturbance on Days 2-5.* All three cell lines were treated with various concentrations of FLT3-IN-3 ranging from 0.5 nM to 4 nM. Each treatment was performed in duplicates. Day 3 data is shown in the graphs on the left and day 5 data is shown in the graphs on the right. Control samples were run with an amount of DMSO that is comparable to the DMSO in the 4nM treatment and the bars presented as 0 nM FLT3-IN-3. Each cell line is color-coated as follows: MV4-11 = purple, PL-21 = blue, and NB-4 = green, and a darker shade represents live cells whereas the lighter shade represents dead cells; **[A]** Day 3 of MV4-11 Treatment; **[A']** Day 5 of MV4-11 Treatment; **[B]** Day 3 of PL-21 Treatment; **[B']** Day 5 of PL-21 Treatment; **[C]** Day 3 of NB-4 Treatment; **[C']** Day 5 of PL-21 Treatment.



Supplemental Figure 2. *Cell Viability Data on Day 5 of FLT3-IN-3 Treatment After Disturbance on Days 4 and 5.* All three cell lines were treated with various concentrations of FLT3-IN-3 ranging from 1 nM to 5 nM. Each treatment was performed in duplicates. Control samples were run with an amount of DMSO that is comparable to the DMSO in the 5nM treatment and the bars presented as 0 nM FLT3-IN-3. Each cell line is color-coated as follows: **[A]** MV4-11 = purple, **[B]** PL-21 = blue, and **[C]** NB-4 = green, and a darker shade represents live cells whereas the lighter shade represents dead cells.