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Identifying Mechanisms Of Resistance And Potential Therapeutic Targets For Pediatric Acute Myeloid Leukemia

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IDENTIFYING MECHANISMS OF RESISTANCE AND POTENTIAL THERAPEUTIC TARGETS FOR PEDIATRIC ACUTE MYELOID LEUKEMIA

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DEDICATION

This work is dedicated to two (often, but not always, distinct) groups of people.

The first is all of those suffering from any sort of debilitating disease. There are a lot of people hard at work to make your life better, even though it may not always seem that way. Our efforts may not be felt on your end, but I assure you that we are here, and are on your side. For many of us, our goal is the end of your struggles and we will work tirelessly to see that goal achieved.
Never give up hope – because we certainly won't.

The second is to my peers, as well as those who will follow in our footsteps. You have undertaken a great task. Great because it often seems insurmountable, but also great because you might just change the world. Research is hard, and on many days seemingly Sisyphean. However, while your resolve will be tested, I urge you to continue on. No matter what path you choose after you are done training, by participating in research you are contributing to the truth upon which future knowledge will be built. With that in mind, do good work, because we are all counting on you.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
ALL	Acute Lymphoblastic Leukemia
AMKL	Acute Megakaryocytic Leukemia
AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
araC	Cytarabine
ara-C	Cytarabine
araCTP	araC-Triphosphate
araU	Uracil Arabinoside
ATRA	All-*trans*-Retinoic Acid
C/EBP	CCAAT Enhancer Binding Protein
C/EBPα	C/EBP Isoform Alpha
CBF	Core Binding Factor
CBS	Cystathionine-β-Synthase
CCG	Children's Cancer Group
CDA	Cytidine Deaminase
CDK	Cyclin Dependent Kinase
CHD	Congenital Heart Defects
ChIP	Chromatin Immunoprecipitation
CI	Combination Index
CN-AML	Cytogenetically Normal AML

CNS	Central Nervous System
CR	Complete Remission
dCK	Deoxycytidine Kinase
DNR	Daunorubicin
DS	Down Syndrome
DSB	Double Stranded Break
EFS	Event-Free Survival
EPO	Erythropoietin
FAB	French-American-British
FL	FLT3 Ligand
FLT3	fml-like Tyrosine Kinase
FLT3-ITD	FLT3 Internal Tandem Duplication
FOG1	Friend of GATA1
G-CSF	Granuloctye-colony Stimulating Factor
GM-CSF	Granulocyte/Macrophace-colony Stimulating Factor
HDAC	Histone Deacetylase
hENT1	Human Equilibrative Nucleoside Transporter 1
HiDAC	High-dose araC
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplant
Jak-STAT	Janus-kinase/Signal Transducer and Activator of Transcription
MDS	Myelodysplastic Syndrome

MEP	Megakaryocyte-Erythroid Progenitor
MM	Multiple Myeloma
MRD	Minimal Residual Disease
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NK	Natural Killer
OS	Overall Survival
Plk1	Polo-like Kinase 1
POG	Pediatric Oncology Group
ROS	Reactive Oxygen Species
SAHA	Suberanilohydroxamic Acid
SCF	Stem Cell Factor
tAML	Treatment-related AML/MDS
TMD	Transient Myeloproliferative Disorder
Topo2	Topoisomerase II
TPO	Thrombopoeitin
VCR	Vincristine
VP16	Etoposide
VPA	Valproic Acid
WBC	White Blood Cell
WHO	World Health Organization

CHAPTER 1. Introduction

1.0 Source Material

Unless otherwise stated, the source material for sections 1.2-1.4 is a combination of five sources: four reviews[31,156,173,174] and one textbook[75]. The reviews are especially well-written and offer timely snapshots of the pediatric AML field and are highly recommended.

1.1 Purpose

Acute myeloid leukemia (AML) is a potentially devastating disease that can affect people of all ages. While there are certain patient groups that typically have favorable outcomes, AML carries a relatively poor prognosis for most age groups when compared to other acute lymphoblastic leukemia (ALL). Furthermore, the treatment for AML typically consists of rigorous chemotherapeutic regimens and bone marrow/stem cell transplant, both of which are associated with severe acute and chronic toxicity and the potential for treatment-related mortality. In order to improve both survival and survivorship after the diagnosis of AML, the development of better treatment options is of paramount importance.

1.2 Hematopoiesis

Like other hematologic malignancies, AML is the result of unchecked proliferation of immature cells from a hematopoietic lineage. Hematopoiesis is the lifelong process by which the cellular components of blood are produced. In general, these components can be classified as either lymphoid (e.g. T- and B-cells) or myeloid (e.g. red blood cells [RBCs] and neutrophils), and each type of

cell plays a specific role within the body. For the sake of clarity, the traditional dichotomous understanding of hematopoiesis will be presented here, although it is important to note that this model has come under question in recent years[96].

1.2.1 Locations of hematopoiesis

As development progresses from embryo to adult, hematopoiesis occurs at varying locations throughout the body. Initially, hematopoiesis begins in the yolk sac of the developing embryo, and occurs exclusively at this site until the development of the fetal liver. As the liver develops, it eventually is the home to the majority of hematopoiesis, although there is a minor role for the fetal spleen for a brief period. Eventually, as the skeletal system and the marrow compartment develop, hematopoiesis begins to occur primarily in red marrow. Though there is a minor role for lymphatic organs in hematopoiesis later in life, red marrow remains the primary site of hematopoiesis until death.

1.2.2 Lymphocytic hematopoiesis and the role of major lymphoid lineages

Though not the focus of the work presented herein, the lymphocytic compartment is responsible for the majority of acquired immunity and cancer prevention in humans, and until recently, deficiencies in this compartment were largely incompatible with post-natal survival. Derived from a common hematopoietic stem cell (HSC), the lymphoid lineage produces T-cells, B-cells, and natural killer (NK) cells. When a HSC is stimulated by the correct external factors, which include extracellular signals from cytokines like notch-ligand and certain interleukins, as well as internal factors, like expression of the proper transcription factors, it develops into a common lymphoid progenitor. From this

state, the decision is made, again by a combination of internal and external factors, to differentiate into one of the terminal lymphoid lineages.

1.2.2.1 B-cells Antibody production, which is required for a rapidly acting adaptive immune system, is the major responsibility of the B-cell lineage. Pre-B cells migrate from the marrow to secondary lymphatic organs like the spleen or lymph node, where they await further stimulation. These cells then undergo recombinatorial rearrangement of the B-cell receptor, which eventually forms the scaffold for the variable region of an antibody. Upon antigen recognition, these pre-B cells become mature B-cells, and can then differentiate into plasma cells for antibody production, or memory B-cells which are long-lived cells that help to preserve immunity. Interrogation of B-cell development has led to great advances in the understanding of DNA repair and cellular maturation processes, however, in depth discussion of these processes is beyond the scope of this work.

Of particular note is the somewhat unique case of plasma cell malignancy. Multiple myeloma (MM) is a disease characterized by uncontrolled proliferation of non-functional plasma cells. The natural history of this disease is quite different from those of other hematologic malignancies, and unfortunately MM is usually fatal. Though not discussed specifically here, as MM is not diagnosed in pediatric patients, good treatment overviews can be found in reviews by Stewart et al. and Mehta et al.[130,194].

1.2.2.2 T-cells Like B-cells, T-cell precursors migrate out of the bone marrow to finish differentiation in a secondary location, in this case, the thymus.

It is in the stroma of the thymus that the highly regulated process of T-cell maturation occurs. Though there are several subtypes of T-cells, the most commonly discussed are those that express either CD4 or CD8. Helper T-cells, or CD4+ cells, represent a variety of cells that act to enhance or reduce the amount of immune activation in response to a stimulus. This compartment is necessary for the full function of the acquired immune system, as CD4+ cells are required for complete activation of CD8+ cytotoxic T-lymphocytes as well as isotype switching in B-cells. In contrast, CD8+ cells, which at terminal maturation become cytotoxic T-lymphocytes, are responsible for the recognition and extermination of virally infected and otherwise transformed (including malignant) cells.

1.2.2.3 Natural Killer Cells Occupying somewhat of a different role in the lymphoid compartment are NK cells. The activation of and target recognition by NK cells is a complicated matter, but the important feature of NK cells is that they are able to target cells without prior exposure to an insult. These cells recognize target cells through a combination of down-regulated major histocompatibility complex I and antibody coating. By recognizing these features, which are common in virally infected and malignantly transformed cells, NK cells are able to help clear potentially dangerous host cells.

1.2.3 Myeloid hematopoiesis – a general overview

The alternative to lymphocyte production is progression down the myeloid lineage. At various stages, the presence of extracellular signaling molecules (discussed in this section), stromal interactions, and transcription factor

expression (discussed in section1.3.2) all combine to drive progression towards a specific lineage. The first major decision after myeloid commitment is whether or not to produce granulocytes. If the granulocyte decision is made, development will progress through the myeloblast stage culminating in either basophil, eosinophil, neutrophil, or monocyte production. The alternative to the granulocyte pathway is the erythrocyte/megakaryocyte lineage, which will be discussed in detail below in section 1.2.3.1.

Just as in lymphoid development, there is an important role for extracellular signaling molecules in myeloid hematopoiesis and lineage determination. Important cytokines for myeloid differentiation include, among others, interleukins 3, 4, and 5, stem cell factor (SCF), granulocyte-colony stimulating factor, and granulocyte/macrophage-colony stimulating factor (G-CSF and GM-CSF, respectively). These cytokines activate a variety of intracellular pathways, but many bind receptor tyrosine kinases, with eventual activation of the Janus-kinase/signal transducer and activator of transcription (Jak-STAT) and mitogen-activated protein kinase (MAPK) pathways. Though a detailed description of these pathways is beyond the scope of this work, these commonly activated pathways have pleiotropic effects, and can cause changes in intracellular signaling cascades, protein localization, and transcription/protein expression.

SCF serves as the ligand for the c-kit receptor, which is highly expressed in most myeloid lineages as well as in many AML cases. Expression of c-kit, and subsequent activation by its ligand, is required for hematopoiesis and survival.

Though less commonly thought to be driving mutations in AML, mutations in c-kit are in fact driving mutations for a type of tumor known as gastrointestinal stromal tumor, or GIST. The colony-stimulating factors G-CSF and GM-CSF play a role in lineage decision, and variations in their levels help drive production of specific myeloid cells.

Another factor important for discussion here, due to its association with malignant phenotypes, is the fms-like tyrosine kinase 3 (FLT3) ligand (FL). The FLT3 receptor can be found on many hematopoietic cell types, and its activation drives enhanced proliferation and survival. Interestingly, knockout of FLT3 is not lethal, and administration of FL alone has little effect. However, FL appears to substantially enhance the effect of many other cytokines. Unfortunately, as discussed below, aberrant activation of the FLT3 axis has deleterious effects.

1.2.3.1 Erythropoiesis and Thrombopoiesis After the common myeloid progenitor stage, cells have an opportunity to differentiate towards what is referred to as a megakaryocyte-erythroid progenitor (MEP). At this point, though there is some disagreement as to how, a cell has to determine which lineage to pursue. In order to differentiate down the erythroid lineage, the master regulator of erythropoiesis, GATA1, must be expressed. The product of the *GATA1* gene on the X chromosome, GATA1, is an essential transcription factor for the development of the erythroid compartment. The presence of GATA1 is an absolute requirement for maturation of erythroid precursors and definitive (late-stage) erythropoiesis, and mice that lack functional GATA1 protein die *in utero* of severe anemia. GATA1 binds and activates many genes important for

erythropoiesis, including the erythropoiesis-stimulating growth factor erythropoietin (EPO). It does this, at least in part, in collaboration with another transcription factor named “friend of GATA1” (FOG1). FOG1 interacts with the N-terminus of GATA1 and enhances the transcription of genes. This interaction is required for definitive erythropoiesis, but is not essential early in the erythroid differentiation process.

Alternatively, the MEP can produce megakaryocytes, which are giant cells that function to produce platelets. Like erythropoiesis, megakaryopoiesis also requires fully functional GATA1 and FOG1 to progress to completion. Uniquely, however, is the requirement for thrombopoietin (TPO), which is produced constitutively in the liver and kidney. After binding the TPO receptor, intracellular Jak-STAT signaling pathways are activated and serve to drive maturation and eventual thrombopoiesis. Once sufficiently mature, megakaryocytes begin the interesting process of endomitosis, in which the cell’s genetic material is replicated and the cytoplasm size increases, but only the nucleus divides. This process continues, and megakaryocytes with ploidy as high as 128N have been reported. The motivations for maintaining such large amounts of genetic material are slightly unclear, but it is thought that the additional DNA allows for more effective transcription and subsequent maintenance of such large cells. Fully mature megakaryocytes’ main function is the production of platelets, the function of which will be discussed below.

1.2.4 Roles of mature myeloid cells

1.2.4.1 Red Blood Cells. The most prominent circulating myeloid cell is

the erythrocyte, or red blood cell (RBC). They play the predominant role in the transport of oxygen from the lungs to the deep tissues of the body, and are what give blood its characteristic red color. These traits are both due to the presence of iron-conjugated heme groups in hemoglobin – a heme containing protein that is present in vast quantities in RBCs. While the absolute number of RBCs is important for some disease states, it is the quantity of hemoglobin that is the most useful clinically, because it is the most correlated with oxygen carrying ability. In fact, anemia is typically defined by a lack of hemoglobin rather than a low RBC count.

The RBC is unique in many ways among cells in the human body, most notably for the lack of nuclei in mature cells. The absence of a nucleus prevents the cell from being able to replenish proteins in response to stress, giving RBCs a finite life span, which is typically on the order of 100 days in a healthy adult. As the RBC ages, membrane changes allow the cell to be recognized by macrophages in the liver, spleen, and lymph nodes, which phagocytose the cell, clearing it from circulation. This constant destruction requires active replenishment, and as a result, RBCs are produced for the duration of a person's life. Of clinical importance is the detectability of recently produced RBCs in the peripheral circulation. These cells, which still possess varying degrees of ribosomal RNA are termed reticulocytes, and can be identified by stains that can detect the remaining RNA, such as methylene blue. As the cell matures further, this RNA is eventually degraded, and thus RNA content can be used to judge the age of the RBC. The quantity of circulating reticulocytes is a useful parameter to

interrogate because it gives an index of how actively the bone marrow is producing new RBCs, i.e., a high reticulocyte count indicates higher rates of production. This has utility during chemotherapy treatment, because it helps an oncologist to predict whether a patient's hemoglobin will trend upward or downward.

Clinically, and most relevant to this work, the most common problem associated with RBCs is anemia, typically defined by low hemoglobin. Low hemoglobin reduces the ability to deliver oxygen to peripheral tissues, and can be fatal in extreme cases. Anemia, when sufficiently acute and of substantial magnitude, typically presents as decreased energy, and is common in cancer patients receiving myelosuppressive chemotherapy. Fortunately, anemia can usually be managed (though not without potential complications) with infusion of packed RBCs and appropriate dosing schedules.

1.2.4.2 Neutrophils Often considered the first line of defense against infection, neutrophils are the most common granulocytes and typically make up the majority of circulating leukocytes. The role of neutrophils is to seek out and destroy insults, as well as enhance inflammatory responses. Primarily directed against invading bacteria, neutrophils are able to phagocytose bacteria and attempt to kill the microorganism using highly reactive oxygen species in a process that has been termed the "respiratory burst". The neutrophil also releases several other pro-inflammatory cytokines that aid in the recruitment and activation of other immune mediators. The pro-inflammatory nature of these cells is offset at least partly by their short life span; neutrophils only live for a few days.

Neutropenia, or low levels of neutrophils, can be a medical emergency and is commonly seen in patients receiving myelosuppressive chemotherapy. When patients' neutrophil counts are depressed, their susceptibility to infection skyrockets, and what are normally benign bacteria can become life-threatening. Unfortunately, unlike RBCs, neutrophils cannot be transfused so the best defense against profound neutropenia is prevention coupled with supportive care (typically prophylaxis against bacterial infection with antibiotics and aggressive, usually broad-spectrum antibiotic administration when infection is suspected). Prevention has historically been achieved through optimization of dosing regimens, careful monitoring of absolute neutrophil counts (ANC), and administration of G-CSF (generic: filgrastim) to increase neutrophil production.

1.2.4.3 Other granulocytes The remaining myeloid cell types, while important in their own right, are mostly relevant to leukemia for the potential of their lineages to become malignant. Monocytes are large circulating granulocytes that migrate to tissue and differentiate into various macrophage subtypes. The principle role for these macrophages is phagocytosis of cellular debris and invading pathogens. After phagocytosis, engulfed proteins are broken down and presented for antigen recognition by the adaptive immune system. Eosinophils are granulocytes that secrete pro-inflammatory markers as well as proteins that help defend against certain parasites. Basophils are the least common form of circulating granulocyte, and function to secrete pro-inflammatory markers involved in allergic responses. Lastly, mast-cells are very similar to basophils, except they extravasate and take up residence in various tissues

throughout the body. Malignant transformation of these last three lineages is rare, but can be associated with poor outcomes and high symptomatic burden [111].

1.2.4.4 Megakaryocytes and Platelets On the opposite end of the spectrum from the small, anuclear RBCs are the megakaryocytes. These massive, multinuclear cells exist to produce platelets, which, like RBCs, lack nuclei and serve somewhat as a delivery mechanism for their contents. Platelets are extremely small cellular fragments that bud off of megakaryocytes (the exact mechanism by which this occurs is somewhat unclear) and serve to maintain hemostasis by providing the cellular components of the clotting cascade. Though their lifespan is only on the order of one week, and typical counts are on the order of 10^5/μL, a healthy individual can easily maintain platelet levels with each megakaryocyte producing approximately 5,000 to 10,000 platelets over their lifespan. Like other cells of myeloid derivation, platelet levels can be jeopardized by myelosuppressive chemotherapy. Because low platelet counts prevent adequate clotting, hemodynamic instability is a major concern for thrombocytopenia-inducing antileukemic interventions. Fortunately, similar to RBCs, platelets can be transfused; however their short life span and their high cost dictate that this is only done when absolutely necessary.

1.3 Leukemia

Leukemia can be loosely defined as an aberrant hyperproliferation of immature blood cells that do not form solid tumor masses (i.e. liquid cancer). In general, leukemia can be either of the myeloid or lymphoid lineages, and

classified as being acute or chronic in nature. Chronic leukemias tend to have more mature cells, and are rare in pediatric patients. Acute leukemias, on the other hand, are typically less mature and commonly occur in patients of all ages and are potentially rapidly fatal if not readily treated; in fact, acute lymphoblastic leukemia (ALL) is the most common childhood malignancy. Similar to AML, and in some cases, on the same disease spectrum, are the myelodysplastic syndromes (MDS). These are a set of diseases that, while not always considered malignant, can be deadly, are sometimes treated with chemotherapy and often progress to AML. Though not discussed in this work, a good review of MDS can be found in reviews by Stone and Fenaux & Adés[40,196]. This work focuses on pediatric AML.

1.3.1 Epidemiology of Pediatric AML

Accounting for approximately 18% of childhood leukemia diagnoses, AML is one of the more common childhood cancer diagnoses. The risk for developing AML in the majority of cases is biological rather than environmental, with the only established pediatric AML cause being *in utero* exposure to ionizing radiation. Other exposures, e.g. maternal chemical exposure and parental age, have only limited evidence supporting their association with AML. In America, the overall incidence of pediatric AML is approximately 7.7 cases per million children. Race appears to play only a very minor role in AML risk among Americans, with Asian and Pacific Islanders having the highest incidence (8.4 per million) and African Americans having the lowest risk (6.6 per million). The risk of childhood AML peaks early in life at 18 per million in infants less than 1 year of age, reaches an

incidence of approximately 4 per million in children aged 5-9. The factors that convey the most risk are genetic. Down syndrome (DS) is the most common genetic risk factor, however, less common diseases, especially those associated with DNA repair deficiencies like Fanconi anemia and ataxia telangiectasia are also associated with an elevated risk to develop pediatric AML. For a more thorough overview of the epidemiology surrounding pediatric AML, please see the 2013 review by Puumala[157].

1.3.2 Biology of Pediatric AML

Originally divided into only a few morphological subtypes, advancements in molecular medicine have allowed for the reclassification of AML subtypes based on the vast array of different morphological, cytogenetic, and genetic variations that can be seen. One of the first major attempts at classification came with the development of the French-American-British (FAB) system in 1976[10]. The FAB system divided AML into 8 different subtypes, M0-M7, which correspond to: acute myeloblastic leukemia, minimally differentiated (M0); acute myeloblastic leukemia, without maturation (M1); acute myeloblastic leukemia, with granulocytic maturation (M2); acute promyelocytic leukemia (APL) (M3); acute myelomonocytic leukemia (M4); acute monoblastic/monocytic leukemia (M5a/b); acute erythrocytic leukemia (M6); acute megakaryocytic leukemia (M7, AMKL). Though less commonly used for prognostic indications, the FAB classification is well-entrenched in modern hematology and is commonly referred to in conjunction with the more modern World Health Organization (WHO) classification.

The WHO classification for myeloid neoplasms represents an attempt at offering a comprehensive classification scheme based on all available clinical, morphologic, cytochemical, immunophenotypic, and genetic data. While substantially more complicated than the older FAB systems, the WHO classification (now in its 4th edition) allows for a much finer differentiation and therefore is capable of offering more accurate prognostic correlations. The major categories are: AML with recurrent genetic abnormalities; therapy related myeloid neoplasms; myeloid proliferations related to Down syndrome; and AML not otherwise specified, which then falls back on the older FAB system. A full discussion of this classification system is beyond the scope of this work, but a 2009 review of the changes performed by Vardiman et al.[210] covers many of the important changes and is a good starting point for those with further interest. Instead, some of the major subtypes with prognostic significance, and significance to the work presented herein will be covered in more detail.

1.3.2.1 Core Binding Factor Leukemia Core binding factor (CBF) AML describes a subset of leukemias that possess genetic alterations in one of the two proteins that make up the family of protein complexes known as CBFs. A heterodimer, CBF is a transcription factor that consists of DNA-binding α- and non-DNA binding β-subunits. In AML, the α-subunit is encoded by the *RUNX1* gene on chromosome 21, also known as *AML1*. The β-subunit is encoded by the *CBFβ* gene on chromosome 16. The CBF transcription factor plays an important role in normal hematopoiesis, and small perturbations of its expression or function can have deleterious consequences, AML being just one (for a more

comprehensive overview of CBF functions in hematologic physiology and disease, see the 2002 review by Speck and Gilliland[190]). Together, two common cytogenetic abnormalities make up the largest group of CBF leukemias: t(8;21)(q22;q22) and inv(16)(p13.1q22), representing approximately 15% and 6% of pediatric AML cases, respectively. Despite these translocations both targeting the CBF complex, each subtype has its own WHO classification, the prognostic implications for which will be discussed below.

1.3.2.2 APL with PML-RARα fusion gene Somewhat unique among the early FAB classifications was the M3 subgroup, which represents APL. As the name suggests, most of the leukemic cells are abnormal promyelocytes, and in most cases, many of the cells will have Auer rods. Though not pathognomonic for APL, the presence of Auer rods, which are fused and typically elongated granules, is easily detected by standard microscopic analyses. This is useful, because it allows for a rapid narrowing of diagnoses which is important as newly presented APL is a medical emergency. More so than other AML subtypes, APL is associated with an extremely high rate of fatal hemorrhage.

Interestingly, almost every case (approximately 95%) of APL has a characteristic t(15;17)(q22;q21) translocation which results in the fusion of the *PML* and *RARA* genes. The *PML* gene product PML is important for nuclear body formation and plays a role in transcriptional regulation and tumor suppression. The *RARA* gene product, retinoic acid receptor alpha (RARα) is a nuclear receptor that normally plays a role in a host of differentiation processes. When these two genes fuse, the resultant protein prevents both of the

physiologic functions. Instead, the PML-RARα fusion protein binds to DNA and acts in a dominant negative fashion to repress gene expression as well as nuclear body formation. This prevents further differentiation of the APL cell, and while not sufficient, is considered necessary for the development of APL. Though APL used to be almost universally fatal, this unique biology allows for the use of targeted therapies (discussed below) that make APL one of AML subtypes with a more favorable prognosis. In approximately 5% of cases, the RARA gene is fused with another partner protein, resulting in variable changes to chemotherapy sensitivity and prognosis. For more information, see the 2005 review by Zhou et al.[233].

1.3.2.3 Therapy-related myeloid neoplasms In a departure from most WHO classifications, myeloid neoplasms (both AML and MDS) that are believed to be sequelae of previous therapy are grouped together into the same category (tAML). Many types of cancer treatments act by inducing structural damage to DNA (these mechanisms are discussed in depth in Chapter 3). In the process of repairing this damage, it is possible that new genetic abnormalities may arise, some of which may aid in the progression to AML. As this process takes time, in many cases years, tAML is less common in children, mostly because by the time tAML develops the patient has reached adulthood. However, tAML is a potential consequence of many types of therapy, especially topoisomerase II poisons and radiotherapy, both of which are commonly used to treat childhood malignancy.

Though tAML is not defined by any specific set of genetic abnormalities, there are trends that arise. The use of certain chemotherapy drugs, especially

anthracyclines and etoposide, are highly correlated with the development of tAML with chromosomal abnormalities involving 11q23. At this locus is the *MLL* gene (officially referred to as *KMT2A*) which encodes a histone methyl-transferase. Rearrangements at this site are common in infant AML, with different fusion partners having different prognostic significance. However, in the context of tAML, the presence of these commonly seen genetic abnormalities help to differentiate tAML from *de novo* primary AML. The biology of tAML is heterogeneous, but in general it is a difficult-to-treat disease for reasons that will be discussed below.

1.3.2.4 FLT3-ITD AML FLT3 is a receptor tyrosine kinase encoded by the *FLT3* gene on chromosome 13. As mentioned above, FLT3 plays an important role in normal hematopoiesis. Like other receptor tyrosine kinases, FLT3 exists primarily as a monomer that upon ligand binding dimerizes and autophosphorylates. This activates an intracellular signaling cascade that has consequences for maturation and proliferation. In approximately 10-20% of pediatric AML cases, an internal tandem duplication of variable length (FLT3-ITD) in exons 14 and 15 promotes ligand-independent activation of FLT3. The unfortunate consequence of this activation is decreased maturation and increased proliferation of myeloid progenitor cells. Similar to FLT3-ITD are point mutations in FLT3 that have similar effects, though these mutations are less common. Because FLT3-ITD is not mutually exclusive with other AML subtypes, but is instead an additional abnormality, AML FLT3-ITD does not get an independent WHO classification. However, FLT3-ITD status has important

prognostic and therapeutic implications and is therefore interrogated as part of standard diagnostic workups. For more information, see the review by Stirewalt and Radich [195].

1.3.2.5 AML with CEPBA mutation The CCAAT enhancer binding protein family (C/EBP) is made up of several related transcription factors, the α-isoform of which (C/EBPα) is commonly mutated in pediatric AML. These proteins play an important role in the differentiation of many tissue types. Specifically, C/EBPα is heavily involved in the maturation of the granulocyte lineage, binding to the promoters and supporting transcription of a variety of genes necessary for this process. Mutations in the CEBPA gene in the context of AML result in the expression of a 30 kDa form of the C/EBPα protein (full length is approximately 42 kDa) which functions in a dominant negative fashion to prevent appropriate promoter binding and gene transactivation. Further information can be found in the 2009 review by Ho et al.[73].

1.3.2.6 AML with NP1 mutation Nucleophosmin, or NPM1, the protein encoded by the *NPM1* gene, has pleiotropic functions, but is most known for its role as a nuclear chaperone that is involved in the import and export of a wide variety of substrates. When mutated, these functions typically cease, and many cellular processes go into disarray. Mutations in *NPM1* are relatively uncommon in pediatric AML (up to approximately 10%, 20% in cytogenetically normal AML [CN-AML]), however their prevalence increases with patient age. Importantly for AML, NPM1 is involved in hematopoiesis, DNA replication and repair, as well as gene transcription. When mutated, these functions are affected to varying

degrees, and *NPM1* mutations are considered to be primary events in the development of malignancy in some cases.[76,112]

1.3.2.7 Myeloid proliferations related to Down Syndrome Down syndrome is a commonly occurring genetic abnormality characterized by constitutional presence of trisomy 21. DS is associated with a host of developmental and health problems, including a substantially increased risk of developing leukemia. The characteristics of DS and associated AML will be discussed in depth in section 1.5.

1.4 Treatment and Prognostic Considerations for Pediatric AML

When treating AML, there are many factors that need to be considered. The first is urgency; AML, especially with certain presentations, is a medical emergency that requires immediate intervention. The second is efficacy; not all interventions are equally effective against each disease subtype. Finally, complicating efficacy, is toxicity. Though advances have been made in the treatment of pediatric AML, the most effective therapies are profoundly toxic even when administered properly. Therefore, it is imperative that measures be taken to maximize efficacy and minimize toxicity. Fortunately, years of experience have provided many effective treatment protocols, most of which build on a framework of induction, consolidation, and, when necessary, salvage and transplant.

1.4.1 Overview

When a patient first presents with AML, it is important to reduce their leukemic burden. Acute leukemia causes morbidity for a variety of reasons, but

some of the most severe are a direct result of the high burden of rapidly proliferating leukemic blasts. Extremely high white blood cell (WBC) counts, or hyperleukocytosis (commonly defined as a WBC count > 100,000 cells/μL), are associated with problems directly resulting from the high cellularity of the blood. When symptomatic, the condition is called leukostasis. There are many pathologies that contribute to the symptomology of leukostasis, but one of the most important is the potentially occlusive nature of the condition. As a result of the high viscosity caused by the relatively rigid circulating blasts, small vessels may become occluded by aggregates of WBCs. This can result in symptoms similar to stroke or pulmonary embolism, including respiratory distress and focal or generalized neurological deficits. Other problems at presentation can be bone pain, which is believed to be the result of increased pressure in the marrow cavities of long bones resulting from hyperproliferation of blasts, and cytopenias. As proliferating blasts can overwhelm marrow cavities, it is possible that physiologic hematopoiesis is prevented, resulting in deficiencies in other compartments. Common manifestations that result in a patient seeking medical care are anemia, which usually presents as fatigue, and thrombocytopenia, which usually presents as uncontrollable bleeding or easy bruising. Alternatively, neutropenia can result in severe infection.

In order to rapidly decrease the leukemic burden, the patient is treated with what is referred to as induction chemotherapy. Typically given in multiple rounds, induction chemotherapy typically consists of moderately high intensity dosing schedules. The goal with induction chemotherapy is to safely induce

what is referred to as remission, or undetectable disease (see discussion on minimal residual disease [MRD] in section 1.4.3.4), and to allow some restoration of normal hematopoiesis. A patient with newly diagnosed AML is typically very ill, and may be less able to tolerate maximal dose chemotherapy, so care must be taken to ensure patient safety.

Once remission is achieved, therapy transitions to a phase known as consolidation. Consolidation therapy is usually maximally intense, both with regard to dosing (high doses) and timing (short latency between doses). The goal of consolidation is to eliminate any remaining leukemic blasts, ideally resulting in a cure. Both the duration and types of treatments used for consolidation vary between AML subtypes.

In the event that a patient's disease does not respond to treatment, and a remission cannot be achieved, their disease is considered primary refractory. Therapy directed at inducing a remission in refractory disease is often referred to as salvage therapy. Highly variable depending on subtype, salvage therapy often consists of somewhat experimental treatments, or treatments that are associated with unfavorable side effect profiles that are preferably avoided. Similarly, if a patient's disease that was once in remission returns, it is considered to be relapsed. Relapse, for many AML subtypes, is not uncommon, and is associated with varying prognoses. In the event of relapse, re-induction is attempted. If unsuccessful, the disease is considered secondary refractory. When chemotherapy alone is likely to be insufficient to achieve a cure, hematopoietic stem cell transplant (HSCT), or bone marrow transplant, may be

pursued.

1.4.2 Prognosis

In the past, AML was associated with an almost 100% mortality rate. Fortunately, with the discovery of new drugs and improvements in supportive care, survival among pediatric AML patients as a group has risen to approximately 70%. With greater treatment experience and understanding of the biology underlying AML has come the ability to identify patients that have higher- and lower-risk disease. This has allowed for the use of less aggressive treatment in those patients with more favorable prognoses, saving them unnecessary toxicity, while still giving appropriate therapies to those whose prognoses are more guarded.

1.4.2.1 Favorable Prognostic Indicators There are several disease characteristics that are associated with a favorable prognosis (survival >70%). Fortunately, CBF AML (t(8;21), t(16;16); inv(16)), which represents one of the largest AML subgroups, is associated with a favorable prognosis. Similarly, APL with the standard t(15;17) cytogenetics is also associated with good outcomes. Mutations in C/EBPα or NP1 are also positive findings, so long as there is not concurrent Flt3 mutation or ITD. Finally, AML in the DS population is associated with one of the most favorable prognoses.

1.4.2.2 Adverse Prognostic Indicators In contrast to those findings listed above, there are several cytogenetic or genetic abnormalities associated with poorer outcomes (survival <50%). Certain translocations, including t(10;11)(p12;q23), t(6;9)(p23;q34), inv(3)(q21126.2), t(3;3)(q21;q26.2),

t(7;12)(q36;p13), t(7;12)(q32;p13), and t(5;11)(q35;p15.5) are poor prognostic markers. Fortunately, these are less common findings. Though not a specific finding, tAML is always considered to have an adverse prognosis. Finally, mutations in Flt3 or Flt3-ITD are less favorable. When found alone, Flt3 abnormalities carry an adverse prognosis. In combination with other, more favorable abnormalities, the net result may be intermediate risk, but Flt3 abnormalities are never favorable prognostic findings.

1.3.2.3 Intermediate risk For many subtypes of AML, prognosis is considered to be intermediate (survival 50-70%). In some cases, this could be the result of underlying biology, but often assignment of intermediate risk is due to inconclusive results regarding outcome for that specific subtype. There are many findings that have had variable outcomes between trials, and therefore it is up to the clinician's judgment to assign risk and subsequent treatment strategy.

1.4.3 Treatment of Pediatric AML

Though there are many variations on standard treatment for AML, an in depth discussion of these variations and their rationale is largely beyond the scope of this work. However, it is important to consider the standards of care when investigating new potential therapies, as any interventions will likely be given in the context of said therapies or after they have failed.

1.4.3.1 Standard Induction and Consolidation Standard induction therapy for pediatric AML has remained largely unchanged for over 20 years, and consists of a "3+7" or "3+10" strategy of administering cytarabine (araC) and an anthracycline (or substitute), typically daunorubicin (DNR). Both araC and DNR

are genotoxic agents that non-specifically target replicating cells. An in-depth discussion of their mechanisms of action and associated toxicities can be found in Chapter 3, but the major toxicities associated with their use can be considered to be myelosuppression (araC) and late cardiac toxicity (DNR). In the typical administration schedule, DNR is given at a dose of 45-60 mg/m^2 for three days and araC is given at a dose of 100-200 mg/m^2 for 7 or 10 days, either twice daily or via continuous infusion. This induction can be repeated depending on protocol. Most protocols include a third drug for induction, often etoposide or thioguanine. The evidence supporting these inclusions is, for the most part, non-conclusive with regards to benefit to overall survival (OS). If complete remission (CR) is obtained, consolidation therapy is appropriate.

Consolidation therapy typically consists of high intensity variants of what was used during induction. For most cases, at least two rounds of high-dose araC (HiDAC) are given. The HiDAC course typically consists of several doses of araC usually in the range of 1-3 g/m^2, usually given every 12 hours for a total of <10 doses. Some protocols treat for 3 consecutive days, while others spread out the doses. In many protocols, dosage is determined at least in part by clinical findings. For most AML subtypes, post-consolidation maintenance therapy is inappropriate.

Part of every standard therapy protocol should be the administration of intrathecal chemotherapy. Though central nervous system (CNS) involvement is uncommon, and CNS relapse does not affect every patient, there is good evidence to suggest that administration of intrathecal chemotherapy to every

patient greatly reduces the occurrence of these unfavorable events. Though there is a dearth of data suggesting the appropriate intrathecal treatment regimen, a combination of methotrexate, araC, and hydrocortisone is considered to be likely ideal, and typically between 4 and 12 treatments are given. It is, however, agreed upon that CNS irradiation is inappropriate despite being effective, and should not be used in lieu of chemotherapy.

1.4.3.2 Role of HSCT The use of HSCT in the treatment of pediatric AML is, in many cases, controversial. Although HSCT is often considered to be a curative therapy, the profound toxicity associated with the procedure prevents its use for low-risk disease. The benefit of HSCT is that the combination of extreme-intensity condition regimens and graft-vs-tumor effects are highly effective at eradicating disease. However, the potential for graft-vs-host disease, the massive supportive care burden, and risk of mortality associated with HSCT are of serious concern. One of the earliest hurdles that HSCT faces is the identification of a suitable donor. Typically, the ideal donor is an HLA-matched sibling, although frequently unrelated donors are required. Prior to transplant, a conditioning regimen of either total body irradiation or myeloablative chemotherapy is undergone by the patient. After conditioning, the cells are infused into the patient, but marrow reconstitution can take weeks, assuming successful engraftment. Current recommendations do not allow for HSCT in first CR for low-risk patients, but for other patients a risk-benefit analysis is appropriate. Although there has been demonstrated benefit to HSCT for high risk patients, treatment related mortality for HSCT in pediatric AML patients is as

high as 41%[16].

1.4.3.3 Treatment of APL and Flt3-ITD AML Both APL and Flt3-ITD AML present opportunities for treatment with agents that are not commonly used for other subtypes of AML. For the treatment of APL, the use of all-trans retinoic acid (ATRA) is the standard of care. When exposed to ATRA, the PML-RARα fusion protein relocalizes and transcription of differentiation-inducing genes is increased. The use of ATRA has helped change APL from being one of the most fatal subtypes of AML to one of the most curable. For the case of Flt3-ITD AML, treatment with the multiple tyrosine kinase inhibitor sorafenib offers potential benefit. Early studies have shown favorable results, although the development of resistance and cross-resistance to other inhibitors remains problematic[78,219].

1.4.3.4 Role of MRD Monitoring At specific stages of treatment, it is important to measure the response to treatment. Historically, this was performed by morphological analysis of bone marrow biopsies/aspirations. Unfortunately, this method is very subjective and offers poor sensitivity for detection of leukemic blasts, and MRD negativity was considered <1/50 cells. However, recent advances have allowed for more sensitive and objective methods for the detection of residual disease. In the event that a specific fusion gene or other genetic abnormality are identified, PCR detection can be used with a sensitivity of approximately 1/10,000 cells. More useful for most cases, though, is multiparameter flow cytometry. By identifying a patient-specific cell surface marker profile, it is possible to rapidly identify as few as 1/1000 cells as leukemic blasts for most patients. The utility of this approach was demonstrated to great

effect in the St. Jude AML02 trial, as well as the recent COG-AAML0431 trial. [29,175,188,212]

1.5 Down Syndrome

Down syndrome, originally described by John Langdon Down in the latter half of the nineteenth century, is a genetic disorder with a host of manifestations. Although an in-depth characterization of the health problems and implications for care of individuals with DS is beyond the scope of this dissertation, the heightened incidence and associated biological mechanisms of AML in this population are of great interest and are highly relevant to the presented work.

1.5.0 Source Material

Unless otherwise stated, most of the general background information presented below was derived from two excellent overviews of DS ([170,216])

1.5.1 Genetics and Incidence of DS

In order to conclusively diagnose a patient with DS, their cells must be trisomic for chromosome 21. Though there are less common mechanisms leading to trisomy, such as translocations or mosaicism, most cases of DS are the result of non-disjunction events at meiosis I of the maternal gamete. In the United States, the incidence of DS in live births is approximately 1 in 1,000. Incidence in other countries varies, largely as a result of elective termination of prenatally diagnosed pregnancies. The only well-established risk factor for DS is advanced maternal age, as nondisjunction events occur at higher frequencies as women age [67]. That being said, there have been efforts to identify modifiable risk factors such as folate supplementation [77], although they have been largely

met with limited success.

1.5.2 Health concerns in individuals with DS

As mentioned above, individuals with DS are at an elevated risk for a large variety of health problems. The most pervasive concerns in DS patients are neurocognitive and psychiatric in nature. Individuals with DS typically suffer mild to moderate cognitive impairment, reaching maximum cognitive ability around adolescence. The difficulties associated with cognitive impairment are frequently compounded by psychiatric problems such as attention-deficit hyperactivity disorder and depression. As a result, children with DS need special care that can persist well into adulthood, and in some cases the requirement is life-long, with only few individuals capable of truly independent living. Other contributing factors to the cognitively impaired phenotype are common anatomical abnormalities that inhibit communication abilities. Individuals with DS commonly have structural differences in the systems involved in speech and hearing, such as macroglossia and Eustachian tube abnormalities or other conductive deficiencies. These need to be identified early, as difficulties in communication can compound the otherwise present cognitive disability. Additionally, a large portion of DS patients have hypothyroidism, which if left untreated can lead to further cognitive impairment.

There are several commonly encountered health problems in the DS population that are especially concerning in the context of leukemia treatment. Approximately half of pediatric DS patients have congenital heart defects (CHD) of varying severity. Although not all CHDs require surgical intervention, the

already decreased cardiac function at least theoretically puts DS children at an increased risk of cardiac dysfunction if treated with cardiotoxic chemotherapy regimens. Another concern results from a combination of anatomical and immunological abnormalities that result in an increased risk of infection. Structural differences in the both the upper and lower airways of DS patients put them at a higher baseline risk for infection. This is in addition to a lower baseline immune function in DS patients. Though there have not been any individual causes of the lowered immune function, it is known that DS patients have below-average levels of both T- and B-cells.

Finally, DS patients have an unusual risk profile for both solid tumors and Alzheimer's disease, though these diseases are of little significance for the pediatric DS population. With the exception of testicular cancer and pediatric leukemias, DS patients have lower than expected incidence and mortality rates for cancer [66,147,198]. Conversely, the prevalence of Alzheimer's disease (AD) in older DS patients is exceptionally high. As the life expectancy for individuals with DS has increased (median survival is approximately 60 years), so has the incidence of diseases that appear later in life, such as AD, which is estimated to affect as many as 60% of elderly DS patients [235].

1.5.3 Leukemia and DS

Despite having a lower incidence of solid tumors, the total incidence of cancer in the DS population is similar to that in the non-DS population due to the increased susceptibility for developing leukemia. Interestingly, this unusually high risk for leukemia is only present in the pediatric DS population and applies

to both ALL and AML. In fact, in a 2005 study, it was found that DS patients represented approximately 2% of ALL cases and 14% of AML cases, giving DS children an approximately 50-fold greater risk of developing leukemia by age 5[230].

Although this work focuses on AML, it is important to acknowledge the unique features of ALL in the DS population. Unlike non-DS pediatric ALL patients, who typically have OS of approximately 80-90%, DS-ALL patients have less favorable outcomes (OS approximately 70%). Their disease also is very different from that in the non-DS population in that commonly seen genetic abnormalities are rare and almost every DS-ALL case is of the B-precursor cell classification, with almost no cases having a T-cell phenotype. An excellent concise review of current DS-ALL treatment and biology can be found in the 2014 article by Izraeli et al. [89].

1.5.3.1 AML in the Pediatric DS Population In contrast to the non-DS population, AML in the DS population is associated with many unique features. DS-AML patients are typically younger than non-DS AML patients, with most cases presenting in patients less than four years of age. Furthermore, depending on the study, as many as 100% of pediatric DS-AML patients have a megakaryocytic phenotype (FAB M7, AMKL) [3,30,45,101,106,161,163,164,175,230,236]. In fact, DS patients have an estimated 500-fold increased risk for developing AMKL compared to non-DS children [236]. This phenotype is almost universally associated with a somatic mutation in the second exon of the *GATA1* gene, the significance of which will be

discussed below. Interestingly, *GATA1* mutations are found almost exclusively in DS-AMKL blasts, with only rare exceptions [2,71,204,215]. Finally, DS-AML patients enjoy better prognoses than their non-DS counterparts, with OS of approximately 80-90% [3,30,45,101,106,161,163,164,175,230,236].

1.5.3.2 Leukemogenesis in DS-AML Unless otherwise stated, this section represents a condensation of information obtained from references [2,13,24-26,32,71,110,124,172,185,187,204,207,215]. Underlying the features common to DS-AML is one of the better understood mechanisms of stepwise leukemogenesis. Likely contributing to both ALL and AML is the effect that constitutional trisomy 21 exerts on fetal liver hematopoiesis. The presence of several genes localized to chromosome 21, as well as what is most likely a number of currently unknown factors, alters the population distribution and growth rates of various hematopoietic progenitors in the fetal liver. Importantly for DS-AML, trisomy 21 appears to give a growth advantage to MEPs, which are both more frequent and clonogenic than in non-DS controls. At this stage, it is possible for a cell to develop a mutation in the *GATA1* gene, which typically causes the transcription of a shorter GATA1s protein, as the result of either alternate splicing or transcription start-site utilization. Lacking the N-terminal transactivation domain, but still able to interact with FOG-1, GATA1s prevents erythroid differentiation, while also rendering the cell unable to complete megakaryocytic differentiation. The combination of GATA1s and the background of trisomy 21 is sufficient to drive a hyper-proliferation of immature megakaryoblasts.

The unchecked proliferation on immature megakaryoblasts in the fetus can have disastrous complications, occasionally presenting as hydrops fetalis. More often, however, this proliferation spontaneously resolves either pre- or postnatally. If detected in an infant, this condition is termed (among other names) transient myeloproliferative disorder (TMD). Whether TMD resolves spontaneously or requires treatment (see below), there is a chance that it may progress to AMKL. Interestingly, it has recently been conclusively shown that AMKL clones are derived from TMD cells, and that they possess similar mutational spectra, albeit at a slightly higher mutational frequency (mean 5.8 non-silent mutations in AML vs. 1.7 for TMD) [136,229]

1.5.3.2 Treatment concerns in DS-AML and TMD As its name implies, TMD is often transient and resolves spontaneously in the majority of cases. This spontaneous resolution has made estimations of incidence difficult, as it has been expected that many cases are clinically silent. A recent study found that, surprisingly, almost every DS neonate had circulating blasts, but only approximately 20% had detectable *GATA1* mutations, indicating TMD[169]. In addition to the approximately 20% of TMD patients that progress to AMKL, TMD itself is associated with potential complications and may require treatment. These typically result from either hyperleukocytosis or organ infiltration by blasts, with pancytopenia and hepatic fibrosis being common signs. In the event of symptomatic or complicated disease, low-dose cytarabine may be indicated (1-3.5 mg/kg/day for 5-7 days)[44]. Overall, TMD is associated with a mortality of approximately 20%.

As mentioned above, DS-AML is associated with a favorable prognosis when compared to non-DS AML. For reasons that will be discussed in Chapter 3 of this work, DS-AML cells are known to be more sensitive to chemotherapy; however, this is at least partially offset by an increase in sensitivity of non-leukemia cells in DS patients. As a result, DS-AML is often successfully treated with lower intensity chemotherapy regimens. For example, the recently finished COG AAML043 trial, which focused exclusively on DS-AML, utilized a cumulative araC dose of 27,100 mg/m^2 araC (Dr. Jeffrey Taub, personal communication), whereas the cumulative araC dose for the St. Jude AML02 trial for non-DS AML was 45,600 mg/m^2 [175]. Though outcomes are generally favorable, those patients who either have refractory disease or suffer a relapse have few options and poor survival of approximately 25% [119,120,199]. Further complicating matters is the poor survival after HSCT, which is only approximately 19% in the DS-AML patients [72]. Therefore, advances in care are necessary to better treat this group of patients.

1.6 Why study pediatric DS-AML?

It is not difficult to make a Utilitarian argument against research directed at improving outcomes for relatively uncommon diseases like DS-AML, or pediatric cancers in general. Though a thorough discussion on the philosophical considerations of such research is well beyond the scope of this work, a good discussion of the considerations for orphan diseases (which are in many ways similar) can be found in the 2005 paper by Gericke, Riesberg, and Busse[54]. Briefly, it is a fairly commonly held belief that there exists a moral obligation to

help every population, regardless of frequency or perceived value to society. Furthermore, while gains in overall survival after cancer diagnosis have been modest at best, certain subgroups, like those with pediatric cancers, have benefited greatly. For example, 5 year survival after diagnosis with pediatric AML has increased from only 19% in 1975 to over 60% today [189]. Moreover, the study of relatively rare diseases that affect relatively homogenous populations or result from common genetic alterations has guided our understanding of some of the most important physiologic and pathological processes that are present in the entire population. Therefore, it is the belief of this author that there is merit in studying pediatric AML, especially in the DS population, not only for the benefit it may provide to those individuals, but also to the population at large. Finally, the NIH has also developed a plan (available at downsydrome.nih.gov) to ensure that adequate research resources are directed for DS individuals, a vulnerable patient population due to their multiple medical problems.

CHAPTER 2 - Overexpression of GATA1 Confers Resistance to Chemotherapy in Acute Megakaryocytic Leukemia

2.0 Preface

This chapter is taken from a previously published work with the same title published in 2013 (PMID 23874683) [18]. It represents a project taken over by this author upon joining the lab. As such, much of the work was done by other lab members, including the microarray and ChIP-on-Chip analyses and the work leading up to those experiments. This author was responsible for generation of the over-expression clones, validation of the array based experiments, back-end analysis of array data, and composing the manuscript. The figure numbers have been adjusted to incorporate what was a supplemental figure in the original manuscript. Finally, the supplemental tables associated with the original publication have been omitted from this dissertation as they are especially cumbersome and are supplemental in nature. They can be found on *PLoS One*'s website.

2.1 Introduction

In the pediatric population, acute myeloid leukemia has a relatively guarded prognosis with five-year survival rates of approximately 50% (www.seer.cancer.gov), despite intensive therapy. Acute megakaryocytic leukemia (AMKL; M7) is a biologically heterogeneous form of AML, representing ~10% of pediatric AML cases and 1-2% of adult AML cases [47,166]. AMKL is considered a very high-risk subgroup with event-free survival (EFS) rates of <35% [5,140]. Remarkably, Down syndrome children with AML, and in particular, AMKL, have extremely high EFS rates of approximately 80% [5,45,106,163,236].

The blast cells of DS AMKL patients almost universally harbor a somatic mutation in exon 2 of the transcription factor *GATA1* gene (localized to Xp11.23), resulting in the introduction of premature stop codons and the synthesis of a shorter GATA1 protein (designated GATA1s, 40-kDa) initiated from a downstream initiation site and distinguishable from the wild-type GATA1 (50-kDa) [215]. Both GATA1s and the wild-type GATA1 proteins show similar DNA binding abilities and interact with a partner protein named “friend of GATA1” (FOG1), though the GATA1s protein exhibits altered transactivation capacity due to the loss of the N-terminal activation domain [215].

GATA1 is a zinc finger transcription factor that is essential for hematopoiesis of the erythrocyte/megakaryocyte lineages. GATA1 acts as an activator or repressor of different target genes by forming distinct activating or repressive complexes with its partner proteins (reviewed in [32]). The pronounced differences in clinical outcomes between DS and non-DS AMKL patients and differences in the *GATA1* gene mutation status in blast cells suggest a potential role for GATA1 in chemotherapy response in both DS and non-DS AMKL cases. In the non-DS population, overexpression of GATA1 in megakaryoblasts from children with AMKL compared to blasts from children with other subtypes of AML was previously observed in gene expression microarray studies [171]. Further, earlier studies demonstrated a worse prognosis for AML patients (adults without AMKL) whose blast cells expressed higher levels of GATA1 than patients whose blasts expressed lower levels of GATA1 [6,186]. Collectively, these studies suggest that GATA1 may contribute to chemotherapy

resistance via regulation of GATA1 target genes in AML, especially in the AMKL subtype.

Bcl-xL, encoded by the long form splice variant of *Bcl-x* transcripts which counteracts apoptotic signals, may be one of these GATA1 target genes. Bcl-xL is a Bcl-2 family protein that is abundantly expressed in both megakaryocytes and erythrocytes (reviewed in [145]). Bcl-xL deficient mice exhibit massive apoptosis of fetal liver hematopoietic cells, suggesting that Bcl-xL prevents apoptosis of hematopoietic cells [133]. Previous studies have established that GATA1 and erythropoietin cooperate to promote erythroid cell survival by regulating Bcl-xL expression [58], and that GATA1 is capable of binding and activating the Bcl-xL promoter during erythroid differentiation [104]. Thus, it is conceivable that GATA1 may also regulate Bcl-xL in megakaryocytes as megakaryocytes and erythrocytes are derived from a common progenitor and both Bcl-xL and GATA1 are expressed in megakaryocytes.

In this study, we confirmed the overexpression of *GATA1* transcripts in non-DS megakaryoblasts compared to non-DS AML blasts. We also demonstrated that GATA1 plays critical roles in sensitivities of megakaryocytic cells to cytarabine (ara-C) and daunorubicin (DNR), the two main drugs used for treating AML, through direct regulation of Bcl-xL. Furthermore, we found that the histone deacetylase (HDAC) inhibitor, valproic acid (VPA), can decrease GATA1 expression and synergize with ara-C in exerting antileukemic activities toward megakaryocytic leukemia cells. Using gene-expression microarray and ChIP-on-

Chip analyses, we identified additional GATA1 target genes which may be downstream targets for AMKL treatment.

2.2 Materials and Methods

2.2.1 Clinical Samples

Diagnostic AML blasts (including blasts with the AMKL phenotype) were obtained from the Children's Hospital of Michigan leukemia cell bank and from the Pediatric Oncology Group 9421 study, as previously described [202]. The diagnosis of AMKL was confirmed by flow cytometry detection of the megakaryocytic antigens CD41 and CD61. Mononuclear cells were isolated on Ficoll-Hypaque gradients to obtain highly purified mononuclear cell fractions consisting mostly of leukemic blasts. Written informed consent was provided by the parent or legal guardian of the patient according to the Declaration of Helsinki. The research protocol was approved by the Human Investigation Committee of Wayne State University School of Medicine.

2.2.2 Cell Culture and Chemotherapy Agents

The Meg-01 megakaryocytic cell line was obtained from the American Type Culture Collection (Manassas, VA). The parental and engineered sublines were cultured in RPMI 1640 with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 2 mM L-glutamine plus 100 U/ml penicillin and 100 µg/ml streptomycin, in a 37°C humidified atmosphere containing 5% CO_2/95% air. Ara-C, DNR and VPA were purchased from Sigma-Aldrich (St. Louis, MO).

2.2.3 shRNA Knockdown of GATA1 in Meg-01 Cells

GATA1 shRNA lentivirus clones were purchased from the RNAi Consortium of Sigma-Aldrich. Meg-01 cells were transduced with the GATA1 shRNA lentivirus. After selection with puromycin, infected Meg-01 cells were plated in soft agar. Colonies were isolated, expanded and assessed for GATA1 expression by western blotting and real-time RT-PCR. Two clones with decreased GATA1 expression (designated GATA1 4-14 and GATA1 5-13) were chosen for further study. A pool of cells from the negative control transduction (lentivirus expressing a shRNA with limited homology to any known human genes) was used as the control (designated GATA1 Neg).

2.2.4 Quantitation of Gene Expression by Real-time RT-PCR

Transcripts were quantitated using primers (Table S1) and Sybr Green (Roche Diagnostics, Indianapolis, IN) as previously described [53] or Taqman probes (Applied Biosystems Inc., Foster City, CA) and a LightCycler real-time PCR machine (Roche Diagnostics, Indianapolis, IN) based on the manufacturer's instructions. Real-time PCR experiments were expressed as mean values from three independent experiments and normalized to GAPDH, with the exception of GATA1 transcript levels post-VPA treatment, which were normalized to RPL13A levels as RPL13A has been reported to be a more reliable housekeeping gene post-HDAC inhibitor treatment [132].

2.2.5 Western Blot Analysis

Whole cell lysates were prepared by sonication in hypotonic buffer (10 mM Tris-Cl, pH 7.0), containing 1% SDS and proteolytic inhibitors, and subjected to

SDS-PAGE. Separated proteins were electrophoretically transferred to PVDF membranes (Thermo Fisher Inc., Rockford, IL) and immunoblotted with antibodies to GATA1 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-xL (Cell Signaling Technology, Danvers, MA), or β-actin (Sigma-Aldrich, St. Louis, MO), as described previously [53]. Immunoreactive proteins were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE), as described by the manufacturer.

2.2.6 In Vitro Ara-C and DNR Cytotoxicity Assays

For determinations of cytotoxicities, the cell lines were cultured in complete medium with dialyzed fetal bovine serum in 96-well plates at a density of 4 x 10^4 cells/ml for 96 hours. Cells were cultured continuously with a range of ara-C and DNR concentrations at 37 °C, and viable cells were determined using the Cell Titer-blue reagent (Promega, Madison, WI) and a fluorescence microplate reader. The IC_{50} values were calculated as the concentrations of drug necessary to inhibit 50% growth compared to control cells cultured in the absence of drug. The data are presented as the mean values ± standard errors from at least 3 independent experiments. Standard isobologram analysis was performed as described previously [225] and combination index (CI) analysis was performed using CompuSyn software (ComboSyn, Inc., Paramus, NJ)

2.2.7 Assessment of Baseline and Drug Induced Apoptosis

The Meg-01 shRNA stable clones (GATA1 Neg, GATA1 4-14, and GATA1 5-13) in logarithmic growth phase in RPMI 1640/10% dialyzed FBS in the presence or absence of ara-C and VPA were harvested, vigorously pipetted and

triplicate samples taken to determine baseline and drug-induced apoptosis using the Annexin V-FITC Kit (Beckman Coulter; Brea, CA), as previously described [35,225]. Apoptotic events were recorded as a combination of Annexin V+/PI- (early apoptotic) and Annexin V+/PI+ (late apoptotic/dead) events. The data are presented as mean percentages of Annexin V positive cells ± standard errors relative to untreated cells.

2.2.8 Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed in Meg-01 cells as previously described [50], with GATA1 C-terminus (C-20 antibody, Santa Cruz) antibody or normal IgG. Standard PCR for the Bcl-xL promoter region was performed with forward (5'-gcatccccgcagccacctcctc-3') and reverse (5'-ccctaaaaattccattccccctccag-3') primers spanning positions –257 to +67. A separate region (exon 3) of the human *GATA1* gene was also amplified with forward (5'tggagactttgaagacagagcggctgag-3') and reverse (5'-gaagcttgggagaggaataggctgctga-3') primers to validate the specificity of the ChIP assays.

2.2.9 ChIP-on-Chip Assay

The ChIP-on-Chip protocol was modified from the Agilent Technologies Mammalian ChIP-on-Chip Kit protocol at Children's Hospital of Michigan. Briefly, genomic DNA from the ChIP assay above was incubated with T4 DNA polymerase to create blunt ends. Linker DNA was ligated to the blunt end DNA, followed by amplification of the samples. The samples were labeled, hybridized to the microarray, washed and scanned according to the manufacturer's protocol. Data were imported into Chip Analytics software (v1.3.1, Agilent Technologies)

for analysis. Normalization was performed using intra-array lowest and inter-array median normalizations. Peak detection was performed using the Whitehead error model (v1.0) and the Peak Shape Detection algorithm (v2.0). Genes with a bound region (peak) were identified, and the associated NCBI Refseq accession numbers were used for subsequent analyses. The ChIP-on-ChIP results were validated by both regular PCR and real-time PCR (see above). We have deposited the raw data at GEO under accession number GSE43018.

2.2.10 Gene Expression Microarray Analysis

Gene expression microarray was performed with the Agilent Whole Human Genome 4 x 44K microarray (catalog G4112F) at Children's Hospital of Michigan. Microarray sample preparation, hybridization, and data analysis were described previously [52]. On each microarray, a labeled GATA1 4-14 or GATA1 5-13 sample was co-hybridized with an oppositely labeled GATA Neg sample. Two arrays were completed for the GATA1 4-14/GATA1 Neg pair and the GATA1 5-13/GATA1 Neg pair, respectively, for a total of four arrays. The two microarrays used for each clone were hybridized in a "dye swap" arrangement with opposite dye orientation to minimize the dye bias effect. Statistical analyses were performed using Rosetta Resolver® [159]. We have deposited the raw data at GEO under accession number GSE42879 and we confirm all details are MIAME compliant.

2.2.11 Construction of Plasmids, Transient Transfection, and Luciferase Assay

The GATA1 expression vector, pPacGATA1, and *Bcl-x* promoter construct, pGL3B-Bcl-x-pro, were prepared as previously described [49,51]. *D.* Mel-2 cells (Invitrogen, Carlsbad, CA) were co-transfected with 1 µg of the *Bcl-x* promoter construct and 125 to 500 ng of pPacGATA1 using Fugene 6 reagent (Roche, Indianapolis, IN), as described previously [49,51]. Luciferase activities were assayed using the Single Luciferase Assay System (Promega) and normalized to total cell protein, measured by the Bio-Rad DC-protein assay kit (Bio-Rad, Hercules, CA).

2.2.12 Production of Lentivirus Particles and Transduction

The pMD-VSV-G and delta 8.2 plasmids were gifts from Dr. Dong at the Tulane University. The transfection was carried out by using Lipofectamine and Plus reagents (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Briefly, RFP (red fluorescent protein) or Bcl-xL cDNA construct from Thermo Fisher Scientific Biosciences (Lafayette, CO), pMD-VSV-G and delta 8.2 were cotransfected into TLA-HEK293T cells and the culture medium was harvested 48 h post-transfection. $2x10^5$ cells were transduced overnight by adding 1 ml of virus supernatant and 4 µg of polybrene (Sigma-Aldrich). Cells were then triple-washed and allowed to grow for 24-48 hours before addition of blasticidin for selection. Cells were selected for at least 7 days before being used for experiments (Invivogen, San Diego, CA).

2.2.13 Trypan Blue Exclusion Assay

An aliquot of the indicated cells was mixed 1:1 with a 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO) and allowed to incubate for 30 seconds. The mixture was loaded into a haemocytometer and total cell counts, as well as trypan blue positive cell counts, were made. Reported results are the percentage of the total cells that were trypan blue positive and represent the average of at least three independent counts.

2.2.14 Statistical Analysis

Differences in transcript levels between distinct AML patient groups were compared using the nonparametric Mann-Whitney two-sample U test. The nonparametric Spearman rank correlation coefficient was used to analyze the relationship between GATA1 and Bcl-xL transcript levels. Statistical analyses were performed with GraphPad Prism 4.0.

2.3 Results

2.3.1 Overexpression of GATA1 in AMKL blasts is associated with chemotherapy resistance

To determine if GATA1 is overexpressed in AMKL compared to other subtypes of AML in non-DS children, real-time RT-PCR was performed to quantify transcript levels of GATA1 in a cohort of diagnostic AML blast samples (12 AMKL cases and 31 non-AMKL AML cases). GATA1 transcript levels were significantly higher in AMKL blasts compared to blasts of other AML subtypes amongst non-DS patients (median 5.5-fold, $p=0.004$) (Figure 2.1A).

To explore the role of GATA1 in chemotherapy sensitivity in AMKL, GATA1 expression was knocked-down using lentivirus shRNA in the non-DS megakaryocytic cell line Meg-01. Two stable clones, designated GATA1 4-14 and GATA1 5-13, showed decreased *GATA1* transcripts [~25% relative to a non-targeted control comprised of a pool of cells infected with a lentivirus negative control shRNA (designated GATA1 Neg)], while at the protein level, GATA1 was reduced to approximately one-third and one-half that of GATA1 Neg in the GATA 4-14 and GATA 5-13 clones, respectively (Figures 2.1B&C). This was accompanied by significantly increased baseline apoptosis in both clones relative to the GATA1 Neg cells, as measured by flow cytometry with Annexin V-FITC/PI staining (Figure 2.1D). Down-regulation of GATA1 also resulted in increased anti-proliferative activity for ara-C and DNR, as measured by the Cell Titer-Blue viability assay. The IC_{50}s for ara-C in the GATA1 Neg, GATA1 4-14, and GATA1 5-13 were 48.3 nM, 10.8 nM, and 12.0 nM, respectively, while those for DNR were 41.6 nM, 27.1 nM, and 18.4 nM, respectively (Figures 2.1E&F).

2.3.2 Bcl-xL is overexpressed in AMKL and is a GATA1 target gene

It was previously reported that GATA1 promotes survival of developing erythrocytes by upregulating Bcl-xL [58]. On this basis, we hypothesized that GATA1 could play a potential role in the survival of AMKL blasts upon treatment with cytotoxic agents by upregulating BcL-xL. To begin to test this possibility, Bcl-xL transcripts were initially measured by real-time RT-PCR in the above cohort of 31 AMKL and 12 non-AMKL AML cases. Consistent with our hypothesis, Bcl-xL transcript levels were significantly higher (median 4.2-fold; p=0.002) in AMKL

cases compared to that in other AML subtypes. (Figure 2.3A) The transcript levels of Bcl-xL closely correlated with GATA1 transcripts in AML and AMKL blasts ($r=0.901$, $p<0.0001$) (Figure 2.3B). Further, in the cell line models, knockdown of GATA1 resulted in decreased Bcl-xL expression at both the transcript and protein levels (Figures 2C&D). Bcl-2 protein was not detected by western blotting in these cells and Mcl-1 levels were comparable to the GATA-1 Neg cells (Figure 2.2A). Finally, the sensitivity induced by GATA1 knockdown was reduced, at least in part, by exogenous expression of Bcl-xL in the GATA1 4-14 clone (Figures 2.2B&C)

To provide evidence that GATA1 directly activates expression of Bcl-xL by binding to the putative GATA1 sites in the *Bcl-x* gene promoter region (Figure 2.3E), ChIP was performed in Meg-01 cells, followed by PCR of the recovered DNA using primers flanking the putative GATA1 binding sites. As can be seen in Figure 2.3F, pulldown with the GATA1-specific antibody GATA1 c-20 resulted in specific precipitation of the *Bcl-x* promoter region (containing the two putative GATA1 binding sites) but not an unrelated region. Activation of the *Bcl-x* promoter activity by GATA1 was further confirmed using a luciferase reporter assay after cotransfection of *D.* Mel-2 cells with increasing amounts of a GATA1 expression vector along with the *Bcl-x* promoter construct. As shown in Figure 2.3G, increasing amounts of the GATA1 construct resulted in dose-dependent induction of luciferase expression driven by the *Bcl-x* promoter. Taken together, these studies provide direct evidence that GATA1 binds to the GATA1 sites in the *Bcl-x* upstream region and transactivates its activity in Meg-01 cells.

2.3.4 Treatment with VPA down-regulated GATA1 and Bcl-xL and sensitized Meg-01 cells to ara-C- induced apoptosis

With currently available treatments, it is not feasible to directly target GATA1 in AMKL cells. Based on a previous report [22], we hypothesized that treatment with VPA, an HDAC inhibitor, could potentially down-regulate GATA1 expression. Indeed, treatment of Meg-01 cells with clinically achievable concentrations (0.5 and 1.0 mM) of VPA resulted in a reduction in GATA1 protein expression, as measured by western blotting (Figure 2.4A), while treatment with comparably toxic doses of DNR did not (not shown). Furthermore, VPA treatment also resulted in decreased expression of Bcl-xL protein (Figure 2.4A). To investigate the mechanism through which VPA modulates GATA1 expression, real time RT-PCR was used to look at GATA1 transcript levels post VPA treatment. Transcript levels were decreased after both 0.5 and 1.0 mM treatments, albeit to different degrees (Figure 2.4B).

To determine the impact of VPA treatment on ara-C cytotoxicity, we measured the viability of Meg-01 cells upon exposure to ara-C, along with increasing VPA concentrations, using the Cell Titer-Blue viability assay. When simultaneously administered with ara-C, VPA at 0.5 and 1 mM significantly enhanced ara-C sensitivity (as reflected in decreased IC_{50}s) by 1.6- and 2.7-fold, respectively (Figure 2.4C). The combined effects of ara-C with VPA on cell proliferation were clearly synergistic, as determined by standard isobologram analysis (Figure 3D) and by calculating CI values. A CI<1 (0.83 and 0.81 for the combination with 0.5 and 1.0 mM VPA, respectively), indicative of synergism,

was calculated for each of the drug combinations. In addition, the combination of VPA and ara-C synergistically induced apoptosis as measured by flow cytometry with Annexin V-FITC/PI staining (Figure 2.4E). Exogenous expression of Bcl-xL in Meg-01 cells abrogated the enhancement of ara-C cytotoxicity by VPA and caused resistance to VPA or ara-C (Figure 2.2B&D)

2.3.5 Identification of additional GATA1 target genes

In order to identify additional GATA1 target genes that may contribute to chemotherapy resistance, oligonucleotide microarray analyses on RNAs from the GATA1 5-13/4-14 clones and GATA1 Neg control were performed. Average log ratios, representing the differences in expression between the *GATA1* shRNA clones and the GATA1 Neg control, were derived for each array feature by combining replicate array data, using the error-weighted algorithm of Rosetta Resolver®. Differentially expressed genes were identified by their p-values, calculated with the Resolver error-model and the replicate data. Using a p-value ≤ 0.005 as cutoff, 3210 differentially expressed features (probes) were identified, with a false discovery rate of 6.4%. Of these, 1521 were down regulated in the knockdown clones, while 1689 were upregulated. Validation of the microarray results was performed using real-time RT-PCR and separate RNA samples from the Meg-01 stable clones. As shown in Figure 2.5A-B, real-time RT-PCR was able to validate both up- and down-regulated genes. A full list of differentially expressed probes can be found in Tables S2 and S3. Interestingly, the down-regulated genes were significantly enriched for genes involved in regulating cell division and cell death, and a large number of the upregulated genes were

associated with chromatin assembly and organization (Tables S4 and S5, respectively), as determined by DAVID gene functional annotation analysis [79,80].

The above oligonucleotide microarray experiment detected both direct and indirect GATA1 target genes in Meg-01 cells. To identify direct targets of GATA1 among the oligonucleotide microarray gene list, a ChIP-on-Chip experiment was performed. Validation of the ChIP-on-Chip analyses was performed using both regular PCR and real-time RT-PCR (Figures 2.5C-D). The gene accession numbers associated with ChIP-on-Chip peaks (described in methods) were compared to the accession numbers for the 3210 differentially expressed genes in the GATA1 knockdown clones. The cross-referencing produced a list of 317 common genes (Figure 2.5E, Table S6) which is likely to be highly enriched for *bona fide* GATA1 target genes. Interestingly, there were many genes in this overlapping group that were found by DAVID ontology analysis to be associated with either regulation of cell death, cell cycle, or proliferation (Table 2.1.).

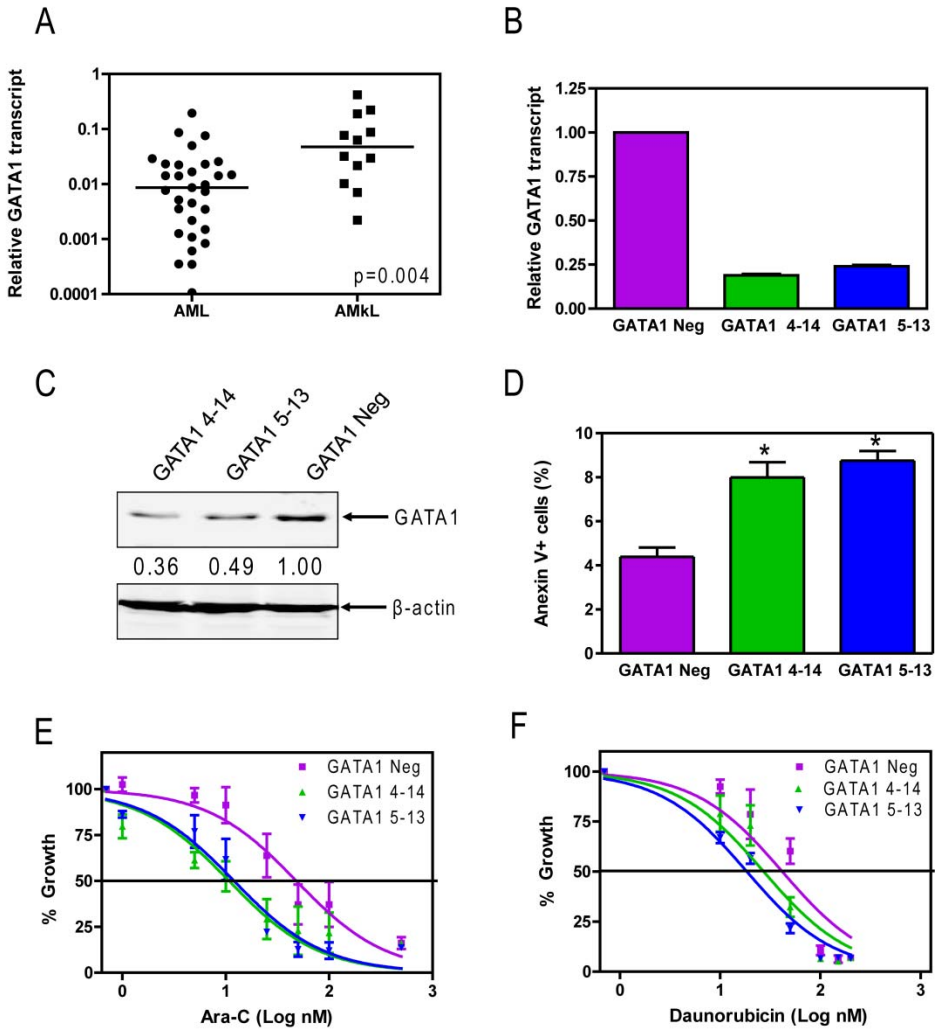


Figure 2.1 GATA1 transcripts are elevated in AMKL blasts and shRNA knockdown increases basal apoptosis and chemotherapy sensitivity. **A**: GATA1 transcript levels in primary non-AMKL AML or AMKL blasts from non-DS patients were quantitated by real-time RT-PCR and normalized to GAPDH transcript levels. Median transcript levels were compared between the two patient groups using the nonparametric Mann-Whitney U test. **B-C**: Meg-01 cells were infected by GATA1 shRNA lentivirus clones. Colonies were isolated, expanded and tested for GATA1 expression by real-time RT-PCR (panel B) and Western blotting (panel C). Two colonies (GATA1 4-14 and GATA1 5-13) with decreased GATA1 gene expression were selected for further study. A pool of cells from the negative control infection was used as the control (designated GATA1 eg). **D**: Baseline apoptosis in the GATA1 4-14, GATA1 5-13 and GATA1 Neg was determined by flow cytometry with Annexin V-FITC/PI staining. *indicates $p<0.05$, error bars indicate standard errors. **E-F**: Meg-01 GATA1 shRNA clones were cultured in 96-well plates at a density of 4x104 cells/mL in the presence of 0-500 nM Ara-C (panel C) or 0-200 nM DNR (panel D) and cell numbers were determined with the Cell Titer-blue reagent and a fluorescence microplate reader. The data represent mean values ± standard errors from at least three independent experiments.

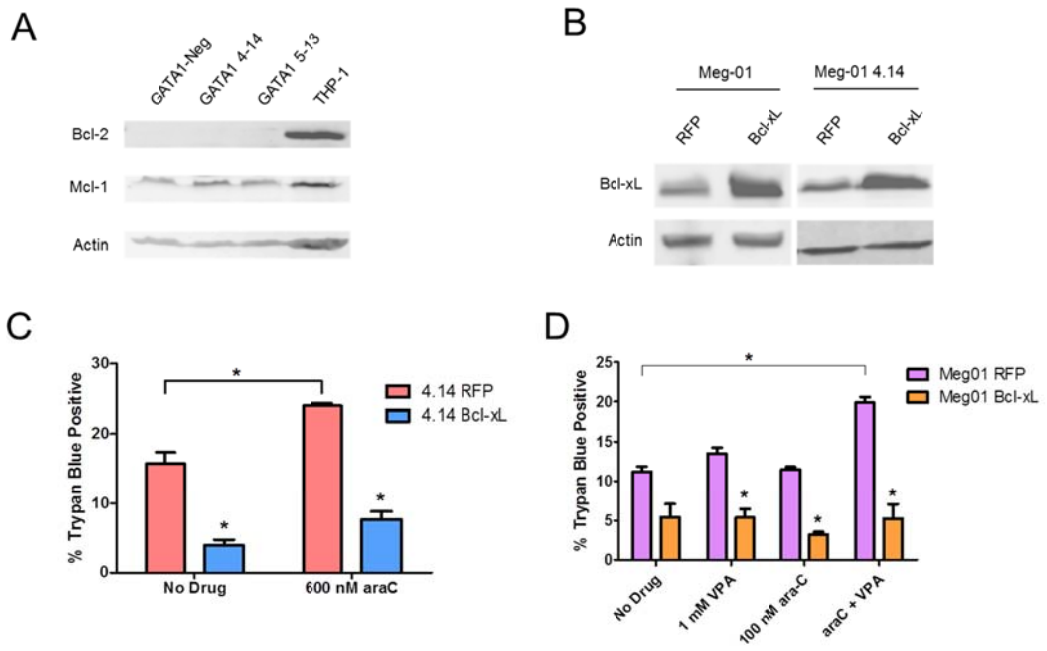


Figure 2.2 Effect of GATA-1 knockdown on Bcl-2 and Mcl-1 expression and overexpression of Bcl-xL overcomes ara-C sensitivity resulting from GATA1 knockdown and conveys resistance to VPA. **A:** Western blots demonstrating the impact of GATA-1 knockdown on Bcl-2 and Mcl-1 expression. 100µg of protein were loaded in each lane, with an excess of THP-1 lysate as a positive control (far right). **B:** Western blots demonstrating overexpression of Bcl-xL in both the parental Meg-01 and Meg-01 4.14 cell lines. **C-D:** Indicated cells were treated for 24 hours at the indicated drug dose and viability was determined using trypan blue exclusion. * indicates $p < 0.05$ compared to RFP or between indicated columns.

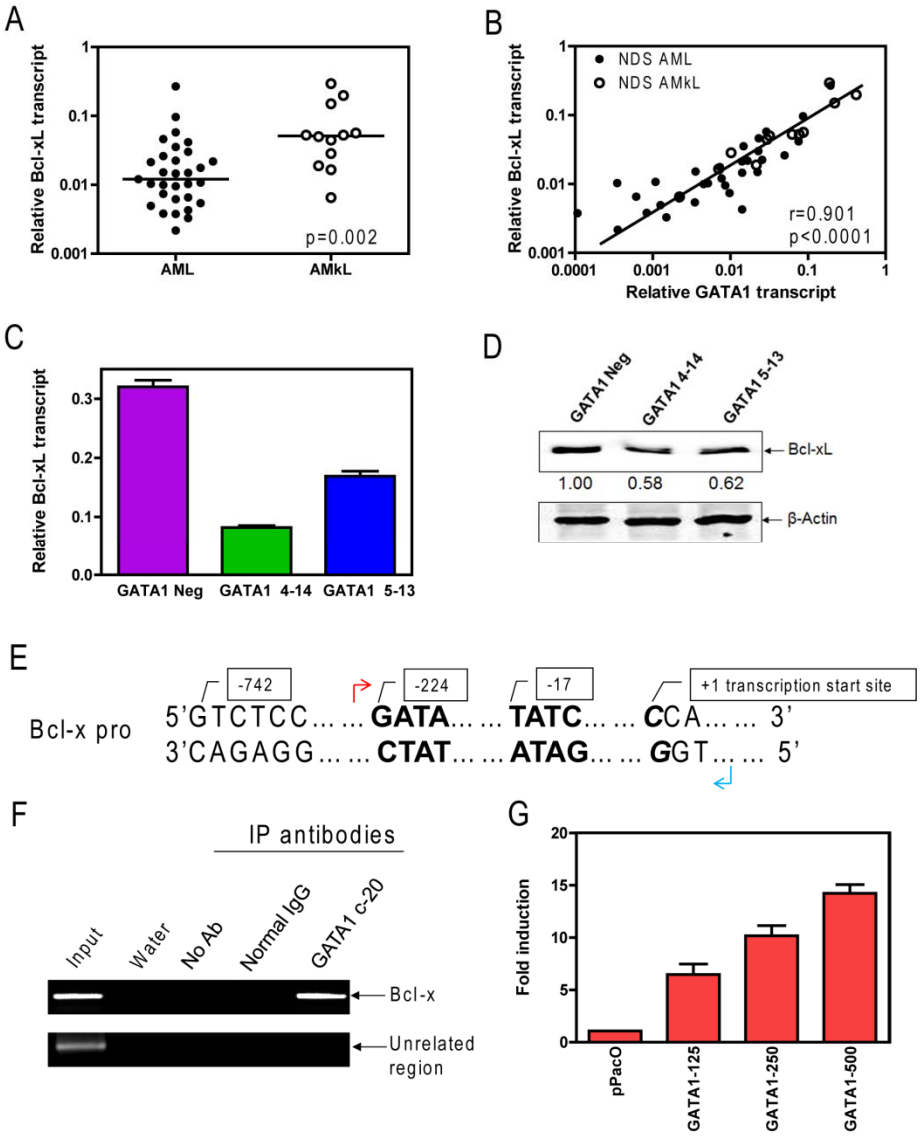


Figure 2.3 Bcl-xL is a bona fide GATA1 target gene in AMKL. **A:** *Bcl-xL* transcript levels in primary non-AMKL AML or AMKL blasts from non-DS patients were quantified by real-time RT-PCR. Median transcript levels were compared between the two patient groups with the use of the nonparametric Mann-Whitney *U* test. **B:** The relationship between *GATA1* and *Bcl-xL* transcript levels was determined by the nonparametric Spearman rank correlation coefficient. **C:** Transcript levels for *Bcl-xL* were quantified by real-time RT-PCR in the Meg-01 shRNA stable clones. Real-time PCR results were expressed as mean values from three independent experiments and normalized to GAPDH. **D:** Whole cell extracts from Meg-01 GATA1 Neg., 4-14 and 5-13 were subjected to Western blotting and probed for Bcl-xL and β-actin. **E&F:** In vivo binding of GATA1 to the putative GATA1 binding sites located in the upstream region of the *Bcl-x* gene in Meg-01 cells was determined by ChIP assays with the use of regular PCR (panel F), as described in the "Materials and Methods". The location of the primers used in 2F are indicated by the arrows in 2E. **G:** *D.* Mel-2 cells were transfected with pGL3Basic-Bcl-x-pro along with 0-500 ng of pPacGATA1 using the Fugene 6 reagent. GATA1 transactivation of *Bcl-x* promoter was determined by luciferase reporter gene assays and normalized to total cell protein.

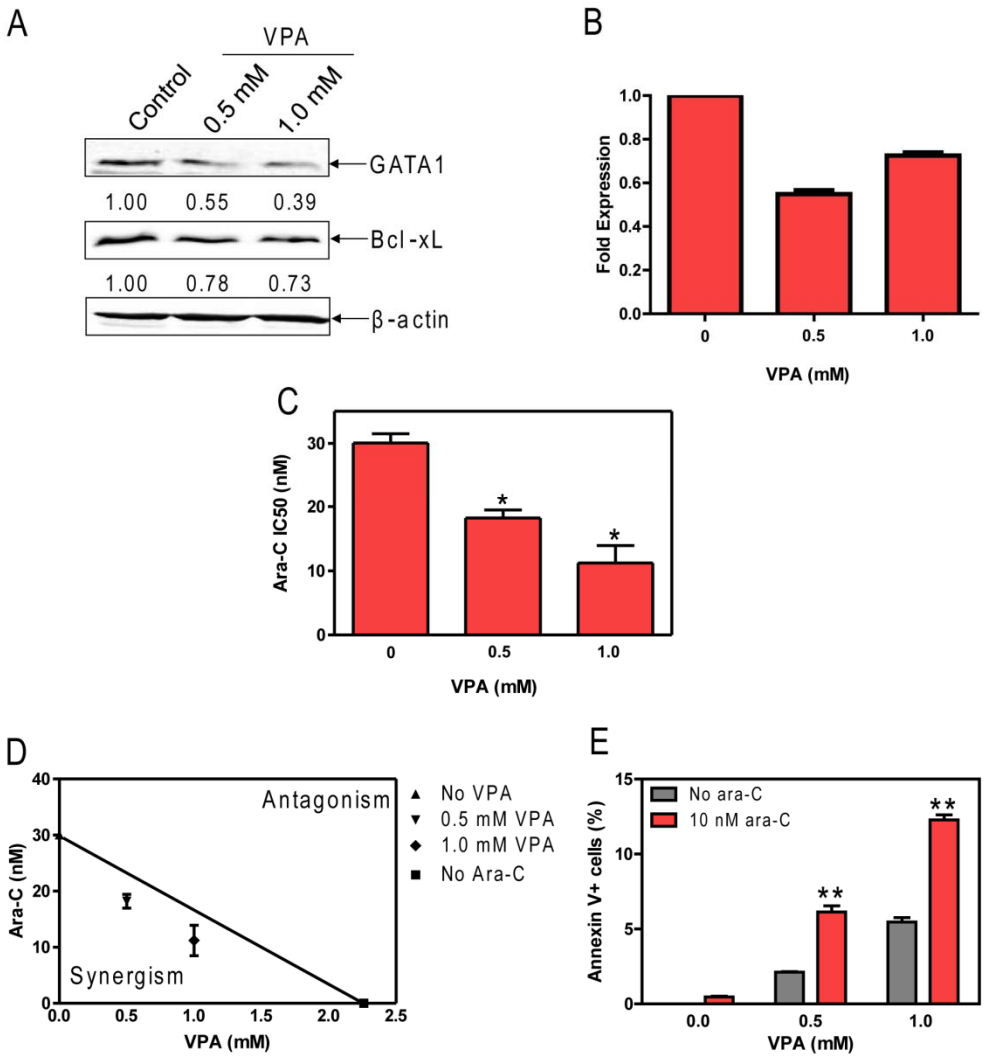


Figure 2.4 Valproic acid causes down-regulation of GATA1 and enhances ara-C induced apoptosis in Meg-01 cells. A: Meg-01 cells were treated with VPA for 48h. Whole cell lysates were extracted, subjected to Western blotting and probed by anti-GATA1, Bcl-xL and β-actin. **B:** Meg-01 cells were treated at the indicated dose of VPA for 48h and RNA was harvested for quantification of GATA1 transcripts by qRT-PCR. Transcript levels for GATA1 in the VPA treated cells were normalized to untreated cells. **C:** Meg-01 cells were treated with ara-C in the presence or absence of VPA and viable cell numbers were determined using Cell Titer-blue reagent. IC_{50} values were calculated as the concentration of drug necessary to inhibit 50% proliferation compared to control cells cultured in the absence of drug. * indicates $p<0.05$ **D:** Standard isobologram analysis of Meg-01 cell proliferation inhibition by the combined treatment of ara-C and VPA. The IC50 value for each drug is plotted on the axes; the solid line represents the additive effect, while the points represent the concentrations of each drug resulting in 50% inhibition of proliferation. Points falling below the line indicate synergism between drug combinations whereas those above the line indicate antagonism. The data are presented as mean values ± standard errors from at least 3 independent experiments. **E:** Apoptosis after indicated treatment for 48 hours as determined by flow cytometry with Annexin V-FITC/PI staining. *indicates $p<0.05$ relative to no ara-C, error bars indicate standard errors.

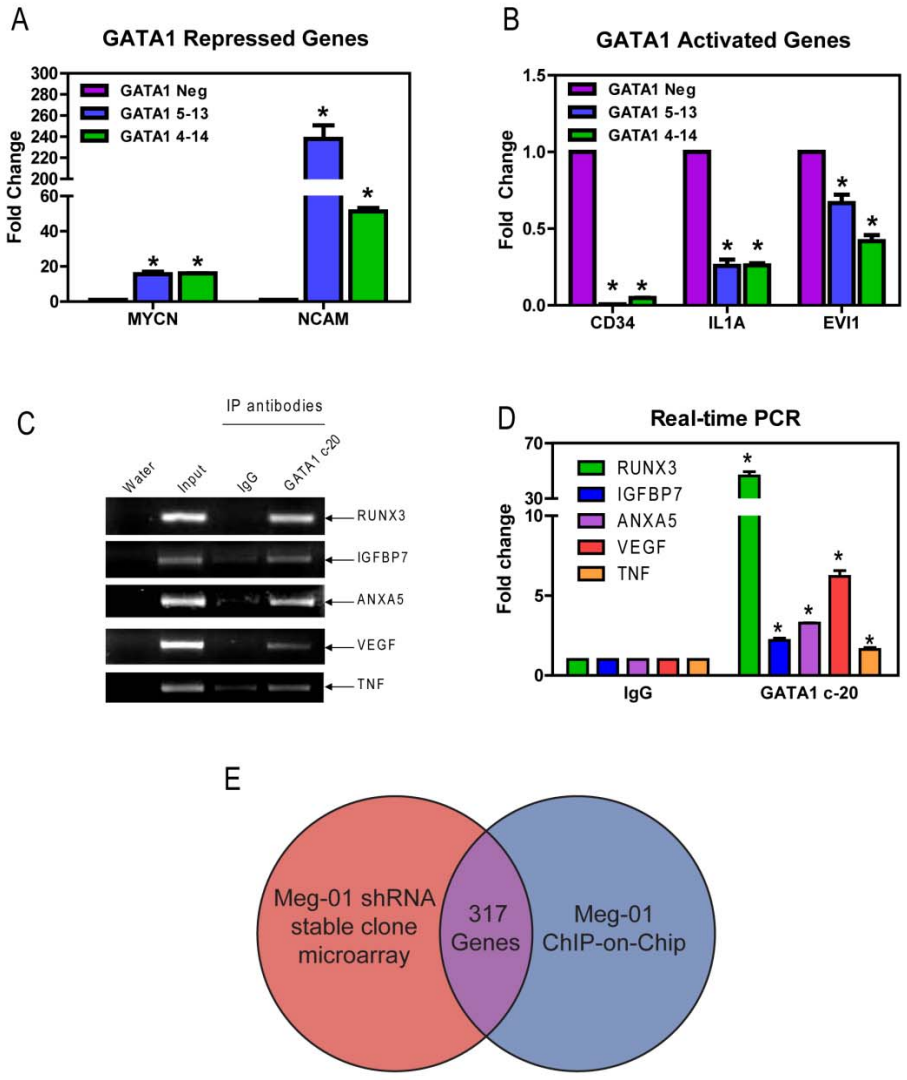


Figure 2.5 Identification of additional GATA1 target genes. A&B: Oligonucleotide microarray analysis was performed with RNA samples form the Meg-01 shRNA stable clones and the results were validated by real-time RT-PCR using Taqman probes. GATA1-repressed (panel A) and GATA1-activated (panel B) transcript levels for MYCN, NCAM1, CD34, ILA1, and EVI1 are expressed as mean values from three independent experiments and normalized to GAPDH. **C&D:** ChIP-on-Chip array analysis was performed in Meg-01 cells and the results were validated by PCR (panel C) and real-time PCR (panel D) using primers against each region (Table S1). **E:** Overlapping genes between oligonucleotide microarray and ChIP-on-Chip array were identified by cross-reference of the two sets of data using GenBank accession numbers.

Table 2.1. Overlapping genes associated with cell-cycle, apoptosis, or proliferation

Accession Code	Name	Fold Change	P-value
NM_006744	RBP4	-3.116	8.89E-16
NM_002253	KDR	-3.081	1.14E-41
NM_000921	PDE3A	-2.489	7.30E-09
NM_030751	TCF8	-2.144	3.70E-03
NM_002371	MAL	-1.911	2.68E-15
NM_021120	DLG3	-1.749	1.02E-07
NM_000459	TEK	-1.464	1.42E-15
NM_001005333	MAGED1	-1.403	5.35E-18
NM_003879	CFLAR	-1.336	1.33E-06
NM_000061	BTK	-1.290	2.70E-03
NM_003292	TPR	-1.274	3.94E-06
NM_001204	BMPR2	-1.242	2.39E-11
NM_004360	CDH1	-1.216	1.50E-03
NM_001154	ANXA5	-1.195	9.00E-04
NM_001006	RPS3A	-1.174	4.08E-07
NM_001605	AARS	-1.125	2.30E-03
NM_138957	MAPK1	1.094	2.60E-03
NM_003318	TTK	1.120	1.20E-03
NM_016542	MST4	1.130	4.50E-03
NM_013239	PPP2R3B	1.146	1.00E-04
NM_003246	THBS1	1.151	4.00E-04
NM_001813	CENPE	1.151	3.50E-03
NM_014881	DCLRE1A	1.156	2.20E-03
NM_006729	DIAPH2	1.173	3.15E-06
NM_018451	CENPJ	1.187	1.00E-04
NM_000876	IGF2R	1.225	4.60E-03
NM_002710	PPP1CC	1.226	4.00E-04
NM_033031	CCNB3	1.236	1.00E-04
NM_032375	AKT1S1	1.247	3.47E-05
NM_000675	ADORA2A	1.247	4.00E-03
NM_019619	PARD3	1.266	1.50E-03
NM_138375	CABLES1	1.277	2.00E-04
NM_002291	LAMB1	1.285	3.59E-07
NM_001104	ACTN3	1.289	1.70E-03
NM_001003940	BMF	1.380	3.43E-05
NM_000599	IGFBP5	1.480	2.80E-03
NM_000600	IL6	1.604	7.75E-08
NM_003239	TGFB3	1.615	3.76E-05
NM_006006	ZBTB16	1.615	1.88E-07
NM_053056	CCND1	1.654	2.84E-08
NM_000852	GSTP1	1.802	7.00E-04
NM_006147	IRF6	1.917	7.00E-04
NM_000417	IL2RA	2.419	3.15E-08
NM_003991	EDNRB	2.662	5.31E-12
NM_005378	MYCN	4.948	2.10E-03

2.4 Discussion

Despite progress in the treatment of AML, there are still AML subtypes such as AMKL that have a poor prognosis. Hence, studies examining the basis for the relative chemotherapy resistance of these subgroups may lead to improvements in therapy. The significantly higher cure rates of DS AMKL patients, who almost uniformly harbor somatic mutations in the *GATA1* gene, suggested that GATA1 may play a critical generalized role in chemotherapy response and resistance [5,45,106,163,215,236]. This is also supported by studies which established an association between high expression levels of GATA1 and a poorer prognosis in adult AML [6,186]. This is particularly relevant to non-DS AMKL since overexpression of GATA1 in this AML subtype has been previously observed [171]. However, the molecular mechanisms by which GATA1 confers chemotherapy resistance in AMKL remain unknown.

This study established that for patients whose leukemic blasts express high levels of GATA1, one potential mechanism of resistance involves overexpression of the anti-apoptotic protein Bcl-xL. A direct role for GATA1 in the expression of Bcl-xL was implicated by knocking down GATA1 in the megakaryocytic cell line, Meg-01. Upon knocking down GATA1, Bcl-xL expression was partially abrogated, accompanied by increased basal apoptosis and sensitivity to ara-C and DNR. Although this finding was not entirely unexpected, as the Bcl-2 family proteins are known to inhibit apoptosis and lead to chemotherapy resistance, and Bcl-xL has a well-documented role in the maintenance of the megakaryocyte lineage [98], it nonetheless suggests a

potential target for patients with AMKL. The Bcl-2 family inhibitor GX15-070 is currently undergoing clinical trials (www.clinicaltrials.gov) and has shown single agent efficacy in leukemia patients [143,182].

Another potential approach to enhance the treatment of AML with high expression of GATA1 involves the use of HDAC inhibitors, combined with standard chemotherapy regimens. While HDAC inhibitors are well known to exhibit modest anti-leukemic activities as single agents, preclinical work from our group has demonstrated their strong enhancement of standard agents [225,226]. In this study, we showed that VPA, an FDA approved anti-epileptic medicine that also acts as an HDAC inhibitor, was able to down-regulate GATA1 along with Bcl-xL, resulting in synergistic induction of apoptosis upon addition of ara-C. Changes in transcript levels post-VPA treatment did not account for the total differences in GATA1 protein seen in this study (Figure 3A-B), suggesting that VPA affects both the transcriptional and post-transcriptional regulation of GATA1. Current clinical trials are investigating the role of HDAC inhibitors as adjuvant therapies in many cancers, including pediatric AML (www.clinicaltrials.gov).

While Bcl-xL appears to be an important GATA1 target, it was of interest to identify additional genes that were regulated either directly or indirectly by GATA1. Using gene expression microarray analysis, we were able to identify over 3000 genes that were differentially expressed upon GATA1 knockdown including genes involved in regulating chromatin structure and cell death. By cross-referencing the differentially expressed genes with regions identified by ChIP-on-Chip, we were able to generate a list of 317 genes corresponding to

bona fide GATA1 targets. In addition to these, other potentially important genes were identified. One such gene is DYRK1A, which encodes the dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A. This gene was recently identified as a driver of megakaryocytic leukemia in a mouse DS AMKL model [127]. Neural Cell Adhesion Molecule (NCAM), which was found to be repressed by GATA1, is associated with poorer prognosis in AML [162] and early death in pediatric AML patients [121]. Vascular endothelial growth factor (VEGF) was identified by the ChIP-on-ChIP to be a direct GATA1 target is indicative of poorer prognosis [61] and is also a potential therapeutic target in AML [126]. Both Evi1 and CD34 were found to be activated by GATA1 and could potentially contribute to the poorer outcome found in GATA1 overexpressing patients. Evi1 has been found to be epigenetically deregulated and associated with poor prognosis in AML [211]. CD34 is a well-established surface marker present on immature hematopoietic cells that despite having variable prognostic capacity in mixed AML backgrounds [93], is of prognostic significance within some subgroups [151,234]. Though not specifically investigated here, this may suggest a potential role for the reduction of GATA1 levels in the differentiation induced by HDAC inhibitors in AML cells [158].

In summary, in this study we were able to further clarify the role of GATA1 in AMKL. Blasts from patients with AMKL overexpressed GATA1 relative to those with other AML subtypes. The finding that GATA1 is able to bind and activate the *Bcl-x* promoter coupled with the high correlation between GATA1 expression and Bcl-xL transcript levels in primary patient samples offers a potential

explanation for why AML patients with high GATA1 expression have been found to have poorer outcomes. Furthermore, treatment with the HDAC inhibitor, VPA, was shown to decrease both GATA1 and Bcl-xL expression in Meg-01 cells and sensitize them to treatment with ara-C. Finally, by combining gene expression microarray and ChIP-on-Chip analyses, we were able to identify additional GATA1 target genes to serve as a basis for future studies of both potential chemotherapeutic interventions and AMKL biology

CHAPTER 3 – Identifying New Therapeutic Options for the Treatment of Down Syndrome Acute Myeloid Leukemia

3.0 Preface

As discussed in Chapter 1 of this work, there remains a subset of DS-AML patients that, despite best clinical efforts, continues to have poor outcomes. This chapter will cover the important background information and preliminary work that culminated in the selection of the wee1 inhibitor MK-1775 for further study, the results of which are presented in Chapter 4 of this work.

3.1 Standard Chemotherapeutic Agents for the Treatment of DS-AML

In order to effectively design new therapies for the treatment of any disease, it is first important to understand the mechanisms by which current therapies work (or do not work). By considering these mechanisms, it is possible to focus research efforts on rationally targeted therapies. The two compounds that have formed the backbone of pediatric AML treatment regimens for decades are araC and DNR.

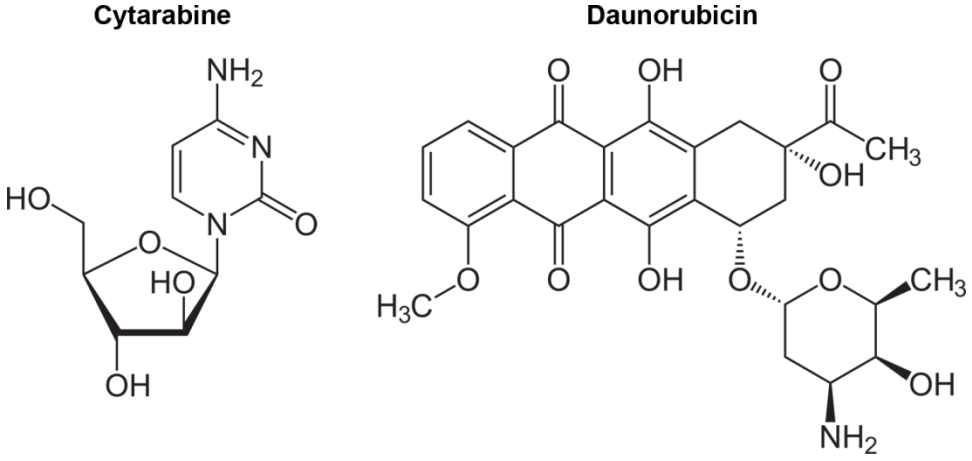


Figure 3.1 Chemical structures of araC and DNR

3.1.1 araC

One of the older anticancer drugs, araC was originally modeled after a compound first isolated from a marine sponge. After observing the effects of the original “spongothymidine”[11], a cytidine analogue with an arabinose replacing the ribose was synthesized and found to have anti-cancer properties[36].

3.1.1.1 Incorporation of araC There are many potential intracellular targets for nucleoside analogues, including interruption of signaling cascades, metabolic processes, and DNA synthesis and maintenance, with araC targeting the last of these. Itself a pro-drug, araC undergoes stepwise phosphorylation inside the cell to become araCTP (see below) and becomes an eligible substrate for DNA polymerases. Despite being initially recognized by the polymerase, as a result of its arabinose sugar moiety, araC is a poor substrate for further elongation. Though there have been somewhat contradictory findings with regards to the robustness of chain termination after araC incorporation and the relative importance of different polymerases, many of these studies were performed using either cell-free systems or low-processivity polymerases, making the importance of these findings difficult to interpret. However, it is generally agreed upon that araC incorporation levels correlate with and are necessary for its toxic effects.[20,46,62,64,88,102,103,144,150,154,167,220]

3.1.1.2 Intracellular metabolism of araC As a nucleoside, the fate of araC is regulated by the same enzymes responsible for normal nucleoside metabolism. Predictably, variations in the expression levels of these enzymes are some of the best-understood mechanisms of resistance to araC[43,105,129].

The main protein believed to be important for araC transport into the cell is the human equlibrative nucleoside transporter 1 (hENT1) encoded by the *SLC28A1* gene. As its name suggests, hENT1 is equilibrative as opposed to concentrative, meaning that araC primarily crosses the plasma membrane by a process of facilitated diffusion. Decreased expression of hENT1 has been associated with resistance to a variety of nucleoside analogues, including araC, in both AML and ALL patients [48,83,192]. Fortunately, there are data to suggest that during courses of high-dose araC plasma concentrations reach levels such that araC transport is saturated and is no longer limited by hENT1[152,218].

Before intracellular araC can be incorporated into DNA, it must first be phosphorylated three times to araC-triphosphate (araCTP). The first of these phosphorylation events is the rate-limiting step, and is catalyzed by the enzyme deoxycytidine kinase (dCK). It has been shown that reductions in dCK activity are not only associated with acquired resistance or relapse[12,92], but that exogenously induced expression can rescue sensitivity to nucleoside analoges[63]. After the initial phosphorylation event, araC is subsequently di- and tri-phosphorylated similar to physiologic nucleosides by enzymes that have not been associated with chemotherapy sensitivity.

Also important for the intracellular levels of araC is the enzyme cytidine deaminase (CDA). Normally involved in pyrimidine salvage, CDA can deaminate araC or its phosphorylated derivatives into uracil arabinoside, or araU. As uracil is not a candidate for incorporation into DNA, araU is inactive. Accordingly, high

levels of CDA are associated with nucleoside resistance, while low levels have been associated with more favorable outcomes [90,184].

3.1.2 DNR and other Topoisomerase II Poisons

The mechanism of action of drugs in this class has been surprisingly controversial, despite almost constant study since the first isolation of DNR in France in 1963 [123]. The biology and relevance to cancer chemotherapy of this class of compounds has been reviewed excellently in [55,137,138], from which much of this overview has been drawn.

Daunorubicin belongs first to a class of drugs known as the anthracyclines, and then to a larger class known as topoisomerase II (Topo2) poisons. The anthracyclines are all derived from *Streptomyces* species bacteria and share similar chemical structure to each other, as well as the prominent anthracycline analogue mitoxantrone. The other predominant drug in the Topo2 poison class is etoposide (VP16), which belongs to the class of drugs known as epipodophyllotoxins.

Type-two topoisomerases are responsible for the decatenation of DNA after replication. Unlike type-1 enzymes which cause single strand breaks and allow for relaxation of supercoils, type-2 enzymes introduce double strand breaks (DSB) in one chain, allowing a second to pass through the gap, followed by ligation of the break. The reason that agents of this class are referred to as poisons is that the presence of their target enzyme, either topoisomerase II α or β, are required for them to exert their toxic effect. In fact, expression levels of Topo2α have been shown to be major determinants of chemotherapy response

[17]. While the exact details are unclear, it is known that Topo2 poisons bind to Topo2 and prevent its decatenation activity. The preponderance of evidence suggests that these drugs do this by locking the enzyme in what is referred to as a “covalent complex” between it and the newly cleaved DNA. The result is not only a DNA DSB, but also a covalently linked DNA-enzyme complex that is difficult to remove.

3.1.3 Cellular effects of araC and DNR treatment

As with most chemotherapeutic treatments, the goal of treating with both araC and DNR is to kill the cancer cell, typically through apoptosis. The exact cellular processes that exert these effects are vast, and in many cases are still being elucidated. In general, however, it is believed that these agents induce apoptosis primarily by elucidating a DNA-damage response. Though a thorough review of these processes is beyond the scope of this work, (excellent coverage of the topic may be found in [7,135,178]), the general trend is that araC induces S-phase DNA damage[37] and DNR induces G2- and M-phase DNA damage[138]. The first response to DNA damage is typically cell-cycle arrest, giving the cell time to repair the damage prior to division or DNA replication. However, if the damage is sufficiently severe, the cell undergoes apoptosis. Alternatively, damage can go unrepaired with two potential outcomes. The first is the introduction of *de novo* mutations or chromosomal abnormalities, and the second is mitotic catastrophe. Mitotic catastrophe occurs when cells with chromosome-level damage undergo mitosis, resulting in non-standard segregation of genetic material. This is typically incompatible with further growth

and results in cell death. However, in either case, if the cell does not die, the altered genetic material is then passed along to the next generation.

3.1.4 Toxicities of araC and DNR

3.1.3.1 Toxicity of therapy with araC Despite being the primary backbone of most therapeutic regimens, araC treatment is associated with profound toxicity. Most patients suffer substantial myelosuppression, resulting in neutropenia, thrombocytopenia, and anemia. While thrombocytopenia and anemia are treatable with transfusion if necessary, neutropenia is not. Neutropenic patients are at a substantially elevated risk for infection, and prophylactic antibiotics directed against both bacteria and fungi are now being used more commonly as supportive care in leukemia protocols. Other adverse effects include corneal toxicity, which can be managed prophylactically with steroid eye-drops, and common chemotherapy side effects like alopecia, nausea, and gastrointestinal toxicity. [75,173]

3.1.3.2 Toxicity of therapy specific to DNR When considering chemotherapy with Topo2 poisons, there are two specific late effects that are of special note. The first is an elevated risk of secondary malignancy, specifically tAML. Though secondary malignancy is a risk of any DNA damaging chemotherapeutic agent, Topo2 poisons (especially VP16) have been associated with a greatly enhanced risk of tAML[39,148]. The second is the well-established risk for cardiotoxicity (e.g. decreased left ventricular function) after treatment with anthracyclines. Cardiotoxicity can manifest either early (within one year) [100] or late [84,228], and though not all cardiotoxicity in cancer survivors is related to

anthracyclines [113], it is still concerning such that anthracycline administration is typically limited by lifetime cumulative doses.

While the effects of Topo2 poisons on secondary malignancy incidence are considered undesirable on-target effects, there is much debate as to the underlying mechanism behind the cardiotoxic effects of anthracyclines. Early work in this field showed that anthracyclines were able to redox cycle due to their quinone moiety, and that this effect was especially prominent in the presence of iron. These findings lead to the belief that anthracyclines exerted their toxic effects through reactive oxygen species- (ROS) mediated damage. A consequence of this belief was that investigators designed iron-chelators to help protect cardiac tissue from ROS generation, one of which was dexrazoxane. Interestingly, dexrazoxane has been shown to also catalytically inhibit Topo2, which could theoretically block the activity of Topo2 poisons [55,137,138]. The debate regarding the mechanism of cardiac toxicity still continues, with recent studies pointing at on-target effects of anthracyclines and their action on Topo2β as well as mitochondrial iron accumulation in cardiac tissue [86,231]. Furthermore, while recent clinical studies have shown benefit of dexrazoxane adjuvant therapy [114], other studies have associated its use with an increased incidence of tAML[176,205], and the FDA has since changed its approval to only apply to adult breast cancer patients who have received high doses of anthracycline (www.FDA.gov).

3.2 Chemotherapy sensitivity in DS patients

As mentioned in Chapter 1 of this work, DS-AML patients have a unique response to chemotherapy compared to non-DS patients. Most importantly, it has been shown that leukemic blasts from DS patients are more sensitive to chemotherapy. Two studies from this group demonstrated that DS-AML blasts generate higher levels of the active araC metabolite araCTP[202,203]. This was at least partially due to increased expression levels of cystathionine-β-synthase (CBS), which is located on chromosome 21. Increased CBS expression impacts the folate cycling pathways involved in nucleoside pool maintenance thereby decreasing the intracellular pools of dCTP. As dCTP inhibits the araC-activating enzyme dCK, elevated CBS levels have been shown to correlate with araCTP levels and increase survival in an *in vivo* mouse xenograft leukemia model[201,202]. A 1999 study replicated these findings, showing that DS-AML blasts were more sensitive to both araC and doxorubicin (an anthracycline like DNR), but that this trend did not hold true for DS-ALL blasts[42]. Another mechanism contributing to this sensitivity is the impact of GATA1 mutations in DS-AML on CDA expression. Two studies from this group demonstrated that CDA is a GATA1-target gene, and that GATA1s mutants are less-capable of activating CDA expression, resulting in higher araCTP levels and higher sensitivity to araC[51,53]. Further, more recent work from this group has demonstrated that DS-AML has a unique gene expression profile compared to non-DS-AML, and that GATA1s also has positive functions promoting the growth and survival of DS-AML blasts[49,222].

The increased sensitivity of DS-AML blasts to chemotherapy, unfortunately, also extends to DS-AML patients, who are at a greater risk for treatment related toxicity. In addition to being more susceptible to infection at baseline as a result of having DS, DS-AML patients have historically suffered from increased treatment-related mortality secondary to infectious complications, although more recent protocols have seen greater success at avoiding these unfavorable outcomes[44,106,161]. Of special concern is anthracycline use in this population. At least partially as a result of the high incidence of congenital heart defects in this population, DS patients are more likely to develop cardiotoxicity, and it that toxicity which may be lethal[100,142].

3.3 Identifying new therapies for DS-AML – approach

In order to further improve outcomes for DS-AML patients, new therapeutic approaches must be developed. As mentioned in Chapter 1, there are subsets of DS-AML patients who have poor outcomes, and transplant is not an attractive option in the DS-AML population. Furthermore, toxicity continues to be a concern for these patients. With the goal of this study being to identify new chemotherapeutic options, a careful approach to maximize the clinical potential of our findings was important.

The first important consideration is that any results obtained should be as rapidly translatable to the clinic as possible. Though there are exciting options emerging from early-stage investigational drugs, agents that are not already under clinical study are less likely to be tested in the DS population even in the distant future. Therefore, we set out to provide a foundation supporting the

immediate inclusion of new agents into DS-AML treatment protocols upon the approval of drugs currently under investigation for other patient groups. Moreover, this limits the investigation to agents that show effects within clinically achievable concentration ranges. Finally, as a result of responsible clinical study design, any agents selected for study must show at least additive effects with araC, as it is unlikely that study arms would withhold this standard of care agent.

The rest of the considerations driving agent selection are of a more practical nature. Though there has been much recent progress in the field of oncology with regards to biologic therapies (e.g. antibodies and other immunotherapies) these treatments are difficult to interrogate *in vitro* using our model system (see below). Also important was that any compound tested needed to be available. Finally, agents were chosen in a way that was perceived to minimize the replication of effort such that either knowledge or perception of extensive study by either collaborators or outside groups precluded inclusion in this study.

3.4 Cell line models

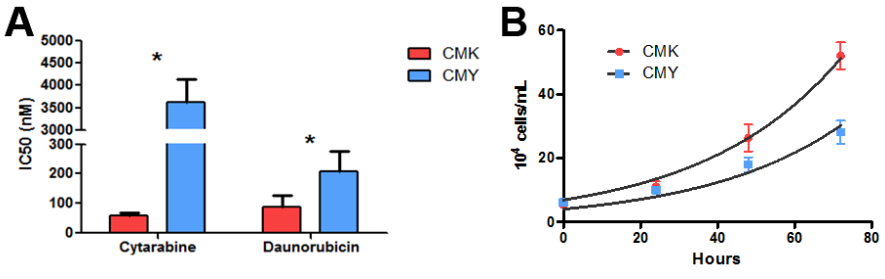
In order to begin investigations into new therapeutic options for DS-AML, model systems needed to be utilized as primary research in patients is neither feasible nor ethical. The model systems used herein are two DS-AML cell lines, derived from DS children with AMKL. The first, CMK, was derived from a patient who presented first with sensitive disease but unfortunately suffered a relapse [181]. The second, CMY was derived from a patient who suffered from primary refractory disease [131]. Though both of these cell lines harbor p53 defects that

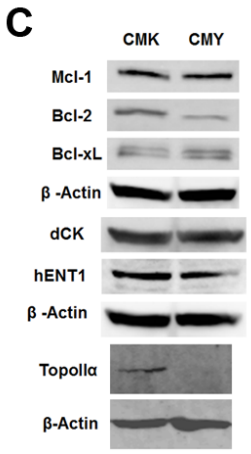
were not present in the primary disease, they are both of the AMKL phenotype with confirmed *GATA1* mutations, and are thus reputable cell line models.

3.4.1 Initial characterization of DS-AML cell lines

The first step in characterizing the CMK and CMY cell line models was determining their response to the standard chemotherapeutic agents, araC and DNR. Utilizing a 3-day 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (methods described in Chapter 4), it was found that CMY was significantly more resistant to both agents than was CMK (Figure 3.2A). Because both araC and DNR are cell-cycle phase dependent (S- and G2/M, respectively), the proliferation rate of the two cell lines was determined by serial cell counts for 3 days. It was found that CMY does in fact proliferate more slowly than CMK (doubling time 33 vs 22 hours) (Figure 3.2B). Next, the expression levels of proteins associated with araC and DNR sensitivity (hENT1, dCK, Topo2α), as well as Bcl-2 family proteins associated with resistance to apoptosis (reviewed in [33]), were interrogated via western blot. Because expression of Bcl-2 family proteins are thought to be able to compensate for one another, it was found that the only substantial difference was in the expression of Topo2α, which was lower in CMY (Figure 3.2C). Finally, it was of interest to determine the response of CMK and CMY cells to treatment with additional agents already under investigation to determine if the relative resistance to araC and DNR was due to CMY cells being globally more resistant or if the resistance was specific to those two classes of agents (Figure 3.2D). Again using a 3-day MTT assay, it was shown that CMY displayed class-wide resistance to the nucleoside

analogues araC, clofarabine, and decitabine, as well as to the Topo2 poisons DNR, etoposide, and mitoxantrone. This class-wide resistance to the Topo2 poisons was not unexpected, as CMY cells possess lower levels of the target protein Topo2α (Figure 3.2C). Interestingly, no cross-resistance was observed for the HDAC inhibitors vorinostat (also known as suberanilohydroxamic acid, SAHA), panobinostat (also known as LBH-589) and mocetinostat (also known as MGCD0103). Similarly, there was no difference in sensitivity to the multiple kinase inhibitor sorafenib. Interestingly, CMY was actually more sensitive to the proteasome inhibitor bortezomib (also known as velcade) than CMK, although it is difficult to make a claim for biological significance with only a 2 nM difference in IC50s. Based on these results, it was determined that CMY is not globally resistant to all treatments compared to CMK, and that it would be possible to find an agent that may be similarly effective in resistant and sensitive cases.





D

Drug	IC50 (nM) CMK	CMY	Fold	
Cytarabine	60	3627	60.9	*
Clofarabine	14	350	25.0	*
Decitabine	50	1099	21.8	*
Daunorubicin	88	209	2.4	*
Etoposide	1251	4432	3.5	*
Mitoxantrone	12	69	5.8	*
Vorinostat	379	449	1.2	
Panobinostat	5	4	0.9	
Mocetinostat	92	127	1.4	
Sorafenib	5533	6101	1.1	
Bortezomib	7	5	0.7	*

* indicates p<0.05

Figure 3.2 Initial characterization of the CMK and CMY cell lines. **A:** CMY cells are more resistant to both araC and DNR than CMK cells as measured by 72 hour MTT assay. **B:** CMK and CMY cells were plated at similar densities and counted every day while the cells remained in logarithmic phase growth. Error bars represent standard error on the mean values from three independent experiments. **C:** Expression levels of the indicated proteins were interrogated using western blots on lysates from untreated, logarithmically growing cells. Representative images are shown. **D:** The sensitivities to the indicated agents of both CMK and CMY cells as measured by 72 hour MTT assays.

3.4 Identifying new agents

From the results shown in Figure 3.2, it was determined that it is unlikely that either nucleoside analogues or topoisomerase poisons would likely produce favorable results. Because araC requires cells to be in S-phase for it to exert its toxic effects, agents that have shown promise in other studies but cause proliferative arrest, like cyclin dependent kinase (CDK) inhibitors and rapalogues, were not eligible for consideration in this study. It was hypothesized that agents that either targeted cells post-S phase or cell cycle checkpoints could be very promising, especially given the pre-clinical successes of Chk1 inhibitors[19,34]. The four targets chosen for further study were the aurora kinases A and B, polo-like kinase (Plk)1, and the wee1 kinase. The first three of these will be covered in this chapter, while the results concerning wee1 can be found in Chapter 4 of this work.

3.4.1 Aurora kinases A and B and Plk1

In recent years, the aurora kinases and Plk1 have been the subject of great clinical interest for the treatment of malignancy. Studies have shown that the aurora A and B kinases as well as Plk1 are overexpressed in AML cells, compared to normal bone marrow cells[65,81,87,165]. Furthermore, preclinical work demonstrated a favorable effect of inhibitors of these kinases on AML cell lines and primary AML blasts, in *in vivo*, *ex vivo*, and *in vitro* experiments[57,65,81,87,97,165,227]. Thus, it is important to understand the mechanism of action of these kinases, as well as the effect their inhibition has on proliferation.

3.4.1.1 Functions of the Aurora A and B kinases and Plk1 The general role played by these kinases is the regulation of the transition between the G2 phase of the cell cycle and subsequent mitotic processes (For excellent reviews on the functions of these proteins, see the reviews by Lens et al., Stebhardt, Goldenssn and Crispino, and Farag [38,56,109,197]). After a cell has finished replicating its DNA, preparations are made to allow for successful completion of mitosis. The processes underlying mitosis are extremely complicated, but there are some major milestones that must be met in order to ensure that proper segregation of genetic material and formation of the proper number of daughter cells occurs. For the purposes of this discussion, emphasis will be placed on the successful alignment of chromosomes at the metaphase plate and the formation of a bipolar spindle assembly.

Under normal conditions, after replication, cells are free to enter into mitosis even in the absence of aurora A kinase and Plk1. However, in the presence of G2 checkpoint activation, these proteins become necessary for further cell cycle progression [116,125,209]. Normally, CDK1 activity is high after S-phase, and in combination with its partner cyclin B, it phosphorylates a host of targets required for mitotic entry. However, in the presence of checkpoint activation, the inhibitory phosphorylation on CDK1 provided by the wee1 kinase is not effectively removed, preventing mitotic entry. To progress, aurora A kinase activates Plk1, which subsequently phosphorylates wee1, leading to its degradation[213] and allows for an increase in CDK1 activity. This starts a feed-forward loop in which CDK1 further activates aurora A kinase and Plk1, allowing

for mitotic entry. In contrast, aurora B kinase is not required for mitotic entry, although, interestingly, inhibition during interphase is capable of disrupting normal mitotic chromosomal organization[68]. After mitotic entry, aurora A kinase and Plk1 cooperate to form a bipolar spindle assembly while aurora B kinase and Plk1 cooperate to regulate chromatid cohesion and chromosome/microtubule interactions. Following initiation of anaphase, all three proteins begin degradation and mitosis completes[109].

Inhibition or down-regulation of these kinases causes interesting, albeit complicated effects. Aurora A kinase inhibition rapidly leads to aneuploidy secondary to ineffective spindle formation and chromosome segregation [74,128]. Aurora B kinase inhibition, on the other hand, leads to bypass of mitotic arrest and tetraploidy, with the potential for further genetic replication, although apoptosis is the likely outcome[91]. Contrary to both of these, is the result of Plk1 inhibition, which leads to robust arrest in early mitosis and subsequent mitotic death[193].

3.4.1.2 Introduction to inhibitors used in this study When studying the effects of inhibiting kinases, results are only as reliable as inhibitors are specific. Fortunately, specific inhibitors for all three of these kinases have not only been developed, but are in early-stage clinical trials, so some pharmacokinetic data are available. To inhibit Plk1, the compound BI6727 was used. This agent has been shown to be safe in phase 1 and 2 clinical trials, with maximal plasma concentrations exceeding 1 μM, although in the more recent study the C_{max} was only approximately 400 nM[183,191]. It is worth noting, however, that at the

conclusion of the Phase 2 trial, it was determined that BI6727 did not have sufficient single-agent activity against urothelial cell carcinomas to be further pursued as a single agent[191].

Barasertib, also known as AZD1152, is a selective aurora B kinase inhibitor. A pro-drug, AZD1152 is converted *in vivo* to the active form AZD1152-HQPA, which has been shown to be safe and possibly effective for elderly patients with advanced AML[94,95,122]. The half-life of this compound is prolonged, at approximately 77 hours, but its maximum plasma concentration is relatively low, approximately 400-500 nM when used alone[94,122], and only approximately 300 nM in the presence of low-dose araC[95].

Finally, alisertib, or MLN8237, is an orally-dosed selective inhibitor of aurora A kinase. Early results have been published for studies investigating the use of this agent in both lymphomas and advanced solid tumors[21,41]. In both studies, MLN8237 was tolerated, showed promising efficacy, and had maximal plasma concentrations between 1 and 2 μM, with a half-life on the order of 15 hours[21,41]. Importantly, MLN8237 was identified in pre-clinical studies by John Crispino's group to be especially promising for the treatment of DS-AMKL, as it was shown to induce polyploidization and differentiation of CMK cells as well as non-DS AMKL blasts[217].

3.5 Effect of aurora kinase and Plk1 inhibition on araC sensitivity in CMK and CMY

In order to begin investigations of the effects of aurora and polo-like kinase inhibition on the araC sensitivities of CMK and CMY cells, standard 3 day

MTT was performed to determine the appropriate concentration ranges for these agents for combination study. Concentration ranges were initially chosen based on preliminary results from clinical trials, with the goal that concentrations that were not clinically relevant would not be pursued. The only agent for which this was problematic was AZD1152-HQPA, as CMY cells were relatively insensitive to this drug at clinically achievable concentrations (see Figure 3.4). However, only minor increases over what is clinically relevant (1,000 nM) were required to generate appropriate response curves.

After identification of the proper concentration range, the effect of the combination of araC and the inhibitor of interest was determined again using 3-day MTT. The effects of the single drugs on CMK and CMY cells could be compared readily by their respective IC_{50}s, but comparison of their interactions with araC required further analysis.

In order to compare the combined effects, standard isobolograms and combination indices (CIs) were used. Isobolograms represent a simple visualization that takes into account the desired parameter (in this case IC_{50}) for each drug and allows for quick assessment of the nature of the effect of the drug combination. Each axis represents the concentration of the indicated drug. Any combination that lies below the line connecting the points representing the IC_{50} of both drugs is considered to be “synergistic”, those on the line are considered “additive”, and those above the line are considered “antagonistic”. It is important to recognize that for a given isobologram, a desired effect level must be pre-determined. For these cases, an effect of 50% viability compared to untreated

cells was used. The same range of araC was tested in the presence of no additional drug, and three increasing concentrations of the second drug, so each plot has 5 total points: each drug alone, and the three points representing the araC concentration required to inhibit 50% of cell growth in the presence of the given secondary drug concentration. For the isobolograms presented in this section, the axes were normalized to the IC_{50} for each drug such that it was equal to 1. The benefit of this approach is two-fold: it allows for better visualization of reproducibility, since each combination point gets both X and Y error bars, and it makes visual comparison between the two cell lines more direct. Alternatively, synergy can be calculated using the median-effect principle from Chou and Talalay. In this system, a CI < 1 indicates “synergy”, CI > 1 indicates “antagonism”, and a CI of 1 indicates “additivity”. For a comprehensive review of the principles of designating synergy in drug combination studies, see Chou’s 2006 review [27]. CIs were determined using the CompuSyn software.

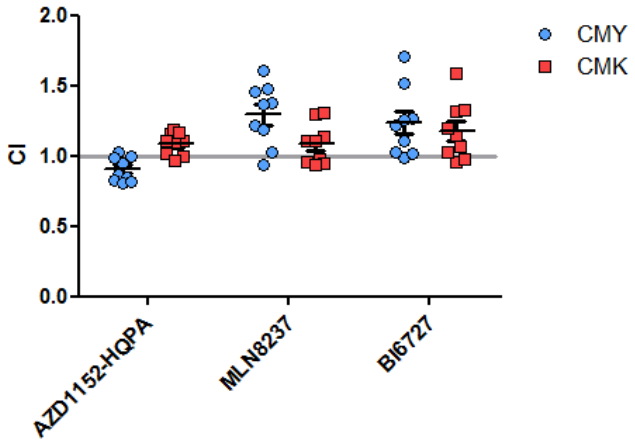


Figure 3.3 CIs for the combination with araC. The CIs representing each point on the isobolograms in Figures 3.4-3.6 are plotted, with each point here representing an individual trial. The CIs were compared to a theoretical median of 1.0 (indicating additivity) using the Wilcoxon Signed Rank Test; p-values and results can be found in the text.

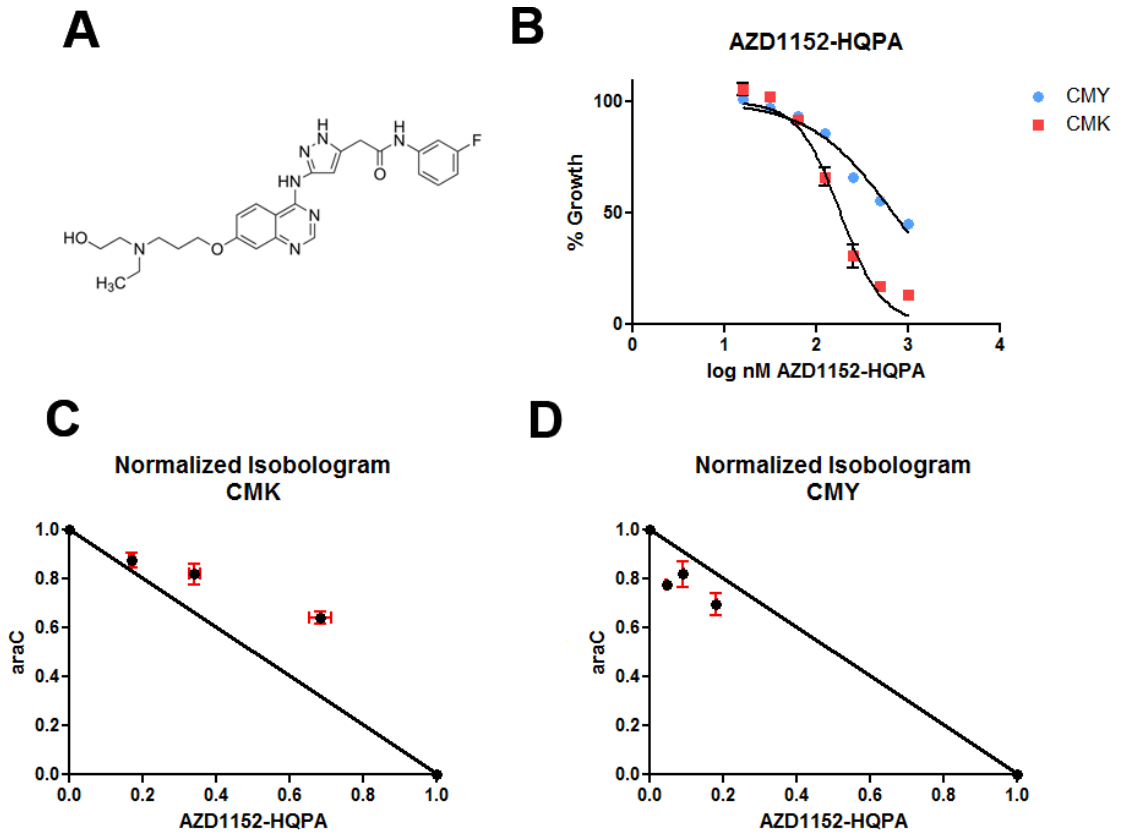


Figure 3.4 The combination of AZD1152-HQPA and araC in CMK and CMY cells. **A:** The chemical structure of AZD1152-HQPA. **B:** The effect of AZD1152-HQPA on the percent of viable CMK and CMY cells as measured by 3-day MTT assay. Data points represent average values from 3 independent experiments while error bars represent standard error of the mean. **C and D:** Normalized standard isobologram analysis of the interactions between araC and AZD1152-HQPA on cell viability. Each axis represents the concentration relative to the IC_{50} of that drug, which was normalized to 1.0. Each point represents the average value from 3 independent experiments, with error bars representing standard error of the mean. X-error bars represent mostly variation in the IC_{50} of AZD1152-HQPA, while Y-error bars represent variation in both the combined effect of both drugs as well as the variation in the IC_{50} of araC.

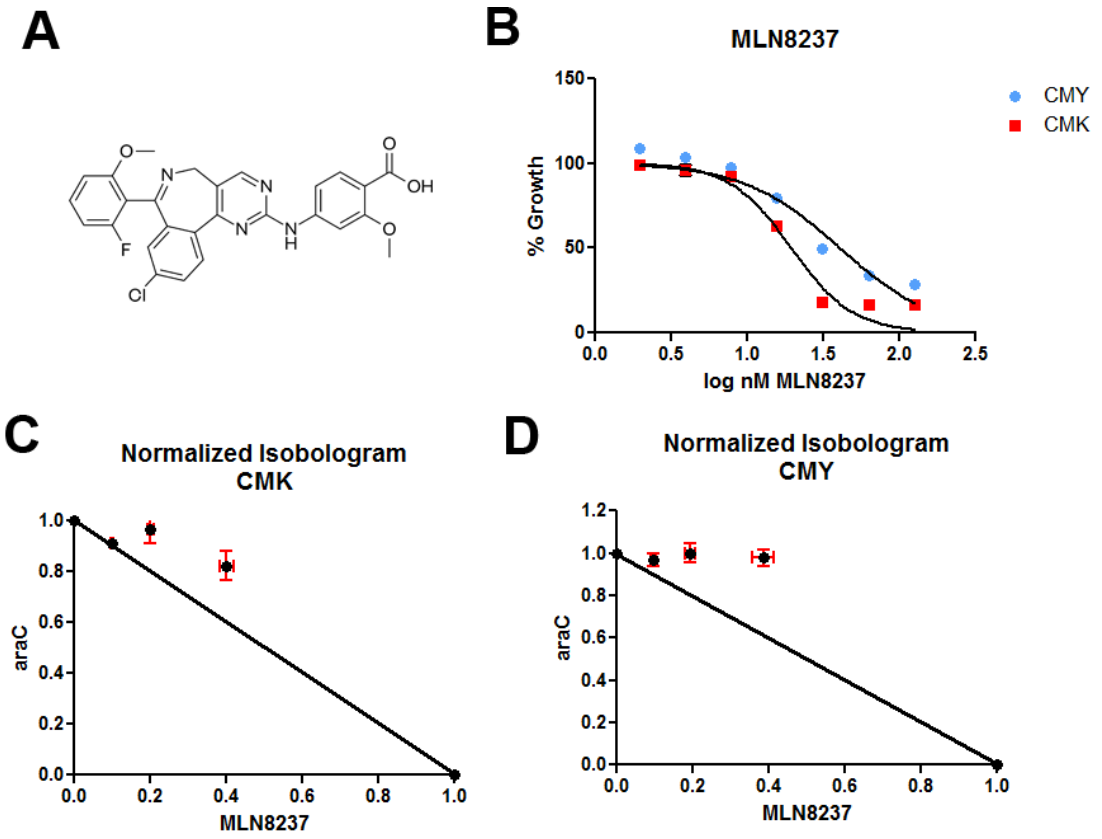


Figure 3.5 The combination of MLN8237 and araC in CMK and CMY cells. **A:** The chemical structure of MLN8237. **B:** The effect of MLN8237 on the percent of viable CMK and CMY cells as measured by 3-day MTT assay. Data points represent average values from 3 independent experiments while error bars represent standard error of the mean. **C and D:** Normalized standard isobologram analysis of the interactions between araC and MLN8237 on cell viability. Each axis represents the concentration relative to the IC_{50} of that drug, which was normalized to 1.0. Each point represents the average value from 3 independent experiments, with error bars representing standard error of the mean. X-error bars represent mostly variation in the IC_{50} of MLN8237, while Y-error bars represent variation in both the combined effect of both drugs as well as the variation in the IC_{50} of araC.

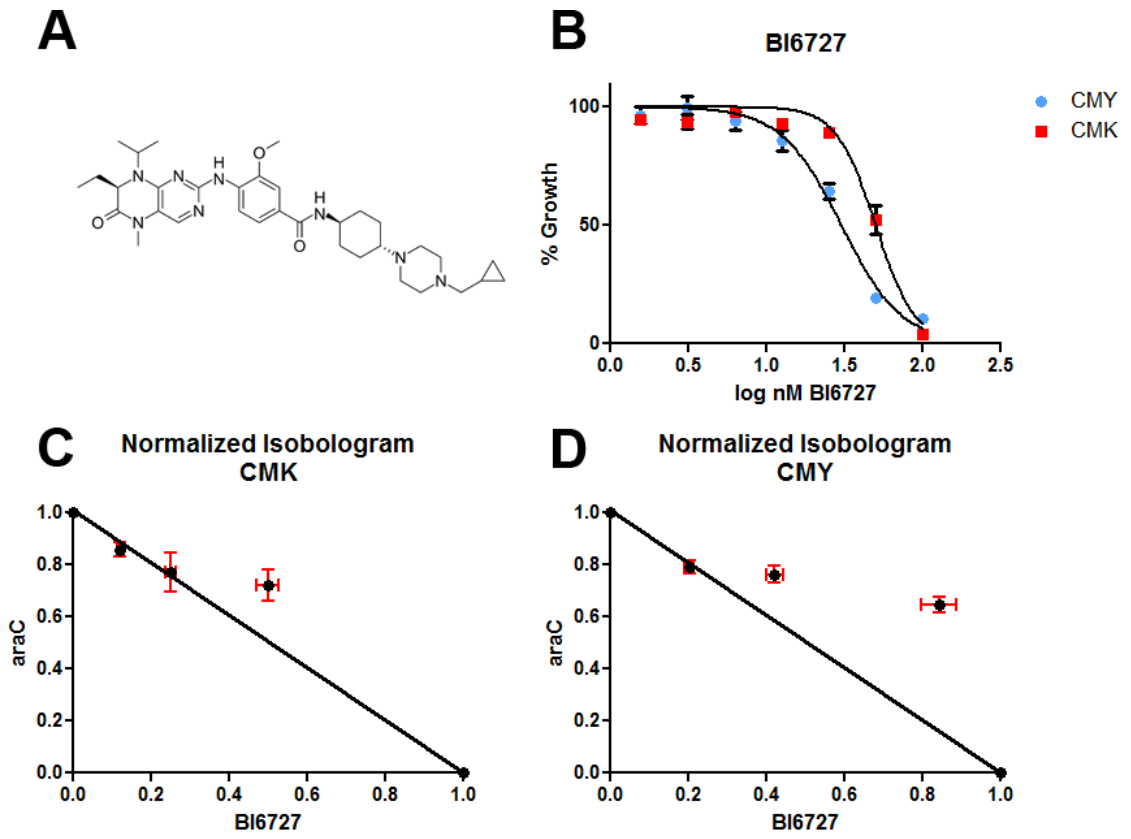


Figure 3.6 The combination of BI6727 and araC in CMK and CMY cells. **A:** The chemical structure of BI6727. **B:** The effect of BI6727 on the percent of viable CMK and CMY cells as measured by 3-day MTT assay. Data points represent average values from 3 independent experiments while error bars represent standard error of the mean. **C and D:** Normalized standard isobologram analysis of the interactions between araC and BI6727on cell viability. Each axis represents the concentration relative to the IC_{50} of that drug, which was normalized to 1.0. Each point represents the average value from 3 independent experiments, with error bars representing standard error of the mean. X-error bars represent mostly variation in the IC_{50} of BI6727, while Y-error bars represent variation in both the combined effect of both drugs as well as the variation in the IC_{50} of araC.

3.5.1 The combination of AZD1152-HQPA and araC in CMK and CMY cells

As seen in Figure 3.4B, CMK and CMY cells displayed different sensitivity to the aurora B kinase inhibitor AZD1152-HQPA, with IC_{50}s of 183 nM (95% 151-216nM) and 689 nM (95% 652-725 nM), respectively. Respecting the maximally achievable plasma concentration of 300-400 nM, combination doses tested were lower relative to the IC_{50} of CMY than CMK due to the relative insensitivity of CMY cells to AZD1152-HQPA (Figure 3.3C-D). It can be seen that the effect of the combination of araC and AZD1152-HQPA trended towards additivity with CMK demonstrating slight antagonism and CMY showing slight synergism. This relationship was similarly seen when CI was used as the metric (Figure 3.3). For both cell lines, their CIs (median 1.1 and 0.87 for CMK and CMY, respectively) were found to be significantly different than a theoretical median of 1.0 by the Wilcoxon Signed Rank test (p=0.02 for both cell lines).

3.5.2 The combination of MLN8237 and araC in CMK and CMY cells

As seen in Figure 3.5B, CMK and CMY displayed different sensitivities to the aurora A kinase inhibitor MLN8237, with IC_{50}s of 19 nM (95% 16-23nM) and 40 nM (95% 29-53 nM), respectively. Though this difference is relatively large (approximately 2-fold), it is possibly insignificant given the achievable plasma concentrations exceeding 1 μM [21,41]. In contrast to the response seen for the combination of araC and AZD1152-HQPA, both CMK and CMY demonstrated an additive-to-antagonistic response to this combination (Figure 3.5C and D). When using CI to determine the nature of the interaction between MLN8237 and

araC (Figure 3.3), only those for CMY (median 1.3, vs 1.1 for CMK) were found to be significantly different than a theoretical median of 1.0 (p=0.01 and 0.16 for CMY and CMK, respectively).

3.5.3 The combination of BI6727 and araC in CMK and CMY cells

As seen in Figure 3.6B, CMK and CMY were differentially sensitive to BI6727, although interestingly, CMK was the more resistant cell line in this case. CMK and CMY had IC_{50}s of 50 nM (95% 38-62nM) and 30 nM (95% 23-36 nM), respectively. Similar to both AZD1152-HQPA and MLN8237, the isobologram analyses (Figure 3.6C and D) did not appear to display any synergism between araC and BI6727, instead trending towards antagonism. In fact, the CIs for these interactions were both found to significantly differ from a theoretical median of 1.0 (CI 1.1, p=0.02 and CI 1.2, p=.008 for CMK and CMY, respectively).

3.6 Discussion

Unfortunately, despite promising preclinical and clinical findings [14,21,41,74,81,87,91,94,95,122,128,183,191,193,217], AZD1152-HQPA, MLN8237, and BI6727 all lacked promise in these preliminary studies. While all three agents demonstrated single-agent efficacy at clinically achievable concentrations in the CMK cell line, only MLN8237 and BI6727 were effective in CMY cells. Furthermore, the combination of these agents with araC resulted in additive-to-antagonistic interactions in each case.

Although these agents were not chosen for further investigation in this study, it is not to say that there is no potential place for them in the clinical setting for the treatment of DS-AML. It is certainly possible that the design of this study

was not optimal to demonstrate a favorable effect of these agents. One potential cause may have been the selection of the 3-day time point. It may be the case that had these experiments been terminated at 48 or 96 hours, different results would have been found, however, the 72 hour time point yielded reproducible results for araC treatment and was thus used here. It is also possible that had a different effect level been interrogated (e.g. IC_{75} or IC_{90}), a different trend may have emerged. While the utility of higher effect levels in investigating chemotherapy drug effects can definitely be argued, it is the opinion of this author that when using *in vitro* cell line models, IC_{50} is more typically communicated in the literature and is therefore a better endpoint for these preliminary-type studies. Further, the experiments presented above were designed around finding a 50% effect size; therefore the concentration ranges tested did not produce results suitable for analysis at higher effect levels, prohibiting robust post-hoc analyses. It is worth noting that, in the context of *in vivo* models, the utility of higher effect levels is much greater, as typically the goal of treatment is disease eradication, not simply a 50% reduction in tumor burden.

In contrast to these agents, which did meet not the relatively strict criteria for further study, a first-in-class inhibitor of the wee1 kinase, MK-1775, did in fact demonstrate favorable effect. The results of the preliminary studies with MK-1775, as well as a further characterization of the mechanism of its effect in DS-AML cells are found in the next chapter of this work.

CHAPTER 4 - Targeting the wee1 Kinase for Treatment of Pediatric Down Syndrome Acute Myeloid Leukemia

4.0 Preface

This chapter is taken in its entirety from a manuscript recently accepted by the journal *Pediatric Blood & Cancer*. Though the text will likely be almost identical to the final published copy, figure numbering has been adjusted to conform to dissertation standards. What was originally an online supplement has been incorporated into this work. This author was responsible for the design and execution of all experiments described herein, with the exception of the *ex vivo* experiment presented in Figures 4.3F (designed by this author, performed by Holly Pitman) and the flow cytometry experiments, which were designed, set up, and analyzed by this author but the steps involving the cytometer were performed by Steve Buck.

4.1 Introduction

Pediatric acute myeloid leukemia (AML) remains a difficult disease to treat and is associated with a relatively guarded prognosis. Patients with Down syndrome (DS), however, typically have much better outcomes, despite having an increased risk of developing AML[223]. This is due to enhanced sensitivity to cytarabine (araC) and daunorubicin imparted by the unique biology of AML in the DS population (DS-AML), in which most cases are the megakaryocytic (AMKL) subtype and have somatic mutations in the X-linked transcription factor *GATA1* gene[51,53,201,203,215].

Despite favorable outcomes, there are still challenges in treating this group of children. Patients with DS-AML who experience either an induction

failure or relapse have dismal prognoses and very few options for salvage[119,120,199]. DS patients with relapsed AML treated on the Pediatric Oncology Group (POG) 9421 and Children's Cancer Group (CCG)-2891 AML studies had an overall survival (OS) rate of 12%, while a Japanese study reported an OS of 25.9% for relapsed and refractory DS AML patients[118,200]. DS-AML patients experience greater adverse toxicity, preventing the use of higher chemotherapy doses, and the high prevalence of congenital heart defects in the DS population makes anthracycline use especially challenging[100,107,141,161]. Following stem cell transplants (SCT), DS-AML patients only had an OS of 19% [72]. These studies all highlight that DS patients with refractory/relapsed AML have extremely chemotherapy-resistant disease. Thus, there is a clinical need for more effective therapies to be developed to treat this subgroup of DS-AML patients.

Inhibition of the wee1 kinase has recently been identified as a potential option for the treatment of several malignancies. The wee1 kinase is responsible for adding inhibitory phosphorylation to the tyrosine-15 residue of CDK1[146,214]. This phosphorylation is required for the activation of S-phase and G2/M cell cycle checkpoints through the CHK1 pathway which inhibits cdc25 phosphatases, which under normal circumstances constitutively remove the inhibitory phosphates, keeping CDKs active[115,149,179,232]. Therefore, preventing this initial phosphorylation event should abrogate these checkpoints that are induced by genotoxic chemotherapy drugs. Indeed, early studies have demonstrated a benefit of combining the first-in-class wee1 inhibitor, MK-1775

(currently in Phase 1 and 2 clinical trials), with standard chemotherapy drugs in a variety of malignancies, including AML[15,23,60,69,70,99,160,208].

In this study, we investigated the potential role for the addition of MK-1775 to araC for the treatment of DS-AML. Using the clinically relevant DS AMKL cell lines, CMK and CMY, and *ex vivo* primary DS-AML blast samples, we determined that MK-1775 was able to synergistically enhance the cytotoxicity of araC. Furthermore, using the cell line models, we determined that MK-1775 enhanced araC-induced apoptosis, likely by enhancing S-phase DNA damage caused by araC. These results support the further development of the wee1 inhibitor MK-1775 for the treatment of DS-AML.

4.2 Methods

4.2.1 Cell Lines, Culture Conditions, and Reagents

CMK cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). The CMY cell line was a gift from Dr. A. Fuse, (National Institute of Infectious Diseases, Tokyo, Japan). The DS-AMKL cell lines CMK and CMY were both cultured in RPMI 1640 with 10% FBS (Life Technologies, Carlsbad, CA, USA) and 2 mM L-glutamine plus 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies), in a 37°C humidified atmosphere containing 5% CO_2/95% air. AraC was purchased from Sigma (St. Louis, MO, USA) and MK-1775 was purchased from Selleck Chemical (Houston, TX, USA). Diagnostic blast cells from DS children with AMKL (n=2) were obtained from the Children's Hospital of Michigan leukemia cell bank. Both patients remain in first remission. Written consent was obtained according to the

Declaration of Helsinki. The research protocol was approved by the Human Investigation Committee of Wayne State University School of Medicine.

4.2.2 Antibodies

Rabbit antibodies directed against wee1, p-CDK1 (Y15), total CDK1, PARP, phosphorylated histone H3(S10) (pH3) and γH2AX were purchased from Cell Signaling Technologies (Danvers, MA, USA). Mouse anti-β-actin was purchased from Sigma. Goat anti-rabbit IRDye 800CW antibody for western blots was purchased from Licor (Lincoln, NE, USA). Goat anti-rabbit-Alexa-488 for flow cytometry was purchased from Life Technologies.

4.2.3 *In Vitro* Cytotoxicity Assay

Viable cells were determined using a standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described[49]. Briefly, cells were added to a 96-well plate and cultured in the presence of increasing drug concentrations for 72 hours. After 72 hours, MTT (Sigma, St. Louis, MO, USA) was added to a final concentration of 1mM. After four hours, formazan crystals were solubilized by addition of 10 mM HCl/10% sodium dodecyl sulfate (SDS). Crystals were allowed to dissolve overnight and plates were read using a microplate reader at 590 nm. For patient samples, 50,000 cells per well were cultured for 48 hours with ITS (Sigma) and conditioned media (20% supernatant from 5637 bladder cancer cell line – a source of GM-CSF) in the presence of various drug concentrations, after which MTT addition occurred as above. The presence or absence of synergistic drug interactions was determined using the Compusyn software (Combosyn, Parasmus, NJ, USA)[27].

Error bars represent standard error on the mean, determined from at least three independent experiments. When indicated, IC_{50}s were compared using non-parametric Mann-Whitney U test using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA)

4.2.4 Lentiviral shRNA Knockdown of wee1 Expression

Lentivirus mediated gene expression knockdown was performed, as described previously[224]. Lentivirus shRNA constructs directed against *WEE1* (designated sh-Wee1) or against no known gene (designated sh-NTC) were purchased from Sigma. Mixed cultures expressing the shRNA of interest were selected for by adding puromycin (Invivogen, San Diego, CA, USA) to the standard culture media. Knockdown was confirmed by both quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot.

4.2.5 Western Blotting

Western blots were performed as previously described[35]. Briefly, cell lysates were prepared in the presence of protease and phosphatase inhibitors (cOmplete Mini and PhosStop, Roche, Indianapolis, IN, USA). Cell lysates were subjected to SDS-PAGE and transferred to PVDF membranes (Pierce Scientific, Rockford, IL, USA) for blotting with antibodies. Membranes were scanned using an Odyssey scanner (Licor). Images are representative of at least 3 identical, independent experiments.

4.2.6 qRT-PCR

qRT-PCR was performed as previously described[35]. Briefly, TaqMan probes from Life Technologies (Carlsbad, CA, USA) directed against either WEE1 or

GAPDH mRNA were used for amplification and detection. Relative transcript levels were determined using the delta-delta-CT method[117] and the experiment was performed on a LightCycler 480 instrument (Roche, Indianapolis, IN, USA)

4.2.7 Flow Cytometry

Flow cytometry for apoptosis was performed using AnnexinV/Propidium iodide (PI) dual staining, as described previously[225]. Analysis of γH2AX and pH3 vs PI was performed using a protocol from Huang and Darzynkiewicz[82], with the following modifications. One million cells per condition were spun at 300 rcf for 4 minutes and resuspended in 0.5 mL of 1% formaldehyde at 4°C for 15 minutes. Samples were spun and resuspended in residual volume, followed by dropwise addition of 1mL of ice-cold methanol and storage at 4°C for at least 30 minutes. Samples were then processed according to protocol, using the antibody dilutions recommended by the manufacturer with overnight primary incubation. Samples were then counterstained with PI and analyzed as above. Histograms were analyzed with FlowJo (TreeStar Software, Ashland, OR, USA). Images are representative of at least 2 identical, independent experiments. Where indicated, statistical analyses were performed using a paired t-Test.

4.3 Results

4.3.1 MK-1775 Has Single Agent Effect in DS-AML

To investigate the potential of targeting wee1 for the treatment of DS-AML, two clinically relevant cell lines were used: CMK and CMY (which were derived from a patient who initially responded to chemotherapy and a patient who had refractory disease, respectively)[131,181]. Each cell line was originally derived

from a pediatric DS patient with AMKL and a confirmed *GATA1* mutation. As can be seen in Figure 4.1A, CMK is much more sensitive (>50-fold) to araC than is CMY. CMY was also significantly more resistant to ara-C-induced apoptosis as measured by AnnexinV/PI staining by flow cytometry compared to the CMK line (Figure 3D&E). CMY demonstrated (using clinically achievable drug concentrations) class-wide resistance to both nucleoside analogues and topoisomerase poisons (Table 4.1). Interestingly, the cross resistance demonstrated by CMY was not seen with MK-1775 (Figure 4.1B) with CMK and CMY having IC_{50}s of 291 and 316 nM, respectively (p=1.0). MK-1775 was able to induce comparable, dose-dependent levels of apoptosis in both cell lines (Figure 4.1C). Knockdown of wee1 expression using lentiviral delivery of shRNA (Figure 4.1D&E) was able to further sensitize both cell lines to MK-1775, suggesting that the effects of this drug are on-target.

4.3.2 Pharmacodynamic Changes of CDK1 Phosphorylation after Treatment with MK-1775

Additional studies examined what dose schedule of MK-1775 would be the most effective for combination treatments. CMK and CMY cells were treated with increasing concentrations of MK-1775 for 24 hours, and phosphorylation status of CDK1(Y15) was interrogated using western blots (Figure 4.2A&B). After 24 hour treatment, it was found that concentrations as low as 100 nM were able to cause near-maximal inhibition of CDK1 phosphorylation. To determine the temporal kinetics of this inhibition, cells were treated with 100 nM MK-1775, followed by drug washout (Figure 4.2A&B). Four hours of treatment was

sufficient to decrease CDK1 (Y15) phosphorylation, but the effect was transient, returning as soon as 1 hour post-drug washout. Based on these findings, it was determined that simultaneous treatment would be utilized for further experiments.

4.3.3 MK-1775 Enhances the Cytotoxic Effects of AraC

The combination of MK-1775 with araC resulted in a synergistic reduction in viable cells (Figure 4.3A&B), with CI values ranging from 0.57-0.74 and 0.7-0.77 for CMK and CMY, respectively (CI values <1 indicate synergism, =1 indicate additivity, and >1 indicate antagonism). Knockdown of wee1 was able to enhance araC-induced cytotoxicity in both cell lines (Figure 4.3C). To investigate whether MK-1775 could enhance araC-induced apoptosis, flow cytometry analysis of AnnexinV/PI dual-staining was used. MK-1775 (100 nM) was able to greatly enhance the apoptosis induced by araC in both cell lines (Figures 4.3D&E). Treatment of two diagnostic DS AMKL samples with varying doses of araC and MK-1775 yielded synergistic inhibition of viable cells, with CIs ranging from 0.28 to 0.65 (Figure 4.3F), confirming that these drug combinations could ultimately be incorporated into clinical protocols.

4.3.4 MK-1775 Enhances AraC-induced DNA Damage in S-Phase

To begin elucidating the mechanism by which the combination of MK-1775 and araC-induced apoptosis, CMK and CMY cells were treated for 24 hours with combinations of both agents and levels of pCDK1(Y15), PARP cleavage, and γH2AX (a biomarker for DNA double strand breaks) were interrogated using western blots. It is well-known that araC can cause replication stress, which results in DNA damage and cell-cycle arrest through the ATR-CHK1

pathway[177]. Therefore, it was important to determine whether MK-1775 could abrogate the inhibition of CDK1 that results from activation of this checkpoint, preventing cell cycle arrest. We hypothesized that MK-1775 would be able to enhance araC-induced DNA damage as inhibition of wee1 alone can cause replication stress, as well as inhibition of DNA repair. Indeed, MK-1775 was able to abrogate the induction of pCDK1(Y15) and increase γH2AX induction by araC in a dose-dependent fashion that paralleled the induction of apoptosis (measured by PARP cleavage) (Figure 4.4).

There have been several reported mechanisms by which MK-1775 can enhance the cytotoxicity of different chemotherapeutic agents including wee1 inhibition, which prevents activation of the G2/M DNA damage checkpoint, allowing cells to progress aberrantly through the cell cycle and resulting in either apoptosis or mitotic catastrophe. MK-1775 can also induce unscheduled mitosis, which typically results in cell death. To assess for these mechanisms, cells were treated with araC, MK-1775, or the combination for 24 hours and p-H3 (a marker for mitosis) and γH2AX status were interrogated using flow cytometry. AraC alone induced S-phase arrest, while MK-1775 alone had little impact on the cell cycle profiles of CMY and CMK cells (Figures 4.5 and 4.6, respectively). While the combination appeared to result in a slight rightward shift of the early-S peak seen from araC alone, possibly indicating abrogation of an early-S arrest by MK-1775, the most notable change seen in the combination treatment was the significant increase in apoptosis as measured by cells with sub-G1 DNA content. Additionally, it does appear that MK-1775 was able to decrease the amount of

G2/M and S-phase after araC treatment in CMY and CMK, respectively (Figures 4.5-4.8). Though MK-1775 alone may have had a small impact on the number of mitotic cells, (Figures 4.5 and 4.6), it did not appear to have caused the degree of unscheduled mitoses reported previously in other cell types, and the modest nature of these changes is unlikely to account for the amount of cell death seen. Interestingly, MK-1775 was able to enhance araC-induced γH2AX primarily in S-phase in CMY cells (Figure 4.5). In CMK cells, the addition of MK-1775 to even low concentrations of araC was able to primarily target cells in S-phase (Figure 4.6, 4.8), however it is unclear whether the increase in DNA damage shown in Figure 4.4 occurred prior to or after the onset of apoptosis. It was expected that if MK-1775 primarily enhanced araC-induced cell death by abrogation of G2/M checkpoint, there would have been an increase in γH2AX+ cells in the G2/M compartment. The dearth of G2/M cells that stained positively for γH2AX therefore is suggestive that the addition of MK-1775 to araC was able to cause sufficient insult to cells that they simply underwent apoptosis directly from S-phase.

Table 4.1 IC50s for nucleoside analogues and topoisomerase II poisons

	IC50 (nM)		
Drug	CMK	CMY	Fold
Clofarabine	14	350	25.0*
Decitabine	50	1099	21.8*
Daunorubicin	88	209	2.4*
Etoposide	1251	4432	3.5*
Mitoxantrone	12	69	5.8*
* indicates p<0.05			

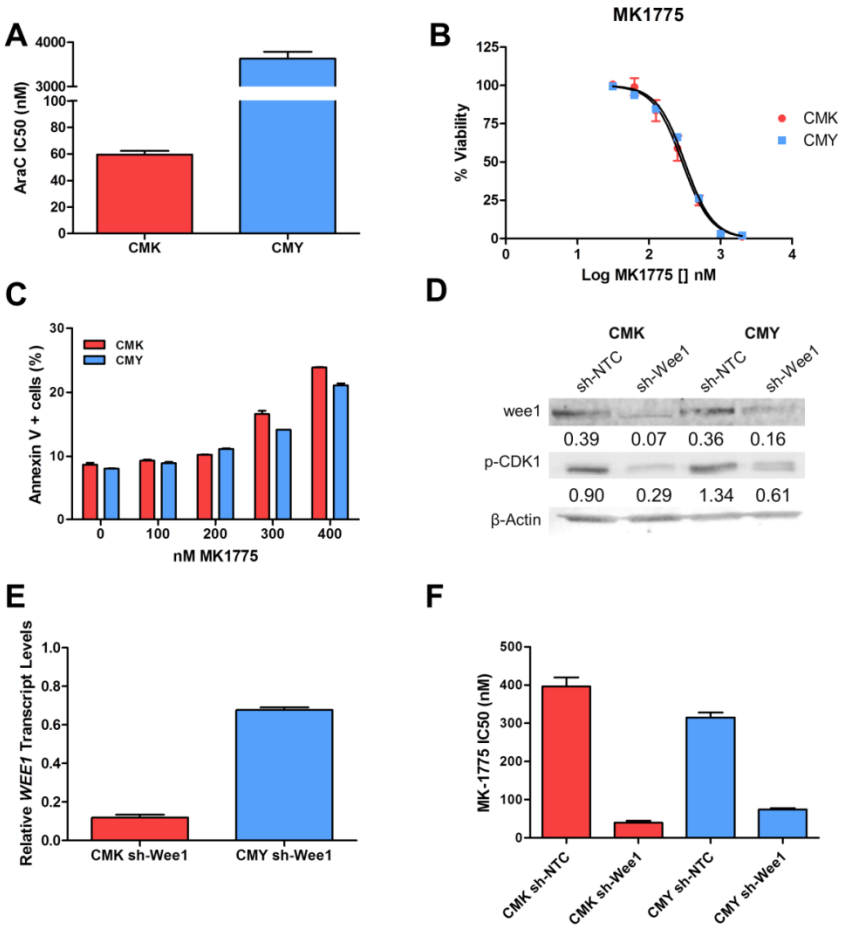


Figure 4.1 MK-1775 has single agent effect against DS-AML. **A:** AraC IC_{50}s for CMK and CMY after 72 hour treatment as determined by MTT. CMY is approximately 60-fold more resistant to araC than CMK. **B:** CMK and CMY cells are comparably sensitive to MK-1775 as determined by a 72-hour MTT assay. **C:** MK-1775 is able to induce comparable, albeit modest dose-dependent apoptosis in both cell lines after 48-hour treatment, as determined by AnnexinV/PI staining. **D-E:** Stable knockdown of wee1 using a lentivirus delivery system and selection by puromycin resulted in reductions in wee1 levels as well as p-CDK(Y15) levels. **F:** Knockdown of wee1 sensitized CMK and CMY cells to MK-1775, as determined by a 72-hour MTT assay.

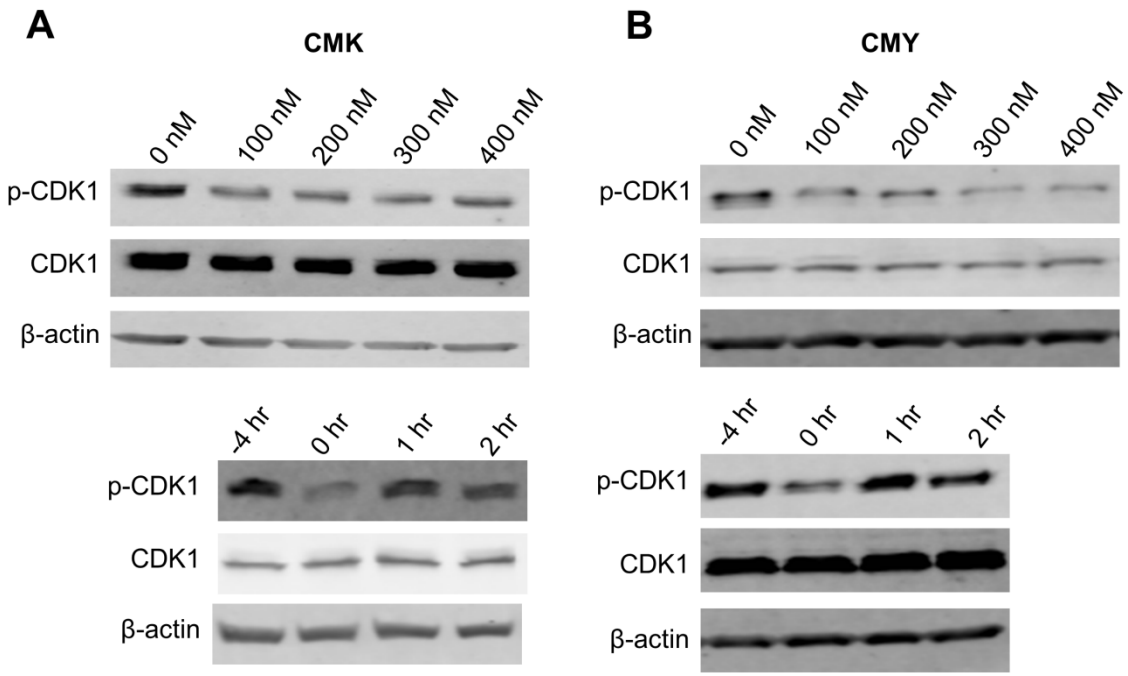


Figure 4.2 Pharmacodynamic changes in p-CDK1(Y15) after MK-1775 treatment. **Top:** CMK and CMY (A&B) cells were treated for 24 hours with increasing concentrations of MK-1775 and probed for CDK1(Y15) phosphorylation by western blot. Treatment for 24-hours with 100 nM MK-1775 was able to cause near-maximal reduction of CDK1(Y15) phosphorylation in both CMK and CMY. **Bottom:** CMK and CMY (A&B) cells were treated with 100 nM MK-1775 for 4 hours followed by drug washout. Treatment with MK-1775 was able to inhibit CDK1 phosphorylation after only four hours, but this phosphorylation returned after only 1 hour.

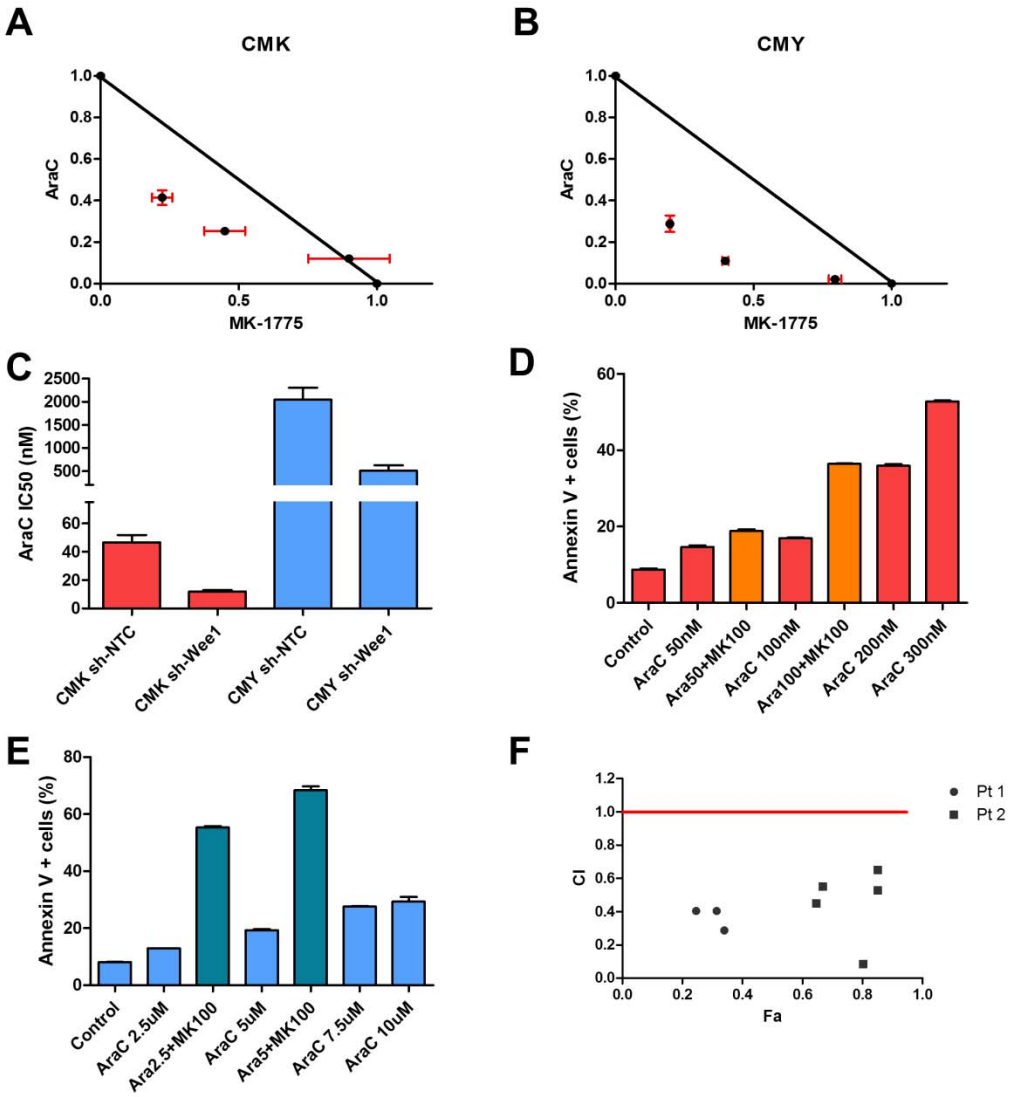


Figure 4.3 MK-1775 synergizes with araC in both cell lines and primary patient samples. **A-B:** CMK and CMY cells were treated with different combinations of araC and MK-1775 for 72-hour and viability was determined by MTT assay. Standard, normalized isobolograms demonstrate the synergistic inhibition of cell viability by the combination of araC and MK-1775. **C:** CMK and CMY –ntc and –shwee1 cells were treated for 72 hours with araC and viability was determined by MTT. Knockdown of wee1 sensitized both cell lines to araC. **D-E:** CMK (D) and CMY (E), were treated with varying doses of araC in the presence or absence of 100 nM MK-1775 for 48 hours and apoptosis was measured using AnnexinV/PI staining. **F:** Various combinations of araC and MK-1775 were tested in 2 primary DS-AML samples *ex vivo* using 48-hour MTT. The CI-Fa plot shows the combination index vs. the fraction affected, with CI < 1 representing synergy.

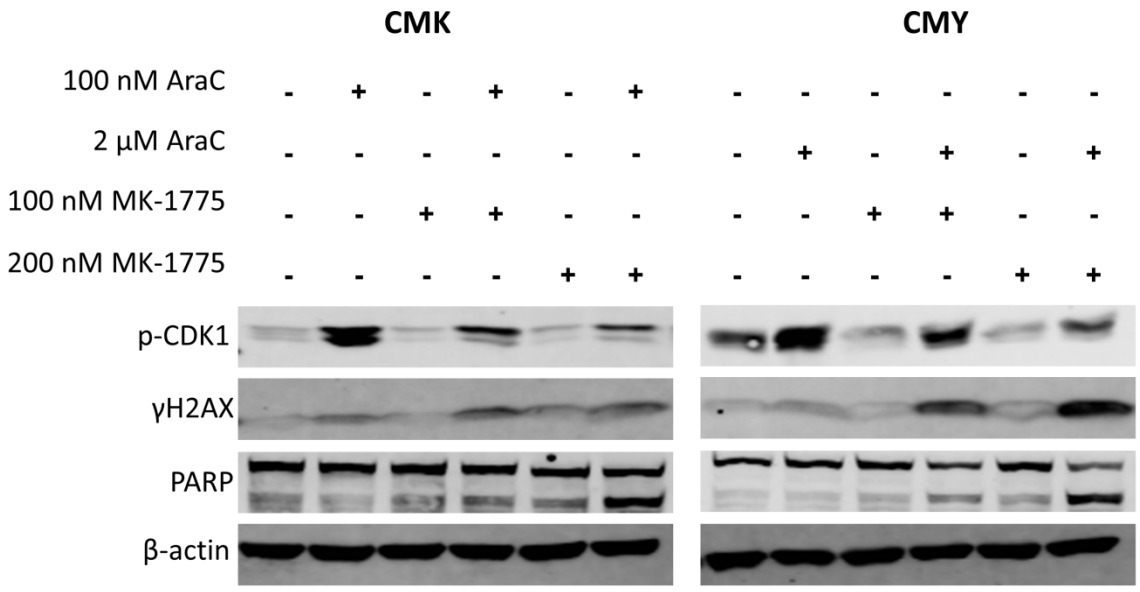


Figure 4.4 MK-1775 can abrogate araC-induced CDK1(Y15) phosphorylation and enhance araC-induced DNA damage. CMK and CMY cells were treated for 24 hours with the indicated drugs. MK-1775 was able to, in a dose dependent fashion, decrease CDK1(Y15) phosphorylation and increase araC-induced DNA damage (γH2AX) and apoptosis (PARP cleavage).

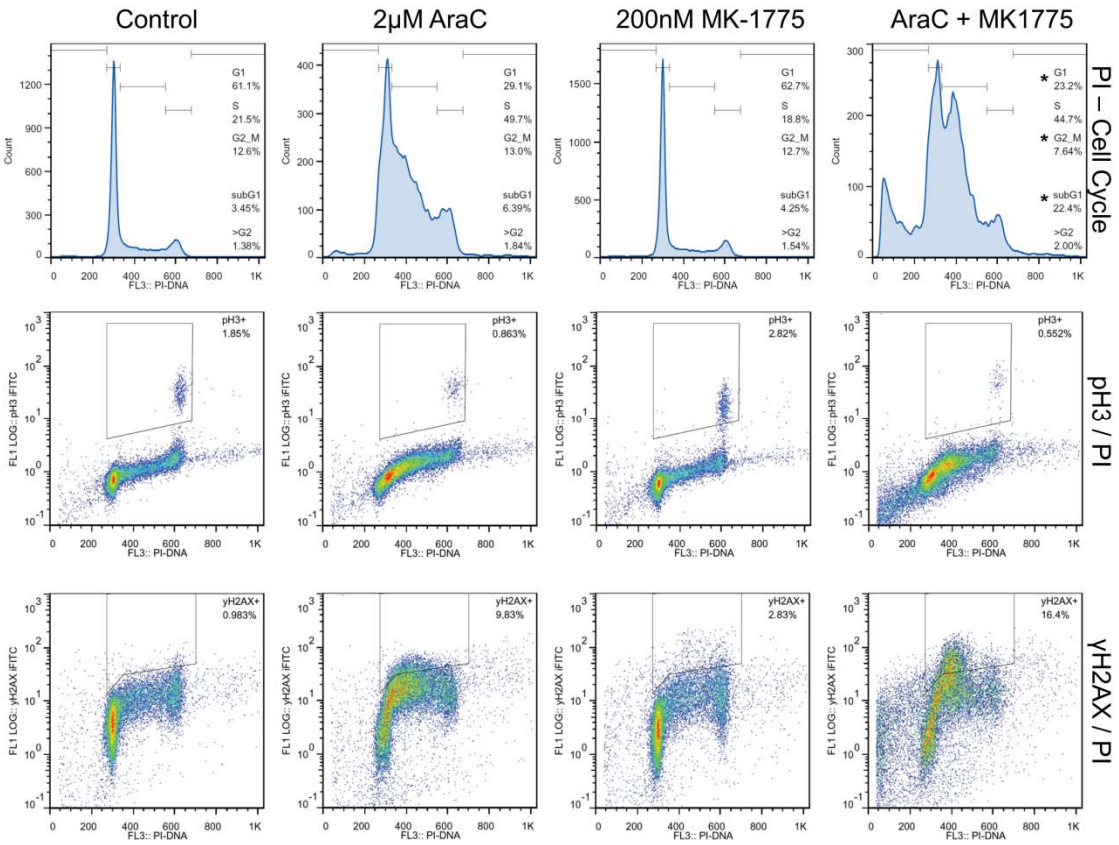


Figure 4.5 MK-1775 effects on cell cycle, mitosis, and DNA damage. CMY cells were treated with the indicated drug for 24 hours and analyzed by flow cytometry. **Top**: The effects of araC and MK-1775 on cell cycle were determined using PI staining. **Middle:** The effects of araC and MK-1775 on mitosis vs. cell cycle were determined using dual pH3/PI staining. **Bottom:** The effects of araC and MK-1775 on DNA damage vs cell cycle were determined using dual γH2AX/PI staining. * indicates $p<0.05$ compared to araC treatment.

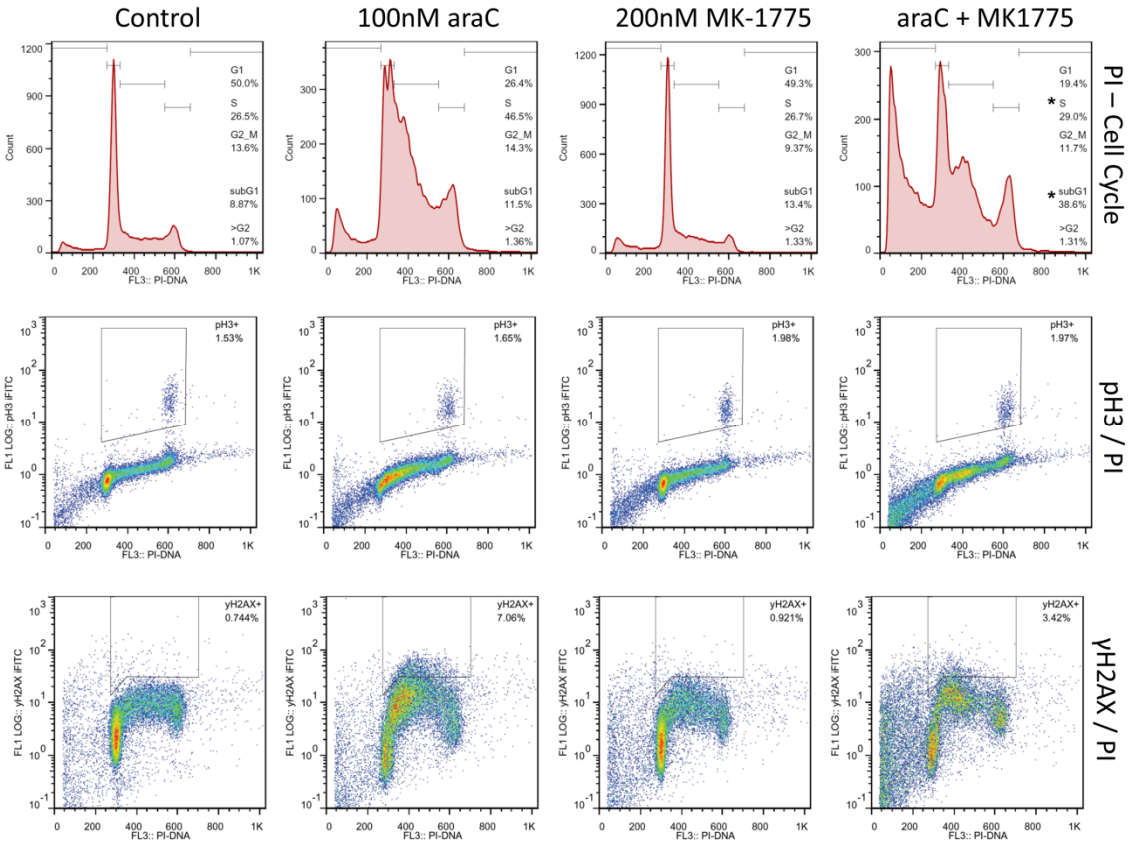


Figure 4.6 MK-1775 effects on cell cycle, mitosis, and DNA damage. CMK cells were treated with the indicated drug for 24 hours and analyzed by flow cytometry. **Top**: The effects of araC and MK-1775 on cell cycle were determined using PI staining. * indicates $p<0.05$ compared to araC treatment. **Middle:** The effects of araC and MK-1775 on mitosis vs. cell cycle were determined using dual pH3/PI staining. **Bottom:** The effects of araC and MK-1775 on DNA damage vs cell cycle were determined using dual γH2AX/PI staining.

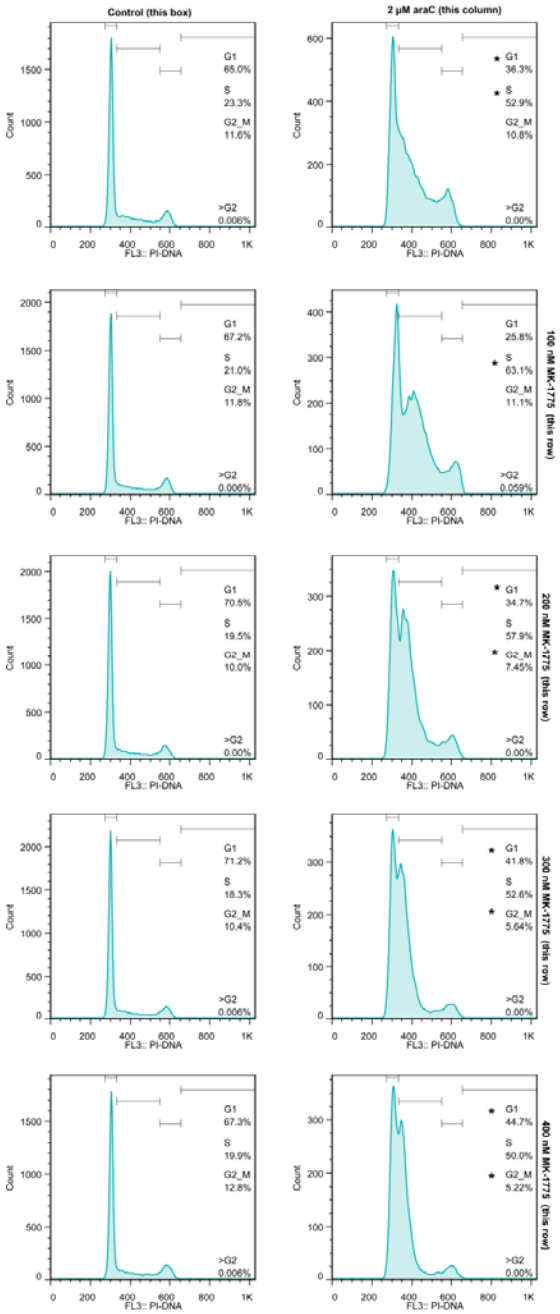


Figure 4.7 MK-1775 can decrease G2/M fraction of viable CMY cells, especially at higher doses. CMY cells were treated with the indicated drug for 24 hours and analyzed by flow cytometry using PI staining. Non-viable (sub-G1) cells were gated out to facilitate analysis of viable cell cycle profile, as higher MK-1775 doses (300 and 400 nM) in the combination treatment resulted sub-G1 populations >50%, obfuscating cell-cycle changes. * indicates $p<0.05$ compared to araC treatment.

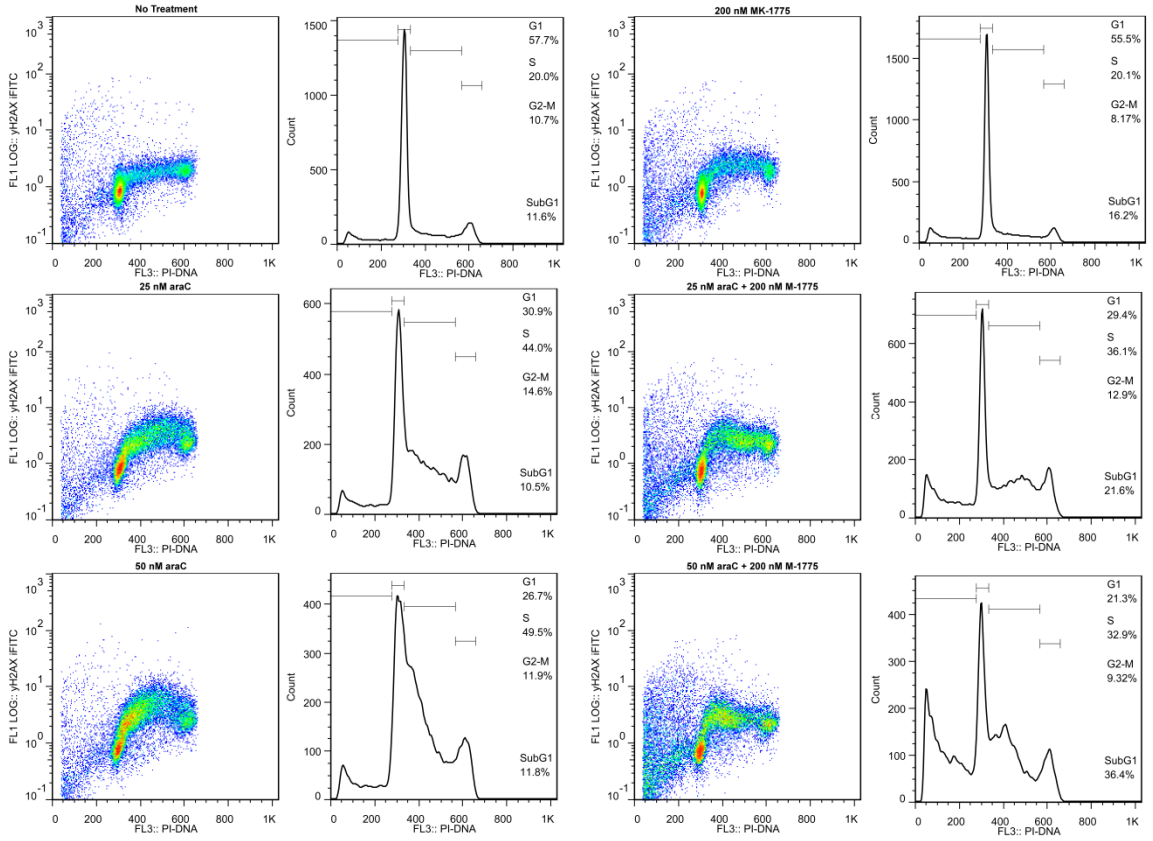


Figure 4.8 CMK cells treated with the combination of araC and lower doses of MK-1775 appear to die out of S-phase. CMK cells were treated with the indicated drug for 24 hours and analyzed by flow cytometry using dual γH2AX/PI staining. The left panel for each treatment shows the two-dimensional histogram while the right panel shows the cell cycle analysis using only PI staining.

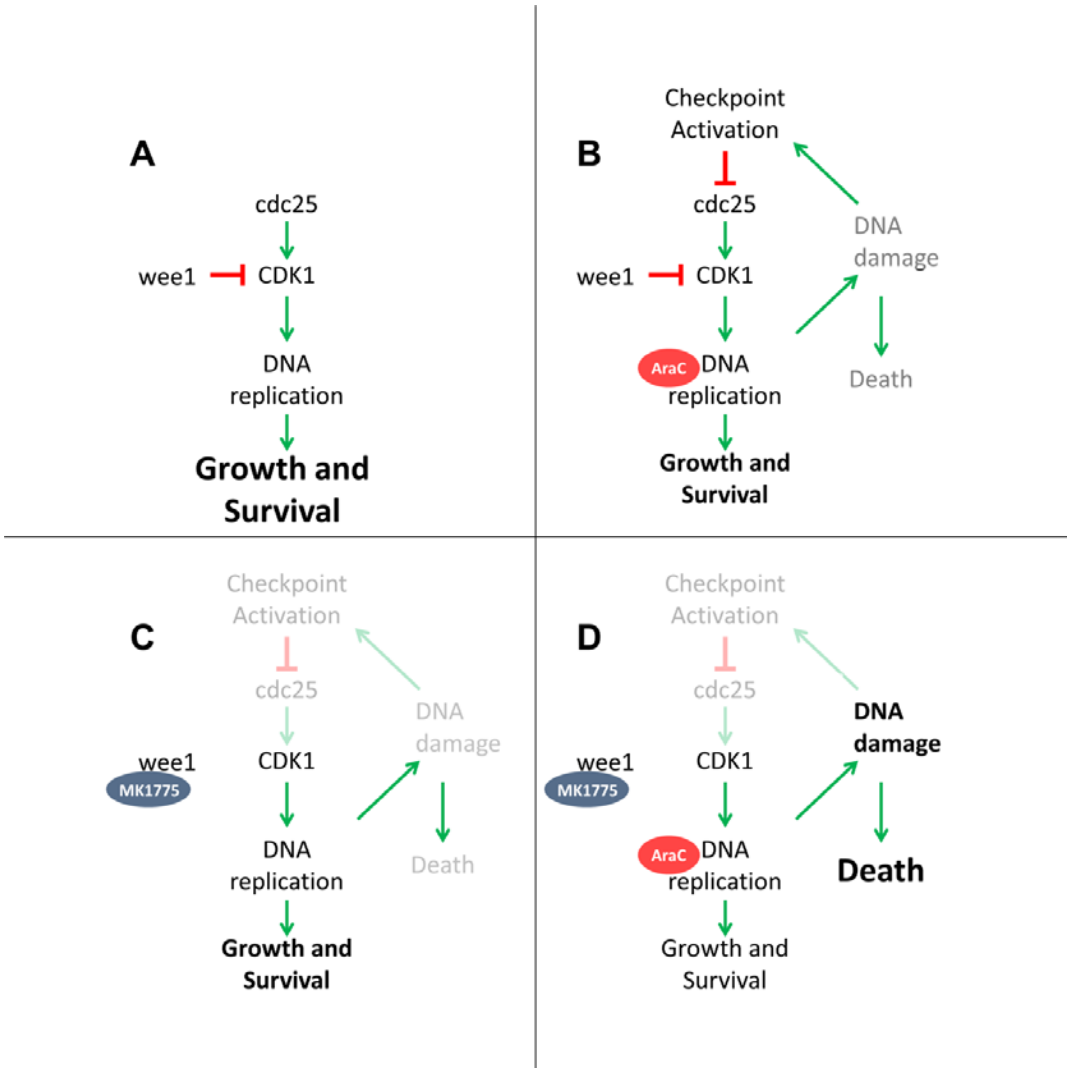


Figure 4.9 Schematic of MK-1775 and araC effects on cell survival. A: In the absence of either drug, wee1 inhibits CDK1 by phosphorylation at Y15. Cdc25 phosphatases constitutively remove this phosphorylation, with the net result being cell growth and survival. **B:** When araC is present, it can incorporate into newly synthesized DNA, causing chain termination and subsequent DNA damage and checkpoint activation. This inactivates cdc25 phosphatases, with the result being cell cycle arrest. In some cells, this leads to cell death, however most cells are able to repair the damage and survive. **C:** In the presence of MK-1775, wee1 cannot inhibit CDK1, so it remains active and DNA replication occurs unimpeded. Unregulated replication results in some DNA damage and cell death due to lack of necessary cofactors and inability to activate cell cycle checkpoints, but the net result is largely cell survival. **D:** In the presence of both drugs, replication continues despite DNA damage induced by araC. The inhibition of wee1 by MK-1775 makes cell cycle checkpoint activation irrelevant. AraC continues to incorporate into newly firing replication forks, resulting in large amounts of DNA damage. The result is then primarily cell death by apoptosis.

4.4 Discussion

DS children with AML have an overall very favorable prognosis with event-free survival rates of ~80%[223]. However, for patients with either refractory or relapsed disease, the prognosis is very poor highlighting the need to develop new effective therapeutic options. Targeting the wee1 kinase for treatment of malignancy is rapidly emerging with several phase 1 or phase 2 clinical trials currently underway (www.clinicaltrials.gov), including one pediatric brain tumor trial. In our study, we provide strong evidence to support the further development of combining the wee1 inhibitor MK-1775 with araC for the treatment of DS-AML.

Early published results from phase 1 trials have indicated that the maximal plasma concentration of MK-1775 is approximately 400-500 nM, with an oral twice daily dosing regimen for 5 doses [108,206]. Though single-agent activity of MK-1775 at those levels appears to be modest, the concentrations used in this study (100-200 nM) for combination studies are well below that threshold. Therefore, even if substantial dose reductions are necessary in this population, our findings are likely to have clinical relevance. Furthermore, based on the results from Figure 4.2 suggesting that araC and MK-1775 should likely be dosed simultaneously, the MK-1775 dosing strategy used previously is likely compatible with the 4-day infusion schedule for araC used in the most recent DS-AML clinical trial, Children's Oncology Group AAML0431. Although this study lacks *in vivo* toxicity and efficacy data, a recently published study using a murine AML model found that the toxicity of the combination was similar to that of araC alone[208].

Most of the prior studies investigating MK-1775 focused on its ability to abrogate a G2/M cell cycle checkpoint to enhance cell death. It was initially reported that this response was only effective in p53-mutant cells, though that notion has been challenged and may represent a cancer- or cell-type-specific phenomenon[1,15,23,60,69,70,99,160,208]. While similar results were expected in our study, it was surprising to see that G2/M checkpoint abrogation did not seem to contribute substantially to the cytotoxic effect of the combination of MK-1775 and araC, and instead cells were likely dying in S-phase (Figures 4.5-4.8). Although it would be ideal if MK-1775 synergizes with anthracycline chemotherapy, the lack of substantial G2/M arrest abrogation, and unfavorable previously published results[208] suggest that this is likely not the case. It is important to note that these findings do not contradict those seen in previous studies (G2/M abrogation, unscheduled mitosis), given that those studies were performed using mostly solid tumor lines. Instead, our results agree with a recently published study that suggests MK-1775 is able to abrogate an intra-S checkpoint[208]. With this in mind, we propose a model (illustrated in Figure 4.9) in which wee1 inhibition by MK-1775 prevents activation of the S-phase checkpoint normally activated by araC. Subsequently, replication forks continue to fire [8,9], allowing for more araC incorporation and subsequent fork collapse. Eventually, a catastrophic level of DNA damage is sustained, and the cell undergoes apoptosis without completing DNA replication. Though further work is necessary to fully confirm this hypothesis, if true, it could have important

implications for the combination of MK-1775 with nucleoside analogues that rely on DNA synthesis for anti-tumor effect.

As a whole, the data presented here support the further development of MK-1775 as a potential adjuvant to araC for the treatment of DS-AML. MK-1775 is able to greatly enhance the anti-leukemic effects of araC, primarily by enhancing araC's DNA damaging effect in S-phase. The anti-leukemic effect was independent of sensitivity to araC alone, and was validated *ex vivo* using primary DS-AML blasts. Based on our results, MK-1775 appears to be an exciting new option for the treatment of relapsed or refractory DS-AML and for further clinical trial development which may also be relevant to non-DS AML patients as well.

CHAPTER 5 – Discussion and Future Directions

Despite great advances in the treatment of AML, it remains deadly for many of the children affected by this disease. Fortunately, by utilizing carefully designed clinical trials and conducting relevant bench research, advances in the treatment of these patients will continue to be made.

Previous studies on the biology of DS-AML blasts found that, at least partially as a result of modulated GATA1 function, these blasts were more sensitive to chemotherapy [51,53,201-203]. The finding that lower GATA1 activity in DS-AML blasts positively impacts outcome for those patients led to the hypothesis that higher GATA1 activity in non-DS AMKL cases may have the opposite effect. Indeed, as the results in Chapter 2 of this work show, this turned out to be the case. GATA1 was overexpressed in AML cases of the AMKL phenotype compared to non-AMKL cases, and knockdown of *GATA1* in a megakaryocytic AML cell line was found to enhance sensitivity to both araC and DNR. One mechanism by which GATA1 conveys chemotherapy resistance is that GATA1 affects the expression of the anti-apoptotic Bcl-2 family protein Bcl-xL. Furthermore, it was shown that treatment with the HDAC inhibitor VPA was able to down-regulate both GATA1 and Bcl-xL sensitizing cells to treatment with araC.

In addition to the additional GATA1 target genes identified for future study, the results in Chapter 2 have potential therapeutic implications. In the non-DS AML population, the AMKL subtype is considered to be high risk with a relatively poor prognosis (EFS < 35%) [5,140,173]; therefore novel therapies that may be

especially useful in this patient group are of interest. Since the completion of the work presented in Chapter 2, novel HDAC inhibitors have been developed clinically, such as panobinostat and vorinostat (reviewed in[134,155]). With new agents, it may be possible to target mechanisms of resistance including overexpression of GATA1, even though such targets had previously been considered “undruggable.” Additionally, the confirmation of Bcl-xL as contributing to the resistance in AMKL offers another potential avenue for therapeutic intervention. There has been great interest in targeting the anti-apoptotic Bcl-2 family proteins in recent years, as inhibition of these proteins has been shown to be effective in a variety of malignancies[33,139]. It does merit note, however, that this excitement is somewhat tempered by the finding that inhibition of Bcl-xL may lead to thrombocytopenia[168,221], highlighting the benefit vs. risk balance which must be considered when developing new cancer therapies.

With regard to DS-AML, the important findings presented in this work include the identification of wee1 inhibition by MK-1775 as an attractive option for further pre-clinical and clinical development. Despite the otherwise promising compounds in Chapter 3 not being as effective as hoped, the results from the MK-1775 are encouraging. As a single agent, MK-1775 demonstrated comparable effects in both the CMK and CMY cell lines, albeit at concentrations toward the high end of what is clinically achievable. However, in combination with araC, MK-1775 was able to enhance the inhibitory effect of araC in a synergistic fashion. Importantly, from the results presented in Chapter 4, it appears that MK-1775 exerts its effects on CMK and CMY cells primarily in S-

phase, a finding that was largely unexpected, as previous studies had largely suggested that inhibiting wee1 targets cells in G2 [1,15,23,60,69,70,99,160,208].

The role of wee1 in human cells is to maintain inhibitory phosphorylation on CDK1 and CDK2 such that CDK activity is controlled appropriately according to the phase of the cell cycle. Though initial studies of MK-1775 were focused on a G2-phase effect, the results presented here, especially in combination with work from other groups, suggest that there is an important role for MK-1775 in the targeting of S-phase cell cycle control [153,208]. Moving forward, it will be important to further clarify the mechanism by which MK-1775 exerts these effects so that its maximal therapeutic potential may be realized.

From previous studies, it is clear that there is a role for CDK1 in the control of S-phase regulation. It has been shown that forced activation of CDK1 by inhibition or knockdown of wee1 causes S-phase DNA damage, as well as increased replication fork firing and decreased fork length[8,9]. Furthermore, it was shown that the shorter fork lengths were thought to be caused by an insufficient supply of necessary cofactors, a phenotype which was partly reversed by adding exogenous nucleosides[8]. This potentially offers mechanistic insight into the recent findings that MK-1775 was able to enhance sensitivity to pemetrexed, an antifolate that reduces available nucleoside pools[208]. If the effect of MK-1775 treatment really is predominantly in the S-phase, it would be expected that it would synergize better with agents that act in S-phase (and activate S-phase DNA damage checkpoints) than agents that act in G2 (activating G2/M DNA damage checkpoints). This is indeed the case, at

least for CMK and CMY cells (Figure 5.1). It was found that for both cell lines, the combination with MK-1775 demonstrated greater synergy with the nucleoside analogues araC, decitabine, and clofarabine, than it did with the Topo2 poisons DNR, VP16, and the mitotic inhibitor vincristine (VCR).

Another question regarding the activity of MK-1775 is whether or not it primarily exerts its effect on CDK1 or CDK2. Traditionally, CDK1 has been considered to be the CDK responsible for driving mitosis, while CDK2 drives DNA replication. This notion was challenged, however, when it was demonstrated that not only can CDK1 perform the roles of CDK2[4], but CDK1 is capable of compensating for CDKs 2,3,4, and 6. In fact, those kinases are all dispensable, but the roles of CDK1 cannot be performed by its family members and CDK1 knockout produces non-viable embryos[180]. Importantly, wee1 is capable of regulating CDK2 as well as CDK1, as the two proteins share homologous N-termini, which include the regulatory tyrosine 15[28,59]. In fact, a constitutively active CDK2 knock-in mutant reproduced many of the phenotypes seen in wee1 depleted cells[85]. Furthermore, pan-CDK inhibition with Roscovitine was able to antagonize the MK-1775 effect more than was specific CDK1 inhibition with RO-3306 in CMK and CMY cells (Figure 5.2). It will be important to use more specific approaches to further assess these differences in the future.

Though these differences in potential mechanisms of action may seem insignificant at first, given the similar phenotypes expected, determining the major mechanisms at play is still important. First, better understanding the

mechanism by which wee1 inhibition enhances araC-induced apoptosis of AML cells allows for better treatment design. It also allows for the potential identification of new drug targets that are in the affected pathway, which might have fewer side effects. Finally, and perhaps most importantly in the short-term, if treatment with MK-1775 predominantly affects S-phase arrest, agents that target S-phase cells should be preferentially combined with MK-1775 in clinical trials.

In conclusion, the work presented in this dissertation describes preclinical efforts at improving outcomes for pediatric AML patients in both the DS and the non-DS populations. The findings presented in Chapter 2 demonstrate a potential for using novel therapies, like HDAC or Bcl-2 family inhibitors to treat AMKL, as well as offering insight into the mechanisms of resistance in a difficult to treat disease. Those findings presented in Chapters 3 and 4 lay the foundation for the use of MK-1775 to enhance the effects of araC in DS-AML. Though there is always more to be elucidated, the studies described herein set the stage for such further work, with the eventual goal of improving outcomes for pediatric AML.

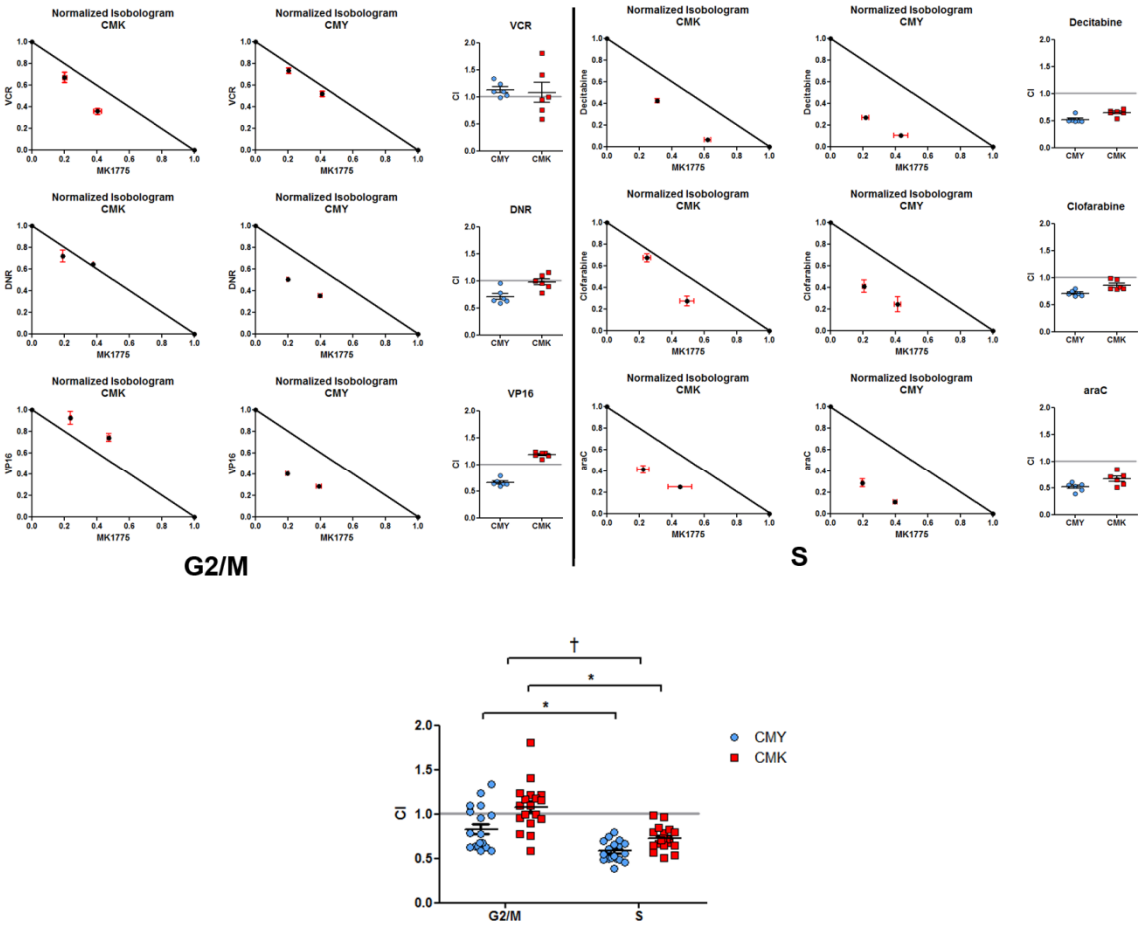


Figure 5.1 Isobolograms and CIs for the combination of MK-1775 with various cell-cycle-specific agents. Construction of isobolograms and CI plots as performed as in Chapter 3. CMK and CMY cells were treated for three days in the presence of increasing concentrations of the indicated drug with or without increasing concentrations of MK-1775 for 72 hours and analyzed using standard MTT assay. **TOP:** MK-1775 was combined with either DNR, VP16, or VCR (left-top) and either decitabine, clofarabine, or araC (right-top). **BOTTOM:** The CIs from the top panel were segregated by cell line and by whether the non-MK-1775 agent targets S- or G2/M-phase cells. Within each cell line, G2/M and S groups were found to be significantly different using the non-parametric Kruskal-Wallis test with Dunn's post-test correction (*). When the cell lines are combined, the G2/M values are significantly different than S values ($p<0.0001$) using the non-parametric Mann-Whitney U test (†).

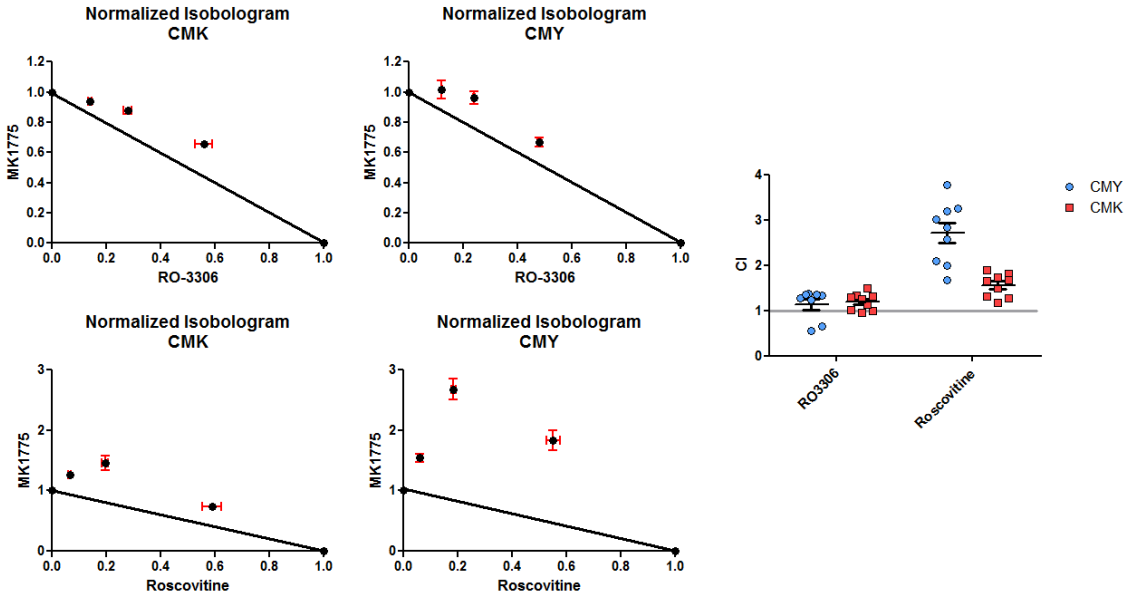


Figure 5.2 Isobolograms and CIs for the combination of MK-1775 and either Roscovitine or RO-3306 in CMK and CMY cells. Construction of isobolograms and CI plots was performed as in Chapter 3. CMK and CMY cells were treated for three days in the presence of increasing concentrations of MK-1775 with or without increasing concentrations of either Roscovitine or RO-3306 and for 72 hours and analyzed using standard MTT assay. Note that the scale for the roscovitine isobolograms and CI plot is much larger than in previous figures.

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ABSTRACT

IDENTIFYING MECHANISMS OF RESISTANCE AND POTENTIAL THERAPEUTIC TARGETS FOR PEDIATRIC ACUTE MYELOID LEUKEMIA

by

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Acute myeloid leukemia (AML) is a potentially devastating disease that can affect people of all ages. While there are certain patient groups that typically have favorable outcomes, in the pediatric population, overall survival (OS) is approximately 70%, leaving much progress to be made. Children with Down syndrome (DS) have a substantially elevated risk for developing AML, especially of the megakaryocytic phenotype (AMKL).

Patients with DS AMKL almost universally harbor a mutation in the gene encoding the GATA1 transcription factor, resulting in the translation of a short-form protein GATA1s, which has been shown to contribute to chemotherapy sensitivity. In contrast, in non-DS patients, overexpression of GATA1 has been correlated with poorer outcomes. In the first half of this work, we investigated the role that elevated GATA1 expression plays on the sensitivity of AMKL cells to chemotherapy. To begin, we confirmed that amongst non-DS patients, GATA1 transcripts were significantly higher in AMKL blasts compared to blasts from other AML subgroups. Further, GATA1 transcript levels significantly correlated

with transcript levels for the anti-apoptotic protein Bcl-xL in our patient cohort. We demonstrated that Bcl-xL is a GATA1 target, and knockdown of GATA1 with shRNA sensitizes cells to chemotherapy treatment and decreases Bcl-xL expression. Treatment of Meg-01 cells with the histone deacetylase inhibitor valproic acid resulted in down-regulation of both GATA1 and Bcl-xL and significantly enhanced ara-C sensitivity. Furthermore, additional GATA1 target genes were identified by oligonucleotide microarray and ChIP-on-Chip analyses.

While OS for DS AML is generally very favorable (approximately 90%), DS patients with refractory or relapsed disease have dismal prognoses, with OS in that group only approximately 25%. Therefore, there is a need for the development of new therapeutic approaches for these patients. In the second half of this work, we investigated the response of 2 DS AML cell lines, CMK and CMY, to the combination of araC and one of either the aurora A kinase inhibitor MLN8237, the aurora B kinase inhibitor AZD1152-HQPA, the Plk1 inhibitor BI6237, or the wee1 inhibitor MK-1775. It was found that MK-1775, in contrast to the other three agents, synergized with araC in antiproliferative MTT assays in both cell lines as well as in ex vivo DS-AML primary patient samples. MK-1775 was able to decrease inhibitory CDK1(Y15) phosphorylation after only 4 hours, and was able to enhance araC-induced DNA damage in S-phase and partially abrogate araC-induced cell cycle arrest.

The work presented in this dissertation describes preclinical efforts at improving outcomes for pediatric AML patients in both the DS and the non-DS populations. The findings presented demonstrate a potential for using novel

therapies, including HDAC or Bcl-2 family inhibitors to treat AMKL, as well as offering insight into the mechanisms of resistance in a difficult to treat disease. Furthermore, these findings lay the foundation for the use of MK-1775 to enhance the effects of araC in DS-AML. Though there is always more to be elucidated, the studies described herein set the stage for such further work, with the eventual goal of improving outcomes for pediatric AML.

AUTOBIOGRAPHICAL STATEMENT

Education

Wayne State University Detroit, MI
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Michigan State University Lansing, MI
2009 *Bachelor of Science*, Biochemistry and Molecular Biology, with Honors
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Publications

1. **Caldwell JT**, Edwards H, Buck SA, Ge Y, Taub JW. (in press) Targeting the wee1 Kinase for Treatment of Pediatric Down Syndrome Acute Myeloid Leukemia. Pediatric Blood & Cancer

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Abstracts

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