

# Centriole Amplification Independently from Aurora A Overexpression Causes Resistance to Alisertib Treatment



A Major Qualifying Project Report

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**Abstract**

Acute Myeloid Leukemia (AML) is a cancer of overproliferative blood cells and is the most common type of blood cancer to occur in adults. AML patients are commonly treated with chemotherapeutic approaches. Although chemotherapy drugs are effective at limiting growth of leukemia cells, the pathways that they target are common in all proliferating cells and as a result, healthy proliferative tissue is also damaged. Currently, the identification and development of therapies that specifically or preferentially impact growth of leukemia cells is in high demand. Alisertib (MLN8237), an inhibitor of the mitotic kinase Aurora A is one potential drug that is currently being investigated clinically as a co-therapeutic for AML. AML cells have been reported to exhibit high levels of Aurora A, and so are hypothesized to be exquisitely sensitive to inhibition of this mitotic regulator. However, the cell biological impact of Aurora A inhibition in AML cells has not been investigated, and it is unclear if Aurora A expression levels alone may be a good predictor of sensitivity to Alisertib.

Our studies have focused on exploring the molecular effects of Aurora A inhibition, and investigating cellular biomarkers, in addition to Aurora A levels, that may predict drug sensitivity in AML patients. Our initial correlative studies in a panel of AML cell lines, does not show a clear correlation between Aurora A levels and Alisertib sensitivity. Instead, our results suggest that an increased centriole number confers resistance of AML cells to Alisertib. We will continue to define centriole number as a potential biomarker through ongoing approaches that will test both the contribution of Aurora A levels and centriole number, both in isolation and in combination, on Alisertib sensitivity.

**Acknowledgments**

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## 1. Introduction

Acute Myeloid Leukemia (AML) is a type of blood cancer that occurs when the bone marrow makes abnormal myoblasts, platelets or red blood cells. These abnormal cells continue to develop and accumulate within the blood vessels, which can result in anemia or easy bleeding. According to the American Cancer Society, AML is the most common type of blood cancer to occur in adults, and is generally seen in adults above the age of 67. The percent of individuals that will survive five years after AML diagnosis is approximately 27%. Most of the individuals will initially experience flu-like symptoms, including fever, sweats or body aches. According to the Seattle Cancer Care Alliance, in order to diagnose AML, several tests are needed, including a bone marrow biopsy, complete blood cell counts or tests for genetic abnormalities. Currently, chemotherapy is the most common treatment given to AML patients. This treatment is generally given in two steps, and the purpose of the treatment is to use chemotherapy drugs to eradicate all of the leukemic cells. However, chemotherapeutic approaches do not specifically target cancer cells but instead target all proliferating cells and cause side effects that impact the health of the patient as stated by the American Cancer Society. Targeted therapies are now being pursued, that may exclusively, or preferentially, target cancer cells through the use of an Aurora A kinase inhibitor called Alisertib.

Aurora A kinase is part of a family of serine/threonine kinases that are vital in cell cycle regulation and mitosis (Fu et al., 2007). Aurora A kinase's function is to control mitotic entry, and recruit components for centrosome maturation. Aurora A plays a critical role in the formation of a bipolar mitotic spindle, which is crucial for the proper separation of the sister chromatids to each of the daughter cells (Dutertre et al., 2002). When Aurora A kinase is overexpressed, centrosome amplification, cytokinesis inhibition and aneuploidy can result. Aurora A kinase has been found to be overexpressed in a variety of cancers, including breast, colon, and AML. Its role within the cell, and together with its overexpression profile in a range of cancers suggest that Aurora A is a promising drug target.

Previous studies have shown that the inhibition of Aurora A kinase function through depletion or inhibition results in mitotic spindle assembly defects (Manfredi, et al., 2011). Loss of Aurora A function disrupts mitotic spindle formation and results in spindles with one or multiple spindle poles. The Aurora A kinase inhibitor that this paper focuses on is Alisertib (or MLN8237). Alisertib has been shown to disrupt the growth of Acute Myeloid Leukemia (AML) cells, and a

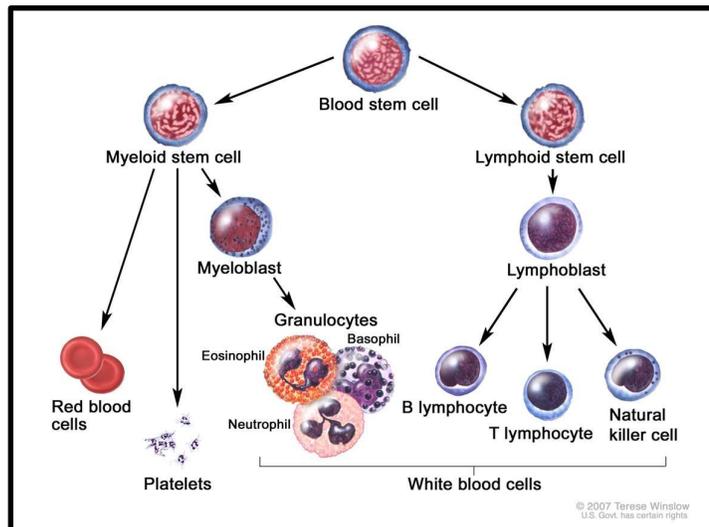
common phenotype of Alisertib-treated AML cells is a monopolar spindle pole (Moore, A.S, et al, 2010). However, not all AML cells exhibit the same degree of Aurora A amplification, or spindle morphology defects and it remains unclear in which contexts Aurora A inhibition may have the greatest therapeutic value. In this Major Qualifying Project we aim to define the cellular implications of Aurora A kinase inhibition with Alisertib, and to investigate cellular biomarkers that predict drug response. In addition biomarkers that could suggest Alisertib would be more or less effective are considered. Lastly, this project explores the possibility that centriole number, independent of Aurora A level indicates the responsiveness of an AML cell to Alisertib.

## 2. Background

### 2.1 Acute Myeloid Leukemia (AML)

The National Cancer Institute defines hematopoietic stem cells, also known as blood stem cells, as immature cells that have the ability to self-renew and to differentiate into any type of blood cell. These cells are generally identified in the bone marrow, the soft, sponge-like tissue in the center of bones. Hematopoietic stem cells first differentiate into blood cells of two different lineages, the lymphoid and myeloid, which give rise to many other types of cells. The lymphoid stem cells further differentiate into T cells, B cells, and natural killer (NK) cells. The myeloid lineage gives rise to megakaryocytes, erythrocytes (MegE), as well as granulocytes (Iwaski and Akashi, 2007). Mature blood cells have short life-spans, and new blood cells are derived from hematopoietic blood cells (Robb, 2007).

According to the Johns Hopkins Comprehensive Cancer Center, blood cancers usually develop in the bone marrow, and it affects both the production and function of blood cells. Each type of blood cancer is due to defects in specific cell lineages. The hematopoietic stem cells replace the normal blood cells as they age and die. However, when an individual has blood cancer, this process is corrupted. Hematopoietic stem cells may not grow or differentiate normally, or the immune system will attack normal tissue. According to the American Society of Hematology, in all three different types of blood cancers - lymphoma, myeloma, and leukemia - the development of blood cells is hindered by the uncontrolled growth of a progenitor.



**Figure 1. Differentiation of Hematopoietic Stem Cells**

Blood stem cells can differentiate into two lineages of blood cells: Myeloid and Lymphoid. Each lineage is responsible for the production of different blood cell types

Retrieved from: Winslow, Terese. National Cancer Institute. 2007

The Mayo Clinic defines acute myeloid leukemia (AML), or acute myelogenous leukemia, as a type of blood cancer that occurs in bone marrow. According to the National Cancer Institute,

AML progresses rapidly and only affects the myeloid lineage of cells, therefore affecting the development of red blood cells, platelets and myeloblasts. These abnormal cells accumulate in the blood vessels and take the space of healthy blood cells. This might lead to anemia and easy bleeding.

### *2.1.1 Prevalence, Risk Factors and Symptoms*

According to the World Health Organization, the incidence of AML worldwide in 2012 was 351,965 people. AML commonly occurs at older ages, with an average of 67 years old, and the lifetime risk for its occurrence is between 0.5 and 1%. According to the National Cancer Institute, the average 5-year survival rate for people with AML is 27%.

During the early stages of AML, patients will experience symptoms similar to the flu including fevers, sweats and body aches. The symptoms can vary based on the deficiency of various blood cell types. For example, patients with low white blood cells will suffer from bacterial or viral infections, and have occurrences of mouth inflammation or sores. To determine if the patient has AML, several tests have to be completed to accurately diagnose the patient. According to the Seattle Cancer Care Alliance, these can include bone marrow biopsies, complete blood counts, and a polymerase chain reaction to test for the presence of a certain chromosomal translocation. Other tests can be used to detect genetic abnormalities, like examining the FLT3 gene for example. The FLT3 gene, when abnormal, has been correlated with poor prognosis in AML patients. In addition to the FLT3 gene, Aurora A kinase has been observed to be overexpressed in AML compared with normal hematopoietic stem cells (Kim et al., 2012).

To date, a high risk factor associated with the development of AML is smoking. Substances present in tobacco do not only affect the cells that are in direct contact with them, such as the lung cells, but it also can affect the cells in the bloodstream, since the smoke diffuses from the lungs to the blood vessels (Lichtman, 2007). There are other risk factors that could increase the probability of AML development, including prolonged exposure to specific chemotherapy drugs such as alkylating agents, platinum agents, and topoisomerases II inhibitors. These can be used as treatments for other cancers. In addition, having blood diseases (i.e polycythemia vera, and idiopathic myelofibrosis) and genetic syndromes (i.e. down syndrome and fanconi anemia) have been linked to an increased risk of developing AML, especially when chemotherapy drugs have been used, as reported in the American Cancer Society. Although a few of the patients present some of these risks factors, their presence alone is not sufficient to cause cancer. AML

commonly develops after an accumulation of mutations in DNA over time, which can happen because of unknown reasons. While translocations are the most common chromosomal changes found in AML, many others can also occur, such as deletions, inversion, insertions or duplications.

### *2.1.2 Current Treatments*

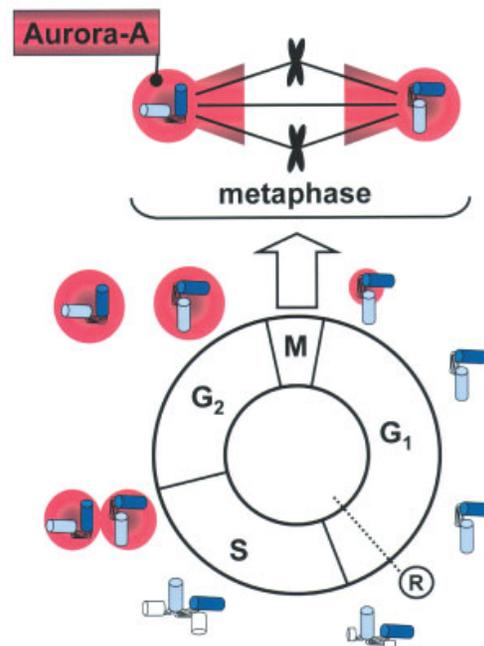
Currently, the most common type of treatment for AML is chemotherapy, which can be followed by a stem cell transplant. The chemotherapy is conducted in two steps: induction and consolidation. Induction is the first phase, and aims to eliminate leukemic cells from the blood, get rid of all signs of disease for an extended time (also known as remission) and to increase the healthy blood count to within a normal range. Generally, doctors will use two or more chemotherapy drugs to treat AML, as each individual drug utilizes different methods to destroy the cancer cells. Therefore, combining drugs can strengthen the treatment's effectiveness. The second step, consolidation, is conducted after the patient has recovered from induction. The second phase is targeted at killing the small population of leukemia cells that may remain after induction. According to the Leukemia & Lymphoma Society, without consolidation, or "postremission therapy", AML has a higher probability of returning.

Common chemotherapy drugs include cytarabine or anthracycline drugs. Cytarabine, once phosphorylated and is incorporated into DNA, will block DNA elongation by inhibiting DNA polymerase, and results in a decrease of DNA replication and repair (Fitzakerley, 2015). Anthracycline drugs primarily act through intercalation. Intercalation inserts an aromatic ring between DNA base pairs, compromises replication, and results in cytotoxicity (Barton et al, 1991). However, both of these chemotherapies can also harm other types of proliferative cells. According to the American Cancer Society, chemotherapy drugs can result in side effects including nausea, hair loss, mouth sores, fatigue, increased bruising and risk of infections.

Although the patient survival has increased over the years, the current treatments are not effective enough in patients older than 60 in which AML is more prevalent. Because of this, there is a need for the discovery of new targets for the development of new effective therapeutics.

## 2.2 Acute Myeloid Leukemia and Aurora A Kinase

In normal human cells, the cell cycle consists of four phases: S phase (chromosomal duplication), M phase (chromosomal separation) and two Gap phases (G<sub>1</sub> and G<sub>2</sub>) that separate both S and M phases (see Figure 2)(van den Heuvel, 2005). The M phase consists of four stages: prophase, metaphase, anaphase, and telophase (O'Connor, 2008). During prophase, the chromosomes begin to condense, the nuclear envelope starts to break down, and the mitotic spindle begins to form. Following prophase, the cell proceeds to pro-metaphase where chromosomes attach to the spindle microtubules and begin to congress towards the center of the cell. Once all chromosomes have fully attached to a bipolar spindle and aligned at the spindle center, the cell is said to be in metaphase. The microtubules nucleated at the centrosomes attach to protein structures known as kinetochores. Attached chromosomes orient so that each replicated chromosome is associated with microtubules nucleated from a single centrosome/spindle pole. This attachment and alignment satisfies the spindle assembly checkpoint and enables the cell to enter anaphase. During this phase, cohesion between replicated chromosomes is lost and the sister chromatids are pulled apart and towards different cell poles. Following anaphase, nuclear envelopes reform around decondensing chromatin and cytokinesis cleaves the dividing cell into two genetically identical daughters (O'Connor, 2008).



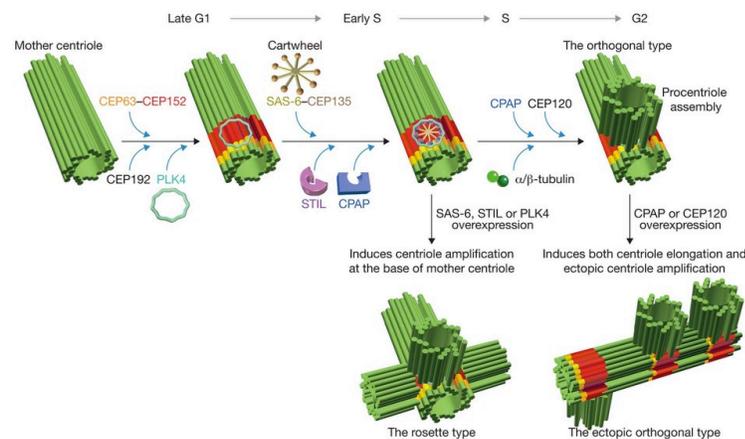
**Figure 2. Aurora A Kinase and Centrosome Separation**

*Centrosomes are duplicated during S phase, move apart upon mitotic entry where they become microtubule nucleating centers that make up functional spindle poles*

Centrosomes are organelles that organize microtubules and are involved in the process of cytokinesis (O'Connor & Adams, 2010). The centrosomes are composed of three parts: two centrioles (a mother and a daughter centriole), a matrix that connects the two centrioles, and pericentriolar material. Centrosomes are duplicated during S phase (Figure 2). The cell, before entering mitosis, contains four centrioles that are organized in two centrosomes. As the cell moves into mitosis, the centrosomes move apart

Retrieved from: Dutertre, Stephanie, Simon Descamps, and Claude Prigent. "On the Role of Aurora-A in Centrosome Function." *Nature.com*. Macmillan Publishers, 9 Sept. 2002. Web. 01 Feb. 2017.

towards opposite sides of the cell, where they nucleate and organize the microtubules of the mitotic spindle. After cytokinesis, each of the daughter cells contains a single centrosome with



**Figure 3. Effect of overexpression of centrosome proteins**

*Overexpression of Plk-4 causes centriole amplification at the base of the mother centriole, also known as the rosette type amplification, in which the daughter centrioles form around the proximal end of the mother centriole.*

Retrieved from: Tang, Tang K. "Centriole biogenesis in multiciliated cells." *Nature cell biology* 15.12 (2013): 1400-1402.

two centrioles. Centriole duplication is mainly regulated by Plk-4, a member of the polo like kinase family, which is localized in the centrioles (Holland, et. al., 2010). The active levels of this kinase controls the number of centrioles that will be formed during mitosis: while decreasing levels of active Plk-4 would cause the cells to progressively lose centrioles, an overexpression of its active levels would cause the creation of multiple centrioles in one cell cycle (Figure 3) (Holland, et. al., 2010).

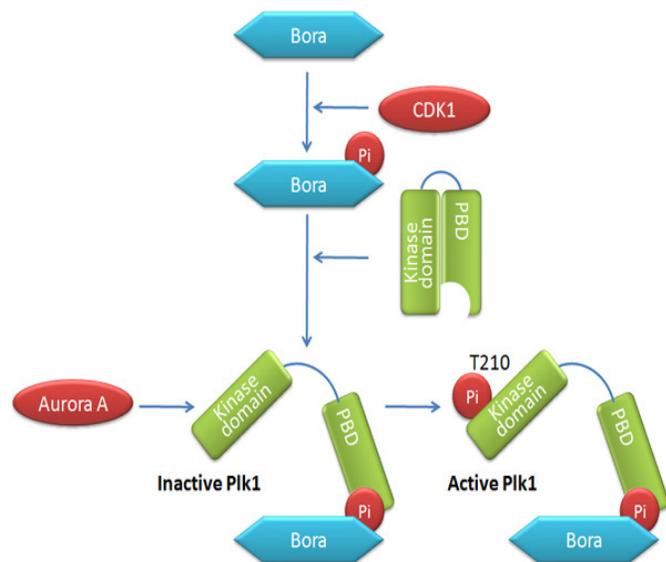
### 2.2.1 Aurora Kinases Function and Regulation

Aurora kinases are a family of serine/threonine kinases that have been implicated in cell cycle control, and are vital during mitosis (Fu et al., 2007). These kinases are enzymes that control the functions of many substrates by phosphorylation. There are three members of the Aurora kinase family: Aurora A, Aurora B, and Aurora C (Fu et al., 2007). The activity of Aurora kinases is closely regulated; as disruption or deregulation of these kinases can lead to genetic instability due to defects in centrosome function, spindle assembly and chromosomal alignment. Aurora A plays a role in mitotic spindle assembly through facilitating centrosome maturation by recruiting various components such as γ-tubulin (Fu et al., 2007). Aurora B regulates spindle assembly, chromosome separation, and is also known to play a role in cytokinesis. For example, when Aurora B kinase is depleted the cell will become polyploid (Fu et al., 2007). According to the National Center for Biotechnology Information, Aurora C has been known to play a role in microtubule organization in centrosome and spindle function during mitosis by forming complexes with Aurora B and centromere proteins. In many human cancers, the expression and activity of the Aurora kinases is increased, suggesting that they may play a role in tumorigenesis, and has become the focus of many anti-cancer drugs and treatments (Fu et al., 2007). The cell

cycle is primarily regulated by cyclin-dependent kinases, also known as CDKs. CDKs are further regulated by phosphorylation, degradation of proteins that inhibit cyclin, and degradation of cyclins, among others (van den Heuval, 2005). The mitotic events of the cell cycle have other regulatory molecules besides CDKs, such as polo-like kinases, and aurora kinases. Aurora A kinase, for example, functions to control mitotic entry, which happens after the activation of CDK1 (Figure 4). This control is done indirectly, by interactions with Polo-like kinase 1 (Plk-1), a cell cycle kinase that regulates processes such as centrosome maturation, spindle assembly and chromatin cohesion (Bruinsma et al., 2014). Plk-1 is located at the centrosomes

and mitotic spindle. Plk-1 is switched on by phosphorylation at residue T210 during G2 phase, reaching its maximum activity during mitosis, after it is phosphorylated at residue T210 (Bruinsma et al., 2014). The phosphorylation of this residue is done by the Aurora A-Bora complex. The phosphorylation of residue T210 causes a change in the conformation of Plk-1 that provides Aurora A enhanced access to residue T210 (Fu, Jiang & Zhang, 2010). Inhibition of Aurora A has been shown to prevent the activation of Plk-1 and lead to the formation of monopolar spindles.

During S phase, Aurora A localizes on duplicated centrosomes, and remains there until the beginning of G1 during the following cell cycle. While located on the centrosomes, Aurora A has three functions. First, as shown by experiments performed in *Xenopus* and in *Drosophila*, Aurora A contributes to centrosome separation. In these two animals, inactivation of Aurora A results in the formation of monopolar spindles, with the centrosomes failing to separate (Duterte et al., 2002). The mechanism by which Aurora A contributes to centrosome separation is through phosphorylation of Eg5, a protein required for centrosome separation to occur. A



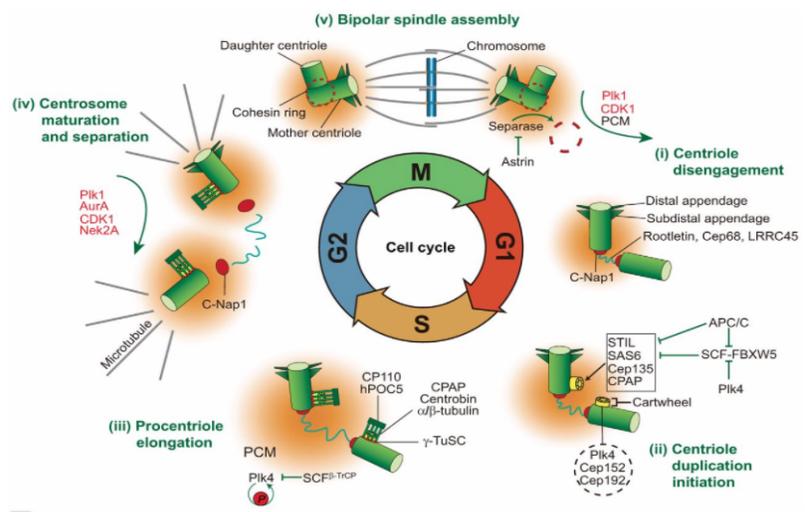
**Figure 4. Role of CDK, Aurora A Kinase and Polo-Kinase 1**

CDK1 activates the Aurora Bora complex, which leads to the phosphorylation and activation of PLK1. This results in the phosphorylation of residue T210

Retrieved from: Fu, J., Jiang, Q. & Zhang, C. (2010) Collaboration of Mitotic Kinases in Cell Cycle Control. *Nature Education* 3(9):82

second function of Aurora A at the centrosomes is to ensure that centrosomes mature after they separate and before mitosis. This maturation includes the recruitment of proteins, such as  $\gamma$ -tubulin and centrosomin, to the centrosome to take part in its structure as well as to preserve its functionality during and after mitosis (Duterte et al., 2002). Lastly, Aurora A recruits and phosphorylates TACC3 at the centrosome. TACC3 is a protein that promotes microtubule stabilization (Lioutas & Vernos, 2013). This interaction contributes to the organization and stabilization of microtubules (Fu et al., 2007).

Both the localization and activity of Aurora A is carefully regulated to ensure that it correctly functions within the cells. In normal cells, Aurora A is down-regulated through APC/C-Cdh1 dependent, proteasome-mediated proteolysis. The degradation of Aurora A by APC/C-Cdh1 requires a destruction box in the C-terminal region and a motif in the N-terminus (D'Assoro et al., 2016). Aurora A is a major contributor to the proper segregation of the daughter cells during mitosis and Deregulation of its localization and activity can lead to abnormal spindle morphology and promote tumorigenesis (Duterte et al., 2002).



**Figure 5. Diagram of Centrosome Duplication and Aurora A Localization**

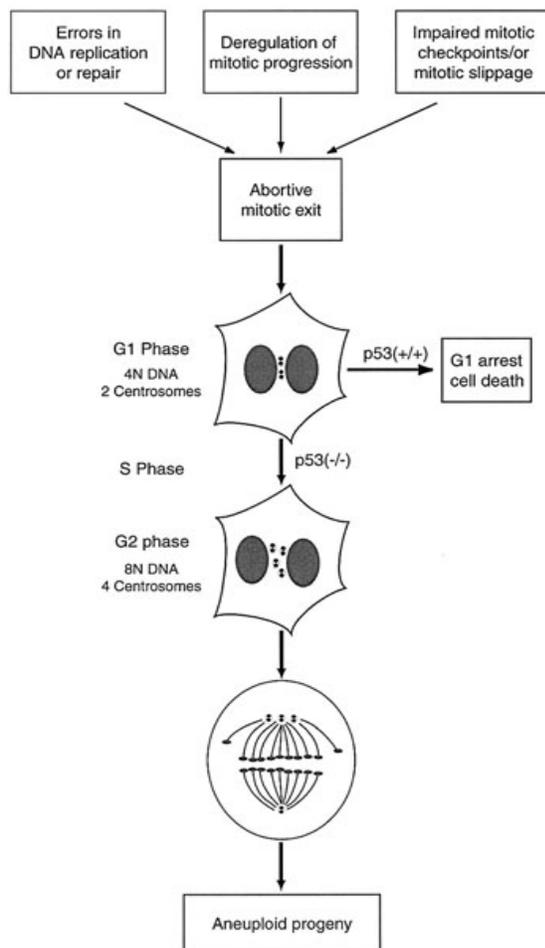
Centrosomes duplicate during S phase, and move apart during G2/M phase. Aurora A contributes to centrosome separation during G2 phase.

Retrieved from: Wang, Gang, Qing Jiang, and Chuanmao Zhang. "The Role of Mitotic Kinases in Coupling the Centrosome Cycle with the Assembly of the Mitotic Spindle." *Journal of cell science*, vol. 127, no. Pt 19, 2014, pp. 4111-4122doi:10.1242/jcs.151753

### 2.2.2. Aurora A Kinase Overexpression and Effects in the Cell Cycle

The Aurora A kinase gene is located in the 20q13 chromosome region, and is amplified and overexpressed in many cancers including breast, colon and ovarian cancers, leading to the overexpression of Aurora A kinase (Duterte et al., 2002). In a large majority of these cancers,

the localization of Aurora A is diffused, being present in other parts of the cell such as the cytoplasm, and not being concentrated in the nucleus (Duterte et al., 2002).



**Figure 6. Diagram of Mitotic Errors**

Errors in DNA replication, mitotic progression, or impaired mitotic checkpoints can result in an abortive mitotic exit. Cells lacking p53 can bypass G1 arrest cell death, and result in centrosome amplification

Retrieved from: Meraldi, Patrick, Reiko Honda, and Erich A. Nigg. "Aurora-A Overexpression Reveals Tetraploidization as a Major Route to Centrosome Amplification in P53<sup>-/-</sup> Cells." *The EMBO Journal*. EMBO Press, 15 Feb. 2002. Web. 28 Feb. 2017

amplification of centrosomes is also correlated with loss or mutation of p53, a tumor suppressor that plays a role during DNA damage response (Figure 6) (Goodsell, 2002). Cells that do not have a functional p53 protein have been shown to have a higher number of centrosomes when

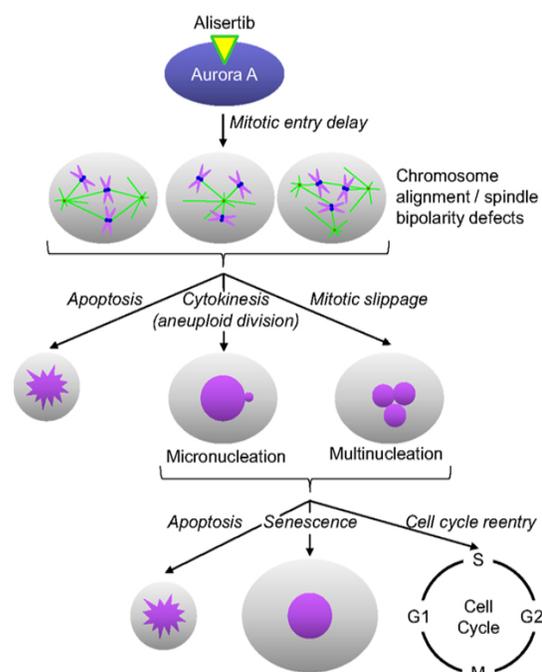
Centrioles are duplicated during S phase concurrent with DNA replication (Figure 5). The cell, therefore, contains four centrioles that are organized into two centrosomes throughout G2 and mitosis. Aurora A kinase activity is not observed until the G2 phase, which implies that the kinase activity of Aurora A is not needed in order to duplicate the centrioles (Duterte et al., 2002). While the activity of Aurora A is not required for centriole amplification, the overexpression of Aurora A has been shown to be sufficient for overamplification of centrioles (Meraldi et al., 2002). Recent studies show that cells overexpressing Aurora A and containing increased number of centrioles also have multiple nuclei, suggesting that these cells experience abnormal mitosis. Overexpression of Aurora A causes cells to form aberrant mitotic structures, and defective anaphases including the presence of anaphase bridges and cytoplasmic connections (Meraldi et al., 2002). These aberrant anaphase defects cause the cells to fail cytokinesis, leading to the formation of tetraploid cells. Some cells that overexpress Aurora A kinase also overexpress Plk1 and Aurora B kinase, which have also been shown to cause an amplification of centrosomes (Meraldi et al., 2002). This

Aurora A, Aurora B or Plk1 are overexpressed. This could explain why the overexpression of Aurora A leads to the formation of multiple centrosomes, as p53 loss would abrogate the checkpoint for aberrant mitotic cells, and permit their continued cycling (Meraldi et al., 2002).

### 2.2.3 Aurora A Kinase Inhibition and Implications

Currently, due to the role of Aurora A within the cell and its overexpression in many cancers, the effect of inhibition of Aurora A is being investigated. It has been observed that Aurora A inhibition can lead to mitotic spindle assembly defects, such as monopolar spindle poles which activate the spindle assembly checkpoint and induce mitotic arrest (Bavetsias & Linardopoulos, 2015). Following a prolonged mitotic arrest, some cells undergo mitotic catastrophe. Other cells that ultimately exit mitosis and enter G1 will senesce or apoptose. However, not all cancer cells respond similarly to Aurora A inhibition and it remains unclear what features of a cancer cell may promote the preferred mitotic catastrophe or apoptosis, over a G1 arrest. There are multiple Aurora A inhibitors in clinical trials, including AT9283, PF-03814735, and Alisertib (or MLN8237).

First, AT9283 is a heterocyclic molecule that inhibits aurora kinases, including both Aurora kinase A and B. This inhibitor is used as a therapy for many solid tumors and leukemic cancers, as it shows a reduction in the proliferative profile of leukemic cancers, as well as an induction of aneuploidy and apoptosis (Qi et al., 2012). Second, PF-03814735 is a reversible inhibitor of both Aurora kinase A and B, and to a lesser extent FLT1, FAK, and TrkA which have been implicated in tumorigenesis in cancers such as leukemia and breast cancer. Small cell lung cancer and colon cancer are the most sensitive to PF-03814735, which works by blocking



**Figure 7. Alisertib Mechanism in vitro**

Alisertib inhibits Aurora A kinase, resulting in mitotic entry delay. This leads to spindle bipolarity defects, which can lead to apoptosis or cell cycle reentry

Retrieved from: Niu, Huifeng, Mark Manfredi, and Jeffrey A. Ecsedy. "Scientific Rationale Supporting the Clinical Development Strategy for the Investigational Aurora A Kinase Inhibitor Alisertib in Cancer." *Frontiers in oncology*, vol. 5, 2015, pp. 189, doi:10.3389/fonc.2015.00189

aneuploidy and apoptosis (Qi et al., 2012). Second, PF-03814735 is a reversible inhibitor of both Aurora kinase A and B, and to a lesser extent FLT1, FAK, and TrkA which have been implicated in tumorigenesis in cancers such as leukemia and breast cancer. Small cell lung cancer and colon cancer are the most sensitive to PF-03814735, which works by blocking

cytokinesis and, therefore, preventing cell proliferation and creating cells that are multinucleated (Jani et al., 2010). Lastly, MLN8237, also known as Alisertib, is the first oral and selective inhibitor of Aurora A kinase. It shows more than a 200-fold increased specificity for Aurora A kinase than Aurora B kinase (Niu et. al., 2015). Alisertib mechanism of action has been studied in both in vitro and in vivo models (Figure 7). The main consequences of Alisertib treatment are either mitotic arrest or apoptosis, which are events that happen after the cells undergo changes in the phenotype related with an inhibition of Aurora A kinase (Niu et. al., 2015). First, cells trying to undergo mitosis under Alisertib treatment experience a delay in its entry, followed by an increase in the number of cells that contain tetraploid DNA content. When the cells enter mitosis they tend to exhibit chromosomal defects such as chromosome misalignment, and form monopolar and multipolar spindle poles. The fate of the cells that undergo a first round of mitosis is either apoptosis, cytokinesis that causes aneuploidy to the daughter cells, or mitotic slippage, which consists in the exit of mitosis without undergoing cytokinesis. In the two possible outcomes that do not represent cell death, the daughter or resulting cell usually expresses micronucleation or multinucleation phenotypes (Niu et. al., 2015). Only a portion of these cells will re-enter the cell cycle (experiencing the same chromosomal misalignments and possible apoptosis) while some others will undergo cell death or stay in a state of senescence, in which cells are metabolically active but do not undergo any type of cell growth (Campisi, 2013).

Previous work has shown that Alisertib, at clinically achievable concentrations, impairs the growth and survival of AML cell

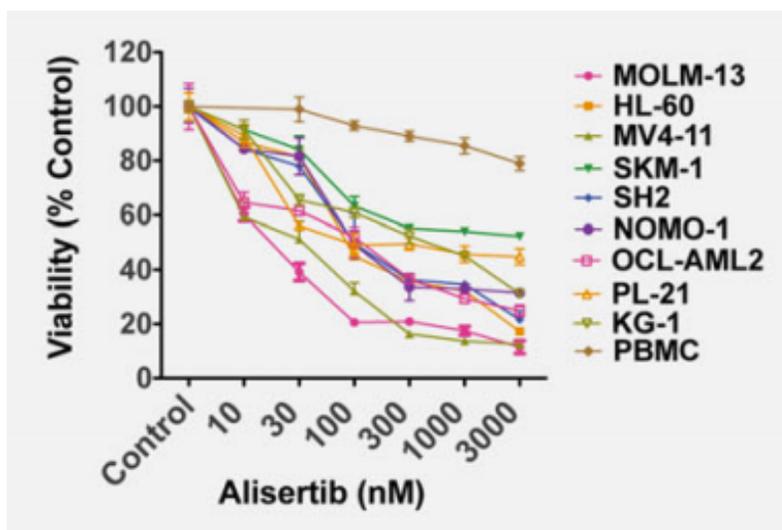


Figure 8. AML Cell Viability at Concentrations of Alisertib

All AML cell lines had significantly larger decreases of cell viability as the concentration of Alisertib increased compared with the control cell line PBMC.

Retrieved from: Kelly, Kevin R. et al. "Targeting Aurora A Kinase Activity with the Investigational Agent Alisertib Increases the Efficacy of Cytarabine through a FOXO-Dependent Mechanism." *International journal of cancer. Journal international du cancer* 131.11 (2012): 2693–2703. PMC. Web. 18 Jan. 2017.

significantly more than normal Peripheral Blood Mononuclear Cells (PBMC) cells (Figure 8). Alisertib was also shown to increase the percentage of AML cells that experienced an induction of apoptosis (Kelly et al., 2012).

Recently, a phase I trial conducted at Massachusetts General Hospital evaluated the safety and tolerability of Alisertib when combined with chemotherapy for patients diagnosed with AML. The treatment during this clinical trial involved infusions of cytarabine for 7 days, and another chemotherapy drug, idarubicin, for 3 days. After the cytarabine infusions on day 7, patients were administered oral doses of Alisertib for 7 days. Overall, the researchers observed that Alisertib was well tolerated. The results showed that overall 86% of the patients that participated in the study achieved complete remission. Within the patient group, 7 out of 8 patients that were over the age of 65 achieved a complete remission. In addition all patients that were diagnosed with high-risk AML achieved complete remission (Fathi et al., 2016).

Despite the success of the clinical trial, in vitro studies have demonstrated that the degree of sensitivity of AML cell lines varied, suggesting that the unique background of individual cell types may be a contributing factor in the cellular response to Alisertib (Kelly et al., 2012). Interestingly, previous research has failed to show a direct relationship between Aurora A expression levels and sensitivity to Alisertib. The reason for this remains unknown. The purpose of this project is to explore biomarkers that indicate drug efficacy and explore the cellular implications of inhibiting Aurora A kinase by Alisertib.

### 3. Materials and Methods

#### 3.1 Subculture of Human Tissue Culture

**Table 1. AML and Control Cell Line Derivation**

(Obtained from ATCC cell lines)

Cell Line	Tissue	Disease	Patient Details	Treatments	Category
RPE-1	Retina, eye	None	Female	None	Non-Transformed
PC-9	Lung	Adenocarcinoma	Unknown	None	Transformed
SAOS-2	Bone	Osteosarcoma	Female, 11 years old, Caucasian	RTG, methotrexate, Adriamycin, vincristine, Cytoxan and aramycin-C	Transformed
K562	Bone Marrow	Chronic Myelogenous Leukemia	Female, 53 years old	None	Transformed
HL60	Peripheral Blood	Acute Promyelocytic Leukemia	Female, 36 years old, Caucasian	None	Transformed
KG1 $\alpha$	Bone Marrow	Acute Myelogenous Leukemia	Male, 59 years old, Caucasian	None	Transformed
U937	Pleura effusion	Histiocytic Lymphoma	Male, 37 years old, Caucasian	None	Transformed
THP1	Peripheral Blood	Acute Monocytic Leukemia	Male, 1 year old	None	Transformed

In the experiments and results discussed below, a variety of AML and control cell lines are utilized. The control cell lines include RPE-1, PC-9 and SAOS-2. RPE-1 act as a negative control, as this cell line is non-cancerous and has not been reported to have any abnormal expression of Aurora A kinase. PC-9 and SAOS-2 both are cancerous tissues, and therefore, may have an overexpression of Aurora A. However, these will act as positive controls and help to determine cellular factors that may be specific to AML (Table 1).

Each cell line was subcultured at a ratio 1:5 every 72 hours. For adherent cell lines (RPE-1, RPE-PLK4, PC9, and SAOS2), the media was aspirated out, and 2 mL of 1XPBS was used to

rinse the cells. The 1XPBS was then aspirated out and 2 mL of trypsin was added to the cells. The cells were left at 37°C for 5 minutes. After the incubation period, 8 mL of media was added to the trypsinized cells. Two mL of this cell suspension was removed, placed into a new T75 flask, and the total volume increased to 10mL with media. All suspension cell lines (U937, THP1, K562, and KG1 $\alpha$ ) were subcultured by moving 2 mL out of the current flask, transferring the 2mL to a new T75 flask, and bringing up the volume to 10mL with fresh media.

### *3.2 Fixing and Staining AML Cells for Immunofluorescence*

#### *Polylysine coverslip preparation*

Coverslips were immersed in 10% acetic acid for 10 minutes in a shaking tray. Afterwards, the acetic acid was removed and the coverslips were washed with water twice by shaking for 10 minutes. Next, the coverslips were incubated in 10% polylysine (diluted in water) for 10 minutes. The 10% polylysine solution was removed and the coverslips were dipped briefly in water, and then air dried in a rack before use.

#### *Immunofluorescence*

In 2mL of media,  $1.0 \times 10^6$  cells of each AML cell line and the RPE cells were plated. Each well received a polylysine coated coverslip. 24 hours after plating the cells, Alisertib was added so the final concentrations were 0nM (untreated), 25nM, 50nM, and 100nM. The cells were exposed to Alisertib for 18 hours. After exposure to Alisertib, the plates containing AML cells were centrifuged at 1000 rpm for 5 minutes to promote adherence of cells to the coverslip. Each coverslip was transferred to a dish containing 1mL ice cold methanol and was incubated at -20°C for 15 minutes. The plates were then centrifuged again at 1000 rpm for 5 minutes. After centrifugation, the methanol was removed, and coverslips were washed with 1mL of 1x PBS. Coverslips were then blocked in 1mL TBS/BSA for 20 minutes at room temperature.

Primary antibodies were prepared by diluting dm1 $\alpha$  (Santa Cruz Biotechnology, Reference Number: SC-32293) and centrin-2 (Santa Cruz Biotechnology, Reference Number: SC-27793R) 1:1000 and 1:200, respectively, in 1mL of TBS/BSA. The coverslips were placed in a humid chamber and 100  $\mu$ L of primary antibody was added to each coverslip for 90 minutes. The coverslips were washed with TBS/BSA for 5-10 minutes. Secondary antibody was prepared at a 1:1000 dilution in DAPI/TBS. The coverslips were incubated with 100  $\mu$ L of secondary antibody in a humid chamber in the dark for 45-60 minutes. The coverslips were then washed with TBS/BSA for 5-10 minutes, and then were mounted on a slide with Molecular Probe Prolong

Gold Antifade Reagent (Reference Number: P36934). Afterwards, each slide was viewed on a Nikon Ti at the objective 60X. To view the staining, channels DAPI, FITC, and TxRed were used to image the DAPI, centrin-2 and dm1-alpha staining respectively. For this experiment, there were three biological replicates, each containing two technical replicates.

### *3.3 Observing Alisertib Impact on Acute Myeloid Leukemia Cells*

#### *Viability Assay*

The five AML cell lines (U937, HL60, THP1, KG1 $\alpha$ , and K562) and three control cell lines (RPE, PC9, and SAOS2) were treated with a range of Alisertib concentrations. 100  $\mu$ L of media containing 6,000 cells were used in each well of a 96-well plate, and 100  $\mu$ L of media or media with Alisertib concentrations of 10nM, 25nM, 50nM, 100nM, and 250nM were added. The viability assay contained three technical replicates in each plate and three biological replicates were done for each cell line. Resistant cell lines to Alisertib, U937 and K562, as well as the three controls were used for additional viability assays at Alisertib concentrations of 500nM, 750nM, 1 $\mu$ M, with the same volume and number of cells.

After three days of exposure to Alisertib, 20  $\mu$ L of Thermo Fisher Presto Blue was added to each well. After 2h, the plate was read on a PerkinElmer 2030 Explorer at 600 A.

For this experiment, there were three biological replicates, each containing two technical replicates.

#### *FACS Analysis*

$2.0 \times 10^6$  cells were plated in 10 cm dishes and media was added up to 10 mL. After 24 hours, the cells were treated with 100 ng/mL Nocodazole and with Alisertib concentrations of 100nM and 250nM for 16h. For only Alisertib treatment, the cells were plated and after 18 hours, were treated with 100 nM or 250 nM of Alisertib for 16h. Then 10 $\mu$ L of Thermo Fisher BrdU was added to the media for 1 hour. Afterwards, the media was collected in 15mL tubes and centrifuged at 1000rpm for 5 minutes, and the supernatant was aspirated. The pellet was resuspended in 150 $\mu$ L of PBS, followed by the addition of 350 $\mu$ L of cold 100% methanol. The 500 $\mu$ L solution was then collected in an Eppendorf tube and stored at -20C.

For analysis, the samples were spun at 800 rpm for 5 minutes, and the supernatant was aspirated. The pellet was washed twice in PBS, spinning and aspirating the supernatant between the two washes. 50  $\mu$ L of 100  $\mu$ g/mL ribonuclease and 200  $\mu$ L of 50  $\mu$ g/mL PI were

added to each sample. The samples were run on the Accuri C6 Flow Cytometer, with a threshold of 50,000 events.

#### *QPCR Protocol*

RNA was extracted from AML cell lines using Trizol (Ambion, Life Technologies) and complementary DNA was synthesized using the Superscript first-strand synthesis system (Applied Biosystems – Life Technologies, Austin, TX, USA). Aurora A expression was then analyzed using primers (Table 2). To establish target-gene expression levels, complementary DNA was quantified using the SYBR green kit (Applied Biosystems – Life Technologies, Austin, TX, USA), gene-specific primers, as well as GAPDH specific primers using relative quantification analysis. The copy number of the gene of interest was normalized to the copy number for GAPDH.

**Table 2. qPCR Primer Sequences**

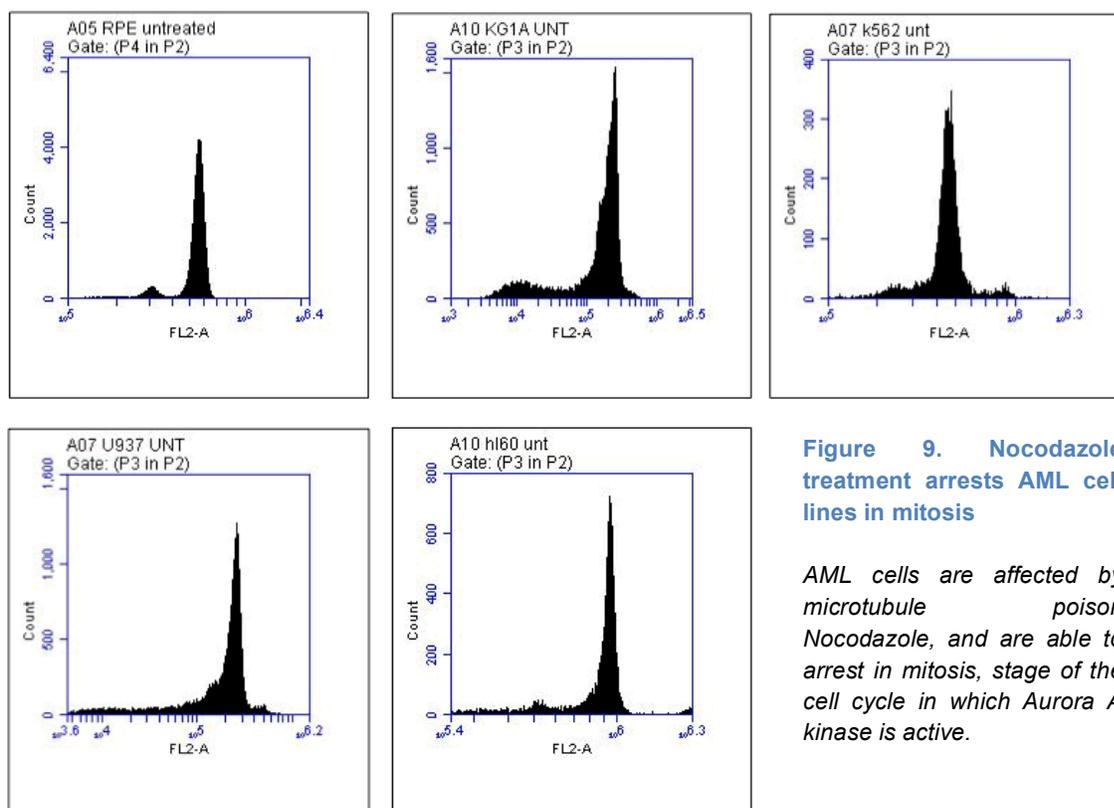
<b>GAPD H</b>	<b>Forward</b>	5' – CCCTCTGGTGGTGGCCCCTT – 3'
	<b>Reverse</b>	5' – GGCGCCCAGACACCCAATCC – 3'
<b>AURK A</b>	<b>Forward</b>	5' – TTTTGTAGGTCTCTTGGTATGTG – 3'
	<b>Reverse</b>	5' – GCTGGAGAGCTTAAAATTGCAG – 3'

For this experiment, there were three biological replicates, each containing two technical replicates.

## 4. Results

### 4.1 AML Cell Lines are responsive to Nocodazole treatment

Failure of consistent response of AML cells to Alisertib induced mitotic arrest may indicate an innate resistance of the cell line to respond to and maintain a mitotic arrest. To examine the susceptibility of the AML cells to mitotic arrest, Fluorescence Activated Cell sorting (FACS) analysis was used to monitor DNA content as a readout of cell cycle progression following treatment with the microtubule poison Nocodazole.



**Figure 9. Nocodazole treatment arrests AML cell lines in mitosis**

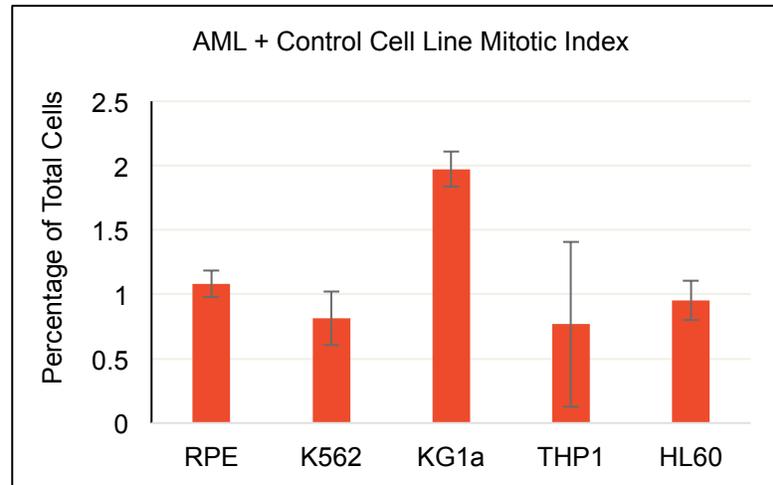
*AML cells are affected by microtubule poison Nocodazole, and are able to arrest in mitosis, stage of the cell cycle in which Aurora A kinase is active.*

Treatment with Nocodazole provided a significant enrichment of both the control cells and AML cells in mitosis, demonstrating that AML cells are responsive to conditions that perturb mitotic progression, leading to an enrichment of cells with 4N/mitotic DNA content. All of the cell lines still show a small percentage of cells that are not arrested, rather in G1 or S phase. Moreover, in some of the AML cell lines such as K562 and U937, we can observe the presence of cells in the region after the G2/M peak, suggesting that there are aneuploid cells (Figure 9).

#### 4.2 AML cells overexpressing total levels of Aurora A also express centrosome amplification

Research studies have correlated the overexpression of Aurora A kinase in human cancers such as breast, colon, and cervical cancer, with poor prognosis (Fu et al, 2013). Some AML cell lines have also been shown to exhibit Aurora A overexpression, usually because of an increase in expression of the Aurora A kinase gene (AURKA) (Kim et al, 2012).

Immunofluorescence microscopy was used in order to determine the percentage of mitotic cells in each of the cell lines. Immunofluorescence analysis using alpha tubulin

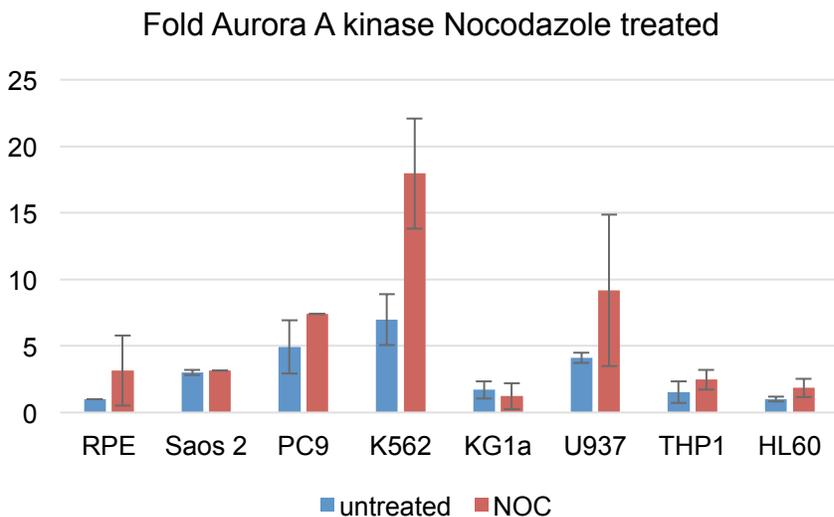


**Figure 10. Percentage of Cells in Mitosis using IF**

and DAPI (DNA) staining to identify mitotic nuclei indicated that both the control and AML cell lines expressed an even number of mitotic cells, around 2% (Figure 10)

*Immunofluorescence analysis was utilized to score mitotic index for AML and control cell lines. All AML cell lines had a mitotic index comparable to RPE-1*

Quantitative PCR was used in order to determine the levels of Aurora A in a panel of AML cell lines as well as RPE cells, used as a control cell line for baseline Aurora A expression levels. KG1 $\alpha$  and THP1 showed a non-significant slight increase of Aurora A kinase expression in comparison to RPE. However, two of the cell lines, U937 and K562, showed a three and eight-fold increase in Aurora A kinase expression, respectively. Aurora A is highly expressed during mitosis and changes in cell cycle distribution can influence the apparent comparative levels of AurA in different cell lines. To control for this possibility, qPCR analysis was used to determine the levels of Aurora A kinase mRNA following a 20h treatment with Nocodazole to enrich for mitotic cells (Figure 11).



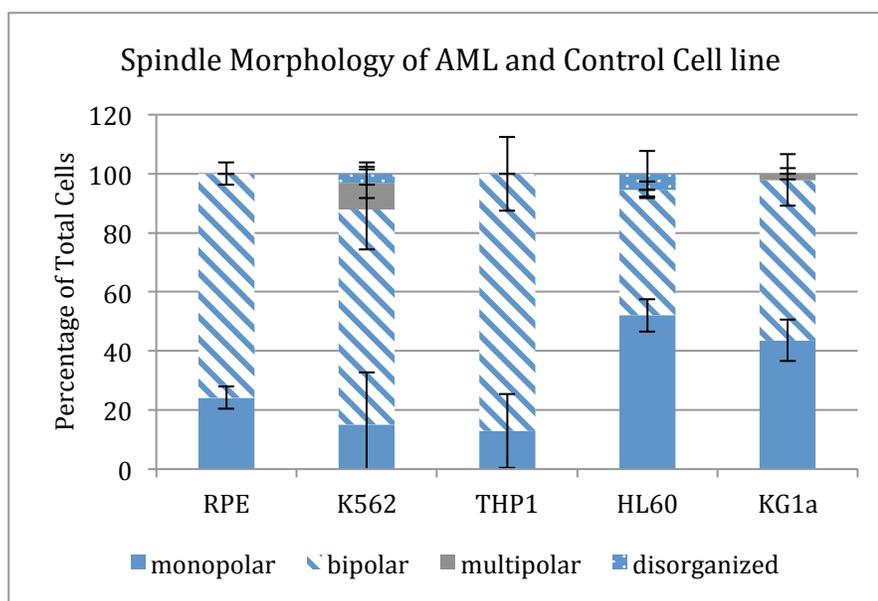
**Figure 11. qPCR of Aurora A Expression in AML Cells over RPE-1**

Quantitative PCR analysis of Aurora A kinase expression in AML and control cell lines. K562 and U937 scored had significantly higher fold expression over the control cell line, RPE-1. Nocodazole treatment caused cell lines to show an increase in Aurora A expression, with K562 and U937 expressing Aurora A the most.

Consistent analysis of asynchronous cells, nocodazole-treated population indicate that K562 and U937 still are the two cell lines that express higher Aurora A kinase expression. RPE-1, SAOS-2, and PC9 cell lines were used as controls cells.

High levels of Aurora A have been demonstrated to corrupt spindle structure and promote overduplication of

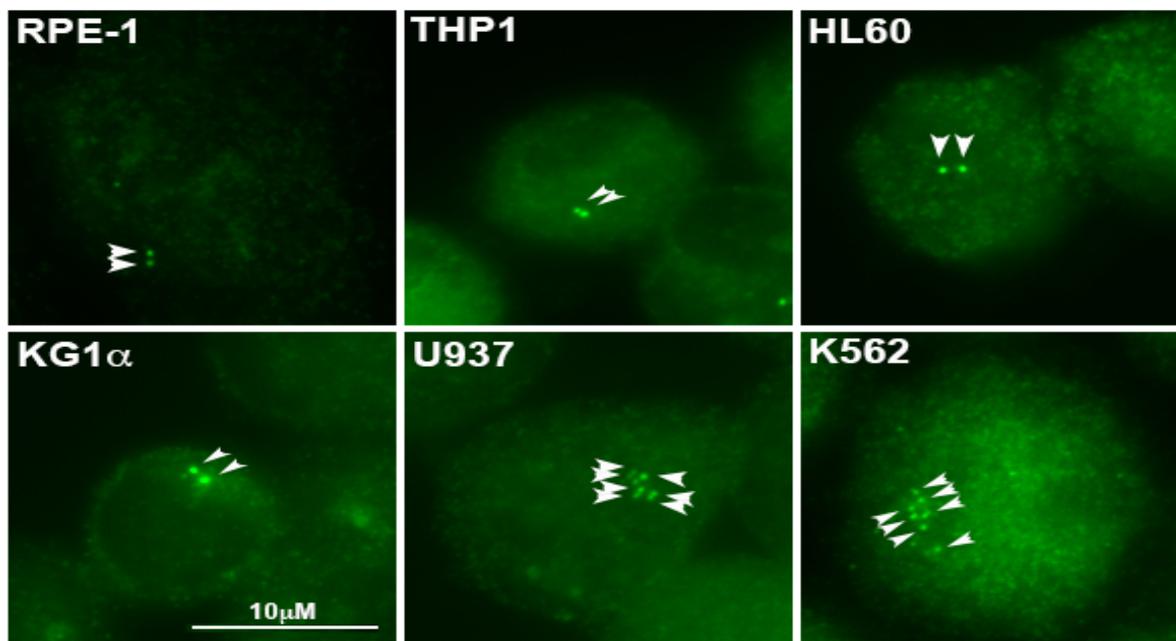
centrioles. Therefore, to characterize how high Aurora A levels may impact spindle assembly, immunofluorescence microscopy was used to monitor spindle structure and centriole number. RPE-1 cells were used as a negative control and mostly expressed a normal mitotic bipolar spindle as well as two centrioles. Although 25%



**Figure 12. Spindle Polarity of mitotic AML cells**

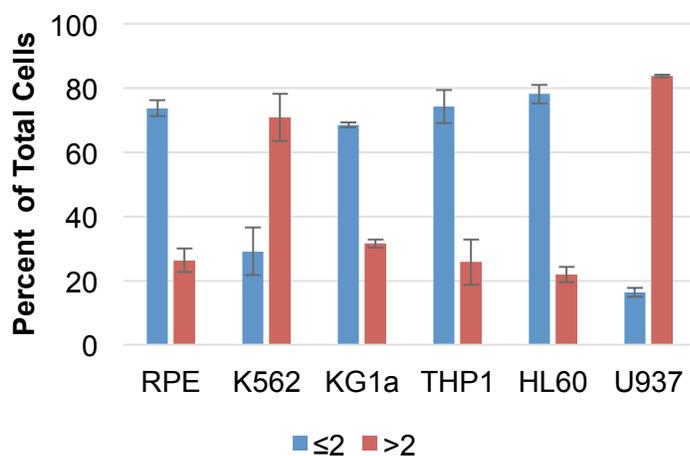
Spindle polarity differed depending on the AML cell line. While some cell lines (K562 and THP1) expressed a majority of bipolar spindles, others (HL60 and KG1a) exhibited a high number of monopolar spindles.

of the cells expressed more than two centrioles, this could be attributed to cells in late S or G2 phases that have already duplicated their centrosomes but haven't entered mitosis yet. Most of AML cell lines expressed a single centrosome with two centrioles, except for K562 and U937.



**Figure 13. Immunofluorescence analysis of centrosome in RPE and AML cell lines**  
Immunofluorescence analysis showed U937 and K562 overexpressed number of centrosomes during interphase

These two AML cell lines that overexpress Aurora A kinase showed centriole amplification, a



**Figure 14. Number of centrioles in control and AML cells from Immunofluorescence analysis**

Immunofluorescence analysis was used to determine the number of centrioles that AML cell lines express. More than 70% of U937 and K562 cells exhibit amplification of centrioles.

and KG1 $\alpha$ , exhibited an increased amount of monopolar spindles during mitosis.

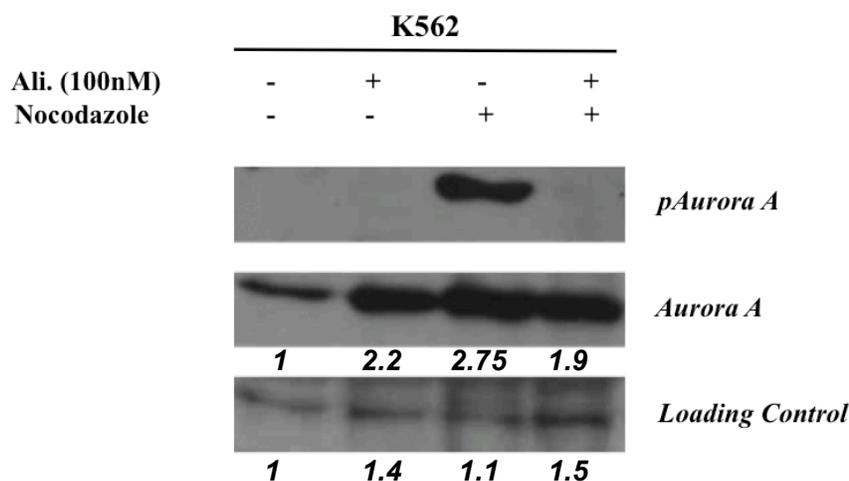
relationship that has been previously characterized. Aurora A overexpression can result in centrosome amplification, and lead to multipolar spindles and abnormal segregation of the chromosomes (Bavestias & Linardopoulos, 2015). Moreover, and consistent with the amplification of centrioles, K562 exhibited an increased percentage of multipolar spindles during mitosis. Additionally, some of the cell lines that express normal levels of Aurora A as well as centrosomes, such as HL60

Given the well-documented role for Aurora A in centriole duplication, regulation of mitotic spindle formation, and mitotic progression, Aurora A has been proposed to be a promising target for novel AML therapeutic approaches (Fu et al, 2007). However, it remains unclear to what extent Aurora A over-expression, or additionally, associated cellular phenotypes, may indicate a likely response of the patient to therapeutic approaches that inhibit Aurora A function.

#### 4.3 AML cell lines that amplify centrosomes are more resistant to Alisertib treatment

Because of Aurora A being suggested as a possible target for the treatment of cancers and tumors that overexpress it, the development of drugs that inhibit Aurora A have been used as part of many clinic trials. Alisertib is characterized for being one of the most specific inhibitors to date and it is being used in AML patients undergoing clinical trials. Because of its specificity, Alisertib was used in our experiments to determine what are the implications of inhibiting Aurora A expression in AML cell lines.

In order to examine the specificity of Alisertib and its capacity to inhibit active Aurora A (phosphorylated Aurora A or pAurA), we performed western blots using the AML cell line that expresses the highest level of Aurora A kinase, K562, in four different conditions: untreated,



**Figure 15 Alisertib inhibits the phosphorylation of Aurora A**

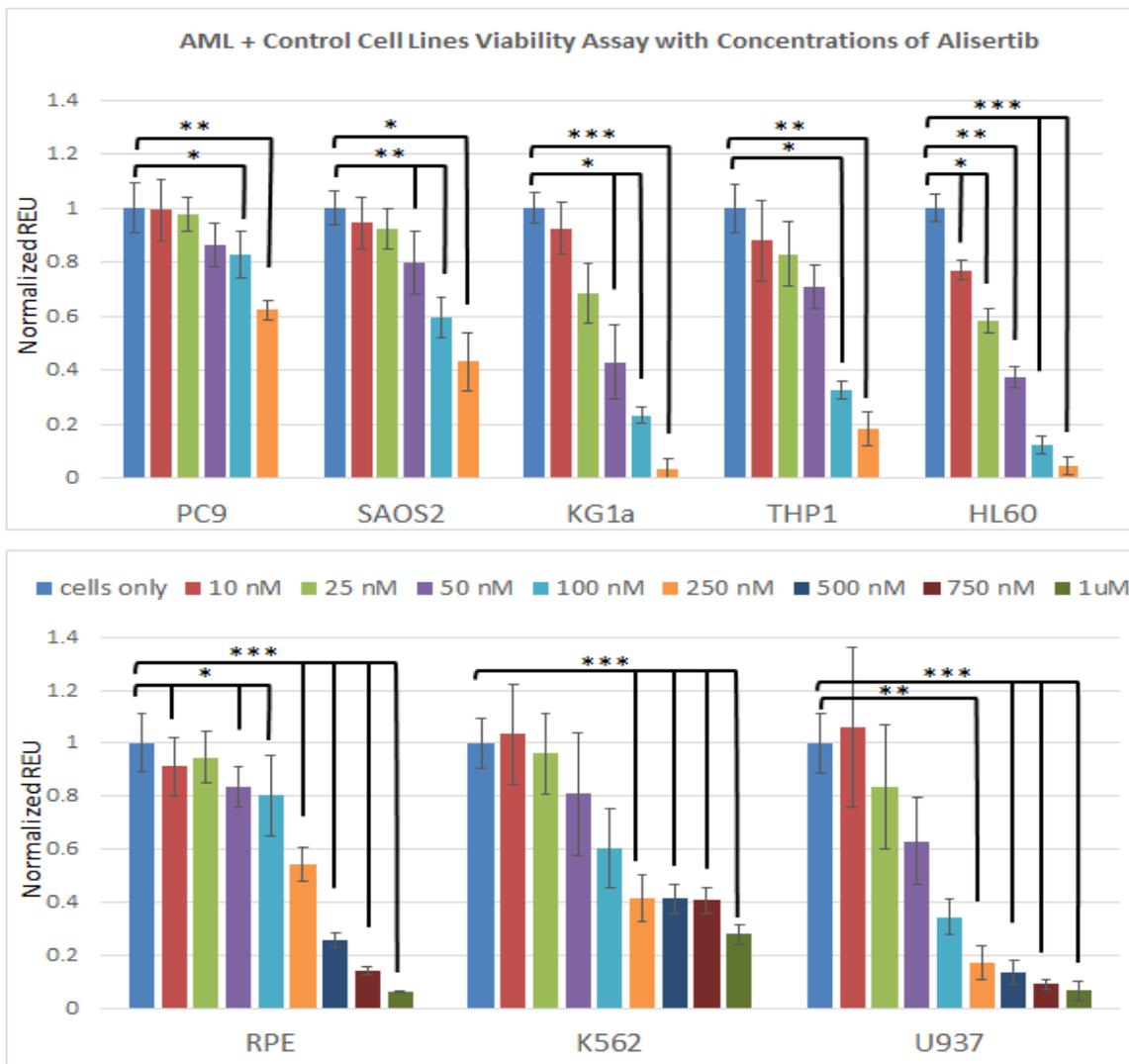
Active Aurora A (*pAurora A*) expression is amplified when treated with Nocodazole. Alisertib treatment caused the inhibition of active Aurora A. The loading control was normalized to the untreated condition for both Alisertib and Nocodazole. Aurora A was quantified by initially normalizing it with the respective loading control and comparing it with the untreated condition.

nocodazole treated, 100nM Alisertib treated, and both nocodazole and 100nM Alisertib treated. Treatment with nocodazole, a microtubule poison, arrests cells in mitosis (Figure 15), the stage of the cell cycle in which Aurora A is phosphorylated and active.

Treatment with nocodazole shows an increase in active Aurora A when compared with the asynchronous cells.

Qualitatively, Alisertib treatment at a 100nM concentration reduces phospho Aurora A to

undetectable levels, in both the presence and absence of nocodazole treatment. The levels of total Aurora A were equal among the conditions. Tubulin was used as a loading control to ensure that the increase in pAurA was not due to the loading of more protein.

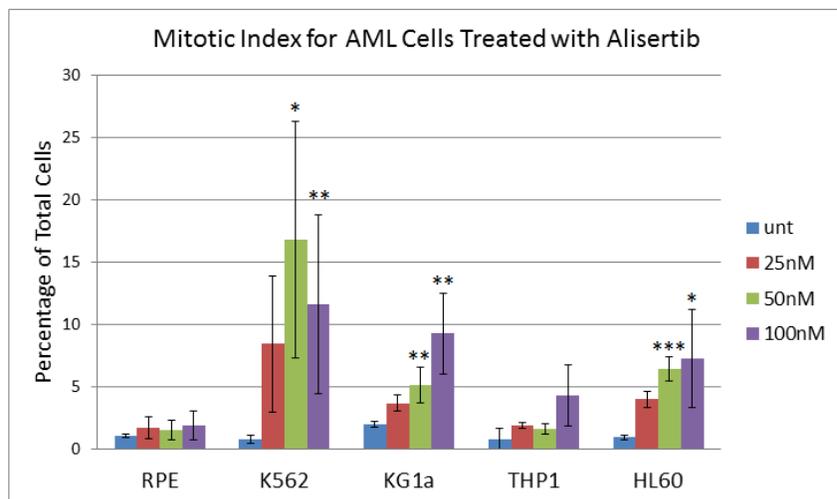


**Figure 16. AML and Control Cell Lines Viability at Concentrations of Alisertib**

Viability assays were used to assess cell viability in each cell line with concentrations of Alisertib. K562 and U937 both scored to have reduced drug sensitivity to Alisertib. Standard Deviation is 1 way ANOVA: P value < 0.05 (\*), P value < 0.01 (\*\*), P value < 0.001 (\*\*\*).

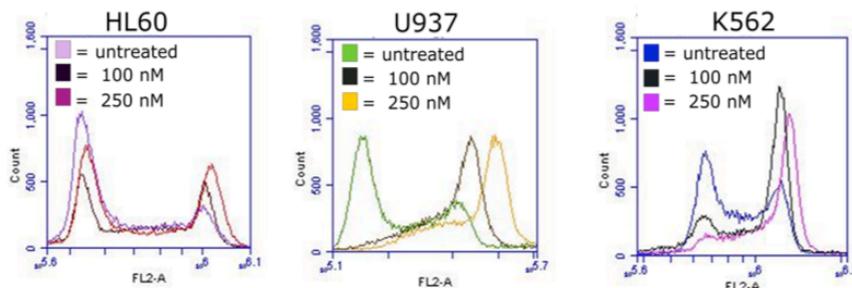
To determine the effects of Alisertib treatment at different concentrations in control and AML cell lines, viability assays were performed (Figure 16). This assay determines the number of viable cells after being treated with Alisertib for 72 hours, which were normalized with their respective untreated condition. Five AML cell lines (KG1 $\alpha$ , HL60, K562, U937, and THP1) and three controls (RPE-1, PC9, and SAOS2) were exposed to increasing concentrations of Alisertib. The

three controls experienced a significant decrease ( $p =$ ) in viability when treating with 250nM concentrations of the drug. In contrast, AML viability to Alisertib concentration was dependent on the cell line. HL60 and KG1 $\alpha$  appeared to be the most sensitive AML cell lines, since they experienced a significant decrease in cell viability when treated with 10nM and 25nM of Alisertib, respectively. Alternatively, K562 and U937 were the most resistant cell lines, and experienced a significant decrease when treated with a 250nM concentration. Because of that, additional



**Figure 17. Mitotic Index for AML Cells Treated with Alisertib**

Immunofluorescence was used to score mitotic index for AML cells treated with varying concentrations of Alisertib. K562, KG1 $\alpha$  and HL60 experienced significant increases in mitotic index at 50 and 100 nM of Alisertib. Standard Deviation is 1 way ANOVA: P value < 0.05 (\*), P value < 0.01 (\*\*), P value < 0.001 (\*\*\*).



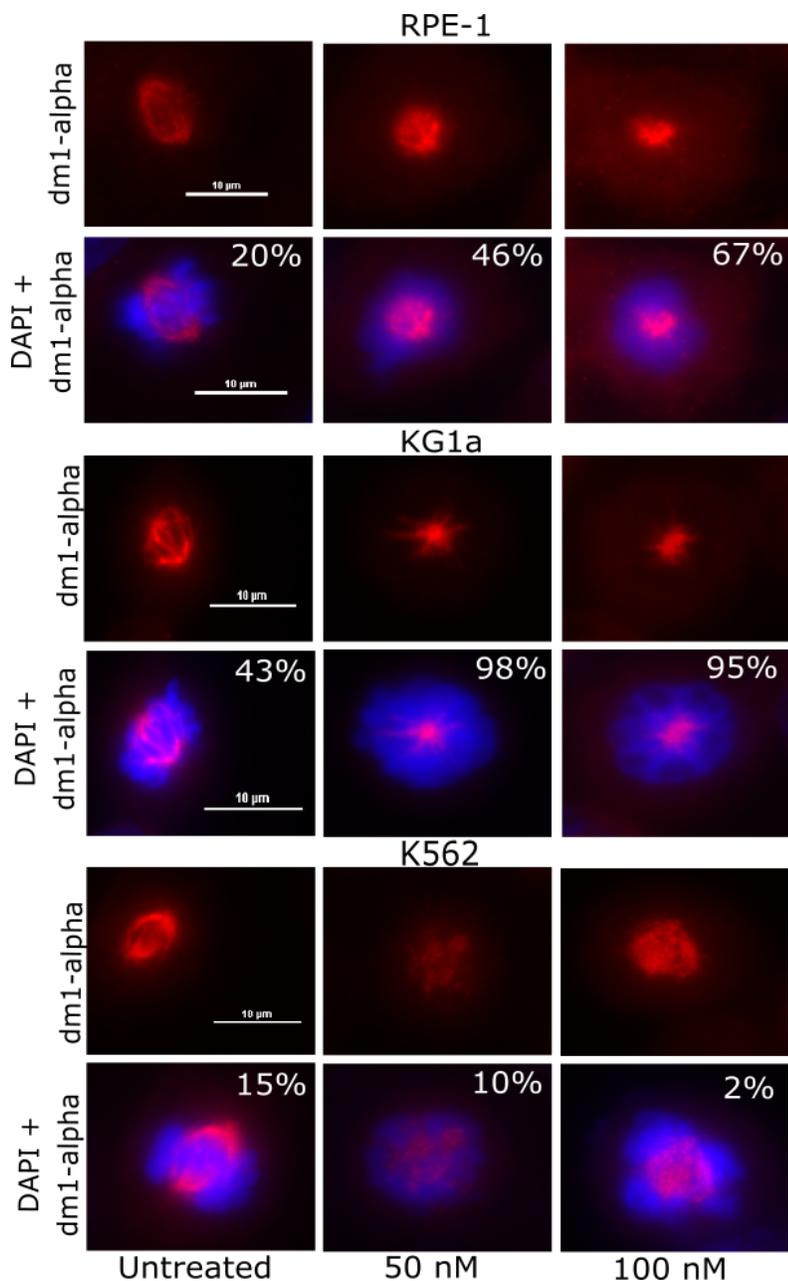
**Figure 18 FACS analysis Cell Cycle of AML cells treated with Alisertib**

*FACS analysis showed an increase in the number of cells in mitosis, specially in the cell lines U937 and K562, after treatment with Alisertib at 100nM and 250nM concentrations. This arrest is dose-dependent.*

viability assays were performed with higher Alisertib concentrations (500nM, 750nM, and 1uM) using these two AML-resistant cell lines and the control RPE-1. RPE-1 and U937 behaved similarly, with only 10% of viable cells at a 1uM concentration. However, the increase in the treatment concentration did not have an effect in K562, which maintained a similar percentage of viable cells.

To further examine the effect of Alisertib treatment on AML cells, immunofluorescence imaging was performed. Cells were left untreated, or treated with 25nM, 50nM, or 100nM of Alisertib for 14h. The percent of cells arrested in mitosis and the spindle polarity of the mitotic cells were examined for all conditions. AML cell lines experienced

an increase in mitotic arrest with increasing concentrations of Alisertib (Figure 17), K562 being the cell line that expressed a higher number of cells in mitosis compared with other AML cell

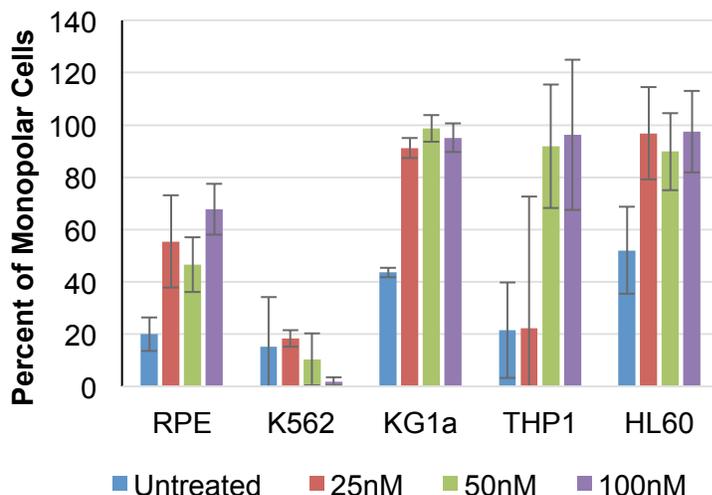


**Figure 19. Immunofluorescence Analysis on Spindle Polarity after Alisertib Treatment**

*Treatment of AML cell lines with Alisertib (100nM and 250nM) caused the formation of monopolar spindle poles in control and representative cell line KG1 $\alpha$ . However, resistant cell line K562 did not increase the number of monopolar spindle poles.*

lines. On the contrary, the control cell line RPE did not experience an increase in mitotic cells. FACS analysis was performed to confirm the increase in mitosis, which positively correlated with the concentration of Alisertib used (Figure 18).

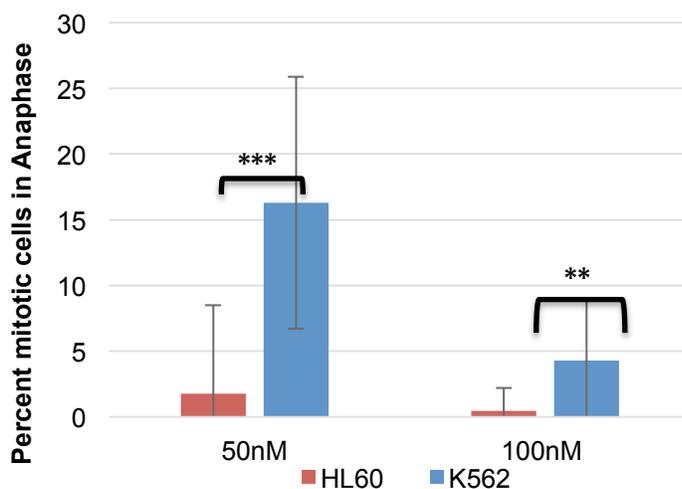
Spindle polarity of mitotic cells was also examined (Figure 19 and Figure 20), exhibiting a dose-dependent increase in monopolarity. RPE-1 cells, used as controls, expressed a 46% of monopolar spindles when treated with 50nM of Alisertib, which was further increased to 67% when the treatment concentration was increased to 100nM. Regarding AML cell lines, most of them already expressed a higher number of monopolar spindles before treatment, which was increased significantly up to 95%. In the case of K562, the opposite results were observed. As the concentration of Alisertib increased, fewer cells were expressing monopolar spindles in favor of multipolar and disorganized spindle expression.



**Figure 20 Percentage of mitotic cells expressing monopolarity**

Treatment with Alisertib causes control and most AML cell lines to express monopolar spindles when they are in mitosis. On the other hand, Alisertib-resistant AML cell line K562, reduced the number of mitotic cells expressing a monopolar spindle.

and 100nM), the resistant AML cell line K562 exhibited x % of cells in anaphase, a significantly higher number than compared with the sensitive AML cell line HL60. Although increasing concentrations of Alisertib limited anaphase cells in both cell lines, K562 maintained a higher



**Figure 21. Percent of Mitotic cells in Anaphase**

Alisertib-resistant AML cell line K562 exhibited a significantly increased number of mitotic cells in anaphase compared with Alisertib-sensitive AML cell line.

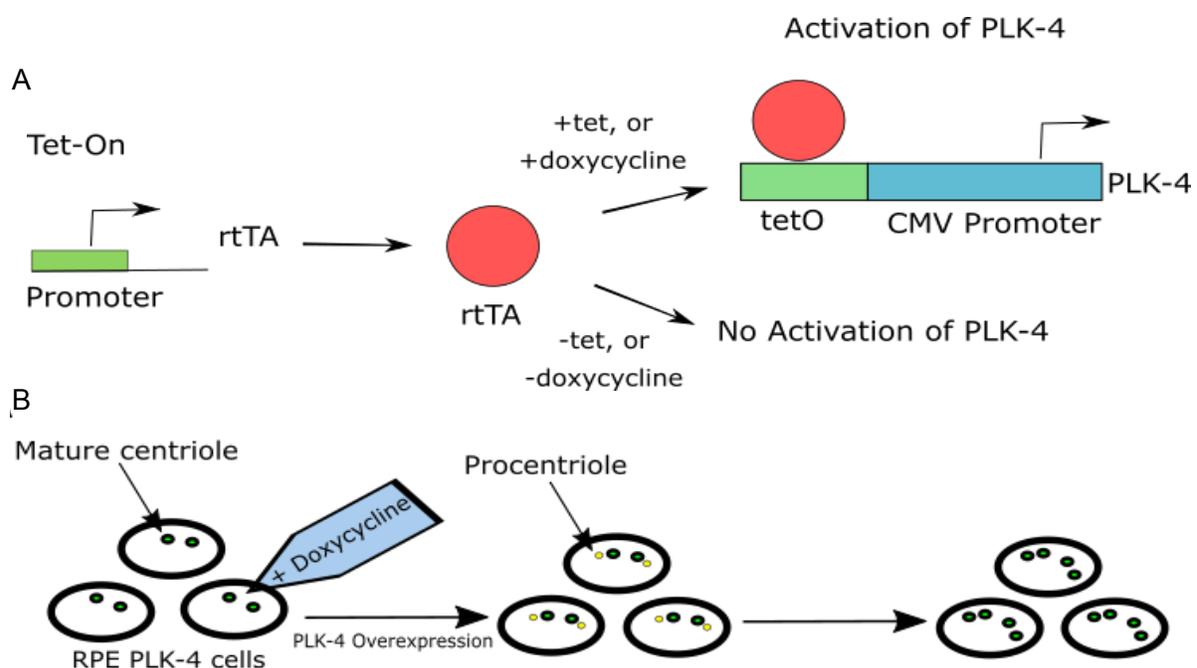
One explanation as to why cells might be resistant to alisertib would be if they failed to maintain a mitotic arrest and were instead able to proceed to anaphase. To determine if this could explain reduced sensitivity in the K562 cell line, we next determined the percentage of mitotic cells that are in anaphase when treating with Alisertib. Sensitive cell line HL60, was examined for comparison. When treated with two concentrations of Alisertib (50nM and 100nM), the resistant AML cell line K562 exhibited a significantly higher percentage of anaphase cells than HL60 at both low and high doses of Alisertib (Figure 21)

#### 4.4 Centriole amplification independently from Aurora A overexpression causes resistance to Alisertib treatment

Because previous experiments depicted a correlation between Aurora A overexpression, centrosome amplification and resistance to Alisertib treatment, we designed new experiments in

which we could amplify centrosomes in an Aurora A independent manner to determine whether

Aurora A overexpression or centrosome amplification is causing the AML cell lines to be less sensitive to treatment. This was done using a RPE-1 cell line that expresses polo like kinase 4 under a tetracycline-on promoter. Polo-like kinase 4 (PLK-4) is a cell cycle kinase involved in



**Figure 22. PLK-4 Overexpression Experimental Layout**

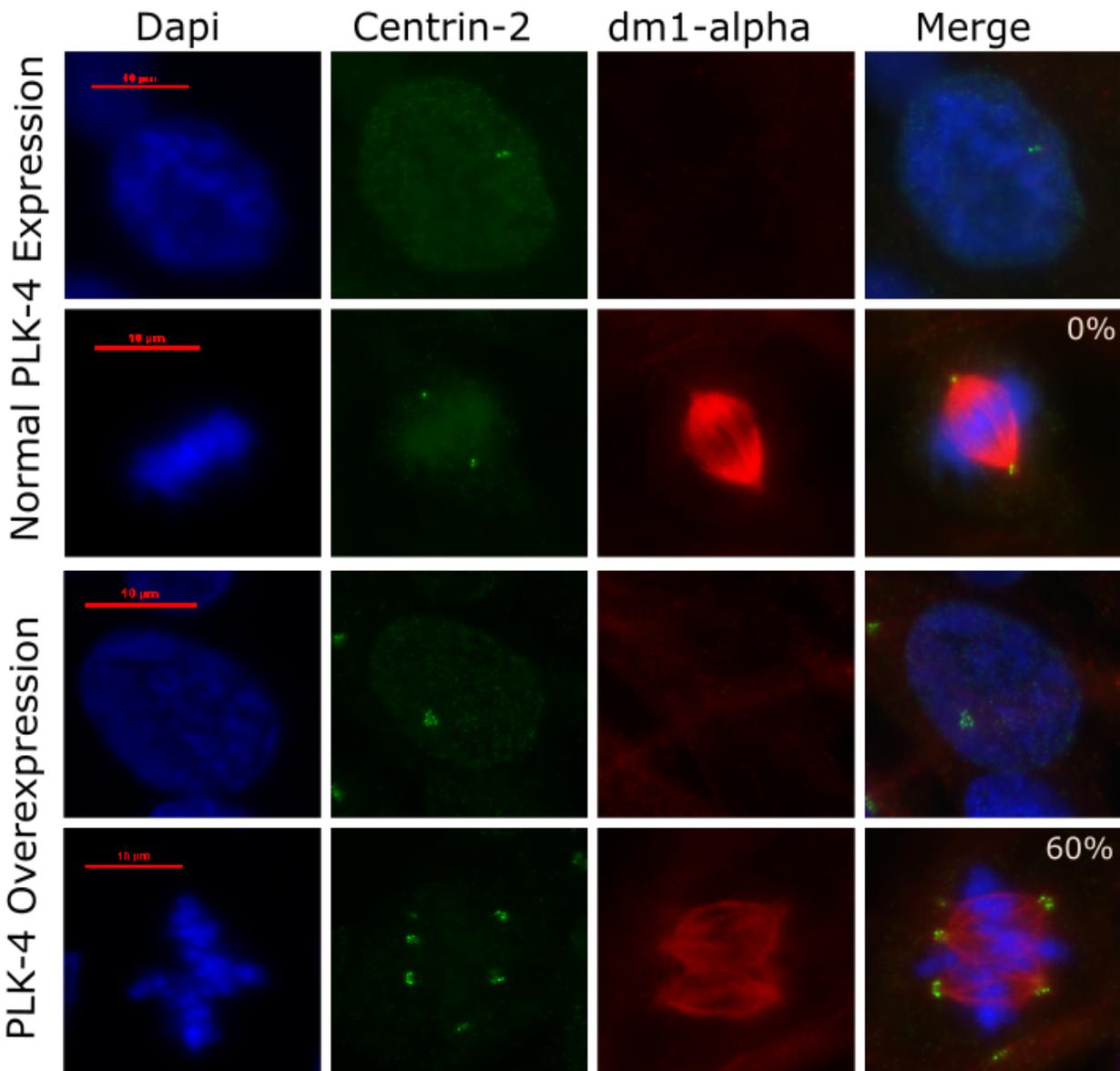
Figure 22A shows the PLK-4 plasmid, and its transcription is activated by the addition of doxycycline.

Figure 22B shows the experimental set-up of the induction of PLK-4 overexpression in RPE-1 cells.

Doxycycline is added to RPE-PLK-4 cells, which results in the overexpression of PLK-4. This leads to the formation of additional procentrioles leading to the development of mature centrioles.

centrosome maturation. When overexpressed, PLK-4 can induce centriole amplification by producing multiple procentrioles that adjoin to the parental centriole (Kleylein-Sohn et al, 2007). In a Tet-on regulated system, the expression of the gene under the promoter is controlled by the presence of tetracycline or doxycycline (Zhou, et. al., 2006). The addition of doxycycline in our experiments inhibits the interaction of the tet-repressor protein with the tet-operator because of a conformational change (Zhou, et. al., 2006). Additionally, the tet-repressor protein binds to the domain of the V16 protein, which is derived from herpes simple virus. In a tet-on system, the tet-repressor protein contains a change in amino acids that causes it to express the opposite phenotype (Zhou, et. al., 2006). This means that in the presence of doxycycline, the reverse tet-activator (rtTA) binds to the tet-operator (tetO) and it causes the expression of the gene (Zhou, et. al., 2006). Doxycycline was used in order to cause the overexpression of Plk-4. To confirm the effects on centriole number as well as spindle polarity, immunofluorescence analysis was used (Figure 23).

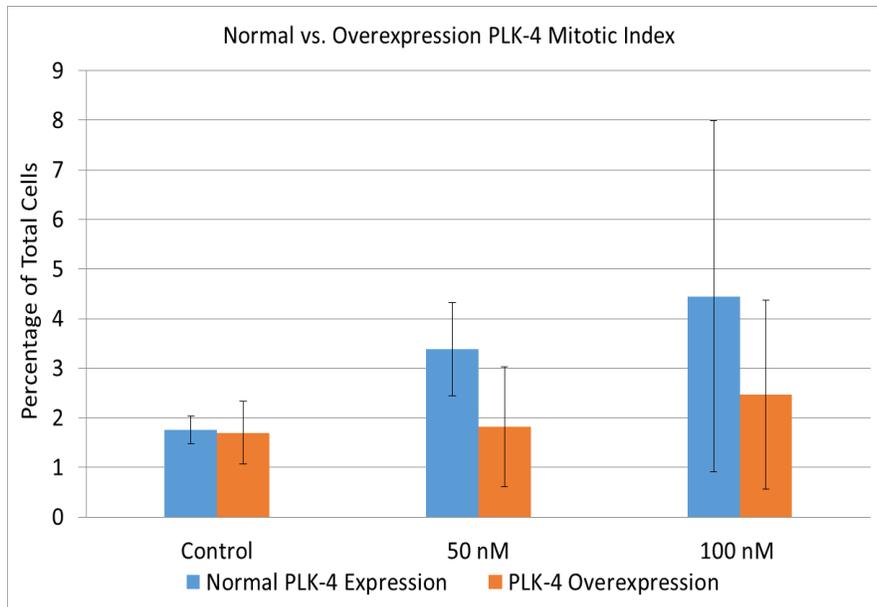
The cells that were cultured without the addition of doxycycline express a single centrosome during interphase, which gets duplicated during mitosis. The addition of doxycycline for 48h caused the amplification of centrioles, in both interphase cells as well as during mitosis. This caused the spindle polarity to also be affected, with 60% of mitotic cells overexpressing PLK-4



**Figure 23. PLK-4 Overexpression and Centriole Amplification Confirmation**

*Immunofluorescence analysis of PLK-4 RPE-1 cells to confirm overexpression of PLK-4 and centriole amplification as a result.*

having a multipolar spindle formation. This data confirms that the induction of PLK-4 in the RPE1 PLK-4 cell line can successfully induce the amplification of centrioles.



**Figure 24. Mitotic Index of Normal and Overexpressed PLK-4 Cells**

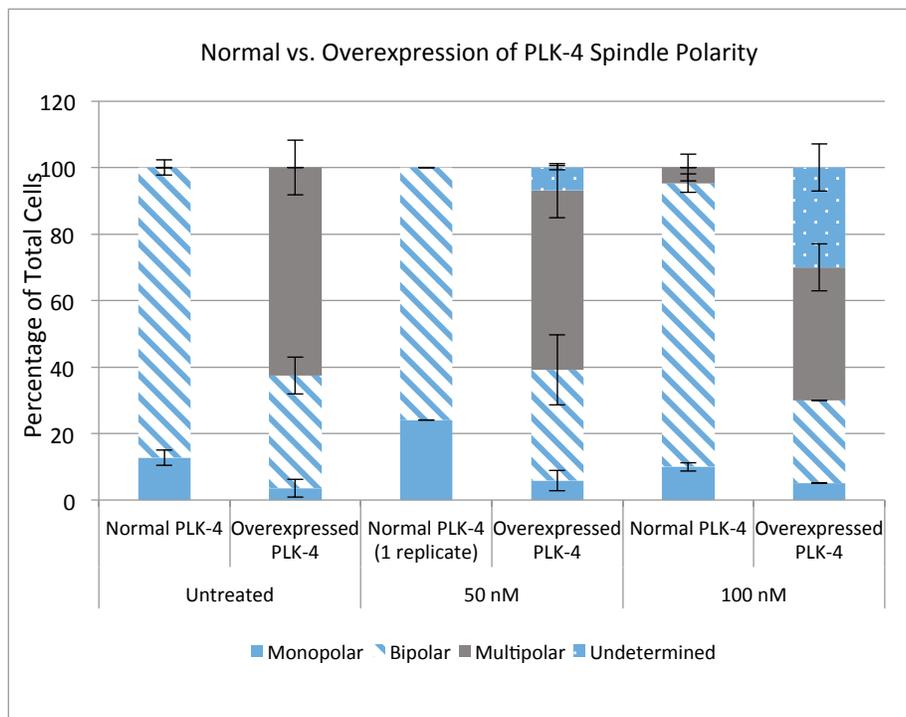
*Mitotic index was scored in normal and overexpressed PLK-4 cells in 50 and 100 nM of Alisertib. Both normal and overexpressed PLK-4 cells showed an increased mitotic index as Alisertib concentrations increased.*

To understand how centriole amplification affects cells treated with Alisertib, immunofluorescence analysis as well as viability assays were performed. First, mitotic index for cells in all conditions was determined. There were no significant differences in the number of cells in mitosis between RPE-1 cells that had normal and Plk-4 overexpression, with about 2% of the cells

being in mitosis. Moreover, treatment with Alisertib causes a slight, but insignificant increase in mitosis in a dose-dependent manner (Figure 24).

Next, we examined the effect of centriole amplification on monopolar spindle formation using Alisertib to inhibit Aurora A kinase in the RPE-1 cells, with or without induction of PLK-4 overexpression (Figure 25). Overexpression of Plk-4 in untreated cells caused an increase in the number of cells expressing multipolar spindles, and a concurrent decrease in cells exhibiting a bipolar spindle. Treatment with Alisertib in the cells that are overexpressing Plk-4 did not cause an increase in monopolarity. On the other hand, there can be seen an increase in the number of cells that express a disorganized spindle pole, as the concentration of Alisertib increases.

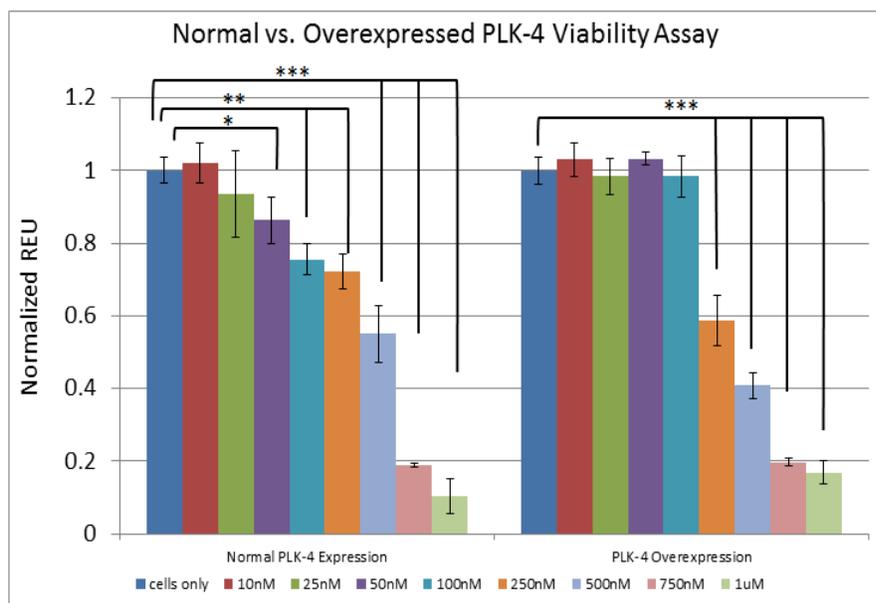
In a couple of the analyzed AML cell lines (K562 and U937) centriole amplification and reduced sensitivity to Alisertib were observed. To examine the role of centriole number in Alisertib sensitivity, without additional factors such as Aurora A overexpression the RPE-1 PLK-4 overexpressed cell line was used. The cell with regular levels of PLK-4 expression behaved comparably to the RPE-1 cells in Figure 12, showing a significant decrease in cell viability at 50



**Figure 25. Spindle Polarity of RPE cells with and without overexpression of Plk-4**

*Overexpression of Plk-4 in RPE cells causes them to express a high percentage of multipolar spindle poles, as well as a disorganized phenotype when the concentration of Alisertib increases*

nM. In the cells with PLK-4 overexpression, there was not a significant decrease in cell survival until 250 nM (Figure 26). This is similar to what was observed in the K562 and U937 cell lines in (Figure 16). These results demonstrate that increases centriole number is a contributing factor in resistance to induced monopolarity and may impact decreased sensitivity of AML cancer cells to Alisertib treatment.



**Figure 26. Normal and Overexpressed PLK-4 Cell Viability Alisertib Treatment**

*Viability assays were used to assess the cell viability of normal and overexpressed PLK-4 cells at concentrations of Alisertib. Overexpressed PLK-4 cells did not show a significant decrease in cell survival until 250 nM. Standard Deviation is 1 way ANOVA: P value < 0.05 (\*), P value < 0.01 (\*\*), P value < 0.001 (\*\*\*)*

## 5. Discussion

Aurora A has been characterized to have several roles during mitosis, such as mitotic spindle assembly, centrosome separation, and G2-M transition, among others. The dysregulation of this kinase has serious effects on the cell, as it causes many defects on mitotic spindle formation, chromosome separation, and increases genomic instability. Aurora A kinase has risen as a possible target for the development of new therapeutics, since it has been shown to be overexpressed in a number of different cancers, such as liver cancer. In this context, research has also revealed that many patients suffering from acute myeloid leukemia also exhibit an overexpression of this kinase. Alisertib, a specific inhibitor of AurA function, is being used in clinical trials in order to determine its effectiveness on AML patients. Our results indicate that AML cell lines respond to Alisertib treatment differently, and suggest the number of centrioles could be affecting the effectiveness of treatment.

Characterization of the AML cell lines started by determining which of those showed an overexpression of Aurora A, and K562 and U937 were the two cell lines to show the highest amount of Aurora A expression during unsynchronized and synchronized states (Figure 11). These two cell lines that overexpress Aurora A, also amplified the number of centrioles (Figure 14) when immunofluorescence analysis was performed. This could be a direct result of Aurora A overexpression, since Aurora A plays an indirect role in centriole duplication. Lastly, spindle polarity was also determined for control and AML cell lines. K562, one of the cell lines that showed amplified number of centrioles, exhibited a number of cells in multipolar and disorganized spindle poles. On the other hand, other AML cell line which express a normal number of centrioles and do not overexpress Aurora A at the high levels of K562 and U937, exhibited a higher amount of monopolar spindles compared with the control RPE-1 (Figure 13).

The effect that Alisertib has on AML was next addressed, after concluding that Alisertib is able to inhibit active Aurora A (phosphorylated Aurora A) in K562, even when the cells are treated with Nocodazole to cause their arrest in mitosis, the stage in mitosis in which Aurora A is active (Figure 15). Cells that overexpress Aurora A and amplify centriole number showed the need for a higher concentration of Alisertib for cells not to be viable. K562, showed the most resistance to Alisertib treatment, while other AML cell lines exhibited a significant decrease in cell viability with lower concentrations of the drug (Figure 16). Treatment with Alisertib caused the arrest of AML cell lines in mitosis, in a dose-dependent manner (Figure 17 and Figure 18). When

determining the spindle polarity of the cells arrested after Alisertib treatment we also see a correlation between those cell lines that overexpress Aurora A more and amplify centrioles and those which do not. While the AML cell lines that express a normal number of centrioles and Aurora A exhibit a monopolar spindle pole, those that overexpress Aurora A and exhibit increased centrioles number decrease the amount of monopolar cells, and show an increase in multipolar and disorganized spindle poles (Figure 19). Moreover, the number of mitotic cells in anaphase after treatment with Alisertib is significantly greater than an Alisertib-sensitive cell line, such as HL60 (Figure 20).

Alisertib is the most specific Aurora A inhibitor up to date. However, as many of the other Aurora A inhibitors, this drug can also be specific for other Aurora kinases, such as Aurora B kinase, at higher concentrations. Based on this, the decrease in cell viability that we observe on some AML cell lines is likely due to inhibition of Aurora A, since a low concentration of Alisertib, such as 25nM, already causes a significant decrease (Figure 16). It is possible that the cell lines that are more resistant and do not show a decrease in viability after an increase in Alisertib concentration are due to Alisertib inhibiting other targets such as Aurora B. There is a need then for performing similar experiments with other Aurora A inhibitors to confirm the results.

Because of these results, and in order to separate the expression of Aurora A to the one of centrioles, we used RPE engineered cells to overexpress Plk-4, which allowed us to induce centriole amplification (Figure 22). This overexpression of centrioles affected the cellular changes when they were treated with different concentrations of Alisertib. Those that expressed amplified centrioles maintained a multipolar spindle phenotype when treated with Alisertib (Figure 25). In addition, cells with PLK-4 overexpression had a reduced Alisertib sensitivity compared to the cells with normal levels of PLK-4 (Figure 26). The cells without PLK-4 overexpression experienced a decrease in cell survival at 50 nM Alisertib, whereas the cells viability was not significantly affected in cells overexpressing PLK-4 until 250 nM. At Alisertib concentrations higher than 250 nM, both cell types were sensitive to the drug and cell survival decreased significantly. From these experiments, we conclude that centriole abundance may play a role in reduced drug sensitivity. Because of these results, two more experiments were designed. In order to ensure that results that we obtained are due to an amplification of centrioles and not the overexpression of Plk-4 itself, HCT116 cells expressing a p53 knockout were used and treated with the drug cytochalasin B. This drug acts by shortening filaments and prevents the completion of cytokinesis, which leads to the acquisition of multiple centrioles after

a cell cycle failure. The cells need to have a defective p53 status, which allows the cells to continue through the cell cycle even with an amplification of centrosomes.

Additionally, live cell imaging is being performed on RPE with or without overexpression of centrioles to understand the effects it has on cell fate when treated with different concentrations of Alisertib. This allows the visualization of the cells during a 16h period of time and the determination of whether cells arrest in mitosis, continue the cell cycle, or exit mitosis.

While centriole number may play a role in Alisertib sensitivity and monopolar spindle formation, the mechanism of this action and other cellular factors that may contribute were not investigated. Therefore, work on this project could address the effects of overexpression of Aurora A in RPE or non AML cell lines and determine the cell's sensitivity to Alisertib. This would help to decipher if Aurora A overexpression has a significant impact on the monopolar spindle pole formation or the sensitivity of the cells to Alisertib. Loss of PLK-4 has been shown to prevent centriole duplication (Holland et al., 2012) Therefore, to further test the impact of centrosome number on Alisertib sensitivity, it would also be interesting to reduce the expression of PLK-4 in K562 (the cell line with an increase in centriole number), to observe the impact of reduced centriole number in these cell lines. This would provide additional insight into the relationship of centriole abundance and drug sensitivity.

To further understand the relevance of centriole abundance and drug sensitivity, in vivo models could be utilized to further assess what factors impact the effectiveness of Alisertib. This experiment would be used to support previous experiments and also explore the potential effects of centriole amplifications in tumor growth and treatment in living models.

These results could lead to further patient studies, where blood samples from each patient are taken and their cellular structure is analyzed. If their cells show signs of centriole abundance, physicians may instruct the patients to use higher dosing or even a treatment different from Alisertib or use a combination of therapies to target the leukemic cells in addition to Alisertib. This would be an initial step to creating patient profile that would indicate drug efficacy based on cellular phenotype.

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