EPIGENETIC DRUGS IN OVERCOMING THE EFFECTS OF BONE MARROW MICROENVIRONMENT-INDUCED CHEMOPROTECTION IN PEDIATRIC AML

by

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ABSTRACT

Pediatric acute myeloid leukemia (AML) is a type of blood cancer, accounting for 18% of childhood leukemia, in which mutated immature myeloid cells are present in the bone marrow. The most common treatment option is induction chemotherapy, however this treatment is associated with high relapse rates contributed by chemotherapeutic resistance provided by the bone marrow microenvironment. New treatment options are needed for pediatric AML in order to improve survival and remission rates. AML is characterized by aberrant epigenetic landscape. Epigenetic changes such as DNA methylation and histone methylation and acetylation can promote leukemia development by altering gene expression at the level of transcription. Azacitidine, a DNA methyltransferase inhibitor, is currently used as treatment for relapsed and refractory AML in adults. Panobinostat, a histone deacetylase inhibitor, is in phase I clinical trials for AML in adults. Our lab previously demonstrated that azacitidine and panobinostat can induce complete remission in a mouse model of KMT2A (MLL) rearranged AML. However, assimilation of these drugs in the chemotherapy regimen is not well described. This research will focus on the use of epigenetic drugs used in combination with chemotherapy to treat pediatric AML. I hypothesize that the epigenetic drug combination will overcome chemoprotection mediated through cell-to-cell contact between the cellular adhesion molecules on AML cells and bone marrow cells. HS5 cells, human bone marrow stromal cells, provided maximum chemoprotection to AML cells in co-culture. This chemoprotection was not evident when AML cells were treated with HS5 cell conditioned media or separated from HS5 cells by a transwell filter. Azacitidine-panobinostat treatment significantly reduced adhesion of AML cells to HS5 cells.
Taken together, these data support the hypothesis that cell-to-cell contact is needed for chemoprotection. I found that the epigenetic drugs, azacitidine and panobinostat, used in combination sensitized pediatric AML cell line and patient samples, to chemotherapeutic drugs (cytarabine or daunorubicin), overcoming the chemoprotection provided by HS5 cells. We also found that using azacitidine-panobinostat treatment in disseminated xenograft models of AML mobilized leukemic cells to the peripheral blood. This treatment in combination with chemotherapeutics is an effective treatment in increasing the overall survival in mice with AML. The data shown here supports my hypothesis that epigenetic drugs can overcome the bone marrow micro environmental chemo protection and that this protection is due to cell interactions within this environment.
1.1 Acute Myeloid Leukemia

Leukemia accounts for 30% of all childhood cancers. Pediatric acute myeloid leukemia (AML) is the second most common pediatric leukemia following behind acute lymphoblastic leukemia (ALL). In the United States, about 730 pediatric AML cases are diagnosed each year with an incidence rate of 7.7 cases per 1 million children [1]. AML accounts for 18% of total pediatric leukemia cases [2]. AML is a very rare disease, however, it surpasses the most common childhood leukemia, ALL, in childhood leukemia mortality in the more recent years [3]. More children with AML are not experiencing good outcomes compared to patients with ALL. Pediatric AML is a disease impacting mostly newborns, infants, and adolescents.

AML arises from mutations in myeloid stem cells, which leads to uncontrollable proliferation and arrested differentiation of these stem cells. These immature cells accumulate in the bone marrow and thereby affect normal hematopoiesis. Thus, new healthy blood cells are not produced causing many other comorbidities, such as anemia, infections and bleeding disorders. Symptoms associated with AML are mostly attributed to the leukemic burden of the disease. These symptoms include fever, infection, anemia, hepatosplenomegaly, thrombocytopenia, bleeding, bone pain, nausea, and lethargy [4]. Anemia is a general symptom caused by lack of healthy red blood cells, leading to lethargy and bleeding [4, 5]. The crowding of unhealthy leukemic cells also limits platelet production which leads to the bleeding
and bruising. The low platelet production is referred to as thrombocytopenia [5]. Hepatosplenomegaly refers to the exceedingly painful and uncomfortable disorders of the spleen and liver. This uncomfortable enlargement of the spleen and liver is due to the increase amount of leukemic cells homing to these areas [5]. Children with AML will experience several bacterial and viral infections, due to their lack of healthy immune cells causing fevers, coughing, and runny nose [5]. The increasing amount of immature myeloblasts give the long bones, in children, the feeling of the bones being full, accompanied by bone pain [5]. Children diagnosed with AML are in constant pain and discomfort. They are also persistently at risk for many different kinds of infection.

This disease is diagnosed through many assessments of the bone marrow and blood stream to determine the mutations that lead to disease development [6]. The blood and bone marrow, of patients, is subjected to flow cytometry to determine different cell surface or cytoplasmic markers characteristic of stages in hematopoietic differentiation. Conventional cytogenetic testing by florescence in situ hybridization and karyotyping is used to identify genetic abnormalities which are characteristic of AML [7]. In pediatric cases, it is also important that all AML patients receive a cerebrospinal diagnostic tap because they have a high risk of cancer cells spreading to the central nervous system even though they are not showing any symptoms [6, 8]. These assessment practices allow the World Health Organization (WHO) to categorize patients into different subtypes. Such classification guides therapy course and predicts prognosis. The major categories are AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy related AML and AML not otherwise
specified [6]. Each of these categories has several more subtypes. This disease is highly heterogeneous and therefore very difficult to treat.

The current standard of care for pediatric AML includes chemotherapy, and bone marrow transplant. Chemotherapy is the initial treatment given as induction therapy. Induction therapy is high doses of a combination of chemotherapy that is given over four or five cycles in order to induce an initial remission. Bone Marrow transplant is usually done after induction therapy to ensure there is no residual disease remaining within the bone marrow. However, this treatment can lead to infection or graft versus host disease which just puts the child at a greater risk. Unfortunately, children diagnosed with AML relapse shortly after initial induction of chemotherapy. Only approximately 60% of children diagnosed with AML achieve long term survival [3]. Currently, there are few targeted therapies used for treatment of pediatric AML [9]. A FMS-like tyrosine kinase 3 inhibitor (FLT3) is the only targeted therapy being used in a clinical setting. It is administered orally and is highly successful for a short period of time. The development of this treatment option provided a better outcome for patients with FLT3 mutations until these cells developed resistance [9]. There are other targeted therapies being studied but these are not being used in clinical trials, and once resistance develops, these targeted therapies are no longer an effective treatment option.

1.2 Difference between Adult and Pediatric AML

The prevalence and poor prognoses associated with AML increases as the age at diagnosis increases [10]. Currently, there are many treatment options available for adult AML, however, adult and pediatric AML differ in the mutations that occur during myeloblast development. For example, patients who develop AML from
myelodysplasia, one subtype of AML, is more common in patients at an older age as opposed to the rare instances of this occurring in children or young adults [10]. This is just one example of the many differences in disease progression between children and adults. Mutations that are commonly present in the instances of pediatric AML are rarely seen in cases of adult AML, on the contrary, some adult AML mutations are not even present in any pediatric case of AML [3]. For example, NRAS is mutated about 30% of the time in pediatric cases, but in adults it is only mutated in about 8% of cases. A mutation in DMNT3A is present in about 30% of adult cases, but there are no documented mutations of this gene in pediatric cases of AML [11]. These differences cause physicians to be skeptical about treating both children and adults in the same manner. However, a treatment regimen effective in adults needs to be preclinically validated in a pediatric model before being used in children. Due to the differing development and classification of AML between pediatric patients and adult patients, treatments used for adults are not always effective in children [3]. Phenomena like this are not uncommon and cause general treatment options to be ineffectual on all patients across the broad spectrum of AML. Subgroup-directed treatment regimens that are designed to treat the specificity of each of the different categories, including age related categories can differ due to the differing mutations associated with different age groups [8, 10].

An additional difference between pediatric and adult cases of AML is the prognosis associated within the different groups of cytogenetic aberrations. The prognosis declines with age in some groups. The c-Kit mutation when found in pediatric cases is associated with good prognosis, however, when diagnosed in adults, this good prognosis associated with pediatric cases is no longer present and the adult
patients are not as fortunate. This age effect is common and independent among all the different groups [10]. The mechanism behind the effect of age on prognosis is still a mystery even though the phenomenon is seen commonly throughout all cases of AML. Using epigenetic drugs in treatment regimens for pediatric cases could be a viable option because there are currently epigenetic treatments being used for adults in trials in order to combat these poor prognoses.

1.3 KMT2a Gene Rearrangement

The high incidence of cytogenetic abnormalities in pediatric AML result in the formation of a fusion protein, which drives uncontrollable proliferation of myeloblasts. 20%-40% of patients with AML have a KMT2a (MLL) gene rearrangement [12]. This gene rearrangement is the most common among pediatric AML and it is the most difficult to treat with an overall 5 year survival rate of 30-60% [13]. In AML, the MLL gene rearrangement has been shown to have over 50 different gene fusion partners [13]. Each of these are associated with a different prognoses and can be considered different subtypes. As mentioned in the last section, the different subtypes would then all need to be treated by a different therapy which would be costly to establish and time consuming.

MLL is an epigenetic modifier that functions as a methyl transferase [14]. Development of AML is associated with hyper methylation and hypoacetylation. Being a very difficult rearrangement to treat, epigenetic drugs could be a viable and logical solution for MLL rearranged AML. Compensation for the lack of acetylation and the excessive methylation that has been reported in the leukemogenisis of AML could be overcome with epigenetic treatments, leading to better prognostic outcomes.
This compensation could occur with the inclusion of epigenetic treatment options in patient treatment regimens.

1.4 Epigenetic Treatment Options

Epigenetics refers to DNA modifications in the form of methylation, histone methylation, and histone acetylation altering gene expression at the level of transcription by upregulating, downregulating, or silencing genes. Dysregulated epigenetic modifications have recently come into the light as important mechanisms in pathogenesis of AML, and specifically MLL rearranged AML. The epigenetic abnormalities are reversible which leads research into the direction of therapies that can more specifically target epigenetic changes [15]. Using specific inhibitors of epigenetic modifiers can offer potential alternative treatment options as opposed to high dose chemotherapy.

DNA methylation is the process by which a methyl group is added to a carbon of cytosines in CpG dinucleotides. From these studies, researchers found that hypomethylation was just as important as hypermethylation [15]. The different methylation patterns found within patients diagnosed with AML are associated with the different subtypes of AML. The unique DNA methylation patterns are also associated with mutations in genes encoded for transcription factors which leads to the association between abnormal transcriptional regulators and changes in epigenetic modifications [15].

The other epigenetic modification that is associated with AML is histone acetylation and methylation. Histone acetylation refers to the transfer of an acetyl group to a lysine residue in histone proteins. The acetylation changes made to the histone alters the ability for transcription factors to bind to the chromatin [15]. Histone
methylation of lysine residues can cause mono, di, or tri methylation with each of these different states resulting in differing functional consequences on the same lysine residue. The two consequences that these alterations are either activation or repression depending on the degree of methylation and the different lysine residues (K). Activation is associated with methylation of histone 3 lysine 4, histone 3 lysine 36, and histone 3 lysine 79. Repression is associated with histone 3 lysine 9, histone 3 lysine 27, and histone 4 lysine 20 [15].

Currently, there are two epigenetic drugs associated with DNA methylation that are being used in treating myeloid malignancies in adults. Azacitidine and decitabine are DNA methyltransferase inhibitors thought to reverse DNA hypermethylation thus restoring the expression of important genes. They have shown success in treatments on elderly patients with AML who cannot withstand the more harsh chemotherapy treatment regimens [15]. This success in elderly patients alone could support success in children if used in combination with other therapies.

Histone deacetylase inhibitors (HDAC) were a part of the beginning research into epigenetic drug development. The focus of these drugs is to be used in combination with other therapies. It was shown that HDAC inhibitor vorinostat in combination with idarubicin and cytarabine improved response rates in patients that were newly diagnosed [16]. Recently, researchers have been exploring the combination of HDAC inhibitors and DNA methyltransferase inhibitors. These combinations, in adults, have been shown to have antagonistic effects and did not improve outcomes in AML patients [16, 17]. Although some of these combinational treatments did not work effectively for adults, the impact on pediatric cases is important to further investigate.
The mechanism by which most of these epigenetic drugs are effective is not well known. Researchers are aware of the alterations of methylation or acetylation at the histone level, but the overall expression changes that happen to make these drugs effective or ineffective is still a mystery. This research shows the expression changes decrease adhesion and allow AML cells to become mobilized and enter the bloodstream to be more susceptible to chemotherapy drugs.

1.5 Cell Adhesion Molecules as Possible Targets

The major families of Cell adhesion molecules (CAM) have been commonly used as targets for different available therapies. This method of treatment have proven to be ineffective on a more general scale. The reason target therapies for CAMs are not as efficient in treatment regimens is because the expression of these molecules differs from patient to patient and cell to cell. The heterogeneous expression makes it difficult to be used as a wide spread treatment option. However, the expression of the CAMs can be altered with the use of epigenetic drugs. The four major families of CAMs are selectins, cadherins, Ig superfamily, and integrins.

Selectins are cell adhesion molecules that bind sugar polymers, so they are considered to be a kind a lectin. There are many different selectins, but one that is most commonly discussed is E-selectin which is expressed by both cancer and hematopoietic stem cells (HSC). E-selectin activates pro survival pathways, such as NFkB, causing it to be a possible part of the chemotherapy resistance that occurs sue to adhesion within the bone marrow microenvironment. One study found that using an inhibitor of E-Selectin, GM-1271, reduced the leukemic burden in mice 2 weeks post treatment [18]. The findings in this study indicate that the alteration in expression of this selectin could lead to increase efficacy of chemotherapy treatments and decrease
the adhesion of these molecules to the microenvironment. Losing the adhesion to the microenvironment would decrease the pro survival signals form E-selectin within the leukemic cells.

Another family of CAMs that could be possible treatment targets are cadherins which are calcium dependent adherins. Many of these molecules have been in the spotlight as targets of different treatment options. N-Cadherin is a CAM that stabilizes cell to cell adhesion by mediating linkages of the actin cytoskeleton via interaction with beta catenin [19]. This interaction has been studied using chronic myeloid leukemia (CML) as the model system. It was found that the interaction between N-cadherin and beta catenin is an important aspect of protection from tyrosine kinase inhibitors in the bone marrow microenvironment for patients with CML [19]. N-cadherin’s role is to stabilize the adhesion between two cells and increase further adhesion. The adhesion mechanisms could increase pro survival signals causing even more protection for leukemic cells within the bone marrow indicating that decreasing the expression of these molecules would have a great impact on furthering treatment options.

The largest, most complex, family of CAMs is the Ig superfamily (IgSF). These molecules are characterized by having one or more Ig-like domains. Members of this family are key factors in cell proliferation, migration and differentiation of all cells. These processes are essential for cell health and survival [20]. IgSF is very instrumental in metastasis and invasion into healthy tissue. These could be potential targets due to the ability of these molecules to activate the NFkB pro survival pathway and evade apoptosis [20]. The expression and implications of this family has not been well studied in AML, or other non-metastatic cancers.
Another important CAM family in the integrin family. They are noncovalently bound heterodimers consisting of an alpha and beta subunit that bind to each other and then either another cell or the extracellular matrix. The intracellular domain interacts with the actin cytoskeleton to propagate signals into the cell [21]. Beta 3 integrin has showed promising evidence that it plays a role in leukemogenesis and adhesion mediated chemoresistance. Integrins have the special ability to be able to bind to many different ligands all leading to different cell signal outcomes. Beta 3 integrin is a part of a subset of integrins that recognize and bind to RGD tripeptide sequences. Beta 3 shows downstream interaction with other integrins and intracellular signaling. The intracellular signaling associated with beta 3 integrin includes activation of NFkB and PI3K-Akt pathways [21]. Galectin 1 induces expression of avB3 and activation of PI3k-Akt signaling which leads to sorafenib resistance [21]. These integrins and their downstream interactions lead to differing levels of resistance, however, beta 3 integrin could prove to be a CAM whose expression should be examined among AML cells after treatment with epigenetic drugs.

This could be an advantage of using epigenetic drugs in combination with chemotherapeutics because the decrease in adhesion between the leukemic cells and cells in the bone marrow microenvironment will allow the cells to be more available to the circulating chemotherapy drugs. Adhesive ability of AML to the bone marrow is what leads to its resistance to current treatment and increased occurrence of relapse. This research will show that the use of epigenetic drugs will overcome this resistance due to decreasing adhesion between the leukemic cells and cells present within the bone marrow microenvironment.
Chapter 2
MATERIALS & METHODS

2.1 Cell Culture Maintenance

Iscove’s Modified Dulbecco’s Media (IMDM), Dulbecco’s Modified Eagle Media (DMEM), Dulbecco’s Modified Eagle Media nutrient mixture F-12 (DMEM F-12), MV4;11 cells, Saos-2 cells, HS5 cells and HS27 cells were obtained from American Type Culture Collection (ATCC), Manassas, VA. Leukemic cell line, MV4;11, were cultured in IMDM culture medium supplemented with 10% fetal bovine serum (FBS), 2 mM/L l-glutamine, 25 U/mL penicillin, and 25 μg/mL streptomycin. HS5 and HS27 cells were cultured in DMEM with the proper supplements described above. Saos-2 cells were cultured in DMEM F-12 supplemented as described above.

Primary AML cells isolated from bone marrow aspirates or peripheral blood of patients from Nemours/Alfred I. DuPont Hospital for Children are stored within the Nemours BioBank. Samples were collected under a Nemours Delaware Institutional Review Board (IRB) protocol approved by the Nemours Office of Human Subjects Protection. Patient samples were passaged in mice following the guidelines of the Nemours Institutional Animal Care and Use Committee (IACUC).

Azacitidine (S1782), panobinostat (S1030), cytarabine (S1648), and daunorubicin (S3035) were obtained from Selleckchem. Powder was dissolved in DMSO to stock concentrations and then serial dilutions were performed to provide more appropriate concentrations for the treatments.
2.2 Determination of IC50 concentrations

Leukemic cells (150,000 per cm²) were plated in a 96-well plate and treated with varying concentrations of cytarabine, azacitidine and panobinostat. Drugs were diluted in IMDM media to the highest concentration, then serial dilutions were performed to reach the lowest concentration, the treatments were then added to the assigned wells which contained the MV4;11 cells. Viability was assessed using the NovoCyte flow cytometer (ACEA Biosciences, Inc.) via a forward scatter by side scatter plot. Live and dead populations were defined using propidium iodide (PI) staining. Many different concentrations of each of the drugs were used in experiments in order to determine the optimal concentration to be used (data not shown).

2.3 Determination of Chemoprotection

HS5, HS27, and Saos-2 cells (25,000 cells per cm²) were plated in a 24-well plate and left to adhere overnight. These cells were chosen because they are present within the bone marrow. Saos-2 cells are an osteosarcoma cell line that have shown chemoprotective effects in vitro with acute lymphoblastic leukemia cell lines [22]. Saos-2 cells have also been used previously to mimic osteoblast effects and are referred to as osteoblast like cells [23, 24]. HS5 and HS27 cells are human stromal cells differing in their origin. HS27 cells originate from the foreskin while HS5 cells originate from the bone marrow. These cell lines are a representative of cells within the bone marrow that could be interacting with leukemic cells and involved in chemoprotection. Media was removed and MV4;11 cells (150,000 cells per cm²) were plated in IMDM and 1.5 µm of cytarabine (determined by IC50 curve and testing multiple concentrations) was added at two times the concentration to the appropriate wells for 48 hours in order to avoid drug dilution. Wells left untreated were used for
control comparison and given only media in place of the drug so all wells contained the same volume. Cell viability was determined via NovoCyte flow cytometer, with analysis performed as described above.

2.4 Sensitivity Assay

MV4;11 cells (150,000 per cm$^2$) were plated in a 96-well plate and were either pre-treated with azacitidine-panobinostat (AP) or left untreated for 48 hours. Cells were then transferred into corresponding wells containing HS5 cells (25,000 per cm$^2$), for co-culture, that were fully confluent. Cells were treated with cytarabine or left untreated for additional 48 hours. Cell viability percentage was determined via flow cytometry, live and dead gates determined as described above.

2.5 Determining the Effects of Soluble Factors on Chemoprotection Using Conditioned Media

HS5 cells (25,000 per cm$^2$) were plated and maintained in a 10 cm dish in IMDM media. After 48 hours, media was collected from the dish. This media was then filtered through a 0.2 µm filtered syringe. The collected media was conditioned media (CM) and diluted to 20% and 40% CM using complete IMDM media. It must be diluted by complete media in order to maintain necessary nutrients present in complete media. MV4;11 (150,000 cells per cm$^2$) were plated in the 20% and 40% conditioned media and treated with cytarabine for 48 hours. Cell viability was determined using flow cytometry.
2.6 Measuring the Impact of Soluble Factors on Chemoprotection Using Transwell Inserts

To determine the role of soluble factors in HS5-mediated chemoprotection, HS5 (25,000 cells per cm²) were plated on a 12-well plate and left to adhere overnight. MV4;11 (150,000 cells per cm²) were placed in a 0.2 μm Transwell filter insert (Corning Inc. 3460) suspended over HS5 cells with or without cytarabine treatment for 48 hours. At the end of incubation, leukemic cells were collected from transwell inserts and cell viability was determined using flow cytometry, live and dead populations gate independently and have been previously confirmed using PI staining.

2.7 Determination of Epigenetic Drugs impact on Adhesion

To determine the effect of AP treatment on adhesion HS5 cells (25,000 cells per cm²) were plated on a 24-well plate and left to adhere overnight. MV4;11 (150,000 cells per cm²) were stained with violet proliferation dye (VPD) and plated on the HS5 cells, then treated with AP or left untreated for 48 hours. Unbound cells were removed from the wells via aspiration. Trypsin-EDTA was used to lift all the remaining cells into suspension. Total cell count in the Pacific blue channel of the NovoCyte Flow cytometer was used to determine percent of Leukemic cells bound to HS5 cells after treatment. Percent bound was determined by the amount of VPD stained cells that remained in the AP treated condition compared to the control condition. VPD enters the cells and is evenly distributed into all of the daughter cells. As the cells proliferate, the dye becomes more dilute, but is still present.
2.8 Identifying Changes in Expression of Surface Molecules

MV4;11 cells were treated with AP pretreatment, or left untreated, were plated. Cells were then washed with 1 mL of 1x PBS and resuspended in FACS buffer. 2 μL of antibody for each corresponding treatment was used and incubated at room temperature in the dark for 15 minutes. Cells were then washed again with FACS buffer. Fluorescence was analyzed using NovoCyte flow cytometer. Determine MFI modulation by: (MFI of molecule Treated - MFI of isotype Treated) / (MFI of molecule Control - MFI of isotype Control). These data are not shown.

2.9 Determining the Effect of Treatment on Patient Derived Xenograft Model

Mice were transplanted with MV4;11 leukemia cells (3.5 × 10^6) via tail-vein injections. Mice were maintained in the Nemours Life Science Center following the guidelines established by the Nemours Institutional Animal Care and Use Committee (IACUC). Disease progression was monitored by flow cytometry of mouse peripheral blood drawn periodically by submandibular bleeds to determine the percent of human cells versus mouse cells to ensure engraftment [25]. When the average percentage of engraftment was 0.4%, the mice were randomly assigned to four treatment groups - vehicle (5% dextrose), APCD (azacitidine-panobinostat pretreatment, followed by cytarabine-daunorubicin treatment), and CD (cytarabine-daunorubicin treatment). The mice were dosed for five days a week with two days of rest. Mice were euthanized with method consistent with the euthanasia guidelines of the American Veterinary Medical Association, when they exhibited disease symptoms: increased leukemic burden, persistent weight loss or hind-limb paralysis. All studies involving mice were approved by the Nemours IACUC. APCD mouse treatments were done starting with
AP Pretreatment, 2.5 mg/kg, on day 21 via IP injections. The treatment was done for five days with two days off. Once the pretreatment concluded, the cytarabine-daunorubicin treatments began on day 28, starting with 2 days of Intravenous injections of a combinational therapy of 50 mg/kg of cytarabine and 1.5 mg/kg daunorubicin (CD) followed by 2 days of cytarabine Intraperitoneal injections. CD mice had treatments beginning on Day 28 with no AP pretreatment, the treatment length was the same for all mice in the study. CD mice were treated for five days with two days off. Dextrose was given to the mice as the vehicle treatment.

2.10 Statistical Analysis

Statistical analyses were done using a Tukey-Kramer non parametric test for multiple comparisons on JMP. Figures were created, and IC50s were determined using GraphPad Prism 7.
Chapter 3

RESULTS

Cell viability of MV4;11 cells in mono culture treated with serial dilutions of epigenetic and chemotherapeutic drugs was analyzed to establish the optimal dosing concentration. At the end of 48 hours of treatment, cells were analyzed by flow cytometry. Live and dead cell populations were gated based on forward scatter vs side scatter plots and propidium iodide exclusion. The percentage of living cell population

Figure 1: Determination of IC50 of Azacitidine, Panobinostat and Cytarabine for MV4;11 Cells. (A, B, C) Representative dose-Response curves used for generating IC 50 concentrations for MV4;11 cells calculated through serial dilutions of (A) azacitidine, (B) panobinostat, and (C) cytarabine. Best fit curve was generated using GraphPad Prism 7.
was calculated and plotted against concentration to yield dose response curves. Representative curves from several trials are shown (figure 1). The half maximal inhibitory concentration (IC50) was calculated from this curve by using Graph Pad Prism 7 software. For the chemotherapy drug, cytarabine, this concentration was 1.5 μM, which was then used throughout experiments described below (figure 1A). The IC50 for azacitidine was 7 μM, (figure 1B), while the IC50 for panobinostat (figure 1C) was 1.3 nM.

Once the IC50 concentration of cytarabine in monoculture was established, the next step was to determine if there is a difference in AML cell death induced by

Figure 2: MV4;11 Cells in Co-Culture Showed Greater Cell Viability. AML Cells (MV4;11) were treated with Cytarabine in the presence or absence of vary cells that are found within the bone marrow. Error bars display the standard deviation from the mean of three different experiments in duplicate. *p< 0.05, level of confidence to determine statistical significance.
cytarabine by comparing treatment in monoculture to treatment in co-culture with different bone marrow microenvironment cell types. This experiment would indicate which type of supported culture provided maximum chemoprotection.

Saos-2 cells, an osteosarcoma line, was used in this experiment to act as immature osteoblast cells. We compared two different human stromal cell lines, HS27 isolated from the foreskin, and HS5 isolated from the bone marrow. Each of these cell lines have been shown previously to provide cancer cells with differing amounts of chemoprotection which is why these cell lines were chosen [22, 26, 27]. When treated with cytarabine in the absence of supported culture, MV4;11 cell viability was reduced to 30%. MV4;11 cells treated in culture supported with Saos-2 cells showed no significant difference from the mono culture with an average viability of 38%. MV4;11 cells treated in culture supported by HS27 cells had an average viability of 50%. This average was accompanied by high variability indicated by the standard deviation bar. HS5 cells provided the maximum amount of chemoprotection in co-culture with a viability of 70% (figure 2). The viability of MV4;11 cells in HS5 co-culture was significantly different from the viability of MV4;11 cells treated with cytarabine alone, indicating that HS5 cells within the bone marrow protected leukemic cells from cytarabine. Due to the high cell viability in co-culture with HS5 cells, these cells were chosen to be the co-culture model for all experiments.
Once I had shown that the bone marrow microenvironment cells, HS5 provide chemoprotection to AML cells, MV4;11, I determined if this protection could be overcome by the use of epigenetic drugs. For this purpose, we used concentrations of AP that were lower than the individual IC50 values of azacitidine and panobinostat in monoculture. Treatments with AP or cytarabine showed 20-30% reduction in MV4;11 cell viability cultured in the presence of HS5 cells. However, when MV4;11 cells were pretreated with AP and then dosed with cytarabine, there was a drastic reduction in cell viability (figure 3). There was a significant difference between AP pre-treatment

Figure 3: Epigenetic Pretreatment Sensitizes Leukemic Cells to Chemotherapy in Co-Culture with HS5 Cells. MV4;11 cells were pre-treated for 48 hours with AP for pretreatment. Cells were then transferred onto HS5 cells and treated with Cytarabine for 48 hours. Error bars display the standard deviation from the mean of three different experiments in duplicate. *p< 0.05, level of confidence to determine statistical significance.
alone, cytarabine treatment alone, and the sequential AP cytarabine treatment indicated (p < 0.001). These results show that the epigenetic drug combination (AP) sensitized leukemic cells to cytarabine. The difference between the two separate treatments indicates that although the chemotherapy alone is not effective in killing

Figure 4: Epigenetic Pretreatment Sensitizes PDX Cell Lines to Chemotherapy in Co-Culture with HS5 Cells. Patient Samples were treated with only AP or pretreated with AP for 24 hours and then treated with cytarabine or daunorubicin for 48 hours. Concentration of Azacitidine was 1 µm and Panobinostat was 1 nm. Concentration of Cytarabine was 1.5 µm. Daunorubicin treatment was 5 nm. Error bars display the standard deviation from the mean of three or more different experiments in duplicate. *p< 0.05, level of confidence to determine statistical significance.
the leukemic cells in co-culture, using it in combination with epigenetic drugs can sensitize the cancer cells to the chemotherapy drugs.

The hypothesis that the epigenetic drugs can overcome the chemoprotection provided by the bone marrow microenvironment was validated in MV4;11 cell line. Patient-derived xenograft (PDX) lines generated by transplanting primary AML samples collected from patients treated at Nemours (NTPL) or at the Dana Farber Cancer Research Institute (DF) were cultured *ex vivo* and used to test the hypothesis stated above. The aim of this experiment was to determine if the AP combination pretreatment and chemotherapy would be more effective than treatment with chemotherapy drugs alone *ex vivo*.

Four different PDX lines from MLL rearranged patients were plated on HS5 cells and pretreated with AP for 24 hours, then treated with cytarabine or daunorubicin for 48 hours. NTPL-146 showed a significant difference in cell viability between treatment with daunorubicin (chemotherapy drug, D) and treatment with AP followed by daunorubicin (APD). This indicates that the combination treatment is more effective than the chemotherapy treatment alone. NTPL-377 also showed a significant cell viability difference between the D treatment and the APD treatment, meaning the combination was once again more effective in killing the leukemic cells than the chemotherapy alone. For DF-2 cells there was also a significant difference between the APD and D treatments indicating the APD combination and the APC combination was more effective than the chemotherapy drugs alone at killing the leukemic cells. DF-5 cells showed that there was a significant difference between the APD and D
treatments showing the more effective treatment in this patients’ case was the combination of the APC and not just the C alone.

Once I determined that the chemoprotection could be overcome by the use of the epigenetic drugs in MV4;11 cells and in PDX lines, I wanted to address the second aim of my study which was to determine which aspect of the bone marrow microenvironment provided the protection. It is important to determine if the protective effects were mediated by soluble factors secreted by the surrounding cells or is it the direct cell-to-cell contact between the leukemic cells and the cells within the bone marrow microenvironment.

The next step was to determine if chemoprotection was present even if the HS5 cells were not. To determine this, a chemoprotection assay was conducted using conditioned media (CM) collected from HS5 cells cultured in complete media for 48 hours. The purpose of this experiment was to determine if there is a protective effect from the factors secreted into the media by HS5 cells in culture. HS5 cells were plated in IMDM, media was collected after 48 hours, and then filtered to remove any HS5 cells. This media was then diluted by complete IMDM as indicated by the percentages of CM and plated with MV4;11 cells, then cells were treated with cytarabine.
Studies have shown that it is not effective to use 100% (CM) in experiments due to the depletion of nutrients and other growth factors in spent media post cell culture, because of this it is suggested not to use over 50% CM [28]. I chose to use 40% and 20% conditioned media for my experiment to determine a dose dependent effect of soluble factors. Neither condition provided enough protection to be comparable to the co-culture of MV4;11 and HS5 cells (figure 5). This experiment

Figure 5: Conditioned Media Does not Provide Chemoprotection to MV4;11 Cells Treated with Cytarabine. MV4;11 cells were plated on HS5 cells or plated in media that was collected from HS5 cells growing for 48 hours and diluted by complete IMDM. All conditions were then treated with 1.5 µm cytarabine to determine cell viability. Error bars display the standard deviation from the mean of three different experiments in duplicate. *p< 0.05, level of confidence to determine statistical significance.
supports that secreted factors are minimally responsible for the chemoprotection of MV4;11 cells by HS5 cells within the bone marrow microenvironment. The protection is not evident when there is no direct cell-to-cell contact between MV4;11 cells and HS5 cells. Once it was initially determined by the conditioned media experiment that there were no protective effects present with conditioned media, the next step was to confirm these results.

Figure 6: No Chemoprotection Present when HS5 and MV4;11 Cells are Treated with Cytarabine are Separated by a Transwell Filter. MV4;11 cells were treated with cytarabine in the presence or absence of HS5 cells. MV4;11 cells were placed in Transwell inserts wherever indicated. Error bars denote SD of the Mean from three independent experiments in duplicates. *p < 0.05 indicates statistical significance.
The aim of this experiment was to determine if MV4;11 and HS5 cells cultured together but separated by a transwell filter would provide more chemoprotection to the MV4;11 cells than the direct cell-to-cell contact established by co-culture. HS5 cells were plated on the bottom of the dish and a transwell filter was inserted into the well, on top of the HS5 cells. The MV4;11 cells were then plated into the transwell insert such that the filter separated them from the HS5 cells, but the exchange of secreted and soluble factors was still possible through the filter. The black bar in figure 6 depicts the cell viability of MV4;11 cells plated in co-culture with HS5 cells without the transwell filter separation. The gray bar shows the MV4;11 cells plated within a transwell filter on top of HS5 cells, with no cell-to-cell contact. The cell viability for the direct co-culture condition treated with cytarabine was 60% while the viability decreased when treated within the transwell filter (30%). Although cultured together, there is no chemoprotection for the cells that do not have the direct cell-to-cell contact (figure 6). This is indicated by a significant decrease in cell viability from the co-culture condition to the transwell condition. This difference has a p value of 0.002. The consistency between the results from the conditioned media experiment and the transwell experiment supports the hypothesis that adhesion is needed for chemoprotection.
The other aspect of the bone marrow microenvironmental protection that remained to be tested was the cell-to-cell contact. The aim of this experiment was to determine if the use of the epigenetic drugs (AP) caused a change in the adhesion of the leukemic cells to the HS5 stromal cells. HS5 cells were used in this experiment because these cells provided a model with maximum chemoprotection. MV4;11 cells were stained and treated with AP and plated on top of HS5 cells. After 48 hours, the cells were washed with PBS and then trypsinized. The cells that remained in the dish following the wash were HS5 cells and the stained leukemic cells that were adhered to

Figure 7: Epigenetic Pretreatment Causes a Decrease in Adhesion of MV4;11 cells to HS5 Cells. (A) MV4;11 cells were stained and plated on HS5 cells, then treated with azacitidine and panobinostat for 48 hours to determine if adhesion is effected by these treatments. The control (100%) is the adhesion level of MV4;11 cells to HS5 cells with no treatment. Treatment adhesion was normalized and compared to control. Error bars display the standard deviation from the mean of three different experiments in duplicate. *p< 0.05, level of confidence to determine statistical significance. (B, C) Phase contrast images of cells in co-culture after wash with PBS. Scale bar = 100 µm.
the HS5 cells. There was an average decrease in adhesion of 40% with the AP treatment compared to the control or untreated condition (figure 7). This supports the hypothesis that the epigenetic drugs impact cell adhesion. Phase contrast images of adherent leukemic cells on HS5 cells post wash with PBS show more leukemic cells clustered on top of the HS5 cells than in panel C. There are far fewer leukemic cells in panel C and these cells are not clustered on top of the HS5 cells.

The studies done on the cell lines provided evidence to support the in vivo evaluation of the epigenetic drugs in combination with chemotherapeutics. To determine if this treatment combination would be effective in mice, MV4;11 cells were injected into immune deficient NSG-B2m mice (day 0) as described previously [29]. AML cells homed to the bone marrow and engrafted there. Once disease was established, leukemic cells were detectable in the peripheral blood. These mice were bled regularly to monitor the engraftment of the leukemic cells. Once the leukemic to mouse cell ratio reached a threshold, the mice were randomly assigned to different treatment groups. These different treatment groups were AP, APCD, and Vehicle (dextrose). The treatment schedule is described in detail in Materials and Methods. The increase in human cell percentage was plotted over time (figure 8A). This percentage was high in AP treated mice compared to CD treated mice when evaluated at the end of treatment. This data indicates that leukemic cells may dislodge from the bone marrow and mobilize into the blood stream, where these cells are more likely to be killed by chemotherapy drugs. Although the percent of leukemic cells in the blood increased, there was no increase in leukemic burden in the mice treated with APCD.
APCD treated mice survived the longest compared to either vehicle treatment alone or CD treatment alone (figure 8B). There was a significant difference in the survival between the two treatment conditions (APCD, CD). APCD mice survived for a median of 57.5 days while the vehicle mice survived a median of 30.5 and the CD mice survived a median of 38.5 days. This indicates that the mice treated with the combination of epigenetic therapy and chemotherapy survived longer and this effect was mediated by sensitization of leukemic cells to chemotherapy by mobilization into the blood stream by AP treatment.

Figure 8: Using Epigenetic Drugs in Combination with Chemotherapeutics Increase Overall Survival in Mice. (A) Graph showing the increase in the percentage of human CD45+ cells in mouse peripheral blood. (B) Kaplan-Meier survival curves indicating the term of survival for mice treated with vehicle, APCD, and CD treatments. AP pre-treatments began on day 21 and was given for 5 days. CD treatment began on day 28 and continued for 5 days. The vehicle for this experiment is dextrose.
Chapter 4

DISCUSSION

The aim of this entire study was to first show that epigenetic drugs can be effective in overcoming the protective effects of the bone marrow microenvironment. The second aim of this study was to determine if the protective effects were mediated by adhesive properties of the leukemic cells to cells present within the bone marrow. The bone marrow microenvironment plays a very intricate role in the protection of leukemic cells due to their tight knit interaction. As seen in figure 2, the supported culture that provided the maximum amount of chemoprotection was the culture with HS5 cells. The increase in cell viability when treated with cytarabine in co-culture shows that MV4;11 cells cultured with HS5 cells had better survival than those cells plated alone in mono-culture. This increase in survival in the presence of cells found in the bone marrow can be related to the instances of relapse for pediatric patients with AML. Patients relapse due to the survival of cells resistant to chemotherapy because of their interactions within the bone marrow microenvironment. Effects similar to this can be seen in other pediatric cancer types such as pediatric acute lymphoblastic leukemia (ALL) [22].

The heterogeneity of AML is what makes the disease so difficult to treat. Once patients are diagnosed there are multiple screenings involved that provide the mutations that are present and put each patient into different categories of the disease [30]. Due to the vast amount of differing categories and subcategories targeted therapies are not a realistic broad spectrum approach to treatment of pediatric AML. The leukemic cells lodge themselves into the bone marrow and thrive off the interactions they experience with the different cells naturally found within this niche.
These cells evade chemotherapy treatments through these interactions, which leads to relapse [31].

Resistance within the bone marrow can be due to many different mechanisms, but we focused on resistance due to microenvironment interactions. Cross talk between cells could initiate pro survival or anti apoptotic signaling that allows the cells to avoid being vulnerable to the chemotherapy drugs. The latter of the two immunities is the one suspected to be at play for AML. The cells are being protected by their interaction with other cells present in the bone marrow microenvironment. Using chemotherapy drugs in combination with epigenetic drugs could be effective in overcoming this protection as seen in figures 3, 4, and 8.

The impact of the AP treatment on the adhesive properties of the leukemic cells effectively mobilizes leukemic cells into the blood stream. In figure 8, at day 28, there is a spike in leukemic cells within the peripheral blood seen APCD treatment, indicated by the blue line, after the conclusion of AP treatment. Adhesion molecules, such as ICAM, VCAM, VLA4, and many more, are responsible for cell homing and migration. AP treatment could be affecting these specific molecules or their ability to interact with their binding partners [31]. Leukemic cells are dependent on their interactions within the bone marrow microenvironment for their survival, so any disruption of those interactions could be detrimental to the survival of leukemic cells [31]. The adhesive interactions between the AML cells and the bone marrow microenvironment are one of the main reasons why this cancer has such a poor prognosis [30, 31].

Many of the mutations that occur during the development of AML come from disruption of the epigenome whether it be from DNA promoter hypermethylation or
aberrant histone H3 hypoacetylation [25]. Using DNA methyltransferase inhibitor, azacitidine, and histone deacytelase inhibitor, panobinostat, in combination could combat the expression changes occurring from the different leukemogenic mutations. Currently, epigenetic drugs are in clinical trials for use as treatments for adults with AML and other leukemias, but not for pediatric cases [25]. This study supports preclinical evidence that the use of epigenetic drugs in combination with chemotherapy is a possible treatment option for children with AML.

The future direction for this study is to examine the specific molecular mechanism of action driving the impact of the AP treatment and if there are alterations in adhesion molecules. In order to conduct this study, a FACS analysis can be performed on both the leukemic cells and the HS5 cells considering both of these cells are in the presence of the AP treatment, and I found that adhesion was decreased with this treatment. Since there is no visible chemoprotection with conditioned media or the transwell filter, the leukemic cells must maintain contact with the bone marrow microenvironment for survival. The decrease in the leukemic cells adhered to the HS5 cells after treatment with AP provide evidence that the contact with the bone marrow microenvironment is disrupted by the treatment of AP. The increase in leukemic cells in the peripheral blood of mice treated with AP show that the cells are detaching from the bone marrow microenvironment and are mobilized into the blood stream. All of this evidence supports the hypothesis that the adhesion molecules are effected in the mechanism of AP. Determining the exact mechanism and which molecules are being altered is the future direction for this research.

An additional future direction for this research would be to determine if there are any global expression changes. An RNA-seq analysis could be performed to
examine changes in the levels of RNA. Because epigenetic drugs alter gene expression via changing the transcript levels, RNA-Seq is a reliable methodology to evaluate the global expression patterns following treatment with azacitidine and panobinostat. This would be an important strategy to determine novel targets of AP.

In order to examine changes in pro-survival signaling mechanisms such as MAPK and Akt, a phospho-proteome array will be useful. The changes in expression of MAPK and Akt could be confirmed using western blotting analysis. Phosphorylation levels of AML cells and HS5 cells in co-culture can be evaluated by flow cytometry using specific markers for each cell line and phosphoflow antibodies. The overall future direction of the lab is to determine how these drugs synergize and if they affect the bone marrow microenvironment, the leukemic cells, or both. To determine the mechanism both, leukemic cells and bone marrow microenvironment cells, should be examined for adhesion molecule and pro-survival protein expression changes.

In conclusion, the data provided here supports evidence that epigenetic drugs could be an effective treatment option to overcome leukemic cell protection within the bone marrow and that this protection is mediated by cell to cell interaction by adhesion molecules.
REFERENCES


Appendix

IACUC
MEMORANDUM

DATE: August 17, 2017

TO: Sonali Barwe, Ph.D.

FROM: Paul T. Fawcett, PhD

SUBJECT: NBR-2009-007 Leukemia xenograft models for preclinical drug testing.

The Institutional Animal Care and Use Committee (IACUC) have reviewed the submitted annual review on the above referenced project and the following decision has been made:

Action: Approved

Date of Action: August 17, 2017

Approval Period: August 17, 2017 through August 16, 2018

Approved Number of Animals: Mice 981

Please submit your Biosafety Classification form electronically to the Alfred I. duPont Hospital for Children Institutional Biosafety Committee via the link:

http://www.nemours.org/pediatric-research/approval/biosafety-committee.html

Please note that the study cannot begin until the Office of Regulatory Compliance in Research Administration has received all approvals.

Please maintain this approval with your project records. A tally of the number of animals approved and the number ordered for the project will be maintained in the Life Science Center. If changes occur in your protocol or if you require more animals than approved, and amendment to your protocol will need to be submitted for consideration.

If you have any questions regarding this memorandum, please contact Paul T. Fawcett, Ph.D. at x 6776 or email: pfawcett@nemours.org.