Université de Montréal

Proscillaridin A Effects on Histone Acetylation and C-MYC Degradation in Acute Lymphoblastic Leukemia

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Mémoire présenté
en vue de l'obtention du grade de Maîtrise en Sciences
en Pharmacologie

juin 2016

Résumé

La leucémie lymphoblastique aiguë (LLA) représente environ 25% des cancers pédiatriques diagnostiqués chaque année. Dans 80 % des cas, une rémission complète est observée. Cependant, les patients résistants aux traitements ainsi que les patients en rechute présentent un mauvais pronostique. Les altérations épigénétiques sont des facteurs essentiels dans le développement et la progression de la maladie, ainsi qu'à la résistance aux traitements. Lors d'un criblage de médicaments approuvés par la FDA, nous avons découvert des molécules ayant des caractéristiques anticancéreux et épigénétiques. Pour évaluer l'activité de ces molécules, nous avons procédé à un criblage secondaire sur plusieurs lignées cellulaires leucémiques. Nous avons découvert qu'une de ces molécules, un glucoside cardiotonique appelé la proscillaridine A, avait une activité anticancéreuse spécifique pour des cellules leucémiques. Nous faisons donc l'hypothèse que la proscillaridine A pourrait avoir des effets épigénétiques et anticancéreux dans des modèles précliniques de LLA. Pour tester cette hypothèse, nous avons traité deux lignées cellulaires de LLA Nalm-6 (LLA pre-B) et Molt-4 (T-LLA) *in vitro* pendant 2 à 96 heures à des doses pertinentes sur le plan clinique. Nous avons alors pu observer une inhibition de croissance qui était dépendante de la dose administrée dans les deux lignées cellulaires, avec des valeurs de 50% d'inhibition de croissance (CI_{50}) de 3.0 nM pour les Nalm-6 et de et 2.3 nM pour les Molt-4. De plus, nos études sur le cycle cellulaire par BrdU démontrent un arrêt en phase G2/M. Nous avons également détecté par immunobuvardage de type western des baisses significatives de l'acétylation de résidus de l'histone 3. Les niveaux d'expression des enzymes responsables de cette acétylation, les histones acétyltransférases CBP, P300 et TIP60 ainsi que de l'oncogène

C-MYC étaient également diminuées. Par des analyses de séquençage de l'ARN, nous avons observé une augmentation de l'expression des gènes impliquées dans les processus d'apoptose et de différentiation cellulaire, ainsi qu'une diminution des gènes impliqués dans la prolifération cellulaire comme en particulier les gènes cibles de C-MYC. Ces résultats prometteurs suggèrent le potentiel prometteur de la proscillaridine A comme nouvelle thérapie pour les patients atteints de LLA.

Mots clés: acétylation, histones, oncogène, leucémies lymphoblastiques aiguës

Abstract

Acute lymphoblastic leukemia (ALL) represents approximately 25% of all pediatric cancers diagnosed every year. In about 80% of cases, pediatric patients will attain a 5-year event-free survival. Unfortunately, patients who are resistant to treatment or who relapse have a poor prognosis. Hence, novel therapeutic approaches are necessary to increase survival rates. Epigenetic alterations, such as DNA methylation and histone modifications, are involved in disease development, progression, and in particular, resistance to treatment. These reversible alterations represent novel targets in ALL. We recently discovered candidate epigenetic drugs in FDA-approved drug libraries. We performed a secondary screen to test the activity of these drugs in a panel of cancer cell lines. We found that a cardiac glycoside, called proscillaridin A, had anticancer specificity against pediatric leukemia cell lines. Thus, we hypothesize that proscillaridin A has some drug repositioning potential in pediatric ALL. To characterize its epigenetic mechanism of action, we treated two ALL cell lines Nalm-6 (pre-B ALL) and Molt-4 (T-ALL) *in vitro* for different time points (2-96h) with clinically relevant concentrations of proscillaridin A and analyzed cell growth, cell cycle, gene expression and chromatin modifications. We observed dose-dependent growth inhibition in both cell lines, where 50% of growth inhibition (IC_{50}) was obtained at 3.0 and 2.3 nM in Nalm-6 and Molt-4, respectively. Our results using BrdU staining indicate a cell cycle block in the G2/M phase. By western blot, we detected significant decreases in histone 3 acetylation levels (H3K14ac, H3K9ac, and H3K27ac). Decreases in histone acetylation were associated with a significant reduction in histone acetyltransferase expression (CBP, P300 and TIP60) as well as the C-MYC oncogene. By RNA sequencing and gene set enrichment analysis, we observed an upregulation of apoptosis and cell differentiation genes, as well as a decrease in cell

proliferation and C-MYC target genes. These promising results illustrate the potential of using the cardiac glycoside proscillaridin A as a novel drug in treatment of relapsed or refractory ALL.

Keywords: acetylation, histones, oncogene, acute lymphoblastic leukemia

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List of initials

FAB: French, American and British
WHO: World Health Organization
FDA: Food and Drug Administration
COG: Children's Oncology Group

List of abbreviations

CLL: Chronic lymphoblastic leukemia
CML: Chronic myeloid leukemia
ALL: Acute lymphoblastic leukemia
AML: Acute myeloid leukemia
MLL: Mixed lineage leukemia
JAK: Janus kinase
mTOR: Mammalian target of rapamycin
HSCT: Hematopoietic stem cell transplantation
CNS: Central nervous system
CSF: Cerebral spinal fluid
MRD: Minimal residual disease
MeCP2: Methyl CpG binding protein 2
NTD: N-terminal domain
CTD: C-terminal domain
MBI/II/III: Myc- box I/II/III
MAX: MYC associated factor X
HSC: Hematopoietic stem cell
DNMT: DNA-methyltransferase
MGMT: O-6-methylguanine-DNA methyltransferase
HAT: Histone acetyltransferase
MBP: Methyl binding protein
MBD: Methyl binding domain
HMT: Histone methyltransferase
HDM: Histone demethylase
HDAC: Histone deacetylase
DNMTi: DNA methyltransferase inhibitor
HDACi: Histone deacetylase inhibitor
DHAC: Dihydro-5-azacytidine
FCDR: 5-Fluoro-2'-azacytidine
HATi: Histone acetyltransferase inhibitor
GFP: Green fluorescent protein
CMV: Cytomegalovirus
ROS: Reactive oxygen species
BET: Bromo domain and extraterminal domain
BETi: Bromo domain and extraterminal domain inhibitors
GSEA: Gene set enrichment analysis
MB-3: α-methylene-γ-butyrolactone 3
SAM: S-adenosyl-L-methionine
ATM: Ataxia telangiectasia mutated
STIM1/2: stromal interaction molecule1/2
SOCE: store-operated calcium entry
NOD/SCID: non-obese diabetic/severe combined immunodeficiency

Acknowledgements

Over the course of the passed two and a half years, I have worked with many people in the Centre de recherche du CHU Sainte-Justine. I have met many inspiring, talented, generous, caring individuals and each one of them has helped me grow to become the person I am today. For this reason, I would like to take some time to thank a few in particular.

First, my research project would have never been accomplished without Dr. Noël Raynal giving me the opportunity to work in his lab. Without your help, I would not be here today writing the acknowledgement section of my Master's thesis. I would also like to thank you for helping me complete the research I have conducted since January 2014; without you guiding me to answer the many questions we have asked ourselves, I would probably still be learning how to use a pipette. With your help, I have become a better scientist; especially with all those journal club meetings, presentations, and scientific conventions I have attended since starting to work in your lab. I can't thank you enough for teaching me what science is about: asking questions and trying to figure out the answers.

I would also like to thank Simon Jacques-Ricard for being the best person in the lab with whom I was able to create interesting decoctions and conduct other non-scientific experiments. Using dry ice, curdled milk, agarose gels, and pretty much anything else we could get our hands on in the lab, we were able to create some very remarkable experiments over the passed two years. I would also like to thank you for supporting Gaspard; I know it was hard for you to always destroy a part of him as you vortexed your tubes, but you did a great job nonetheless.

I would also like to thank Elodie Da Costa for being an awesome workbench partner and a great friend. Du coup, merci de m'avoir appris à parler français quoi! Thank you for having a great sense of humor, making me laugh, and joking around with me on a regular basis. Thank you for letting me borrow your bench top, pipette tips, pens, and western blot trays when you were either not in the lab or not paying attention. I know I could be a little bit annoying when I was invading your bench with papers and books on a daily basis, so I thank you again for not complaining too much. On a more serious note, I would also like to thank you for giving me advice and helping me out with my research; without that, I would not be where I am today. I will always remember that Spanish language we invented: Bueno lucko Elodita aveco tono projecto de researcho!

Next, I want to thank Annie Beaudry for teaching me how to properly conduct different lab experiments and procedures that helped me complete my research project. Thank you for being patient with my many questions about how to make solutions and how to properly run western blots. I would also like to thank you for supporting my ridiculous sense of humor and my constant use of the word "jerky" along with another word I cannot write in this manuscript (I am confident we all know what the word I'm referring to actually is).

Last but not least, I would like to thank my buddy Gaspard. Although you were silent most of the time, smelled like beta-mercapto, and took up literally half my workbench, I really loved having you around.

Chapter 1: Introduction

1.0 Leukemia

Leukemia is a malignant disease affecting normal developmental and maturation processes of white blood cells and lymphocytes [1, 2]. It is described as a progressive loss in normal cell maturation and an increase in cell proliferation, leading to abnormally high levels of undifferentiated cells in the bone marrow and blood stream [1, 2]. The accumulation of undifferentiated white blood cells and lymphocytes also disrupts the bone marrow niche, and consequently affects the maturation and production of other hematopoietic progenitor cells [1-3].

Depending on the blood cell affected, leukemia is separated into two major groups: lymphoblastic and myeloid. Whereas lymphoblastic leukemia only affects T- or B-lymphocytes, referred to as T-ALL or B-ALL, respectively, myeloid leukemia affects all other white blood cells, such as basophils, neutrophils, eosinophils, and monocytes [2]. Leukemia can also be classified as being either acute or chronic, depending on the maturity of the cell affected and the rate of disease progression. In cases of acute leukemia, patients are diagnosed with a rapidly progressing disease [2]. On the other hand, patients diagnosed with chronic leukemia are portrayed as having a slower rate of disease development and progression [2]. There are four main classes of leukemia: chronic lymphoblastic leukemia (CLL), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML) [2, 4].

Pathophysiology of each leukemia subtype varies drastically from one main class to another [2, 4]. Hence, each leukemia subtype will have different biological characteristics and some will be more prevalent in certain age groups compared to others [2]. For instance, CLL is most frequently observed in elderly patients and affects cells in the bone marrow compartment and blood stream [2]. Patients can either be diagnosed with an aggressive or a dormant form of CLL, the former requiring therapeutic intervention while the latter does not [2]. Differences in cell biology, such as cell surface markers and chromosomal aberrations, can help classify patients in either the dormant or aggressive CLL categories [2]. In addition, presence of certain mutations or disorders in important tumor suppressor genes – like p53 for instance – are linked to the aggressive CLL subtype and result in a poor prognosis [2, 5].

On the other hand, CML only affects myeloid cell development in the bone marrow, which eventually leads to higher concentrations of undifferentiated myeloid cells in the blood stream [2]. Peak incidence for CML patients is observed between 50 and 60 years of age [2]. The most common chromosomal aberration involved in development of CML is a translocation between chromosomes 9 and 22, also known as the Philadelphia chromosome, forming the BCR-ABL fusion gene [2, 6, 7]. This translocation is observed in approximately 90% of CML patients [2, 6, 7]. There are three main phases in CML that correlate to disease progression and influence prognosis as well as overall survival: a chronic, an intermediate, and a blastic phase [2, 8]. As disease progresses from the chronic phase to the intermediate and blastic phases, there are increasing numbers of immature white blood cells in the bone marrow and blood stream [8]. Eventually, patients are placed into the blastic phase category upon the occurrence of white blood cell infiltration into different organs or greater than 30% blast

counts in their blood stream or bone marrow [8]. The blastic phase is deadly in most cases, usually leading to patients succumbing from their disease within 6 months [2, 8].

Alternatively, ALL is a malignant blood disorder affecting solely T- and B-lymphocytes [2, 9, 10]. ALL essentially alters development of T- and B- cells by inducing an increase in the cell proliferation and survival pathways [2, 10]. Interestingly, ALL is very rarely diagnosed in adults but has very high prevalence in children [2]. Like chronic leukemia subtypes, ALL is associated with numerous chromosomal translocations leading to formation of fusion proteins playing critical roles in disease initiation and progression by affecting important cellular pathways [2, 9, 11].

Like the other leukemia subtypes, AML is described as having an interruption in cell differentiation leading to accumulations of AML blast cells in the bone marrow that will eventually spill into the blood stream [2, 11]. AML is a disease affecting primarily adults, but in rare cases affects children as well [2, 11]. Pediatric cases of AML are associated with many chromosomal abnormalities, the most common one being translocations encompassing the mixed lineage leukemia (MLL) protein [2, 11].

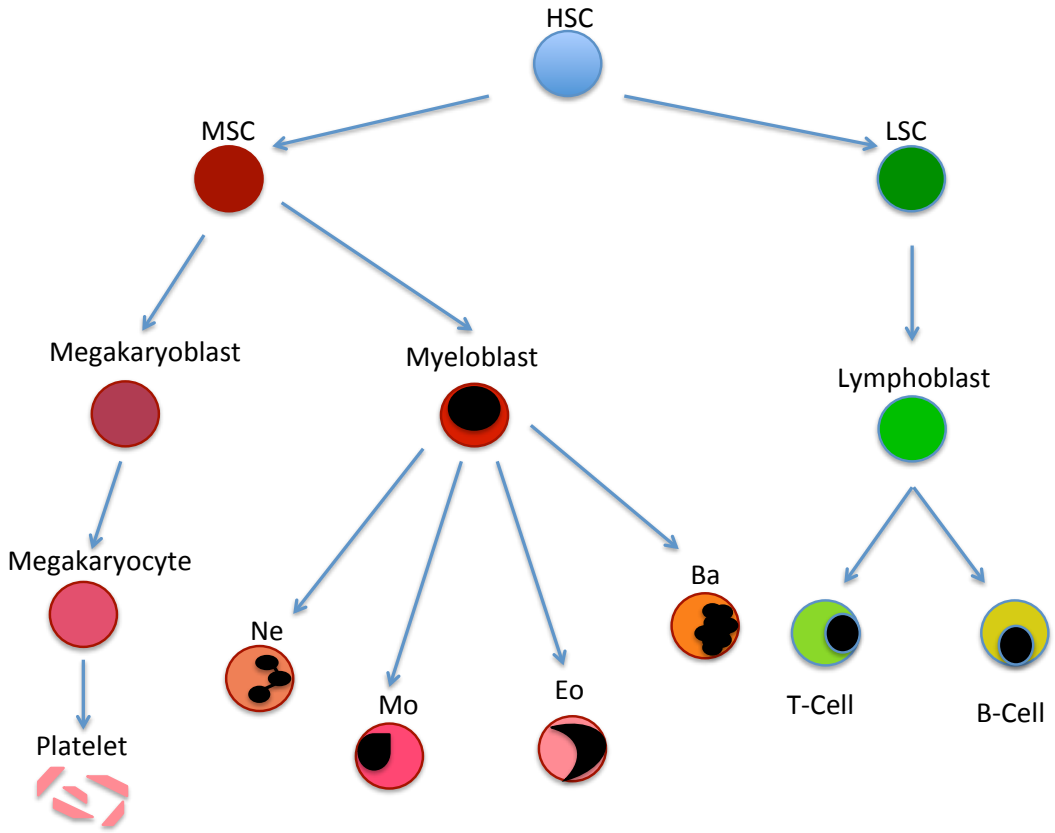

Figure 1: Normal and abnormal hematopoiesis.

Differentiation of hematopoietic stem cells into either the myeloid or lymphoid lineages. Cells affected in myeloid and lymphoblastic leukemia are myeloblasts and lymphoblasts, respectively. HSC: hematopoietic stem cell, MSC: myeloid stem cell, LSC: lymphoid stem cell, Ne: neutrophil, Mo: monocyte, Eo: eosinophil, Ba: basophil. [12]

2.0 Pediatric Leukemia

Leukemia is the most common pediatric cancer diagnosed every year, representing approximately 30% of all newly diagnosed cases in pediatric patients around the world [11]. The two most common leukemia subtypes diagnosed in pediatric patients are ALL and AML [11]. Approximately 85% and 15% of acute leukemia cases in children are ALL and AML, respectively [13, 14].

The following sections describe the biology behind pediatric ALL and AML development and current treatment methods for each leukemia subtype.

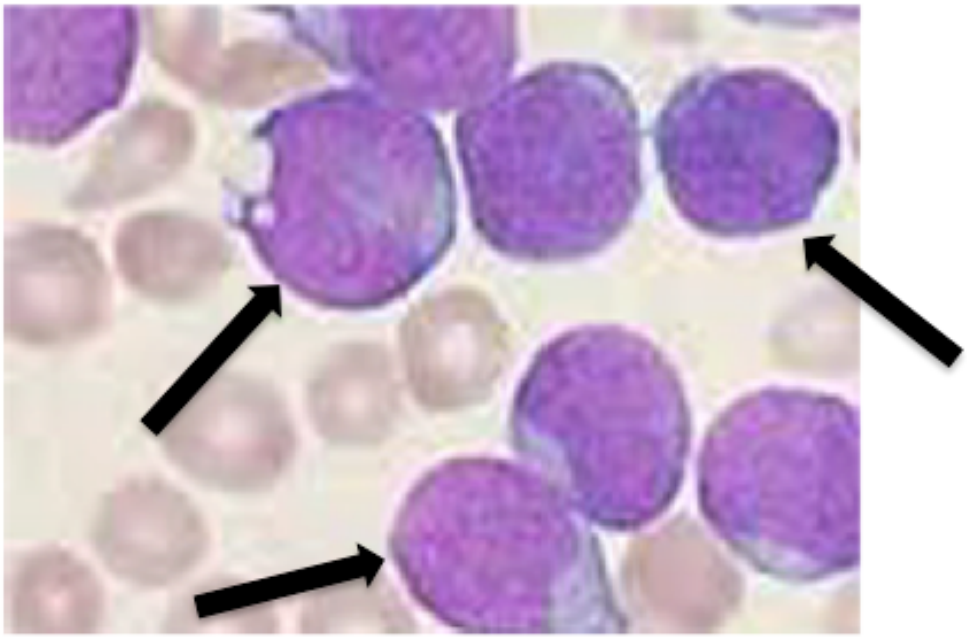

Figure 2: Bone marrow accumulation of lymphoblasts in a pediatric ALL patient.

In patients with ALL, there is an accumulation of leukemic blasts in the bone marrow, which disrupts development of other blood cells. Leukemic blasts are described as having very large nuclei, as shown by the black arrows.

2.1 Pediatric ALL, Current Treatments, and Relapse

2.1.1 Biology of ALL

Acute lymphoblastic leukemia (ALL) is characterized as a blood malignancy that induces a decrease in differentiation and an increase in proliferation in lymphoid progenitor cells [15, 16]. These events lead to an overpopulation of the bone marrow compartment and blood stream with immature leukemic cells, also called "blasts". As a consequence, accumulation of blast cells affects normal blood cell development and impairs hematopoiesis [15, 16]. Depending on the lymphoid progenitor affected, ALL can be subdivided into two main categories: B- and T-ALL, the most common form in children being B-ALL [16, 17]. ALL accounts for approximately 25% of all pediatric cancers diagnosed every year with incidence peaking in children between the ages of 2 and 5 [18, 19]. Although current treatment methods cure over 85% of pediatric patients diagnosed with ALL in developed countries worldwide, patients who either relapse or experience resistance to treatment have a very poor outcome portrayed by an overall survival of approximately 25% [10, 20]. Hence, because of its prevalence, ALL is the leading cause of death by disease in children.

Molecular mechanisms underlying B- and T-ALL include inactivation of tumor suppressor genes and irregular activation of oncogenes and signal transduction pathways [21-23]. These genetic aberrations affect key regulatory processes, producing highly proliferative blast cells with unlimited self-renewal and cell-survival properties [21-23]. Chromosomal lesions, such as abnormal chromosomal numbers and structural abnormalities – especially translocations – are often identified in the pediatric ALL population [21-23]. Evidently, these chromosomal lesions differ between the two ALL subtypes.

The most common chromosomal translocation observed in pediatric B-ALL is the translocation between ETS variant 6 (ETV6), located on chromosome 12, and Runt-related transcription factor 1 (RUNX1), located on chromosome 21, termed the ETV6-RUNX1 fusion protein [24]. This translocation accounts for roughly 25% of all pediatric B-ALL cases [24]. In normal cells, both proteins act as transcription factors required in hematopoiesis and normal cell differentiation [24]. Fortunately, patients with the ETV6-RUNX1 translocation have an elevated survival rate [25]. The TCF3-PBX1 fusion protein, made up transcription factor 3 (TCF3) and pre-B cell leukemia homeobox 1 (PBX1), accounts for approximately 5% of B-ALL cases, and is associated with a good prognosis, despite the slight risk in CNS relapse [26]. For this reason, patients diagnosed with TCF3-PBX1 leukemia are given higher doses of CNS preventative therapies [26]. Next, translocations involved in forming the BCR-ABL1 fusion protein, formed from the fusion between breakpoint cluster region protein (BCR) and Abelson murine leukemia viral oncogene homolog 1 (ABL1), account for about 3% of cases and are correlated with good patient outcomes due to the recent introduction of imatinib in their treatment regimens [27]. Finally, translocations involving the mixed lineage leukemia (MLL) protein, which occur at a much higher frequency in patients under two years old, account for about 5% of B-ALL cases [19, 24, 28].

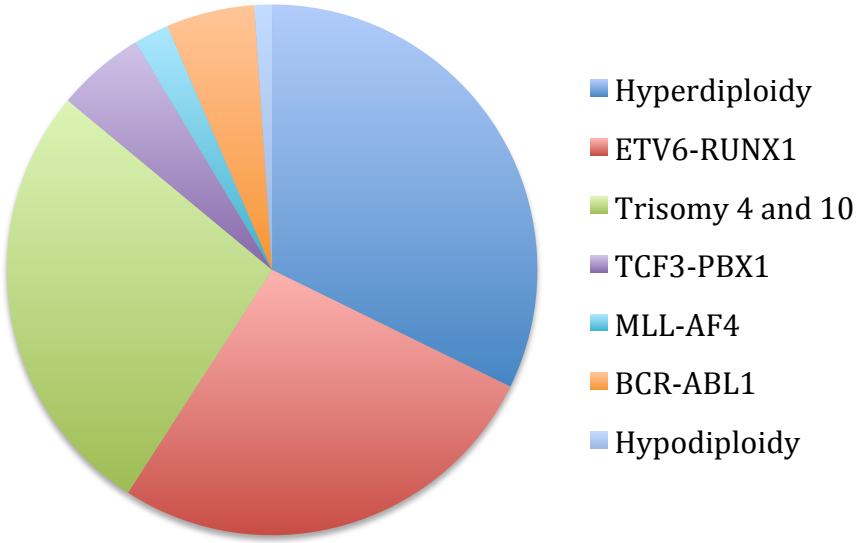

Figure 3: Pediatric B-ALL subtypes.

Pie chart of pediatric B-ALL subtypes.

Chromosomal translocations in T-ALL are not as well characterized as their B-ALL counterparts. Pediatric patients are classified into four categories, each of which is associated with upregulation of different oncogenes [24, 29]. The four major oncogenes present in T-ALL are HOX11, LYL1, TAL-LMO2, and HOX3 [24, 29]. Translocations between the T-cell receptor and the HOX11 oncogene represent a distinct subset of patients part of the HOX11 category. Other than these noticeable chromosomal translocations, T-ALL can also be illustrated by abnormal NOTCH1 signaling that impairs T-cell differentiation in approximately half of T-ALL cases [30].

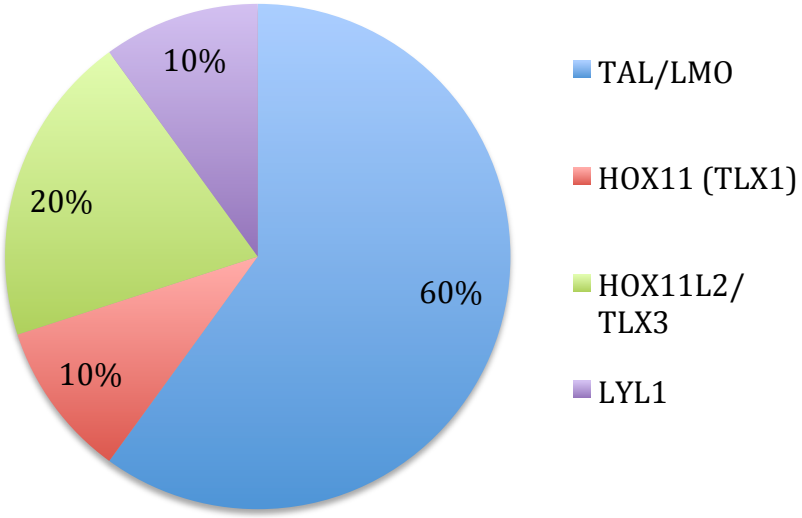

Figure 4: Pediatric T-ALL subtypes.

Pie chart of the four major oncogene subtypes in pediatric T-ALL.

In addition, chromosomal and genetic alterations also take part in development of acute leukemia in children. For example, Down's syndrome (trisomy 21) increases the risk to develop pediatric ALL or AML significantly [31]. Patients with Down syndrome are frequently associated with ETV6-RUNX1 chromosomal translocation and are often categorized as high-risk mostly because of frequent relapse rates and lower overall survival [32].

Gain or loss of entire chromosomes, termed hyperdiploidy and hypodiploidy, respectively, are also frequently observed in pediatric patients [19, 24]. Chromosomes X, 4, 6, 10, 14, 17, 18, and 21 are the ones most often gained in ALL patients with hyperdiploidy and make up approximately 30% of all cases [19, 27]. These patients are associated with positive outcome [27]. Hypodiploidy, on the other hand, is much less frequent, observed in approximately 1% of patients, and is correlated to a poor prognosis [27].

2.1.2 Treatment of pediatric ALL

Because ALL is such a diverse disease, the diagnostic of the correct subtype is critical in determining which treatment methods a patient should undergo and whether or not a given treatment will fail [33]. Hence, at the time of diagnosis, risk stratification for a patient is established and is an important aspect in attaining overall survival [33, 34]. Therefore, prior to starting treatment, patients are labeled as standard, intermediary, or high-risk [11]. Two important clinical characteristics considered at the time of diagnosis are age and white blood cell count [11, 33-35]. Favorable outcomes are observed in patients between the ages of 1 and 10, whereas unfavorable outcomes are observed in patients under 1 year of age and older than 10 years of age [33]. Also, white blood cell counts greater than 50,000 cells per μL of blood are predicted to have an unfavorable outcome [33].

Another important characteristic examined is leukemic cell penetration into sanctuary sites, defined as areas in the body that are difficult to target with conventional chemotherapy. Two common sanctuary sites in leukemia are the central nervous system (CNS) and testis [11, 33]. Penetration of leukemic cells into these organs is correlated with an unfavorable outcome and is usually involved in patient relapse [11, 33].

After establishing risk stratification, patients undergo treatment according to their risk category [11, 33]. Generally speaking, current treatment methods include three main stages [11, 33]. The first step in ALL treatment is induction therapy, lasting between 4-6 weeks; the goal of induction therapy is to eliminate all leukemic blasts from the bone marrow [11, 33]. Four medications are used in induction therapy: vincristine, prednisone, L-asparaginase, and an anthracycline (either doxorubicin or daunorubicin) [11, 33]. The usual two-drug regimen consists of vincristine followed by prednisone, which leads to remission in about 85% of

patients [11]. Addition of a third drug – either L-asparaginase or an anthracycline – further increases the remission rate to approximately 95% [11]. In high-risk patients, a fourth drug, usually an anthracycline or methotrexate, is added to the regimen in order to attain remission [11].

Since ALL penetration into the CNS is correlated with increased rates of patient relapse, CNS preventative therapy has also been established in treating the disease [11]. CNS preventative therapy includes intrathecal methotrexate injections followed by low to moderate cranial irradiation to eliminate any leukemic cell that crossed the blood-brain barrier [11]. CNS preventative therapy is very efficient, and leads to good prognosis in a majority of cases with minimal cytotoxicity if started at the same time as chemotherapy regimens [11, 36]. Unfortunately, some patients develop secondary cancers due to side effects of treatment, such as non-Hodgkin lymphoma and cancers affecting the brain and thyroid organs [37].

Induction therapy is followed by the second phase in ALL treatment called consolidation therapy. The goal of consolidation therapy is to eliminate any remaining leukemic cell population from the patient's bone marrow and blood stream in order to prevent any further disease progression or relapse [11, 33]. Treatment protocols differ in length and intensity, usually lasting 6-9 months, but can last longer if a patient is in the high-risk category [33]. During this phase, patients receive higher doses of drugs formerly used in induction chemotherapy. Different classes of cytotoxic drugs not previously used during induction therapy, such as mercaptopurine, etoposide, cyclophosphamide and cytarabine, can also be added to treatment regimens in order to prevent resistance [11, 33].

The third and final stage of ALL treatment is maintenance therapy, where patients receive low-dose of chemotherapy drugs for approximately 2-3 years in order to prevent

disease relapse [11, 33]. During this phase, treatment protocols include weekly methotrexate and mercaptopurine administrations, in addition to prednisone and vincristine on a monthly basis [11, 33].

2.1.3 Personalized Treatments for ALL

Nowadays, personalized approaches exist taking patient risk stratification into account. Depending on chromosomal aberration identified in ALL patients, treatments can be administered to specifically target cancer cells with minimal effects on healthy cells. Several classes of these targeted therapies exist for ALL, with a few of these currently undergoing clinical trials [9, 27, 34, 38].

First, monoclonal antibodies can be used in treating specific ALL cases having cell surface receptors or markers that can potentially be targeted [9, 38]. A monoclonal antibody can either be conjugated or unconjugated; the former being attached to a radioactive or cytotoxic compound whereas the latter is not [9, 38]. An unconjugated monoclonal antibody identifies a target on the surface of the cell and initiates either antibody-dependent cell mediated toxicity or complement-dependent cytotoxicity [9]. On the other hand, conjugated monoclonal antibodies identify and bind onto their target cell and get internalized along with their conjugate. This phenomenon enables the release of the cytotoxic compound into the cell, causing cell death [38]. Numerous examples using unconjugated monoclonal antibodies exist in treating ALL. For instance, rituximab, a chimeric murine and human monoclonal antibody, specifically targets CD20 surface antigen present on B-lymphocytes enabling its use as a target in B-ALL cases [38]. CD20 expression is observed in over 50% of pediatric B-ALL

cases, and is usually upregulated after chemotherapy treatment [9, 39, 40]. The monoclonal antibody alemtuzumab specifically targets CD52 surface antigen present on T- and B-ALL cells and can be used in relapsed or refractory cases of pediatric ALL [9, 38]. Although response rates for alemtuzumab are generally very low, patients with the t(12;21) translocation have a slightly higher sensitivity to treatment compared to patients without this translocation [9, 41]. Another popular monoclonal antibody is epratuzumab. Epratuzumab targets CD22 surface antigen present primarily on pre-B cells, and can therefore be used in treating pediatric pre-B ALL [9, 38]. Recent studies suggest it also has promising effects on relapsed B-ALL cases in combination with chemotherapy, inducing a complete response in the majority of cases [9, 38, 42, 43].

Compared to unconjugated monoclonal antibodies, conjugated monoclonal antibodies appear to be more efficient in treating pediatric ALL [38]. Inotuzumab ozogamicin is a monoclonal antibody attached to calicheamicin, an anti-tumor antibiotic showing promising results in preliminary tests on patients with refractory ALL [38, 44]. Its mode of action specifically targets CD22-positive cells, which incorporate calicheamicin leading to cellular toxicity [38]. Additionally, SAR3419 is a monoclonal antibody linked to the natural cytotoxic compound dregeanin DM4 [45]. SAR3419 is used to target CD19, a cell surface marker expressed on all immature B-lymphocytes and can therefore be used in treating pediatric B-ALL cases [9, 45]. It was also recently identified as having synergistic effects when administered in combination with chemotherapy drugs presently used in induction therapy in pediatric ALL [46].

Second, another important group of compounds used in targeted ALL treatments are tyrosine kinase inhibitors. Because constitutive expression of tyrosine kinase inhibitors plays

an important role in development of leukemia and eventually resistance to treatment, targeting these receptors is a great way to also target development and progression of the disease [9, 34]. Specific molecules can target the two major groups of tyrosine kinase receptors in pediatric ALL: the BCR-ABL (in Philadelphia positive ALL, referred to Ph+ ALL) and the FLT3 tyrosine kinases [9, 34].

The recent discovery and approval of imatinib in 2013, a BCR-ABL tyrosine kinase inhibitor, greatly changed the overall long-term survival of pediatric patients with Ph+ leukemia from approximately 40% to 85% [9, 47]. In fact, when administered with conventional chemotherapy, patients treated with imatinib had similar long-term survival as patients undergoing hematopoietic stem cell transplantation [48]. Unfortunately, like many other types of treatments for pediatric ALL, resistance to treatment develops [49, 50]. Although very rare in children, point mutations of the ABL domain appear to be one of the leading causes of resistance to treatment and eventually causing death of the patient [9, 34, 49, 51]. After discovery of imatinib, development and approval of dasatinib and ponatinib, second and third generation BCR-ABL tyrosine kinase inhibitors respectively, quickly went underway in order to counter the resistant ALL cases [34, 51-53]. These two drugs show promising results in recent clinical trials on targeting BCR-ABL resistant cells. In addition to being more effective against Ph+ ALL, they are more efficient at crossing the blood-brain barrier and targeting leukemic cells in the central nervous system to prevent any potential relapse [9, 54].

FMS-like tyrosine kinase 3 – also known as FLT3 – is a tyrosine kinase receptor usually present in CD34+ pre-B progenitor cells that is essential in normal hematopoiesis [9, 34, 55]. However, when abnormally expressed by activating mutations, FLT3 upregulates proliferation pathways leading to B-ALL development [9, 34]. Although FLT3 gain-of-

function mutations are rare in pediatric ALL cases, therapies targeting mutant forms of the protein consist mainly of lestaurtinib in combination with chemotherapy are administered to patients diagnosed with FLT3-driven ALL [9, 56]. Although this combination has shown promising results in certain cases, it still needs to be improved in order to attain better complete response rates on a regular basis [9].

Third, drugs that target nucleic acid synthesis are also used in treating certain cases of pediatric ALL. Initially, this drug family was rarely used in treating pediatric ALL since they are not specific and extremely toxic, but recent improvements have enabled use of these compounds in either high-risk or relapse cases [9, 57]. Clofarabine, a molecule inhibiting DNA polymerase and DNA repair among other things, is sometimes used in combination with conventional chemotherapy in relapse or resistant cases [58]. In other instances, clofarabine is used as a monotherapy in patients that already received high doses of cytotoxic therapy [59-61]. More recently, the Children's Oncology Group (COG) is evaluating clofarabine administration in combination with drugs used during induction therapy for high-risk pediatric ALL patients [9]. Another compound in this category is the prodrug nelarabine; once it enters a cell, it is metabolized to a nucleoside analog and rapidly gets phosphorylated [9]. It is then inserted into the DNA sequence, prevents DNA synthesis, and induces apoptosis [9, 62]. Interestingly, since nelarabine is more potent in T-cell malignancies compared to B-cell malignancies, it is more often used on T-ALL patients who either relapse or are unresponsive to treatment [9, 63]. It is currently undergoing a clinical trial on a potential addition in induction therapy for pediatric T-ALL cases [9].

Fourth, compounds that inhibit serine and threonine kinases, responsible for irregular growth pathways, are also used in targeted therapy against pediatric ALL. Overexpression of

the serine/threonine kinases leads to cell proliferation and development of leukemia [9]. There are several types of serine/threonine kinases that can potentially be targeted for therapy in pediatric ALL [9, 34]. First, the Janus kinase (JAK) family of tyrosine kinases are great targets in high-risk pediatric ALL patients in which the JAK-STAT pathway is aberrantly expressed [9, 34]. The JAK pathway is usually overexpressed in high-risk B-ALL patients, as well as in children with ALL and Down syndrome [9, 34, 64]. Unfortunately, not much is known about the efficacy of JAK-STAT inhibition in pediatric ALL since clinical trials are currently underway [9, 34].

Another type of serine/threonine kinase that is studied in pediatric ALL cases is the mammalian target of rapamycin (mTOR). Aberrant activation of the PI3K/Akt/mTOR pathway is involved in accelerated cell growth and proliferation, which leads to development of leukemia [9, 34, 65]. Hence, targeting the mTOR pathway using mTOR inhibitors is an efficient way to target ALL with a hyperactive mTOR signaling pathway. In recent years, preclinical and clinical studies have determined a synergistic effect between mTOR inhibitors and chemotherapy drugs used in induction and maintenance therapies, such as cyclophosphamide, methotrexate, doxorubicin, etoposide, asparaginase, prednisone, and vincristine [66-70]. Several mTOR inhibitors exist in treating pediatric ALL. mTOR inhibitors, such as rapamycin or any of its analogs, have been greatly studied *in vitro* on ALL cell lines – especially pre-B ALL – and seem to have positive effects [34, 71]. In addition, combination studies in Ph+ leukemic cell lines demonstrate synergistic effects when rapamycin is combined with daunorubicin *in vitro* [72]. Not only did the combinational approach demonstrate a higher cytotoxicity in these cell lines, but it also increased autophagy and blockage of the cell cycle when compared to daunorubicin monotherapy [72].

Development of steroid resistance in pediatric ALL patients is one of the leading causes of treatment failure [68]. Interestingly, rapamycin treatment can actually sensitize steroid-resistant cells, which can potentially lead to countering steroid-related resistance [68]. These preclinical results provided a rationale to pursue clinical trials in pediatric ALL patients. Hence, ongoing clinical trials in combining rapamycin or its analog temsirolimus with cytotoxic compounds have been studied in relapsed or resistance pediatric ALL (NCT01403415) [73].

Fifth, targeting a cancer cell's survival ability is another suitable approach for chemotherapy. In normal cells, there is a balance between apoptosis and cell survival mechanisms. In cancer cells, however, the balance is shifted towards an interruption of the apoptotic pathways and upregulation of survival genes and their downstream effectors [9]. Since there are many genes involved in pro-survival mechanisms in leukemic cells, several agents can be administered to patients depending on their gene expression profiles [9, 34]. Bortezomib, a proteasome inhibitor, prevents ubiquitin-dependent proteasome degradation, which subsequently inhibits the action of NF-κB and activates pro-apoptotic and cell cycle regulators such as *p53, p21, p27,* and *Bax* [9, 34]. The overall effect of bortezomib treatment is to increase effects of chemotherapy drugs by lowering the apoptosis threshold of leukemic cells [9, 34, 74-76]. Treatment of ALL cell lines with bortezomib has shown positive effects, prompting its use in numerous clinical trials in pediatric ALL patients [77]. As a monotherapy, bortezomib has been shown to be inefficient. As demonstrated in a phase I combination clinical trial with reinduction chemotherapy drugs, it has very promising effects in resistant pediatric ALL [9, 34, 78, 79]. The subsequent phase II part of this trial further demonstrated

positive effects of bortezomib combination with reinduction cytotoxic treatment in both B- and T-ALL resistant patients [34, 79].

Another pro-survival target in pediatric ALL are heat shock proteins (HSPs) – more specifically Hsp90. These proteins are involved with leukemia cell formation by trafficking and stabilizing oncogenes required in development of a malignant proliferative phenotype with abnormal survival properties [9, 80, 81]. In addition, elevated amounts of Hsp90 in patients are correlated with poor prognosis; hence, targeting these proteins in high-risk cases can potentially increase overall survival [81]. In preclinical studies, tanespimycin, an Hsp90 inhibitor, repressed growth of ALL cells *in vitro* and has even been shown to synergize with imatinib in Ph+ ALL cell lines [9, 82]. More recently, Akahane and colleagues demonstrated that NVP-AUY922, a much stronger inhibitor for the Hsp90 protein, promotes apoptosis in T-ALL cells [83]. This product is already in clinical trials for solid tumors and can potentially be an interesting candidate drug in treating T-ALL pediatric patients [84]. Despite recent advances in Hsp90 inhibitor development, these drugs are not frequently used in clinic since a therapeutic window has not yet been established [83, 85].

Other anti-apoptotic proteins frequently overexpressed in pre-B-ALL and T-ALL are members of the Bcl-2 family. Not only are these proteins important in regulating apoptosis, but they also play key roles in resistance to treatment during induction therapy [86]. Development of navitoclax and obatoclax, two Bcl-2 family inhibitors, was necessary in order to treat patients with highly resistant forms of ALL [87]. Both inhibitors show promising effects *in vitro* on pediatric ALL cell lines in addition to clinical studies on adult patients suffering from ALL and AML [88-91]. However, clinical trials have yet to be conducted on

pediatric patients [88-91]. Thus far, preclinical anti-Bcl-2 therapy has been shown to be more effective on t(4;11) ALL cell lines compared to all other forms of the disease [92].

2.2.4 Relapsed and Resistant pediatric ALL

While the majority of pediatric ALL patients are cured with current chemotherapy regimens, approximately 20% experience a relapse, with boys relapsing more frequently than girls [9, 11, 34]. Research on relapsed ALL patients has identified leukemic blast populations that have acquired chromosomal aberrations and genetic mutations, making them more resistant to chemotherapy treatment compared to blasts isolated at initial diagnosis [93-98]. Furthermore, genomic studies on blast cells present at relapse have determined an overexpression of the DNA repair and cell survival gene pathways compared to blasts at diagnosis [93, 99].

ALL relapse is primarily caused by expansion of a clonal population leading to treatment resistance [93]. Recent studies on ALL relapse have concluded it is a clonal disease. In fact, studies by Mullighan and colleagues have determined that cells present at relapse are also present at initial diagnosis at a very low concentration. This cell population then undergoes clonal expansion, populate the bone marrow and sanctuary sites in the body, and drive disease relapse [93, 95].

There are many factors that determine how clonal expansion affects the development of chemoresistance. Mutations and deletions of tumor suppressor genes may appear in the relapse blast cells that were not present in the initial cell population [93]. For example, a study by Hof and colleagues determined that p53 tumor suppressor gene, deleted in over 10% of the

relapse population, was correlated to a poor prognosis [100]. Deletions of the zinc finger protein IKZF1 were commonly identified in patients with relapsed Ph+ B-ALL [101, 102]. In these studies, IKZF1 deletions were correlated with a negative prognosis compared to patients diagnosed with Ph+ B-ALL in which IKZF1 was still present [93, 101, 102]. Resistance to prednisone and mercaptopurine treatment was also linked to deletions of *MSH6*, a gene in the mismatch repair pathway, leading to overall poor patient outcomes [103, 104]. Additionally, prednisone resistance has been linked to mutations of glucocorticoid receptors in the relapse population [95, 104, 105]. In general, a patient's response to prednisone administration is a determining factor in development of treatment resistance or disease relapse [68]. Hence, patients having a decreased response to prednisone treatment also have higher rates of resistance and relapse after initial induction therapy [68]. In other cases, epigenetic mechanisms play critical roles in development of resistance to treatment [106].

At the time of relapse, protocols are implemented for risk stratification [33, 93, 107]. Time since complete remission is the most important factor in determining a positive or negative prognosis [33, 93, 107]. Patients can be placed into one of three different categories depending on the time between complete remission and relapse [33, 107]. Generally speaking, patients who relapse less than 36 months after remission are considered the most difficult to treat and have the worst prognosis with an overall survival rate of less than 30% [33, 93, 107, 108]. On the contrary, patients relapsing after three years are easier to treat, as demonstrated by an overall survival rate exceeding 50% [33, 93, 107]. Another risk factor taken into consideration are the sites of relapse [33]. Most commonly, isolated marrow relapse occurs in approximately 60% of the time and is correlated to a negative prognosis [33, 109]. The majority of remaining extramedullary relapse cases are located in the CNS and testicles,

comprising approximately 25% and 5%, respectively [33, 109]. In some rare situations, a combination of bone marrow and extramedullary relapse can occur, faring a negative prognosis [33, 109]. Furthermore, because patients diagnosed with T-ALL have a greater chance of relapsing early compared to patients with B-ALL, they are more often correlated with a poor outcome [93, 109, 110]. Unfortunately, between 7-23% of patients with T-ALL relapse will have a 5-year overall survival, indicating the need for new types of therapies [93, 109, 110].

With the development of novel chemotherapy drugs that are capable to reduce the incidence of CNS and testicular relapse, isolated extramedullary relapse – or relapse occurring in sites other than the bone marrow – is uncommon nowadays [111]. Patients with isolated CNS relapse are defined as having leukemic blasts in the cerebral spinal fluid (CSF) without any blasts present in the medullary compartment of the bone marrow [111]. Similarly, patients with isolated testicular relapse do not have blast cell accumulations in the bone marrow but will experience enlargement of one or both testicles due to blast infiltration [111]. According to the majority of research on relapse patients, the main reason why these two organs are ideal sanctuary sites for leukemic blasts is the inability for chemotherapeutic agents to penetrate the blood-brain and blood-testes barriers [111]. Other studies on testicular relapse cases points towards a cooler temperature in the testes compared to the rest of the body as another potential reason of relapse, which may lead to decreased efficacy of certain chemotherapy drugs at that location [112].

Some important factors at the time of diagnosis can actually predict which patients are predisposed to isolated extramedullary CNS or testicular relapse [111]. For instance, in patients with T-ALL, certain chromosomal translocations such as t(4;11) forming the MLL-

AF4 fusion protein and t(9;22), and presence of blast cells in the CNS at the time of diagnosis are all predictors of CNS relapse [113, 114]. In addition, patients diagnosed at an older age and who have a delayed response to induction therapy are also more prone to isolated CNS relapse [113, 115]. Moreover, patients diagnosed with T-ALL during the early puberty years are more prone to developing an isolated testicular relapse [116].

Once risk stratifications have been established, there are multiple ways to go about treating patients experiencing a relapse. Depending on their risk stratification, most patients undergo strict treatment regimens that will decrease blast counts in their sites of relapse. For example, treatment schedules for high-risk patients will include multi-drug chemotherapy combinations prior to undergoing hematopoietic stem cell transplantation (HSCT) once second remission is achieved [73, 117-120]. On the other hand, low and intermediate risk patients will undergo intensive rounds of chemotherapy without HSCT [73, 117, 118]. Importantly, the cytotoxic drugs used in relapse reinduction were ones not previously used in the initial induction phase [93]. Unfortunately, finding the best drug combinations for relapse patients is extremely difficult and not much progress has been made over the past 20 years in increasing overall survival [93]. In cases of isolated CNS and testicular relapse, intensive chemotherapy regimens using high-doses of methotrexate, cytarabine, steroids and asparaginase are undertaken [121, 122]. In some testicular relapse cases, removal of the affected enlarged testicle is sometimes performed [118, 123]. Usually, unilateral or bilateral testicular irradiation is also completed to prevent any subsequent disease [118, 123].

Unfortunately, even after breakthrough discoveries in pediatric ALL treatment, many patients experience multiple relapses. In addition, their chances of overall survival decrease for every ensuing relapse from approximately 44% to 27% in second and third relapses,

respectively [93, 124]. Another point that raises concern is the inability to treat some patients adequately after first relapse since they experience high toxicity and many devastating side-effects due to treatment [93, 125, 126]. Hence, novel treatment methods need to be developed and implemented in order to achieve better overall survival rates for pediatric ALL patients, preferably using less cytotoxic compounds.

2.2 Pediatric AML, Current Treatments, and Relapse

2.2.1 Biology of pediatric AML

Acute myeloid leukemia, also known as acute non-lymphoblastic leukemia, is a heterogenous malignant disease affecting myeloid, erythroid, and megakaryocyte cell precursors [11, 127, 128]. Overall, long-term survival in pediatric AML cases has dramatically improved over the years, reaching roughly 50-65% in developed countries worldwide [11, 27].

AML can be subdivided into seven different classes in adults and children according to the French, American, and British (FAB) and World Health Organization (WHO) classification systems depending on the blood cell progenitor affected and its level of differentiation [129]. For a detailed list of AML subtypes according to FAB classifications, see table 1.

Table 1: The French, American, and British (FAB) classifications of AML

FAB Classification	AML Subtype
M0	Undifferentiated AML
M1	Acute myeloblastic leukemia without maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M5	Acute monoblastic leukemia
M6	Erythroblastic leukemia
M7	Acute megakaryoblastic leukemia

AML is a multifactorial disease involving cooperative mechanisms between several cellular pathways in order to trigger disease onset [130, 131]. Development and progression of AML requires two important genetic abnormalities classified as being either type 1 or type 2 mutations [14, 132, 133]. Type 1 mutations affect genes involved in the signal transduction pathways which increases cell proliferation and inhibits apoptosis, whereas type 2 mutations

include chromosomal rearrangement of transcription factors resulting in formation of fusion proteins that impair differentiation processes [14, 134]. Consequently, when both type 1 and 2 mutations are present at a given time, the resulting groups of cells acquire a high self-renewal capacity and an uncontrollable proliferative ability leading to onset of AML [134].

Like ALL, there are numerous predispositions associated with AML. For instance, peak age incidence for pediatric patients is either in the first four weeks after birth (also referred to as congenital leukemia) or during adolescence, with boys being more prone to developing the disease [11]. In addition, children with Down syndrome have a higher risk of developing AML; however, these patients are very well treated, with overall survival attaining 85% [135, 136].

After diagnosing pediatric AML, patients undergo risk stratification assessments in order to determine appropriate treatment methods to attain long-term survival [11, 27]. The most important risk consideration is age: adolescent patients have a worse prognosis compared to patients below two years of age [11]. In addition, obese patients tend to be negatively correlated with long-term survival [11]. Patients with M0 and M7 subtypes are usually correlated to prominent treatment responses [11]. Other characteristics like the t(8;21) and t(15;17) chromosomal translocations are generally correlated with positive outcomes [11]. Another important risk factor taken into consideration prior to beginning treatment is high blast concentrations at diagnosis, which is usually indicative of a resistant disease associated with very high death rates [137]. Because onset of AML is linked to many mutated genes, one way to improve treatment methods is to identify and target these mutations prior to beginning chemotherapy. The ability to detect chromosomal aberrations and genetic mutations in AML is critical since they are also found to play important roles in relapsed and resistant AML

[127]. For example, high levels of AML1-ETO or CBFB-MYH11 fusion proteins are known to be associated with decreases in both patient survival and treatment responses [127, 138]. Hence, by specifically targeting these fusion proteins in personalized therapies, patients can potentially achieve better outcomes.

2.2.2 Treatment of pediatric AML

AML treatment consists of induction and consolidation therapies. First, induction therapy specifically aims at decreasing leukemic cell concentrations in order to attain remission [11, 139]. Induction therapy in AML involves combining cytosine arabinoside with either doxorubicin or daunorubicin [11, 139]. For higher risk AML patients, additional drugs are administered, such as etoposide and thioguanine [11, 139]. Similarly to ALL therapy, CNS directed therapy is also given to patients in order to prevent leukemic blast homing into the CNS [127].

Once remission is attained, the second phase of treatment, termed consolidation therapy, is initiated. Consolidation therapy involves administration of cytosine arabinoside and etoposides at very high doses [11, 139]. In some patients, certain cytogenetic aberrations are correlated with negative disease outcomes. Hence, in order to increase their quality of life and overall survival, hematopoietic stem cell transplantation is conducted immediately after induction therapy [11, 93, 127]. Furthermore, in cases where minimal residual disease (MRD) is elevated at the end of the induction phase, patients undergo hematopoietic stem cell transplantation (HSCT) in order to prevent any occurrence of relapse [73, 93, 101, 140].

2.2.3 Relapse of pediatric AML

Despite recent treatment advances, approximately 35-45% of pediatric patients diagnosed with AML will experience a relapse [9, 141-143]. The two most common relapse sites are the bone marrow and CNS, the former being much more prevalent than the latter [127]. Patients who experience a relapse immediately undergo reinduction therapy. In AML, drugs used in treating relapsed patients include cytarabine, fludarabine, and anthracycline [144, 145]. In addition to these drugs, patients also receive CNS targeted therapy to limit leukemic cell infiltration into sanctuary sites [127]. Ideally, once second remission is attained, patients receive HSCT [127].

3.0 Epigenetics

Epigenetics is the study of any cellular event that changes gene activity or expression without changing the actual DNA sequence [146]. These changes in gene expression can then be passed down to daughter cells during meiosis and mitosis [146-149]. Many types of epigenetic modifications exist in order to conduct cellular epigenome reprogramming that are regulated by a variety of different enzymes [146, 147, 149, 150]. Other than methylation and acetylation – the two most studied types of modifications – epigenetic regulation can also be directed by phosphorylation, ubiquitination, and sumoylation [146, 147, 149, 150].

The field of epigenetics can be separated into three main classes: DNA methylation, chromatin modifications, and microRNAs [146, 151]. Overall, epigenetic changes are necessary for normal cell development, differentiation, and survival; hence, any deregulations in epigenetic pathways may have drastic consequences on the cell's phenotype [146, 147, 149, 150]. Unlike somatic mutations, changes to a cell's epigenome are actually reversible and therefore constitute interesting therapeutic targets [152, 153].

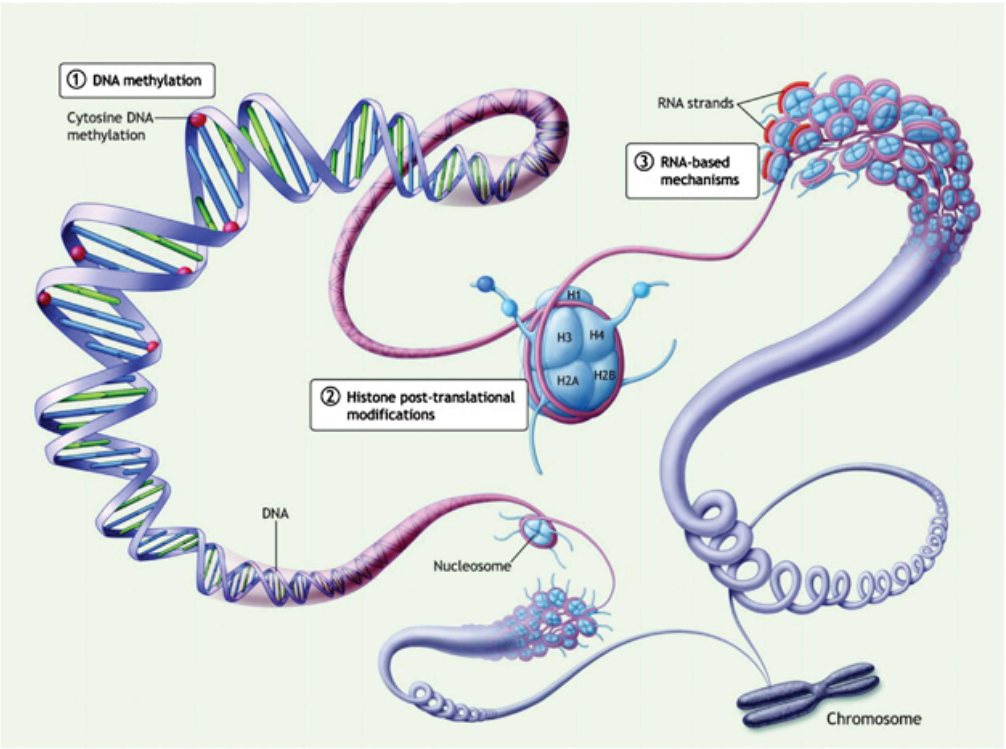

Figure 5: The three classes of epigenetic modifications.

(1) DNA methylation affects cytosine residues (red circles) of CpG sites on primarily on promoter regions. (2) Post-translational histone modifications occur at different histone tail residues. H3 and H4 subunits are most studied regarding gene expression patterns. (3) RNA-based mechanisms by short noncoding RNA sequences, such as micro-RNAs also regulate gene expression levels. These sequences are transcribed from DNA but do not undergo translation [154].

Epigenetic proteins can be functionally characterized as being writers, erasers, or readers [155, 156]. Epigenetic writers are enzymes that transfer epigenetic marks onto histones or DNA residues [155, 156]. Popular epigenetic writers are histone and DNA methyltransferases (DNMTs) and histone acetyltransferases (HATs) [156]. On the contrary, epigenetic erasers remove epigenetic marks from histones and DNA (i.e. histone and DNA demethylases and histone deacetylases) [156]. Finally, epigenetic readers are proteins that bind onto these epigenetic marks, thereby transmitting an epigenetic signal by either activating or silencing gene transcription through recruitment of other proteins [156]. There exist many

types of epigenetic readers that bind to the multitude of epigenetic marks previously mentioned. For instance, the bromodomain proteins recognize histone residue acetylation, whereas Methyl CpG binding protein 2 (MeCP2) binds onto methylated DNA residues [156].

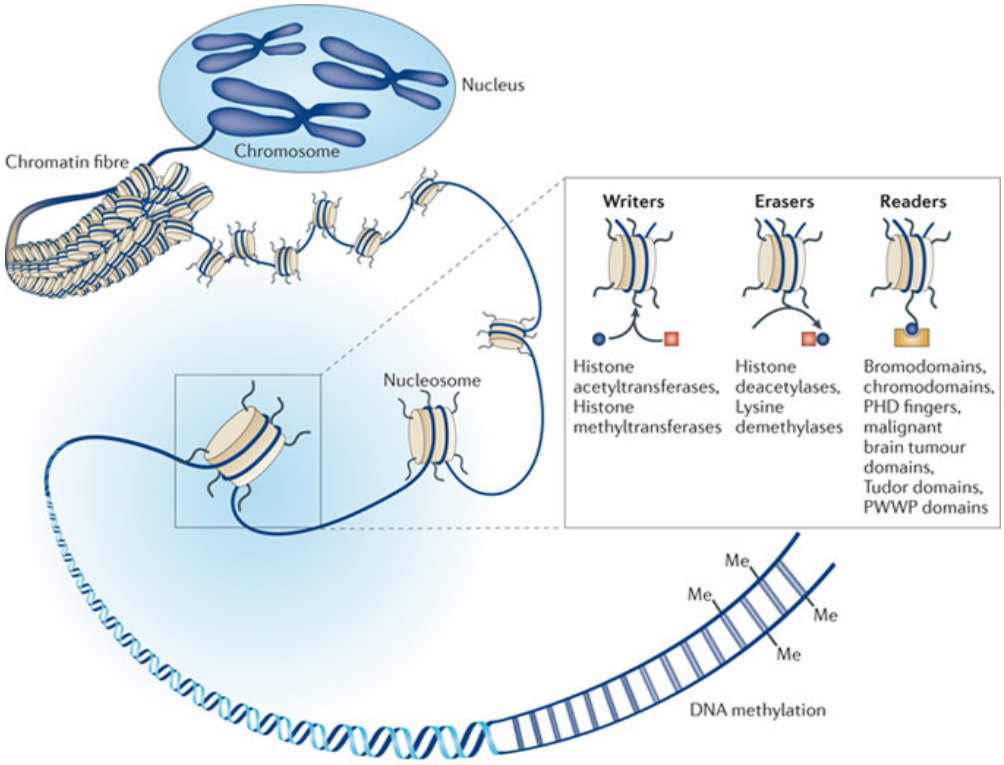

Figure 6: Examples of epigenetic readers, writers, and erasers.

Depending on their function, epigenetic enzymes can be classified as being writers, erasers, or readers [157].

3.1 DNA Methylation

DNA methylation refers to covalent addition of a methyl group by a DNMT enzyme to cytosine residues located primarily on CpG dinucleotides. The methyl group donor for this reaction is S-adenosyl-L-methionine (SAM) [158]. Areas in the genome are enriched in CpG sites, which are refered to as CpG islands. These genomic DNA sequences are defined as stretches of approximately 200 base pairs comprising of more than 50% cytosine and guanine repeats [159]. CpG islands are scattered throughout the human genome, but are more concentrated in repetitive sequences, imprinted genes, and over half of gene promoters [160-162]. In normal cells, DNA methylation is required for maintaining a given gene expression pattern when genetic material is passed from mother cell to daughter cells [151]. It is also necessary in genetic imprinting and X chromosome inactivation [160, 163-165]. In addition, CpG island methylation is necessary for cell differentiation and normal cell development [162]. It has also been shown to play important roles in expressing tissue-specific genes [162].

There are four main types of DNMTs: DNMT1, DNMT2, DNMT3a, and DNMT3b. DNMT1 is required for maintaining a cell's methylation patterns as it divides into two daughter cells thereby transmitting epigenetic information from one generation of cells to the next [160, 166, 167]. Hence, as a cell divides, DNMT1 uses the parental strand as a template to appropriately conserve methylation patterns [160, 165]. On the other hand, DNMT3a and DNMT3b are enzymes responsible for addition of new methyl groups to cytosine residues that were not previously methylated, also known as "de novo" DNA methylation. Interestingly, DNMT2 does not methylate DNA sequences, but targets cytosine residues in transfer RNA (tRNA) instead [168].

DNA methylation can lead to gene silencing by a variety of different mechanisms. First, DNA methylation acts as a direct transcriptional silencer mainly by recruiting DNA methylation readers, also known as methyl-binding domains (MBD) and other chromatin repressor complexes onto CpG islands of gene promoters [150, 169-171]. There are five MBD proteins identified thus far, with the most studied one being MeCP2 [169]. Once bound onto methylated cytosine residues, these MBD proteins are able to recruit additional co-repressors that induce changes in chromatin structure, leading to gene silencing [169]. Types of co-repressors recruited by MBD proteins include histone methyltransferases (HMTs) and histone deacetylases (HDACs) [172]. In this manner, DNA methylation alters chromatin dynamics that will influence gene expression. In order to activate gene transcription, transcription factors bind onto unmethylated CpG islands in promoter regions. Hence, by methylating CpG islands, transcription factors are blocked and cannot accomplish their function [169, 170, 173]. Another means of gene silencing induced by DNA methylation is recruitment of methyl-binding proteins (MBPs), which bind onto methylated cytosine residues and prevent the binding of transcription factors [169, 170, 173].

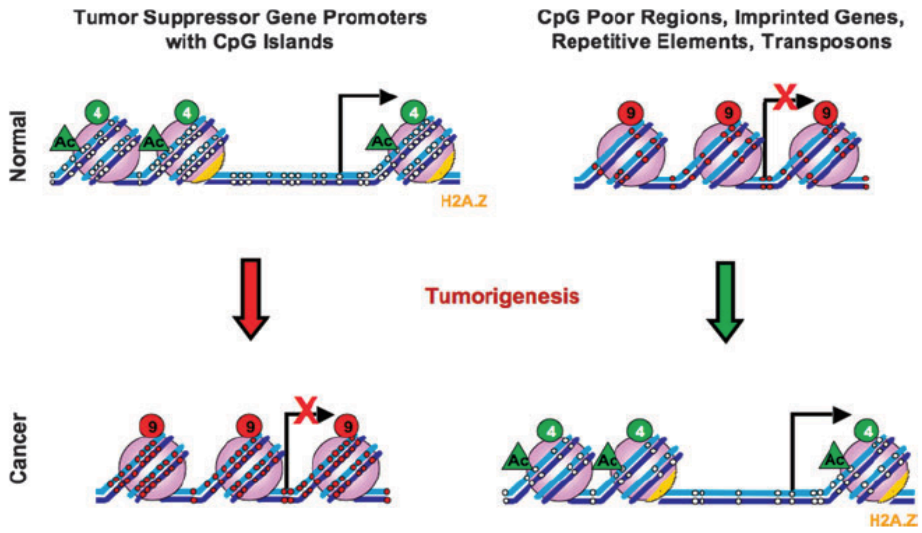

Figure 7: Differences in DNA methylation levels between normal and cancerous tissues.

In normal tissue, lack of DNA methylation on promoter regions of tumor suppressor genes allows transcription to be initiated and expression to be turned on. In regions of repetitive elements, oncogenes, and transposons, promoter hypermethylation allows gene expression to be turned off. In cancer, a hypermethylated promoter for tumor suppressor genes is indicative of transcriptionally inactive genes. On the other hand, promoters for imprinted genes, repetitive elements, transposons, and oncogenes are hypomethylated, allowing gene expression. Figure taken from Sharma and colleagues [150].

3.2 Histone Post-Translational Modifications

The ability for a cell's nucleus to contain very long strands of DNA would not be accomplished without the presence of histone proteins [174, 175]. DNA is tightly wrapped around histones to form nucleosomes [147, 174, 175]. The nucleosome is formed by a histone octamer [147, 174]. Each octamer is made up of four histone proteins that form the nucleosome core: H2A, H2B, H3, and H4 [147]. Nucleosomes are connected to one another by linker DNA and are packed together to form chromatin [147, 174].

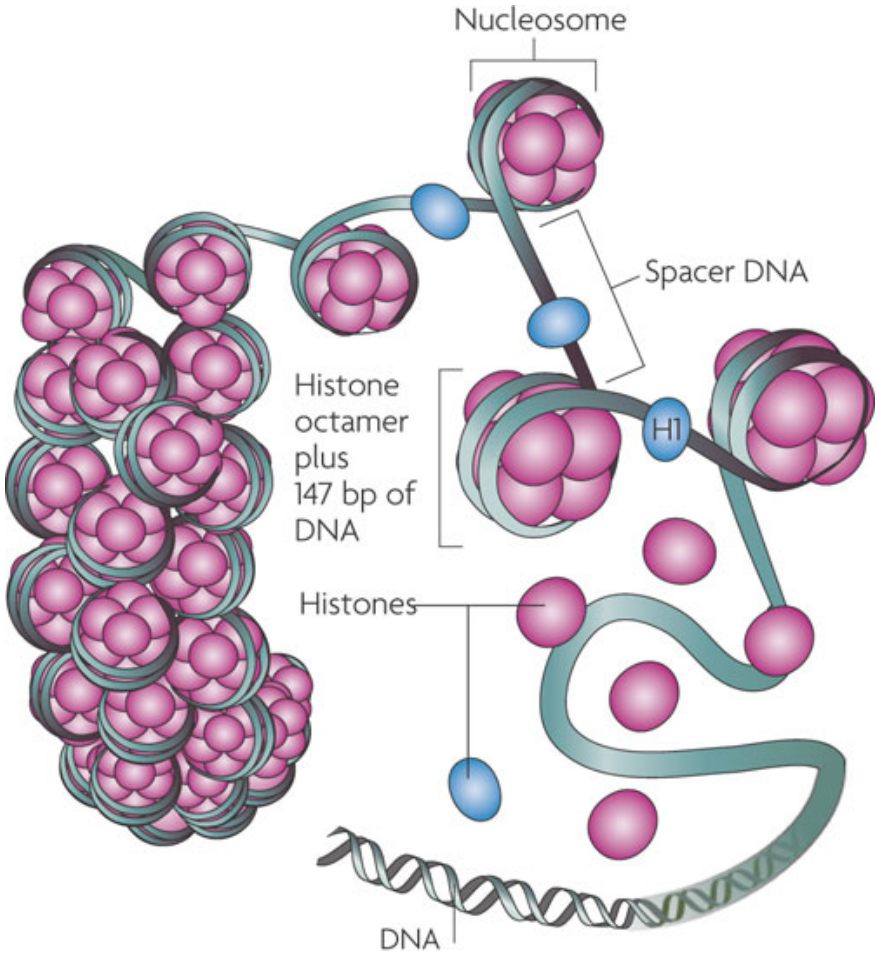

Figure 8: Nucleoprotein complex of DNA and histone forms chromatin.

DNA is wrapped around a histone octamer made up of two copies of each histone subunit forming a nucleosome. Each nucleosome is separated by spacer DNA [176].

Each histone contains a C-terminus region, which forms the globular portion of each nucleosome, and an N-terminus region, forming a tail that can be covalently modified post-translationally by a variety of different enzymes. Depending on the post-translational modifications, chromatin can be either loosely or tightly organized, referred to as euchromatin and heterochromatin, respectively [147, 174, 177]. Whereas euchromatin favors gene transcription, heterochromatin is so tightly configured that gene transcription is almost non-existent [147, 174, 178]. The two most commonly studied histone modifications are acetylation and methylation [147, 150]. Other post-translational modifications that play a role in gene expression changes include phosphorylation, sumoylation, and ubiquitination [147, 150].

Fascinatingly, two models exist regarding the effects of histone acetylation on structural chromatin changes and regulation of gene expression. The first model describes acetylation of histone H3 and H4 subunits playing key roles in changing chromatin polarity [147]. The abundance of lysine residues on histone tails makes them inherently positive in polarity [147]. Hence, when lysine residues are not acetylated, they are tightly compacted with the negatively charged DNA strands to form heterochromatin [147]. On the other hand, when lysine residues are acetylated, the positive charges are neutralized, supporting the loose chromatin structure favoring gene transcription [147]. In the second model, however, acetylation of lysine residue 14 on histone 3 (H3K14ac) by P300 induces nucleosome eviction, mediated by the histone chaperone Nap1 [179]. Once the nucleosome is removed, transcription factor complexes are assembled and gene expression is turned on [179]. Luebben and colleagues demonstrated the importance of H3K14ac in mutation studies where they

mutated lysine residues on histone H3 and realized that transcription was inexistent, demonstrating the necessity of H3K14ac in nucleosome eviction and gene transcription [179].

Acetylation and methylation of histone tail residues is regulated by enzymatic reactions involving epigenetic writers and erasers [2, 147]. HATs and HDACs are enzymes involved in adding and removing acetyl groups on histone residues, respectively [2, 147]. Histone methyltransferases (HMTs) and histone demethylases (HDM), on the contrary, are involved in adding and removing methyl groups, respectively [2, 147].

Compared to DNA methylation, histone modifications are more complex in terms of activating or repressing gene transcription [150, 174]. Two factors come into play when studying gene expression changes from histone modifications [150, 174]. First, the type of modification present on a tail residue will determine whether gene transcription will be active or repressed [150, 174]. Second, the position of the modified tail residue will influence formation of either euchromatin or heterochromatin [150, 174]. In most cases, histone tail acetylation is associated with an increase in gene expression [150, 180]. Histone tail methylation, on the other hand, can either be an activating or repressive mark, depending on the position of the histone residue [150, 181]. In addition, when referring to methylation, histone residues (either lysine or arginine) can either be mono-, di-, or trimethylated; the number of methyl groups added to a given residue can also determine whether genes are transcriptionally active or repressed [150, 181].

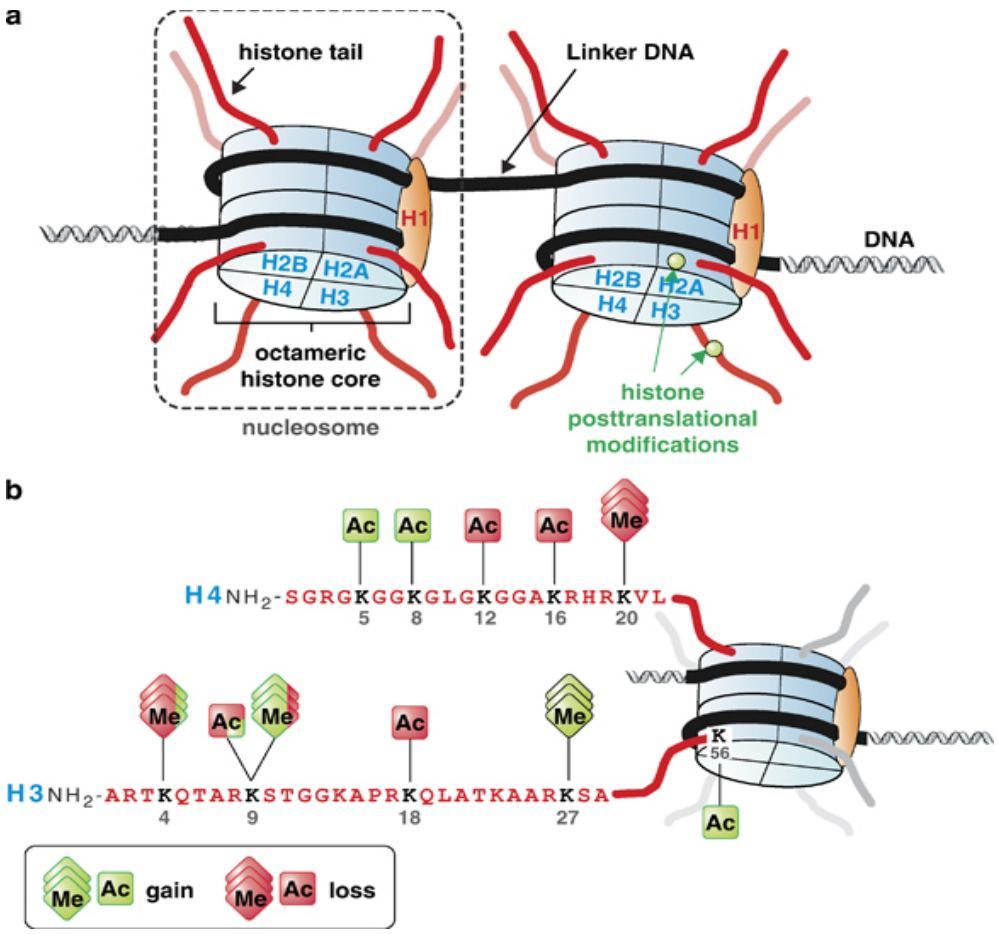

Figure 9: Histone tail modifications.

a) Schematic representation the four histone subunits (H2A, H2B, H3, and H4) forming an octamer. Linker DNA strands connect adjacent nucleosomes. Green circles show possible post-translational histone modifications. **b)** The most studied histone tail modifications on H3 and H4 subunits. The two most commonly studied histone tail modifications are acetylation (Ac) and methylation (Me). With regards to methylation, histone tail residues can be mono-, di-, or trimethylated. Loss and gain of acetylation or methylation is depicted as either green or red, respectively. Most commonly studied histone residue affected by post-translational modifications is lysine (K). Figure taken from Fullgrabe and colleagues [182].

3.2.1 Histone Acetyltransferases

With regards to activation of gene transcription, histone acetylation is by far the most studied post-translational modification. HATs will be classified under MYST, GNAT, or CBP HAT families [2, 180, 183]. In addition, all HATs share one common feature: they all use acetyl-CoA as an acetyl group donor in their enzymatic reactions [184, 185].

The MYST family of HAT enzymes has five key members in mammals: HBO1, MOF, MORF, MOZ, and TIP60 (table 2) [147, 183-186]. The defining feature of all HATs in this family is the presence of a MYST domain and a histone acetylation reader domain termed "bromodomain". This particular domain is a fusion between an acetyl-binding motif and a zinc finger. Depending on their binding partners, MYST HATs exhibit different functions, either oncogenic or tumor suppressive. For example, members of this family are able to act as transcriptional co-activators for important proteins regulating cell proliferation and survival pathways such as NF-κB and the oncogene C-MYC [184, 185]. They can also assume apoptotic and anti-proliferative functions by activating the apoptosis pathway via p53 acetylation [185]. They have also been linked to activating genes involved in response to DNA damage and repair, such as ataxia telangiectasia mutated (ATM), a protein involved in p53 and and γ-H2AX phosphorylation [183]. Once phosphorylated, both proteins activate and recruit DNA repair machinerie to the necessary sites [185].

Table 2: The MYST HAT family and their target histone subunits.

HAT Enzyme	Target Histone(s)	Complex
MOZ	H2A,H2B,H3,H4	-
TIP60	H4, H3, H2A	TIP60 complex
HBO1	H3,H4	HBO1 complex
MOF	H4*, H3, H2A	MSL complex
MORF	H4,H3	-

*: Preferred target

There are three major enzymes that make up the GNAT family of HATs in humans: GCN5, PCAF, and HAT1 (table 3) [183, 186, 187]. Members of the GNAT family contain a HAT domain and a bromodomain and preferentially acetylate residues on the H3 histone subunit [183]. Along with the MYST family of HATs, GNAT also acetylates the tumor suppressor p53, thereby activating its apoptotic functions [188].

Table 3: The GNAT HAT family and their target histone subunits.

HAT Enzyme	Target Histone(s)	Complex
GCN5	H3*, H4	STAGA and TFTC complex
PCAF	H3*, H4	PCAF complex
HAT1	H4, H2A	HAT1 complex

*: Preferred target

Members of the CBP HAT family are slightly different from members of the other two classes for two main reasons (table 4). First, the structure of CBP HATs differs significantly

when compared to the other two classes [189]. Unlike the other two classes, CBP and P300 contain cysteine-histidine rich regions (C/H rich-2 and C/H rich-3 regions) [190]. Second, dissimilarities in the interacting proteins – and consequently in protein complexes – are also observed [189]. Other than acetylating histone residues, these proteins are also involved in acetylating p53 tumor suppressor gene, thus mediating apoptosis [183]. It has been recently shown that CBP and P300 are involved in acetylating proteins that shuttle between the nucleus and the cytoplasm [191].

Table 4: The CBP and P300 HATs and their target histone subunits.

HAT Enzyme	Target Histone(s)	Complex
CBP	H2A, H2B, H3, H4	-
P300	H2A, H2B, H3, H4	-

Although all HATs share very similar core domain structures across all three families, each enzyme will have specific histone and residue targets on histone tails, primarily lysines on either histones H3 or H4 [183, 190, 192]. For example, members of the GNAT family are more specific for H3 tail residues, whereas MYST members prefer H4 substrates [183, 190, 192]. The CBP/P300 family does not have a specific preference on any histone subunit and acetylates H2A, H2B, H3, and H4 subunits at equal frequencies [183, 190, 192]. In addition, the ability for HATs to form protein complexes with members same family further increases both activities and their targets [183, 190, 192].

A very important relationship exists between histone acetylation and epigenetic readers, since initiation of gene transcription is often dependent on both of these proteins [106, 155, 193, 194]. Three main types of histone acetylation readers have currently been

discovered, with the most characterized family being the bromodomains [155]. Recently, two other types of histone acetylation readers have been discovered: the PHD finger domain of DPF3b zinc finger protein and the double pleckstrin homology domain of RTT106 chaperone [195, 196]. Overall, epigenetic readers have a strong affinity for consecutive acetylated histone residues and are occasionally required for transcription factor activity to be completed [155].

3.2.2 **Histone Deacetylases**

HDACs are epigenetic erasers that remove acetylation from histone tail residues. They can be separated into four main classes, depending on their tertiary peptide sequences, cellular localization, and functions. HDACs 1, 2, 3, and 8 are located in the cell nucleus, making up class I [2, 174, 197, 198]. Class I HDACs play key roles in deacetylating histone proteins in the nucleus [2, 174, 197, 198]. Class II HDACs (4, 5, 6, 7, 9, and 10) are present in the cytoplasm and the nucleus, functioning as a deacetylating enzyme on non-histone proteins [2, 174, 197]. The Sirtuin family of enzymes (sirtuin 1-7) makes up HDAC class III [2, 174, 197]. Finally, HDAC11, a cytoplasmic protein, is the only enzyme making up class IV [2, 174]. Since it exhibits sequence homology to both classes I and II, HDAC11 is considered a hybrid between both HDAC classes [2, 174, 197]. Interestingly, HDACs of different classes are known to interact with each other when performing their functions; in some cases, they even form protein complexes on histone tails [2, 174, 197]. In order to accomplish protein deacetylation, HDACs require cofactors [2, 174, 197, 198]. Classes I, II, and IV require Zn^{2+}, while the sirtuin family requires NAD^{+} [2, 174, 197, 198].

4.0 The C-MYC Oncogene in Leukemia

In cells displaying a normal phenotype, *C-MYC* is a master transcription regulator that plays important roles in a variety of different cellular processes like cell cycle progression, protein synthesis, cell adhesion, and apoptosis [199, 200]. Hence, expression levels of the C-MYC transcription factor are tightly regulated via mRNA and protein mechanisms. Short C-MYC mRNA half-life and stability triggers its rapid degradation when normal cells do not present necessary upstream signals [201]. Additionally, post-translational modifications like ubiquitination, acetylation, and phosphorylation of specific residues on the C-MYC protein modify its half-life, thereby regulating the amount of proliferative MYC-target genes activated at a given time [201-206]. Hence, all these factors aimed at maintaining C-MYC at low levels prevent cells from undergoing cellular transformation into a hyperproliferative phenotype [201].

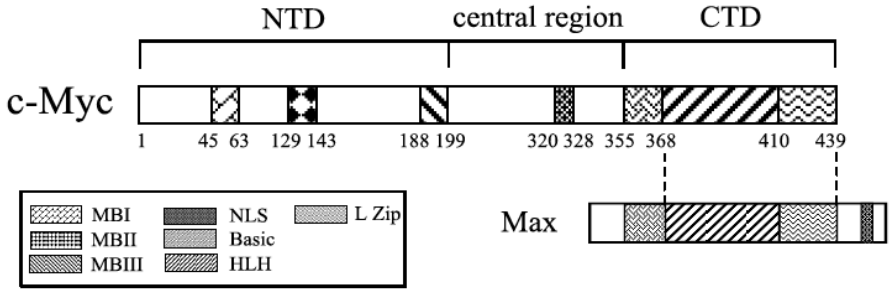

Figure 10: Structure of the human C-MYC oncogene.

The C-MYC oncogene is divided into three main regions identified as N-terminal domain (NTD), the central region, and the C-terminal domain (CTD). The NTD contains the three Myc-box regulatory motifs. The central region contains the nuclear localization sequence (NLS). The CTD contains the basic helix-loop-helix domain, as well as the leucine zipper. Depicted in the figure is the interaction between C-MYC and Max in the C-terminal domain. Figure taken from Chen and colleagues [200].

The human C-MYC protein is a member of the helix-loop-helix leucine zipper transcription factor family. It contains a transactivation domain in the N-terminal portion [200]. In addition, there are three MYC-box segments (MBI, MBII, MBIII) that contribute to the function of the protein [207, 208]. The C-terminal region contains the important helix-loop-helix region that enables its association and dimerization with the MYC-associated factor X (MAX), forming a protein complex that activates transcription of MYC-target genes [201, 209-211].

C-MYC is required in hematopoiesis and lymphopoiesis [212]. Many hematopoietic progenitors are in a C-MYC protein equilibrium, which is required to drive normal blood cell development [212, 213]. C-MYC plays a critical role in determining whether a hematopoietic stem cell will undergo self-renewal or differentiation [212, 213]. In cases of C-MYC overexpression, a hematopoietic stem cell (HSC) will differentiate instead of self-renewing [212, 213]. On the other hand, when C-MYC expression is low, HSC undergo self-renewal [212, 213]. Furthermore, C-MYC expression is necessary for both B- and T- lymphocytes differentiation and receptor formation [212, 214-217].

In tumor cells, all the regulatory mechanisms controlling C-MYC mRNA and protein stability are dysfunctional [211, 218]. The *C-MYC* promoter is frequently the target of many cell-signaling pathways that are aberrantly overexpressed, such as WNT, RAS/RAF/MAPK, JAK/STAT, and NF-κB [201, 211]. Hence, these changes induce an increase in both C-MYC protein levels and function, leading to increases in C-MYC target genes and forcing cells to proliferate at very high rates [201]. In other cases, C-MYC is constitutively expressed because of activation mutations or chromosomal translocations [201, 218-221]. For these reasons, C-

MYC expression and function are greater than any other oncogene in human cancers, especially in sarcoma, leukemia, lymphoma, and myeloma [201, 222-225].

Before upregulating expression of its target genes, the MYC-MAX complex recruits additional proteins to transcription start sites. Some of these proteins regulate chromatin dynamics by acetylating histone tail residues (HATs GCN5 and CBP) thereby exposing transcription start sites, while others influence activity and recruitment of RNA polymerases and other transcriptional regulators [201, 211, 226]. Overall, the MYC-MAX complex is involved in cellular transformation by increasing expression of genes involved in cell proliferation, cancer metabolism, and protein biosynthesis, thus inhibiting expression of important tumor suppressor genes [201, 211, 227].

Although C-MYC is involved in normal blood cell development, it is oftentimes implicated in initiation, progression, and resistance to treatment in B- and T- ALL. *In vitro* studies conducted in the 1980s linked high *C-MYC* expression to development of different types of blood malignancies, especially leukemia [228-230]. In these studies, leukemia initiation was correlated with the ability to block cell differentiation and initiate abnormal proliferation pathways [212, 228-230]. Three chromosomal translocations (t(8;14), t(8;22), and t(2;8)) are involved in activation of C-MYC leading to leukemia in approximately 5% of adult and pediatric patients [231]. In other cases, C-MYC protein stability is significantly increased, thereby stimulating its overall activity [232]. In pediatric B-ALL, *C-MYC* can occasionally be associated with the *TEL2* oncogene, which further amplifies development of the disease [233]. Cooperation between C-MYC and another oncogene is also observed in T-ALL patients. Mutations in the NOTCH1 signaling pathway, which are observed in almost

50% of T-ALL cases, induce increases in C-MYC mRNA and protein levels, consequently leading to target gene activation [225, 234].

Unfortunately, the structure of the C-MYC protein and the lack of an active site or ligand-binding domain have prevented development of strong inhibitors [200, 207, 235, 236]. Recently, many groups have tried developing small molecule inhibitors that will either target transcription of the *C-MYC* gene, the C-MYC/MAX dimerization, or the binding of the C-MYC/MAX complex onto DNA transcription start sites [200].

Another way to indirectly inhibit C-MYC in cancer is to target histone acetylation. C-MYC is recruited by bromodomains that bind to acetylated histone residues [237, 238]. Hence, several researchers have developed a rationale to target these bromodomain proteins in order to decrease *C-MYC* activation by preventing its binding and transcription initiation [237, 238]. There are three members of the bromodomain and extra-terminal (BET) family of proteins that associate with C-MYC, termed BRD2, BRD3, and BRD4, with the latter being the most pertinent in terms of C-MYC association and activation [207, 239].

Hence, the link between BET and C-MYC proteins prompted the development of JQ1, a selective BET bromodomain inhibitor (BETi), which blocks C-MYC recruitment onto acetylated histone tail residues and ultimately blocks transcription of C-MYC target genes [240]. Many *in vitro* and *in vivo* studies showing promising effects of JQ1-targeted C-MYC inhibition on different types of cancers have recently been published [207, 240-244].

JQ1 treatments have shown promising results on B- and T-ALL leukemic cell lines and patient samples – including pediatric patients – as well as animal mouse models [239, 243, 245, 246]. In pediatric precursor B-ALL, Da Costa and colleagues demonstrate that not only does JQ1 inhibit C-MYC transcription, but it also decreases its stability [243]. In high-risk

pediatric B-ALL models, Ott and colleagues discovered that JQ1 inhibits both C-MYC and IL-7, two proteins that play crucial roles in development and progression of both B- and T-ALL leukemia [239]. In AML, Herrmann and colleagues discovered that JQ1 actually targets leukemia stem cells and leukemia progenitor cells, two key players in development of leukemia and resistance to cytotoxic therapies [246].

Despite all these promising results, cells develop resistance to JQ1. In fact, some leukemia cells do not respond to JQ1 treatment, particularly cells that are associated with resistant and relapsed diseases [106, 242, 247]. Despite their promising results, Ott and colleagues mention that mice treated with JQ1 eventually die of leukemia, which was, upon further analysis, most likely associated with upregulation of C-MYC protein levels [239]. In another study, Rathert and colleagues determined that upon JQ1 treatment, AML cell lines and patient samples overexpress Wnt signaling genes that confer resistance to treatment [247]. Taken together, these studies clearly show a major problem in JQ1 treatments despite promising results initially published. Therefore, novel means to inhibit C-MYC must be developed in order to target leukemia cancer cells.

5.0 Epigenetic Aberrations in Cancer

Over the course of the past ten years, epigenetic alterations have been linked to development and maintenance of many types of cancers [147, 248, 249]. At first, many researchers believed that cancer was a disease based solely on genetic mutations. However, in 1983, the first studies – lead primarily by Andrew Paul Feinberg and Bert Vogelstein – linking DNA methylation to cancer were published [146, 250-253]. Many research groups then focused on epigenetic mechanisms leading to cancer development and subsequent therapies.

Cancer is frequently described as having global hypomethylation, with hypermethylation at promoter regions [1, 2, 147]. Levels of general hypomethylation can lead to expression of genes not usually present on a normal basis, like certain oncogenes and transposable elements that may insert themselves into different parts of the genome [161, 254, 255]. Once transposable elements are actively transcribed, they are able to increase a cell's oncogenic potential by either leading to certain chromosomal abnormalities or inserting themselves directly into oncogene sequences [161, 254, 255]. On the other hand, the genes usually affected by hypermethylation patterns are very frequently tumor suppressors [147, 256]. Hence, because DNA methylation patterns are extremely important in disease development, they are used for risk stratification in pediatric neuroblastoma and predict a complete response in adult CML [257-259].

Two studies by Esteller in 2007 and 2008 established the link between histone modifications and DNA methylation, and their subsequent effect on gene expression in cancer [260, 261]. These studies revealed that two histone modification marks, H4K16ac and H4K20me3, are reduced in areas of DNA hypomethylation in repetitive elements, or areas of

the genome that are usually silenced [261]. Furthermore, decreases in H3K9ac and H3K4me2/3 as well as increases in H3K9me2/3 and H3K27me3 were observed in hypermethylated promoter regions of tumor suppressor genes in cancer cells [260].

Overall, changes in a cell's epigenome are important players in cancer development and progression [147, 256]. These changes lead to a complete dysregulation of a cell's normal gene expression patterns that lead to a high proliferative potential and an increase in cell survival pathways [147, 256].

5.1 Epigenetic Aberrations in Pediatric Leukemia

Both in AML and ALL, epigenetic modifications lead to silencing of critical tumor suppressor genes or activation of certain oncogenes resulting in development of malignant cells [262, 263]. Recent whole genome analyses by Downing et al. have demonstrated the importance of epigenetic alterations in the development of pediatric cancers in two independent studies, including pediatric leukemia [262, 263]. In both studies, they discovered that some epigenetic histone-modifying enzymes, such as *CREBBP*, *EED*, *EP300*, *EZH2*, *PHF6*, and *SETD2* are the ones most frequently mutated in AML and ALL, resulting in epigenetic deregulation of histone methylation and acetylation levels [262]. In addition, fusion proteins containing epigenetic enzymes are also found in pediatric leukemia, which might accentuate degrees of histone acetylation or methylation. For example, translocations involving the HMT *MLL* occur in approximately 5% and 10% of pediatric AML and ALL cases, respectively [264]. In both leukemia subtypes, a general upregulation is observed in the HOX gene family (*HOXA3*, *HOXA5*, *HOXA7*, *HOXA9*, and *HOXA10*) [264]. Translocations

involving HATs, CBP with either MOZ or MORF, are also observed in certain cases of the M5 AML classifications [1, 265].

Abnormal DNA methylation patterns also play an important role in silencing or activating genes in pediatric leukemia. For instance, genes involved in regulating cell cycle checkpoints, such as *p15*, *p16*, and *p21*, and the *FHIT* and *PTEN* tumor suppressor genes are silenced in pediatric leukemia cases [266]. Other genes silenced in leukemia include *ER*, *SDC4*, and *MDR*, and calcitonin [266, 267]. Examples of oncogenes hypomethylated in leukemia cases are *LINE-1*, *DEK*, and *KRAS* [161, 267, 268].

5.1.1 Epigenetic Aberrations in Pediatric ALL

Aberrant DNA methylation in pediatric ALL is one of the hallmarks underlying disease development and progression [10, 95, 262, 263, 267]. Increases in DNA methylation in pediatric ALL patients are frequently observed around promoters of tumor suppressor genes. For instance, many cell cycle regulators like *p21* (*CDKN1A*), *p16* (*CDKN2A*), and *p15* (*CDKN2B*) are found to be hypermethylated in pediatric ALL compared to healthy cells [269-271]. In 2014, Chatterton and colleagues compared the methylation status between B-ALL and healthy patient samples and discovered a hypermethylated state in 325 genes, whereas 45 genes were hypomethylated [272]. Amongst the differentially expressed genes between the two populations – which included various kinases, cytokines, and transcription factors – they observed upregulation of genes involved in cell signaling, cell-to-cell interactions and survival. Another study by Nordlund and colleagues in 2013 identified promoters of important transcription factors, such as *NANOG*, *OCT4*, *SOX2* and *RESR*, to be hypermethylated in a large cohort of patients with pediatric ALL [273].

To further emphasize the importance of DNA methylation in pediatric ALL, Figueroa and colleagues performed genome-wide cytosine methylation analyses in pediatric B- and T-ALL patient samples. They determined a correlation between promoter CpG island methylation and genetic subtype of ALL (B- or T-ALL), highlighting the importance to distinguish methylation patterns at diagnosis prior to starting treatment in order to treat specific subsets of patients [267]. Overall, they discovered that genes involved in development of B- and T-cell receptors were silenced by hypermethylated promoters [267]. They also illustrated that genes involved in cell signaling (*TIE1*, *MOS*, and *CAMLG*) cell cycle regulation (*DGKG*), cell proliferation (*MCTS1*), transcription factors (*PROP1*. *TAF3*, *H2AFY2*, *ELF5*, and *CNOT1*) and HOXA gene clusters (*HOXA5* and *HOXA6*) share a similar methylation signature between both ALL subtype [267]. Musialik and colleagues discovered that genes involved in B-cell development are silenced in some cases of B-ALL. When analyzing patient samples, they determined that promoters for genes involved in early hematopoiesis (*HOXA4* and *HOXA5)*, were hypermethylated and silenced in approximately 20% of their patient samples with B-ALL [274]. The gene with the most hypermethylated promoter was *TAL1*, a transcription factor regulating hematopoiesis in early progenitor cells [274].

Not only does DNA hypermethylation play key roles in development and progression of pediatric ALL, but it is also implicated in relapse and drug resistance [94, 275]. Whole-genome methylation studies found that genes were generally hypermethylated in relapse patients compared to newly diagnosed patients [105]. In addition, DNA hypermethylation levels in relapsed patients may play a role in activating the WNT pathway. Common WNT target genes include cadherins proteins (*CDH1* and *CDH11*) as well as SOX genes (*SOX2*,

SOX8, *SOX11*, and *SOX21*) making cells prone to proliferating and surviving after chemotherapy treatment [105, 276].

In addition to changes in DNA methylation, aberrant histone modifications are also a determining factor in development of a malignant phenotype [275, 277]. Gain- or loss-of-function mutations in histone modifying enzymes are frequently observed in pediatric ALL cases [275]. Many HAT enzymes, such as GCN5, MYST, and HBO1, were recently discovered to be upregulated in B-ALL cases, causing general increases of histone acetylation levels [277]. It is important to note that in order to activate transcription, C-MYC is recruited to areas of the genome rich in histone acetylation by bromodomains [207]. Hence, when HATs are dysfunctional, so is C-MYC target gene expression. Occasionally, HATs acetylate and stabilize non-histone proteins that may have roles in development of leukemia. Upregulation of GCN5 has been associated with acetylating and stabilizing the E2A-PBX1 fusion protein, thus increasing its stability and downstream gene activation [278].

Some HATs, such as TIP60, P300, CBP, and GCN5, have also been linked to stabilizing the C-MYC oncogene by acetylating certain lysine residues on the protein [205, 206, 279]. TIP60 and GCN5 specifically target lysines K323 and K417, whereas CBP/P300 acetylate lysines K143, K157, K275, K317, K323, and K371 [205, 206, 279]. Two of these lysines (K323 and K417) are substrates of both acetylation and ubiquitination. Hence, when these residues are acetylated, ubiquitination is inhibited, allowing proteins to avoid proteasomal degradation [211, 279]. For this reason, acetylation does not only increase stability of C-MYC, but it also inhibits its degradation. Unfortunately, the exact mechanism of C-MYC acetylation is not yet characterized. Moreover, studies linked high C-MYC expression

to decreased overall patient survival and overall in a variety of different cancers, including breast cancer, gastric cancer, and leukemia [280-282].

In relapse studies, it was determined that mutations in the CBP HAT domain have been correlated to poor patient outcome and resistance to treatment [283]. Mullighan and colleagues identified a mutation in CBP HAT domain in approximately 20% of relapse cases correlating loss of function mutation and resistance to prednisone treatment [283]. Additionally, they identified other mutations in epigenetic regulating enzymes such as *EP300*, *EZH2*, and *CTCF* present in the relapse patient samples that were either not present at initial diagnosis or they were at a very low concentration [283]. However, it is still unclear if these mutations are drivers or passengers in ALL. A study on whole genome transcriptome analysis performed on relapsed leukemia patient samples found deregulation of the epigenetic modifier PRMT2 as well as chromatin modifying proteins CBX3 and MIER3 [284]. Moreover, Inthal and colleagues linked CBP HAT domain mutations to cases of hyperdiploid pediatric ALL [285].

Numerous other studies have linked overexpression of HDAC protein levels to poor patient outcomes [286, 287]. Moreno and colleagues discovered an upregulation of HDAC6 and HDAC9 in B-ALL and HDAC1 and HDAC4 in T-ALL [286]. Elevated levels of HDAC4 have also been shown to play key roles in development of resistance to prednisone in T-ALL patients [287]. Other studies conducted on ALL patient samples associated an increase in HDAC3, HDAC7, and HDAC9 activity with a poor patient outcome [286, 288].

Furthermore, leukemia is a disorder characterized by the formation of fusion proteins [147, 275]. Numerous fusion proteins involving histone-modifying enzymes exist in pediatric ALL, and some are actually fundamental in disease development, progression, and relapse [147, 283, 285, 289, 290]. In ETV6-RUNX1 and TCF3-PBX1, two subtypes of B-ALL, a

gain-of-function mutation of the HMT protein NSD2 induces a conversion of H3K36me1 to the silencing histone mark H3K36me2 on promoters of lymphoid developmental genes, such as *CD69*, *CD52*, *NKD2*, and *CD200* just to name a few [291].

The MLL fusion proteins play important roles in leukemia development [264, 292-296]. In normal cells, MLL has a dual function, primarily in normal hematopoiesis: it acts as a transcription factor and an H3K4 methyltransferase involved in transcriptionally active chromatin [292, 294, 295, 297]. In order for MLL to activate transcription of its target genes, it recruits CBP and MOF in order to further loosen the chromatin structure and upregulating MLL-target gene expression [298]. The H3K79 methyltransferase DOT1L interacts with MLL-AF9, MLL-AF10, MLL-AF17, and MLL-ENL to increase MLL-target gene reactivation. The most studied MLL target genes are the HOX gene family, consisting of HOXA and HOXB clusters, involved in normal hematopoietic differentiation into the different blood cell lineages [299, 300].

In leukemia, MLL is frequently involved in formation of gain-of-function fusion proteins that lead to development and progression of leukemia, primarily by increasing expression of HOX genes [292, 297]. MLL fusion proteins affect approximately 10% of pediatric ALL and over 60% of infant ALL patients [264]. To date, there are over 50 fusion proteins identified with the most frequently observed fusion partners being ENL and AF9, proteins functioning as transcriptional activators by recruiting the SWI-SNF chromatin-remodeling enzyme [114, 294, 298]. Other popular MLL fusion proteins include AF4 (associated with pro-B pediatric ALL), AF6, AF10, and AFX1 [114, 294, 299]. Moreover, a study by Zangrando and colleagues identified a unique subset of genes that are upregulated in

MLL-rearranged pediatric ALL; this gene list included MEIS1, a protein known to upregulated expression of HOX genes required to transform cells into a cancerous state [264].

5.1.2 Epigenetic Aberrations in Pediatric AML

Recently, sequencing technologies have enabled scientists to discover novel mutations in epigenetic enzymes targeting both DNA methylation and histone modifications that play roles in development of the disease [301-303]. For example, DNA methylation enzymes frequently found mutated in adult AML are DNMT3a, TET2, IDH1, and IDH2 [304]. Other enzymes affecting chromatin modifications are ASXL1 and EZH2, as well as all MLL protein translocations [304].

As in the majority of cancers, DNA methylation levels in AML patients are abnormally high in promoter regions and generally low in other parts of the genome [305]. Since numerous chromosomal translocations observed in AML affect histone modifying enzymes, post-translational modifications in histone tail residues are more commonly associated to disease onset compared to DNA methylation levels [306, 307]. Despite drastic differences in AML subtypes, when comparing any type of leukemic blast to a normal myeloblast, there is a global hypermethylation in the malignant phenotype [304, 308]. Generally speaking, these high methylation levels demonstrate the important roles of altered DNA methylation levels in development of AML [304, 308].

The most common chromosomal translocations in AML involve the histone methyltransferase MLL, forming various MLL-fusion proteins involved in H3K4 methylation, thereby activating gene transcription [293, 304, 309]. Frequent targets for MLL targeted upregulation in AML patients are the HOX genes [304, 309]. Two other histone modifying

enzymes involved in translocations are the HATs MOZ and CBP [310]. In addition, chromosomal translocations involving RUNX1/RUNX1T1 and PML/RARA form protein complexes that change chromatin dynamics to inhibit gene expression [311-313]. Although the mechanism is not yet elucidated, these fusion proteins also recruit DNMT enzymes to their complexes, which increase DNA methylation levels and repress gene transcription [314, 315].

EZH2, an H3K27 trimethylase part of the polycomb repressive complex, is mutated in about 2% of AML patients [304, 316]. The complexity in EZH2 function is still not well characterized, but recent research on this subject matter point to a dual role as an oncogene and tumor suppressor [317, 318]. In AML, EZH2 gain-of-function mutations are associated with increased levels of silencing chromatin marks, as well as recruiting DNA methyltransferases to promoters of tumor suppressor genes and genes involved in cellular differentiation [319-321]. On the other hand, studies on EZH2 loss-of-function mutations have reported a shift to a more stem-like state, potentially giving rise to leukemic stem cells. Consequently, formation of these cancer stem cells are involved in resistance to treatment and disease relapse [317, 319-321].

6.0 Epigenetic Therapies

Although cure rates have dramatically improved over the last 40 years, there are still many aspects of treatment that need to be advanced in order to increase remission and reduce both relapse rates and short- and long-term toxicity. As mentioned previously, epigenetic marks are chemically reversible modifications and can therefore be targeted by drugs that are known to induce epigenetic changes [152, 153]. Hence, epigenetic drugs – or epi-drugs – can be used to target DNA methylation patterns or induce chromatin remodeling [147, 152, 153, 249]. By this approach, epi-drugs induce their effects by targeting epigenetic readers, writers, or erasers.

Currently, the US Food and Drug Administration has approved five epigenetic drugs for cancer treatment: two DNA methyltransferase inhibitors (DNMTi) azacytidine and decitabine, as well as two histone deacetylase inhibitors (HDACi) vorinostat and romidepsin (see table 4) [322-325]. It is important to mention that all four epigenetic drugs approved thus far are only used in adults, and no epi-drugs are approved for pediatric patients.

Table 5: Approved epigenetic drugs.

Name	Class	Indication
Decitabine	DNMTi	Myelodysplastic Syndrome and AML
Azacitidine	DNMTi	Myelodysplastic Syndrome and AML
Vorinostat	HDACi	Cutaneous T-Cell Lymphoma
Romidepsin	HDACi	Cutaneous T-Cell Lymphoma
Belinostat	HDACi	Peripheral T-Cell lymphoma

Adapted from Mack and colleagues [326].

6.1 DNA Methyltransferase Inhibitors as Epigenetic Therapy

Decitabine (5-aza-deoxycytidine) and azacytidine (5-aza-cytidine) both act as nucleoside analogs that are incorporated into dividing cells to form a complex with DNMTs leading to their degradation, thereby inhibiting their function [327, 328]. When treating patients with DNMTi, low-dose treatment induces an epigenetic effect, while high-dose treatments induce a cytotoxic effect due to formation of DNA adducts [324, 329]. Therefore, by treating patients with low-dose DNMTi, cytotoxic side effects are limited [324, 329].

As mentioned previously, promoter regions of tumor suppressor genes in cancer cells are found to be hypermethylated, leading to epigenetically silenced gene expression. Other than silencing many tumor suppressor genes, DNA methylation changes also affect key cellular pathways [330]. Some genes frequently silenced by abnormal DNA methylation patterns in cancer include *p16* and *DAPK* involved in regulating the cell cycle and apoptosis, respectively. In addition, genes involved in suppression of the WNT signaling pathway and DNA repair are two additional targets of DNA hypermethylation leading to gene silencing [330].

DNMTi were first introduced in the 1980s as a potentially novel drug for treating hematological diseases, including pediatric leukemia [331-334]. The most common side effect of DNMTi treatment is myelosuppression [329]. Despite leading to increases in survival and overall positive effects, decitabine has some negative points, such as non-specific demethylation of target regions, very low stability in solution and short half-life [147, 335]. Unfortunately, remission duration for patients treated with DNMTi is generally short, and

development of resistance to treatment is oftentimes observed [335]. In order to improve DNMT inhibition, researchers began working on several other DNMTi analogs that are more stable, more specific, and less toxic than the two original FDA approved drugs [336, 337]. These novel drugs, which include DHAC (dihydro-5-azacytidine), FCDR (5-fluoro-2`-deoxycytidine), zebularine, and SGI-110, are currently in clinical trials [336, 337]. Therefore, the need to combine DNMTi treatment to other types of epigenetic or cytotoxic treatments may be the key in treating certain sub-groups of patients.

6.2 Histone Modifying Drugs As Epigenetic Therapy

In normal cells, a balance exists between histone modifying enzymes that add and remove epigenetic marks, enabling cells to proliferate at a normal pace, differentiate, and eventually activate the apoptosis pathway [336, 338]. Abnormal expression or activity of one or more of these histone-modifying enzymes may induce malignant cellular transformation that leads to cancer development, mainly by silencing tumor suppressor genes or activating oncogenes [336, 338]. The diversity of histone-modifying enzymes enables researchers and clinicians to target specific histone modifications, enabling the use of histone-modifying drugs as epigenetic therapy [336, 338].

6.2.1 Histone Deacetylase Inhibitors (HDACi)

HDACi are divided into four different classes according to their chemical structure [147, 338]. The four classes of HDACi are short-chain fatty acids, hydroxamic acids, cyclic peptides, and benzamides [336]. The vast majority of HDACi, whether they are already

approved by the FDA or presently in clinical trials, target HDAC classes I and II [338, 339]. Their general mechanism of action is to block the catalytic domain of HDAC enzymes [338]. This effect will inhibit HDAC recognizing and binding onto its targets [147, 338]. Overall, HDACi induce gene reactivation, cell differentiation and apoptosis *in vitro* and *in vivo* [147, 338, 340]. They also inhibit colony formation and cell cycle progression [338, 340]. Currently, there are only three HDACi approved by the FDA for the treatment of cutaneous T-cell lymphoma: vorinostat, romidepsin, and belinostat [147, 338, 341, 342]. HDACi increase expression of differentiation and tumor suppressor genes in many cancers [336, 343]. It is also found to inhibit cell cycle progression by specifically activating *p21* and the p53 pathway [277]. Vorinostat and other HDACi also exert their effects through induction of oxidative stress, and increase in gene expression of cell cycle regulators and cell-surface death receptors [198, 277].

FDA-approved and newly developed HDACi are currently in numerous clinical trials for treatments of many types of cancers including melanoma, meningioma, leukemia, and prostate cancer (NCT01265953, NCT02032810, NCT01798901, NCT02282917). These drugs are potent anticancer agents in preclinical models, but when administered to patients they have limited effect. Hence, recent evidence has emerged questioning the mechanism and efficacy of HDACi. Because these compounds increase histone acetylation, they can actually potentiate effects of C-MYC recruitment by bromodomains. One study in particular focused on deleting HDACs in lymphoma and leukemia that overexpress the MYC oncogene *in vivo* determined that enhancements of oncogenicity were observed in these animals [344, 345].

6.2.2 Histone Acetyltransferase Inhibitors (HATi)

Due to the fact that HATi have been studied less thoroughly compared to HDACi, they are much less characterized [336]. Development and testing of certain HATi *in vitro* on ALL cell lines have demonstrated efficacy, but only curcumin, a naturally occurring molecule that targets P300/CBP has been integrated into clinical trial testing on pancreatic, colon, and breast cancers (NCT02724202, NCT00094445, NCT00192842) and has shown promising results on prostate cancer *in vitro* [277].

Structural differences between HAT families have permitted the development of molecules that target specific HATs. For instance, α-methylene-γ-butyrolactone 3 (MB-3) specifically targets GCN5. MB-3 can potentially be used as a supplement to chemotherapy drugs in ALL patients harboring the E2A-PBX1 translocation since GCN5 stabilizes it by acetylation [278]. Another molecule currently studied is garcinol, the naturally occurring P300 and PCAF inhibitor. It acts as a HATi by binding onto two sites located on HATs, the acetyl-CoA and the histone binding domains, thereby suppressing cell growth [346, 347].

Apart from curcumin, CBP/P300 inhibitors include ICG-001, chetomin, and C646. ICG-001 inhibits interactions between CBP and β-catenin specifically but has no effect on P300 and β-catenin interactions. When tested against pre-B ALL, ICG-001 induced differentiation and eliminates all resistant cancerous cells when combined with conventional chemotherapy regimens [348, 349]. Moreover, chetomin is a molecule that blocks the interaction between CBP and P300 with HIF-1, which induces cell differentiation in a glioma cell model [350]. Finally, compound C646 specifically targets ETO driven AML by inducing cell cycle arrest and apoptosis [351, 352]. It has also been demonstrated that C646 sensitizes melanoma cells to chemotherapy drugs when treated in combination [351]. Other molecules

targeting TIP60 are also currently being studied. For instance, anacardic acid sensitizes HeLa cells to radiotherapy, whereas NU9056 and TH1834 decrease cell viability in *in vitro* models of prostate and breast cancer cells, respectively [353-355].

6.3 Combinational Epigenetic Therapies

In general, treating cancer is impossible with single-agent therapy because of tumor heterogeneity and rapid development of resistant clones in both leukemia and solid cancers [356]. The same is true concerning epigenetic therapies; as a single agent, epigenetic drugs do not show strong treatment responses when administered to patients. However, in combination, the potential of epigenetic therapy can be efficiently applied. Malignant cancer cells become addicted to certain levels of DNA methylation and specific histone tail modifications in order to survive [357, 358]. Hence, by targeting more than one epigenetic mark, malignant cells lose the ability to express certain key proteins required for their survival. Interestingly, several groups have shown promising results in studies that combine epigenetic drugs to either immunotherapies or conventional chemotherapy regimens.

Another fantastic new method in treating many cancers nowadays revolves around immunotherapy. Thus far, patients responding to treatment show elevated overall survival rates and extremely promising results [359]. Unfortunately, most patients do not respond to immunotherapy treatments and additional measures to ensure increased response need to be implemented [111]. The main reason why immunotherapy is ineffective in these patients is because cancer cells find ways to evade the host immune system [360-363]. They do so by either silencing cell surface receptors critical for tumor cell recognition and destruction or overexpressing molecules that generally suppress the immune system [360-363].

The ability for epigenetic drugs to induce reversible changes in gene expression has made them interesting candidates for combinational approaches with immunotherapy. Many preclinical studies have shown extremely promising results in combining epigenetic drugs and immunotherapy. For example, pre-treating cells with epigenetic drugs increases expression of antigen-presenting molecules on the surface of tumor cells [364-368]. They have also been shown to increase presentation of tumor specific antigen and activating ligands for NK cell-mediated cytotoxicity [364, 369-371]. All these factors lead to detection and destruction of tumor cells by the patient's immune system. Many ongoing or completed clinical trials studying the effects of epigenetic therapy in combination with immunotherapy demonstrate the interest in utilizing both methods of treatments for a variety of cancers.

Table 6: Selected clinical trials testing epigenetic drugs in combination with immunotherapy treatment.

Clinical Trial Identifier	Cancer	Epigenetic Drugs and immunotherapy
NCT02546986	Advanced or metastatic NSCLC	Azacytidine + Pembroluzumab
NCT01928576	NSCLC	Azacytidine or Azacytidine + Entinostat and Nivolumab
NCT01038778	Metastatic kidney cancer	Entinostat + Aldesleukin
NCT01241162	Neuroblastoma and sarcoma	Decitabine + Dendritic cell vaccine
NCT01673217	Recurrent ovarian epithelial/peritoneal/fallopian tube cancers	Decitabine+ NY-ESO-1 peptide vaccine
NCT01834248	Myelodysplastic syndrome and AML	Decitabine + DEC-205/NY-ESO-1 fusion protein vaccine

As mentioned previously, epigenetic dysregulation in cancer cells is one important reason cells develop resistance to conventional induction chemotherapy treatment. One means

to counter resistance in cancers is inducing epigenetic resetting or preconditioning prior to or during chemotherapy treatment [372, 373]. However, before combining epigenetic with chemotherapy drugs, the exact mechanism of resistance needs to be characterized in order to avoid antagonistic actions between them [372, 373]. For example, in some pediatric glioblastoma cases, the O-6-methylguanine-DNA methyltransferase (MGMT), DNA repair enzyme, is silenced by hypermethylation; treating these patients with DNMTi will activate this gene and trigger resistance of these cells to cytotoxic therapy [374]. So far, synergy between DNMTi and chemotherapy drugs like paclitaxel and cisplatin have been established in lung and prostate cancer cell lines [375-377].

Along with DNMTi, HDACi are also promising combination drugs in many cancers. Because HDACi facilitate drug-induced apoptosis, combination treatments with chemotherapy drugs are promising to counter resistance and reset chromatin to a non-resistance state [198]. In general, HDACi are known to synergize extremely well with multiple cytotoxic drugs by facilitating apoptosis-mediated cell death, primarily by upregulating apoptosis and downregulating cell survival pathways [338, 378]. For example, after pre-treating cancer cells with either trichostatin A or vorinostat – two HDACi – Kim and colleagues showed the effects of sensitizing cancer cells to chemotherapy; this type of treatment can also be applied to resistant cancer cells *in vitro* or *in vivo* [379]. Qian and colleagues determined a synergistic relationship between HDACi and three cytotoxic compounds, carboplatin, docetaxel, and paclitaxel, in ovarian cancer studies *in vitro* and *in vivo* [380]. Other known synergistic cytotoxic partners for HDACi are gemcitabine, cisplatin, etoposide, and doxorubicin [379, 381-383]. All in all, preclinical and clinical studies have demonstrated promising results in DNMTi and HDACi.

Instead, other groups have opted to combine epigenetic drugs targeting distinct epigenetic regulators. The idea behind combining epi-drugs targeting different regulators of a cell's epigenome has been shown to improve efficiency compared to monotherapy [153, 384]. In a phase 1 and 2 clinical trial, Garcia-Manero and colleagues tested the combination between the DNMTi decitabine and the HDACi valproic acid in patients diagnosed with acute and chronic leukemia [385]. In their study, they determined a synergistic relationship between the two epigenetic drugs and promising results in terms of gene re-expression of several cell cycle regulators and overall patient survival [385]. In some cases, treating with a DNMTi as monotherapy is not sufficient. A study by Si and colleagues demonstrated that despite treating with DNMTi, some genes were not re-expressed because of silencing chromatin marks [386]. This phenomenon was overcome by combining DNMTi to epi-drugs targeting histone modifications, thus demonstrating the importance in using a combinatorial epigenetic therapy approach to target a bigger subset of cancer cells harboring both aberrant histone modifications and DNA methylation levels [386].

Table 7: Selected clinical trials testing epigenetic drugs in combination with cytotoxic or other epigenetic drugs.

Clinical Trial Identifier	Cancer	Epigenetic Drugs
NCT00867672	AML	Decitabine + Valproic Acid
NCT02190695	AML-MDS-CMML	Decitabine + Carboplatin + Arsenic Trioxide
NCT02159820	Ovarian Cancer	Decitabine + Paclitaxel
NCT01928576	NSCLC	Azacytidine + Entinostat

Finally, combinations of epigenetic drugs with non-cytotoxic molecules have also been shown to have promising results. These drug combinations aim to increase response to treatment – and potentially synergizing with the epigenetic drug – while limiting toxic side

effects altogether. The isoflavone genistein, a compound found in soybeans, is a prime example of utilizing these non-toxic molecules shown to have interesting cancer preventative properties in treating the disease [387, 388]. Prior to being used as a potential anticancer drug, several studies demonstrated genistein's ability to synergize with chemotherapy medication and its ability to increase expression of *p15* and *p16* tumor suppressors in cancer cell lines [389-392]. These findings prompted Raynal and colleagues to test genistein in combination with decitabine in their preclinical models for leukemia, in which they found an interesting synergistic combination [393]. Clinical trials using the decitabine-genistein combination are highlighted in table 8.

Table 8: Clinical trials testing the combination of decitabine and genistein.

Clinical Trial Identifier	Cancer	Drugs
NCT01628471	NSCLC and advanced solid tumors	Decitabine + Genistein
NCT02499861	Pediatric relapsed or refractory cancers	Decitabine + Genistein

7.0 Background on Project

Development of new drugs is a very costly and extremely long process. In order for a drug to become commercially available, numerous steps must be completed [394]. The first step in drug development is identifying a potential target protein or pathway that is dysregulated in cancer cells [394]. Researchers then develop drugs to target these proteins or pathways in their preclinical studies and animal models [394]. If results are promising, the drugs enter clinical trials [394]. Once validated in humans for toxicology and indication, drugs are approved by the US Food and Drug Administration (FDA) [394]. Overall, development of a new drug costs over one billion dollars and may take up to 15 years before finally being FDA-approved [395-397].

In order to avoid the lengthy and costly process, drug repositioning – also known as drug repurposing – can be conducted to develop new anticancer therapies [395, 396]. This process consists of using drugs that are already approved by the FDA for one indication in order to treat other disease types, such as cancer [395, 396]. Because drug repositioning uses FDA approved drugs that have a long history of preclinical and toxicological studies, they can quickly be developed or incorporated into treating cancer without passing through rigorous testing [395, 396]. The primary benefits from drug repositioning include much lower cost and reduced side-effects compared to cytotoxic therapies currently used [395].

7.1 Screening FDA approved drugs for epigenetic and anticancerous properties

In 2010, Si and colleagues developed a model to evaluate reactivation of gene expression in colon cancer cells after decitabine treatment [386]. In their model, they attached a green fluorescent protein (GFP) to a hypermethylated cytomegalovirus (CMV) promoter. Hence, if GFP expression is increased, it implies that the drug treatment induces epigenetic changes in either a cell's chromatin structure or DNA methylation levels. Raynal and colleagues used the same model in 2012 when they demonstrated two important findings [398]. First, they characterized DNA methylation levels as the long-term epigenetic signal in determining whether gene expression is turned on or off [398]. Second, they demonstrated the importance of chromatin modifications in transient gene expression changes, implying that chromatin can potentially be an important target in epigenetic therapies against different cancers [398].

The same hypermethylated CMV-GFP model was used in 2016, when Raynal and colleagues screened over 1,100 FDA-approved drugs to assess for epigenetic and anticancer properties using two dose schedules [399]. They separated the eleven most efficient drugs that re-expressed GFP into three different classes, from highest to lowest, respectively [399]. The first class contained drugs that induced the highest GFP expression; the three FDA-approved epigenetic drugs decitabine, azacitidine, and vorinostat were placed in this category [399]. Next, the second group of drugs inducing most gene re-expression contained five cardiac glycosides (ouabain, lanatoside C, digoxin, digitoxin, and proscillaridin A) [399]. Interestingly, all the cardiac glycosides used in their study stimulated GFP re-expression [399]. Finally, the third class contained an antibiotic (oxyquinoline), an anti-cancer drug (arsenic trioxide) and a drug used in treating chronic alcohol abuse (disulfiram) [399].

Interestingly, they also demonstrated changes in intracellular calcium levels as a potential anticancer mechanism of the drugs displaying newly identified epigenetic properties; these same changes in calcium levels were not observed in FDA-approved epigenetic drugs [399]. Hence, they suggested that calcium signaling is a key regulator in activating tumor suppressor gene expression in cancer cells [399].

7.2 Cardiac Glycosides

Cardiac glycosides are naturally occurring compounds that can be subdivided into two major categories: the cardenolides and bufadienolides [400, 401]. All cardiac glycosides share a similar molecular structure consisting of a steroid ring; the steroid ring defines the active portion of the molecule [402]. The only structural difference between cardenolides and bufadienolides is the lactone moiety which is made up of a four-membered or five-membered ring in cardenolides and bufedienolides, respectively [403]. Both classes act as sodium-potassium (Na^+/K^+) channel blockers, leading to increases in intracellular sodium levels. Increases in intracellular sodium levels induce a diminished activity of the sodium-calcium (Na^+/Ca^{2+}) pump, thereby leading to increases in calcium levels and cardiac contractility in patients suffering from arrhythmias and congestive heart failure [400, 401, 403].

Interestingly, cardiac glycoside intake has been correlated to anti-cancerous properties. It has previously been shown that patients taking cardiac glycosides during or prior to chemotherapy treatment not only have less invasive cancers, but also present a decreased mortality risk after induction phase therapy is completed [404-407]. Other epidemiological studies have demonstrated a decreased risk in developing leukemia and urinary tract cancers in patients who are treated with cardiac glycosides [408, 409]. In other

studies, cardiac glycosides were found to block lung cancer cells in the G2/M phase, thereby sensitizing them to radiotherapy *in vitro* [410, 411]. In addition, a study by Felth and colleagues demonstrated synergistic and additive effects of different cardiac glycosides with cytotoxic chemotherapeutic drugs on colon cancer cell lines, further demonstrating diverse anticancer properties [412].

Recent studies suggest that cardiac glycosides also activate downstream signaling pathways leading to cell proliferation, growth, motility, differentiation, and apoptosis [413-417]. There are two main means of triggering signaling pathways induced by cardiac glycoside treatment. First, cardiac glycosides induce oscillations in intracellular calcium levels by activating the phospholipase C and IP3 pathway [418-420]. Depending on the calcium concentrations and the duration of the calcium oscillations, cells will either differentiate, proliferate, or apoptose [421]. Second, calcium changes are able to trigger the RAS-RAF-MAPK cascade. Once activated, RAS induces the release of reactive oxygen species (ROS) from mitochondria [403, 422]. These ROS will activate NF-κB, a protein that will induce transcription of genes involved in cell differentiation [403, 422].

On the other hand, mechanisms inducing anticancer effects by cardiac glycosides have not yet fully been characterized, but there are a few potential speculated mechanisms that explain the anticancer properties of cardiac glycosides. For example, Kometiani and colleagues believe that cardiac glycosides increase expression of the cell cycle regulator p21 via the MAPK pathway [423]. Some groups have also uncovered an inhibitory role against topoisomerase enzymes, while others have identified upregulation of important pro-apoptotic genes in T-cell lymphoma, prostate and lung cancers [424-428]. Another means by which cardiac glycosides are able to induce anticancer effects is by inhibiting glycolysis. Because

glycolysis is upregulated in cancer cells, inhibition of this energy-producing pathway can specifically target these cells for destruction [429, 430].

All in all, many potential mechanisms underlie the antitumor effects of cardiac glycosides; perhaps some mechanisms are activated in specific cell types compared to others but these findings have yet to be fully understood.

7.3 Proscillaridin A

Proscillaridin A is a member of the bufadienolide cardiac glycoside family extracted from the Scilla plant [431]. Interestingly, this cardiac glycoside in particular has been shown to have anticancerous activities in many cell lines during drug-repositioning studies [400, 432, 433]. For example, proscillaridin A induces apoptosis and antiproliferative actions in various breast cancer cell lines *in vitro*, primarily by fluctuations in calcium levels [400, 432]. Furthermore, Bielawski and colleagues determined that proscillaridin A inhibits topoisomerase enzymes in breast cancer cells, which ultimately leads to cell death [434]. Denicolaï and colleagues revealed that proscillaridin A inhibits growth of glioblastoma cell lines *in vitro*, in addition to significantly decreasing tumor volume and increasing survival of mice transplanted with glioblastoma cancer cells [433].

Unfortunately, proscillaridin A, along with other bufedienolides, have a very small therapeutic window – with plasma concentrations between 1 and 10nM in humans – and doses need to be very low to limit cardio- and neurotoxicity [435-437]. In a study by Daniel and colleagues, cardiac glycosides of the bufadienolide family specifically target T-lymphoblasts while completely avoiding healthy non-malignant cells [438]. Despite the high toxicity risk, proscillaridin A has evident anti-tumor effects that can be used to treat cancer patients.

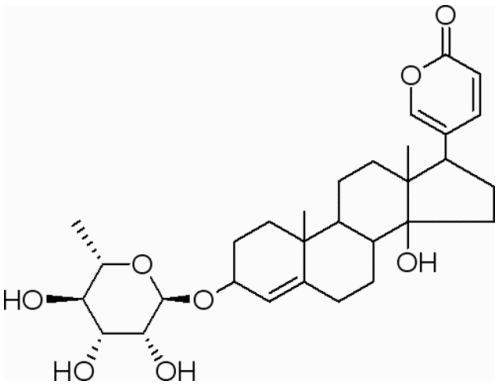

Figure 11: Molecular structure of proscillaridin A

[433]

8.0 Hypothesis

We hypothesize that the cardiac glycoside proscillaridin A will induce anti-leukemic effects by an epigenetic mechanism and can therefore be repositioned to treat pediatric leukemia.

Chapter 2: Article

Title: Targeting histone acetylation and C-MYC degradation in acute lymphoblastic leukemia

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(*In preparation for Oncogene*)

1. Introduction

Acute lymphoblastic leukemia (ALL) is characterized by genetic and epigenetic aberrations that induce a loss of differentiation and an increase in proliferation of lymphoid progenitor cells [15, 16]. Depending on the lymphoid progenitor affected, ALL can be subdivided into two main categories: B- and T-ALL [16, 17]. In children, ALL accounts for approximately 25% of all pediatric cancers with incidence peaking between the ages of 2 and 5 [18, 19]. Although current treatment methods cure over 85% of pediatric patients, those who relapse or become resistant to treatment have a very poor outcome with an overall survival of approximately 25% [10, 20]. Because of its high prevalence, relapsed or refractory ALL is the leading cause of cancer-related mortality among children [439]. Thus, there is an urgent need to develop new therapeutic strategies to increase cure rates for relapsed or resistant pediatric ALL.

Molecular mechanisms underlying B- and T-ALL development include inactivation of tumor suppressor genes and activation of oncogenes [21-23]. Genetic lesions, such as abnormal chromosomal numbers, translocations, and mutations are often identified in the pediatric ALL population [21-23]. Not only are genetic lesions involved in the development of

ALL, but epigenetic alterations have been demonstrated to have prominent roles in pathogenesis as well. Aberrant DNA methylation of promoter CpG islands, defined as stretches of approximately 200 base pairs comprising more than 50% cytosine and guanine repeats, is one of the hallmarks underlying disease development and progression [10, 95, 159, 160, 262, 263, 267, 440]. For instance, hypermethylated CpG islands in promoter regions are frequently observed in tumor suppressor genes, whereas promoters for oncogenes are shown to be in a hypomethylated state, correlated to gene silencing and activation, respectively. In addition to changes in DNA methylation, aberrant histone modifications are also a determining factor in the development of a malignant phenotype [275, 277]. Chromosomal translocations and gain- or loss-of-function mutations in histone-modifying enzymes involved in adding or removing acetyl or methyl groups on histone tail residues are frequently observed in pediatric ALL cases [275, 277, 283-285, 289, 290]. These epigenetic modifications are also linked to treatment resistance and disease relapse [94, 275]. Interestingly, because DNA methylation and post-translational histone modifications are reversible chemical reactions, they have become promising targets for treatment.

Currently, there are five FDA-approved drugs – two DNA methyltransferase inhibitors (DNMTi) and three histone deacetylase inhibitors (HDACi) – targeting epigenetic modifications in adult cancer patients. Notably, none of these drugs are approved for pediatric patients. Two DNMTi, decitabine and azacitidine, decrease overall DNA methylation by blocking enzymes involved in adding methyl groups to CpG islands, subsequently reactivating hundreds of silenced tumor suppressor genes. HDACi, such as vorinostat, romidepsin, and belinostat are drugs that lead to general increases of histone acetylation levels. Because increases in histone acetylation are correlated to increases in gene expression, the properties of

HDACi permit overall epigenetic upregulation. Unfortunately, these epigenetic drugs have limited effects in cancer patients. Hence, the need to develop or discover additional epigenetic drugs is evident, especially in treating pediatric cancers like ALL.

Histone acetylation is catalyzed by histone acetyltransferases (HAT) and is associated with euchromatin and transcriptionally active regions. Histone acetylation is removed by histone deacetylases (HDAC), which create condensed chromatin leading to gene silencing [147]. Recently, decreasing histone acetylation has gained popularity because of its involvement in stimulating activity of oncogenic enhancers. Recent epigenomic studies on pediatric cancers, including leukemia, have demonstrated that histone acetylation attracts bromodomains proteins, which transmit acetylation signals and then recruit oncogenic transcription factors, such as C-MYC [106, 194, 441, 442]. Therefore, histone acetylation represents an attractive target for epigenetic therapy in pediatric ALL.

In order to avoid the lengthy and costly process of approval, drug repositioning – also known as drug repurposing – can be conducted to develop new anticancer therapies [395, 396]. This process consists of using drugs that are already approved by the FDA for one indication in order to treat other disease types, such as cancer [395, 396]. Epigenetic drug screening on more than 1,100 FDA-approved molecules was conducted to discover drugs with new epigenetic and anti-cancerous properties in a preclinical colon cancer model [399]. One group of drugs in particular, cardiac glycosides, exhibited the most epigenetically driven gene reactivation and robust anti-cancerous properties [399]. Surprisingly, all drugs exhibiting newly discovered epigenetic properties were shown to upregulate gene expression of tumor suppressor genes by activating calcium-calmodulin kinase pathway [399]. The exact

mechanisms of action of these compounds and their tumor specificity have yet to be determined.

These findings encouraged us to perform a secondary screen on a number of these drugs to explore potential epigenetic mechanism and identify cancer specificity among a panel of cancer cell lines. Amongst the four compounds tested, we determined that the cardiac glycoside proscillaridin A demonstrated the greatest anticancer activity towards pediatric leukemia cell lines. Proscillaridin A induced epigenetic effects by decreasing expression of HATs TIP60, CBP, and P300 in addition to histone acetylation levels specifically on the H3 subunits. In addition, we noticed a significant decrease in both C-MYC protein levels and expression of its gene targets. Therefore, we showed that proscillaridin A induce a rapid degradation of HAT and histone acetylation levels resulting in degradation of the C-MYC oncogene in ALL cell lines. In the present study, we demonstrate, for the first time, the anticancerous effects of proscillaridin A in pediatric cancer cell lines targeting acetylation to reprogram ALL cells both at the levels of oncogenic transcription factors and core histone proteins.

2. Materials and Methods

Cell Culture and Drug Treatments

REH (non-T; non-B) leukemia cell line was purchased from American Type Culture Collection. Thp-1 (AML) leukemia cell line was purchased from American Type Culture Collection. Molt-4 (T-ALL) leukemia cell line was purchased from the American Type Culture Collection. Nalm-6 (pre-B ALL) leukemia cell line was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. REH, Molt-4, and Nalm-6 cell lines were cultured in Gibco RPMI 1640 supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Thp-1 cell line was cultured in Gibco RPMI 1640 supplemented with 10% fetal bovine serum and 0.05mM of 2-mercaptoethanol. Proscillaridin A was dissolved in 100% dimethyl sulfoxide at stock concentration of 100 mM and stored in -20°C. ORFLO MOXI Mini Automated Cell Counter was used to analyze growth inhibition after treatment.

Cell Cycle Analysis

To analyze effects of Proscillaridin A on cell cycle by Fluorescence-activated Cell Sorting (BD FACS Canto II), cells were treated with for 48 hours at three different concentrations (2.5, 5, or 7.5 nM). Cells were then harvested, fixed, and stained with bromodeoxyuridine (BrdU) and/or 7-amino-actinomycin (7-AAD) (BD Pharmingen BrdU Flow Kit). Untreated, BrdU-only, and 7-AAD-only stained cells were used to set up compensation controls.

Analysis of Whole Cell Proteins by Western Blot

Cell pellets were lysed in cold whole cell lysis buffer (50mM Tris-Cl pH7.4, 5mM EDTA, 250mM NaCl, 50mM NaF, 0.1% Triton, 0.1mM Na_3VO_4, and 1mM PMSF). Lysis buffer was supplemented with protease inhibitor cocktail (PIC) and phosphatase inhibitor cocktail (phospho-stop). Protein extracts were separated by SDS-PAGE and transferred onto a polyvinyl difluoride (PVDF) membrane. The following primary antibodies were used to detect expression levels of isolated proteins: TIP60 (1:5000, Active Motif cat#61546), CBP (1:2500, Bethyl Labs cat#A-300-362A-T), P300 (1:5000, Active Motif cat#61402), HDAC1 (1:5000, Cell Signaling cat#5356), HDAC2 (1:5000, Cell Signaling cat#5113), HDAC4 (1:5000, Cell Signaling cat#7628), C-MYC (1:5000, ABCAM cat#AB32072), C-MYC 323 acetylation (1:5000, Millipore cat#ABE27), BRD4 (1:2500, ABCAM cat#AB128874), actin (1:5000, Sigma-Aldrich cat#A2228), and α-tubulin (1:5000, Active Motif cat#39528). Membranes were tagged with secondary rabbit (goat anti-rabbit HRP purchased from BIO RAD) or mouse (goat anti-mouse HRP purchased from BIO RAD). Proteins of interest were detected using BIO RAD ECL. Quantifications of western blots were conducted using the ImageJ program.

Analysis of Histone Proteins by Western Blot

Cell pellets were lysed in either cold Triton Extraction Buffer (0.5% Triton, 2mM PMSF, 0.02% NaN_3, 10mM sodium butyrate) used for histone acid extraction (ABCAM protocol). Lysis buffer and TEB were supplemented with protease inhibitor cocktail (PIC) and phosphatase inhibitor cocktail (phospho-stop). Protein extracts were separated by SDS-PAGE and transferred onto a polyvinyl difluoride (PVDF) membrane. Membranes were tagged with primary antibodies and incubated overnight at 4°C. The following primary antibodies were used to detect histone acetylation and methylation marks on H3 subunit: H3K27ac (1:5000,

Active Motif cat#39134), H3K9ac (1:5000, Active Motif cat#39917), H3K14ac (1:5000, Active Motif cat#39698), H3K9me3 (1:5000, Active Motif cat#39765), H3K27me3 (1:5000, Active Motif cat#39155), and H3 total (1:5000, Active Motif cat#39763). The following primary antibodies were used to detect histone acetylation marks on H4 subunit: H4K16ac (1:5000 Active Motif cat#39167), H4K8ac (1:5000, Active Motif cat#39172), H4 total acetylation (1:5000, Active Motif cat#39244), and H4 total (1:5000, Active Motif cat#39244). Membranes were tagged with secondary rabbit (goat anti-rabbit HRP purchased from BIO RAD) or mouse (goat anti-mouse HRP purchased from BIO RAD). Proteins of interest were detected using BIO RAD ECL. Quantifications of western blots were conducted using the ImageJ program.

RNA Extraction and RNA sequencing

QIAshredder was used prior to RNA extraction to homogenize cell lysates and eliminate any debris. Total RNA was extracted using RNeasy Mini Kit according to instruction manual. Upon RNA extraction, 10 μg of purified extract were treated with DNAse to eliminate any potential DNA contamination. Upon DNAse treatment, RNA quality and yield were verified with Bioanalyser chips using Agilent RNA 6,000 Nano kit according to manufacturer's instructions. The mRNA libraries were created using TruSEq Stranded mRNA LT using 25 ng of mRNA extract. RNA sequencing was performed using HiSeq2500 on "rapid run" mode. Gene set enrichment analysis was conducted using the "GSEA – Broad Institute" program available online: www.broadinstitute.org/gsea.

3. Results

Proscillaridin A affects cell viability in a secondary drug screen on ALL and AML cell lines

In order to identify the drug inducing the greatest effects on cell viability, we performed a secondary drug screen by identifying four candidate drugs from the initial drug screen conducted by Raynal and colleagues. Initially, we examined effects of disulfiram (anti-alcohol abuse), pyrithione zinc (anti-dandruff), digitoxin (cardiac glycoside), and proscillaridin A (cardiac glycoside) on REH (non-T, non-B ALL) and Thp-1 (AML) cell lines (Fig. 16). The IC_{50} values after 48 hours for disulfiram, pyrithione zinc, and digitoxin were 45 nM, 100 nM, and 25 nM in REH and 10 μM, 192 nM, and 50 nM in Thp-1 cell lines, respectively (Fig. 16A and B). Surprisingly, the IC_{50} values for proscillaridin A were 3.4 nM and 15.3 nM in REH and Thp-1, respectively (Fig. 16A and B). Because these IC_{50} values fall within the cardiac glycosides' narrow therapeutic window, we decided to pursue our research with proscillaridin A on B- and T-ALL cell lines in order to compare its effects on the two main branches of pediatric ALL.

Proscillaridin A decreases histone subunit 3 (H3) acetylation and HAT protein expression after treatment in B- and T-ALL cell lines

We then explored the possibility of epigenetic effects of proscillaridin A on two leukemia cell lines, Nalm-6 (B-ALL) and Molt-4 (T-ALL). To do so, we treated both cell lines for 24 – 96 hours with proscillaridin A at clinically relevant concentrations (5 nM). In both cases, we identified significant decreases in histone acetylation levels of the H3 subunit on specific lysine residues (Fig. 12A and 17A). In Molt-4 cell lines, H3K9ac levels rapidly

decreased after 24 hours to about 50% relative to untreated cells and continued to decrease after 72 and 96 hours of treatment (Fig. 12A and C). H3K27ac steadily decreased from 24 hours, reaching approximately 10% after 96 hours of treatment (Fig. 12A and C). H3K14ac was the one least affected after treatment, reaching its lowest acetylation levels after 48 hours and increasing slightly at further time points (Fig. 12A and C). Similar effects on histone H3 acetylation were observed in Nalm-6 cell line after proscillaridin A treatment (Fig. 17A). Interestingly, proscillaridin A treatments do not affect acetylation on H4 residues, demonstrating a specific target for the H3 subunit in both cell lines (Fig. 12B and 17B). We also examined levels of H3 methylation on lysines K9 and K27 and observed no significant changes in both cases (Fig. 17C).

In order to determine the cause of these reduced histone acetylation levels, we investigated enzymes involved in modifying post-translational histone acetylation. We first examined HDACs, the family of enzymes involved in removing acetyl groups from histone tail residues. After 48 hours of proscillaridin A treatment, we identified no significant changes in HDAC protein expression by western blot (Fig. 17D). We then assessed the expression of HATs. When testing for TIP60 protein levels, a HAT involved in acetylating both histone (H3K14ac and H4) and non-histone proteins, we discovered an overall decrease after treatment in both cell lines, even at very short time points (figure 12D) [345]. In Nalm-6, levels of TIP60 protein expression begin to decrease after 16 hours, attaining its lowest expression after 96 hours of roughly 20% (Fig. 12D and E). In Molt-4, a much more potent effect of proscillaridin A treatment is observed; a significant decrease in TIP60 protein levels are observed commencing at the 8-hour mark and reaching nearly inexistent levels after 48 – 96 hours (Fig. 12D and E). We next sought to investigate other HATs involved in H3 subunit

acetylation. To do so, we examined CBP, P300, and GCN5 in both cell lines (Fig. 12F and G and Fig. 18). We found that CBP decreases significantly to roughly 30% after 48 hours and continues to decrease thereafter in Molt-4 after treatment with proscillaridin A (Fig. 12F). In addition, P300 also decreases in Molt-4 in a time-dependent manner after treatment, reaching approximately 20% of control values after 96 hours of proscillaridin A treatment (Fig. 12G). Interestingly, significant changes in CBP and P300 were not observed in Nalm-6 cell line (Fig. 18A and B). In both cell lines, however, we found that proscillaridin A treatment does not alter GCN5 protein levels (Fig. 18C).

Proscillaridin A reduces expression of the C-MYC oncogene in B- and T-ALL cell lines

As previous studies have demonstrated, in order for the C-MYC oncogene to upregulate target gene expression, it is recruited to acetylated histone tail residues by bromodomain proteins [240]. Since there were significant decreases of histone acetylation after treatment, we investigated potential changes of gene expression of the C-MYC oncogene and BRD4, the bromodomain protein involved in C-MYC recruitment (Fig. 13). We discovered a prominent decrease in C-MYC in both Nalm-6 and Molt-4 cell lines (Fig. 13A). In Nalm-6, significant deregulation in C-MYC protein expression is observed after 48 hours treatment (Fig. 13A). On the other hand, a significant C-MYC reduction in Molt-4 is observed even after 16 hours of proscillaridin A treatment and continues to decrease thereafter (Fig. 13A). When testing protein levels of the BRD4 bromodomain, we determined no change in expression in Molt-4, whereas a slight non-significant increase was observed in Nalm-6 (Fig. 13B).

Lysine acetylation is an important post-translational modification that leads to increased C-MYC stability [205, 206, 279]. Our findings demonstrating decreases in TIP60, CBP, and P300 – otherwise known as HATs involved in acetylating and stabilizing the C-MYC oncogene at lysines K143, K157, K275, K317, K323, K371, and K417 – encouraged us to investigate C-MYC acetylation levels. By western blot, we discovered a decrease in lysine 323 acetylation that occurs prior to C-MYC loss in both Nalm-6 and Molt-4 cell lines after 24 – 96 hours of treatment (Fig. 13C). This interesting finding demonstrates that instability of the C-MYC oncogene is produced subsequently to proscillaridin A treatments by decreasing HAT expression levels, which can be one potential cause for degradation and diminished oncogenic protein function.

In order to determine which C-MYC target genes are effectively downregulated, RNA sequencing was performed on Molt-4 cell line after 48 hours of proscillaridin A treatment. In order to compare untreated control cells to cells treated with proscillaridin A, we performed a gene set enrichment analysis (GSEA) on C-MYC target gene data sets. We observed a significant decrease of C-MYC target genes after proscillaridin A treatment (Fig. 13D). We then classified these genes according to their function and determined that the majority are involved in mRNA processing and post-translational regulation of gene expression (Fig. 13E). In addition, some C-MYC target genes involved in mTOR and E2F signaling, two oncogenic pathways usually found to be upregulated in T-ALL, were also reduced (Fig. 13E). These interesting RNA sequencing data show noticeable effects of proscillaridin A not only on the C-MYC protein, but also on the cross-talk between C-MYC and other oncogenic pathways (Fig. 13E). We then selected five noteworthy genes involved in development and progression of certain cancers, including leukemia, that were identified as having significant decreases in

normalized read counts (RC) amongst all C-MYC target genes present (Fig. 13F). We identified CDC45, a gene involved in cell proliferation [443]. We also identified APEX1, a gene involved in single-strand DNA repair [444]. In solid tumors, APEX1 is overexpressed and is correlated with cell survival, cell growth, and development of a tumorigenic phenotype [444]. In addition to these functions, it is a regulator of Notch1 activity, a pathway oftentimes upregulated in pediatric leukemia cases. We also identified RAN, a gene involved in activating the PI3K/Akt/mTORC and Ras/MEK/ERK pathways, both playing roles in leukemia development and progression [445, 446].

Proscillaridin A downregulates genes involved in oncogenic pathways and upregulates genes involved in cellular differentiation

We next considered studying effects of proscillaridin A treatment on cell differentiation. In order to do so, we first selected all genes upregulated by proscillaridin A by one or more standard deviations (Fig. 19A). By doing so, 491 genes were classified as being upregulated in our data set. From this gene list, we identified 67 upregulated genes involved in cellular differentiation pathways (Fig. 14A). We then considered the top 12 most upregulated genes involved in cell differentiation. We discovered that a number of these genes involved in T-cell development are upregulated after treatment (Fig. 14A). From this group, we discovered two genes involved in T-cell differentiation (SGK1 and BCL6) and five genes involved in T-cell activation pathways (DUSP10, LIF, PLK2, FOS, and JUN).

Interestingly, two of the most upregulated genes in our RNA sequencing samples were FOS and JUN. In most cases, both these genes are associated with oncogenic functions [447]. However, in T-cells, they form a protein complex known as AP-1 that plays a role in T cell

differentiation and activation when interacting with the NFAT protein family [448-450]. Because FOS and JUN are the second and third most upregulated differentiation genes in our samples, respectively, we believe an upregulation of the AP-1 protein complex occurs in proscillaridin A treated cells that drives cell differentiation. In addition, proscillaridin A upregulates expression of the NF-κB signaling pathway; in T-cells, this pathway interacts with and activates AP-1, which can potentially mediate cell differentiation (Fig. 14A) [448].

We next examined genes downregulated after 48 hours of treatment with proscillaridin A from our RNA sequencing data. We selected all genes that decreased by two or more standard deviations when compared to untreated controls (Fig. 19B). By this means, we obtained a total of 612 downregulated genes in our data set. We then classified these genes according to protein function (Fig. 14B). We determined that many gene families are downregulated, including genes involved in response to interferon-α (IFN- α), cholesterol homeostasis, and metabolism. Additionally, we also observed dowregulation of genes involved in the mTOR and E2F signaling pathways (Fig. 14B). When investigating oncogenic pathways by GSEA, we compared untreated controls to cells treated with proscillaridin A and determined that both the mTOR and E2F signaling pathways were in fact downregulated in all our samples (Fig. 14C and D). Hence, we concluded that proscillaridin A does not only target the C-MYC oncogene and its target genes, it also affects other signaling pathways that are present in T-ALL patients.

We then sought to determine specific genes downregulated in each of these oncogenic pathways identified. Among the E2F targets, we discovered five genes that play a role in leukemia development and progression that are shown to have significantly lower normalized read counts after treatment (Fig. 14C). These genes play a role in increasing cell proliferation

(MCM7) and inhibiting normal lymphoid development and cell cycle progression (HMGA1) [451]. Other genes include PAICS, shown to be upregulated in relapse cases, and MYBL2, a protein that interacts with micro-RNAs in AML cases leading to formation of a malignant phenotype [105, 452]. Among all mTOR target genes, we detected three of which were particularly interesting (Fig. 14D). For instance, MCM2, a gene upregulated in relapsed or refractory ALL cases, and XBP1, a gene involved in maintaining leukemic cell survival, were both found to have decreased RC values after treatment [453, 454]. The fact that multiple oncogenic pathways are inhibited after proscillaridin A treatment prompted us to investigate tumor suppressor genes. Among the 491 genes upregulated by one or more standard deviations, we discovered a significant increase in the normalized read counts for two tumor suppressor genes, PTEN and CDKN1a, after proscillaridin A treatment (Fig. 14E) [455, 456]. All in all, we concluded that proscillaridin A has a dual role in upregulating cell differentiation while downregulating oncogenic signaling pathways involved in development and progression of T-ALL.

Clinically relevant concentrations of proscillaridin A induced growth inhibition and cell cycle arrest in Nalm-6 and Molt-4 cell lines

Next, we examined upregulation of genes induced by proscillaridin A treatment. To do so, we selected all genes that increased by two or more standard deviations from our RNA sequencing data when compared to untreated controls (Fig. 19B). We then classified genes from this set depending on their functions (Fig. 15A). We noticed that genes involved in TGF-β signaling, an important pathway involved in T-cell differentiation, were upregulated after proscillaridin A treatment (Fig. 15A) [457]. In addition, we discovered that genes involved in

the P53 pathway and TNF-α signaling via NF-κB are the ones most upregulated after treatment (Fig. 15A). The NF-κB signaling pathway is involved in many cell-regulating mechanism such as proliferation, survival, and apoptosis. We believe that in this case, upregulation of NF-κB is involved in inducing apoptosis. Conversely, to test this hypothesis, we performed GSEA on all genes that were differentially expressed upon proscillaridin A treatment. We found that genes involved in apoptosis and NF-κB signaling were upregulated after treatment, suggesting that NF-κB signaling may be mediating cell death (Fig. 15B). Interestingly, we also found that genes involved in oxidative phosphorylation were decreased, implying a change in cellular energy pathways that may cause cells to produce less ATP and stop growing (Fig. 15B).

In order to determine the cytotoxic effects of proscillaridin A treatment in Nalm-6 and Molt-4, we first determined IC_{50} values after 48 hours of treatment. To do so, we treated both cell lines with different concentrations of proscillaridin A ranging from 0.5 nM to 100 nM. We discovered IC_{50} values of 3.0 nM and 2.3 nM in Nalm-6 and Molt-4, respectively (Fig. 15C). Most importantly, these concentrations of proscillaridin A fall into the clinical range, which is approximately 1 – 10 nM in human patients. By flow cytometry, we determined a block in the cell cycle in the G2/M phase (Fig. 15D and E). In Nalm-6, there are significantly less cells in S phase and higher amounts in G2/M phase that was dose dependent (Fig. 15D and E). Since Molt-4 cells are more sensitive to proscillaridin A, there was a significantly greater amount of apoptosis after 48 hours that was not present in Nalm-6 cell line (Fig. 15D and E). In both cases, the optimal dose to induce G2/M block and limiting excessive cytotoxicity is 5 nM (Fig. 15D and E).

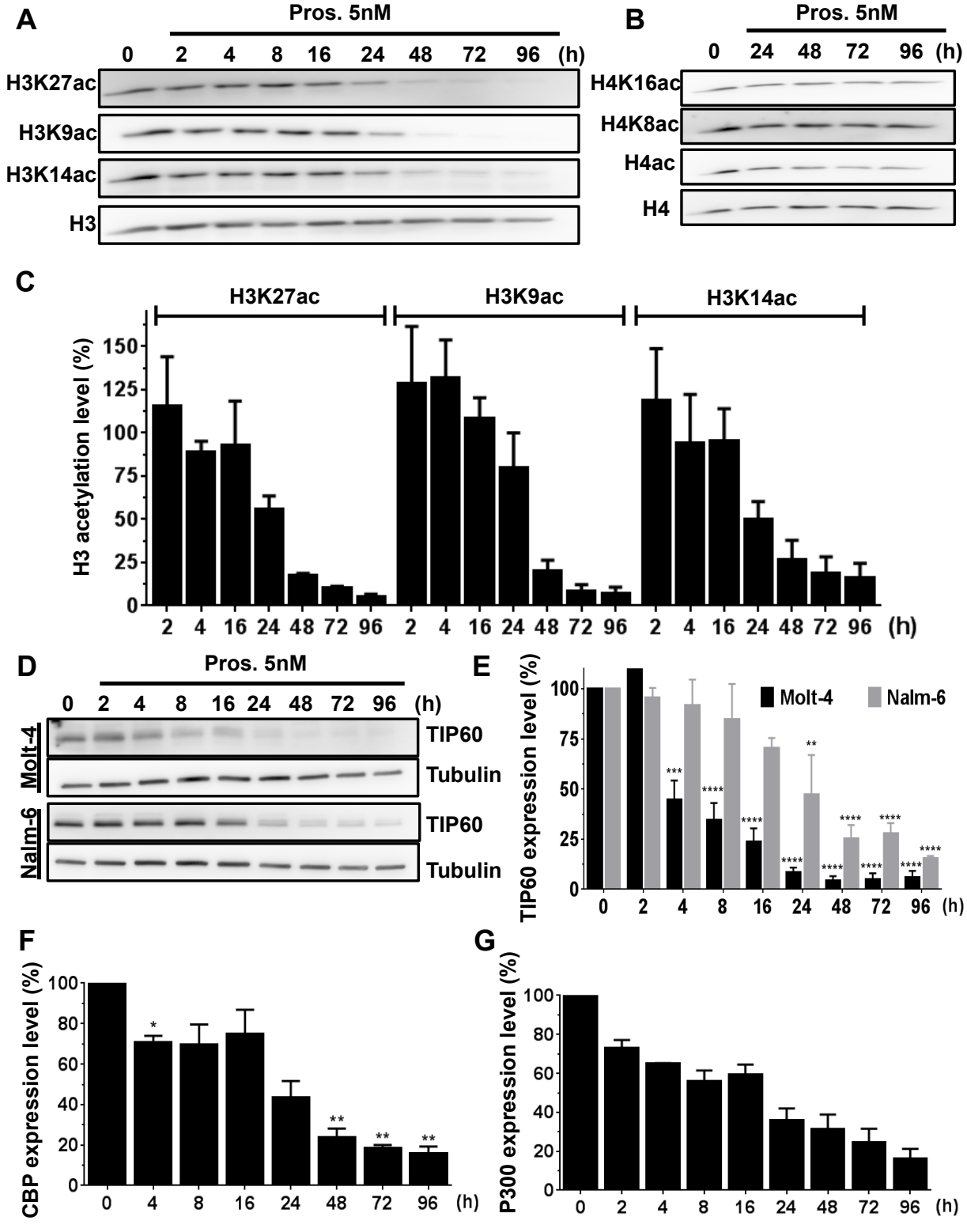

A
Pros. 5nM
0 2 4 8 16 24 48 72 96 (h)
H3K27ac
H3K9ac
H3K14ac
H3
B
Pros. 5nM
0 24 48 72 96 (h)
H4K16ac
H4K8ac
H4ac
H4
C
H3K27ac
H3K9ac
H3K14ac
H3 acetylation level (%)
0 25 50 75 100 125 150
2 4 16 24 48 72 96 2 4 16 24 48 72 96 2 4 16 24 48 72 96 (h)
D
Pros. 5nM
0 2 4 8 16 24 48 72 96 (h)
Molt-4
TIP60
Tubulin
Nalm-6
TIP60
Tubulin
E
Molt-4
Nalm-6
TIP60 expression level (%)
0 25 50 75 100
0 2 4 8 16 24 48 72 96 (h)
F
CBP expression level (%)
0 20 40 60 80 100
0 4 8 16 24 48 72 96 (h)
G
P300 expression level (%)
0 20 40 60 80 100
0 2 4 8 16 24 48 72 96 (h)

Figure 12: Proscillaridin A treatment decreases histone 3 acetylation levels and expression of HATs in Molt-4 and Nalm-6 leukemic cell lines.

A. Western blot depicting decreases in histone 3 lysine residue acetylation (K9, K14, and K27) after 2 – 96 hours of proscillaridin A treatment (5 nM) in Molt-4 cell line.
B. Western blot depicting histone 4 lysine residue acetylation (K8, K16, and total acetylation) after 24, 48, 72, and 96 hours of proscillaridin A treatment (5 nM) in Molt-4 cell line.
C. Quantification of histone 3 lysine residue acetylation shown in figure 12A.
D. Expression of TIP60 after 2 – 96 hours treatment with proscillaridin A (5 nM) in Molt-4 (top) and Nalm-6 (bottom) cell lines by western blot.
E. Quantification of TIP60 protein levels, expressed as a percentage, in Molt-4 (black) and Nalm-6 (grey) cell lines.
F. Western blot quantification of CBP in Molt-4 cell line after 4 – 96 hours treatment with proscillaridin A (5nM).
G. Western blot quantification of P300 in Molt-4 cell line after 2 – 96 hours treatment with proscillaridin A (5nM).

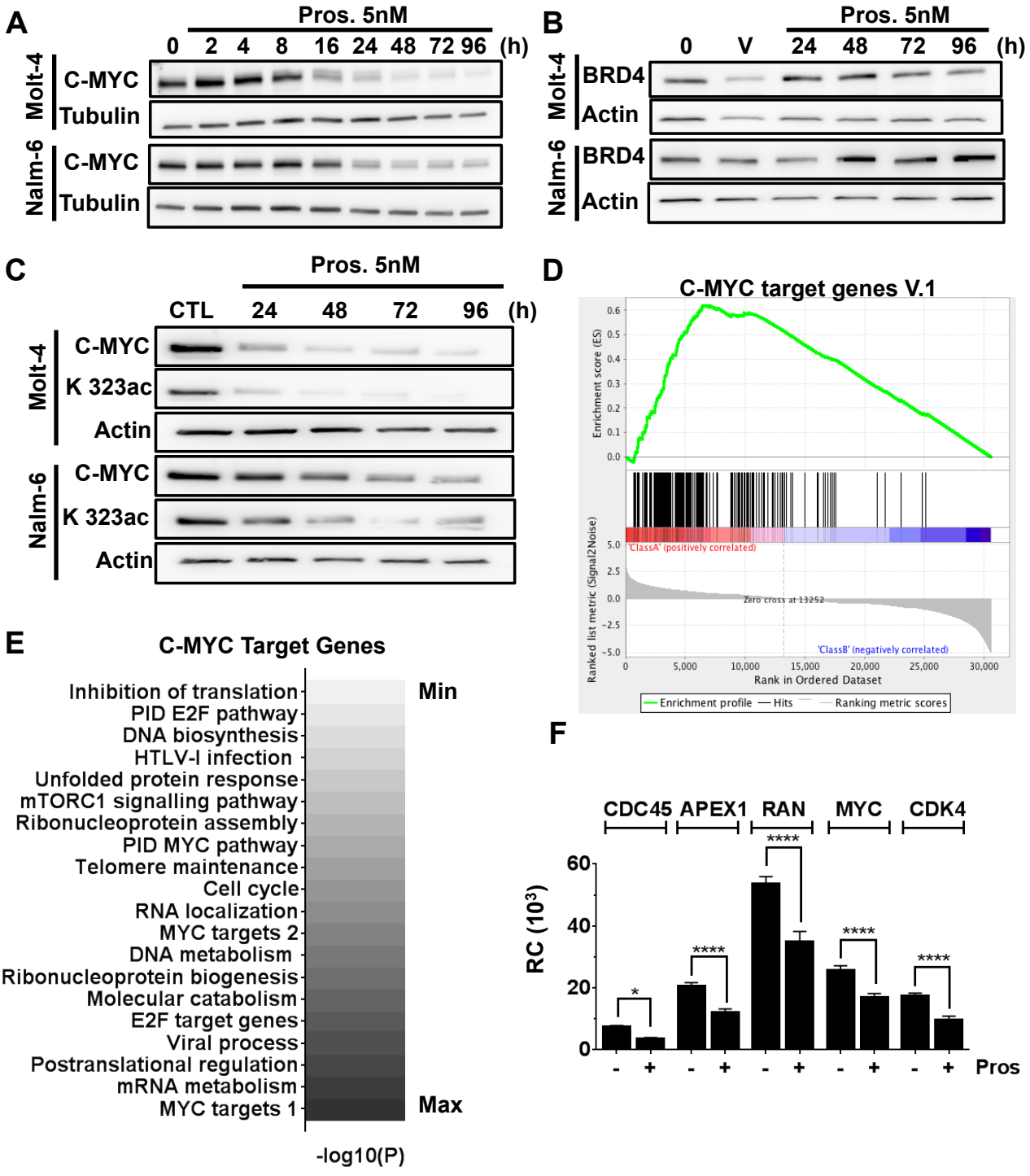

A
Pros. 5nM
0 2 4 8 16 24 48 72 96 (h)
Molt-4
C-MYC
Tubulin
Nalm-6
C-MYC
Tubulin
B
Pros. 5nM
0 V 24 48 72 96 (h)
Molt-4
BRD4
Actin
Nalm-6
BRD4
Actin
C
Pros. 5nM
CTL 24 48 72 96 (h)
Molt-4
C-MYC
K 323ac
Actin
Nalm-6
C-MYC
K 323ac
Actin
D
C-MYC target genes V.1
Enrichment score (ES)
'ClassA' (positively correlated)
Zero cross at 13252
'ClassB' (negatively correlated)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
E
C-MYC Target Genes
Inhibition of translation
PID E2F pathway
DNA biosynthesis
HTLV-I infection
Unfolded protein response
mTORC1 signalling pathway
Ribonucleoprotein assembly
PID MYC pathway
Telomere maintenance
Cell cycle
RNA localization
MYC targets 2
DNA metabolism
Ribonucleoprotein biogenesis
Molecular catabolism
E2F target genes
Viral process
Postranslational regulation
mRNA metabolism
MYC targets 1
Min
Max
-log10(P)
F
CDC45 APEX1 RAN MYC CDK4
RC (10³)
Pros

Figure 13: Proscillaridin A treatment decreases expression of the C-MYC oncogene and C-MYC target genes in Nalm-6 and Molt-4 leukemic cell lines.

A. Western blot depicting expression of the C-MYC oncogene after proscillaridin A treatment (5 nM) after 2 – 96 hours in Molt-4 (top) and Nalm-6 (bottom) cell lines.
B. Western blot depicting expression of BRD4 after proscillaridin A treatment (5 nM) after 2 – 96 hours in Molt-4 (top) and Nalm-6 (bottom) cell lines.
C. Western blot depicting C-MYC and C-MYC acetylation on lysine 323 in Molt-4 (top) and Nalm-6 (bottom) cell lines after 24 – 96 hours of treatment.
D. RNA sequencing data depicting gene expression data sets of C-MYC target genes after 48 hours treatment with 5 nM of proscillaridin A in Molt-4 cell line. The red color indicates untreated cells while the blue color indicates treated cells. Each black vertical line is a gene in the list.
E. RNA sequencing data on genes obtained from the C-MYC oncogene data set classified according to gene function in Molt-4 cell line.
F. RNA sequencing analysis depicting selected C-MYC target genes significantly decreased after proscillaridin A treatment (5 nM) expressed as the number of read counts (RC).

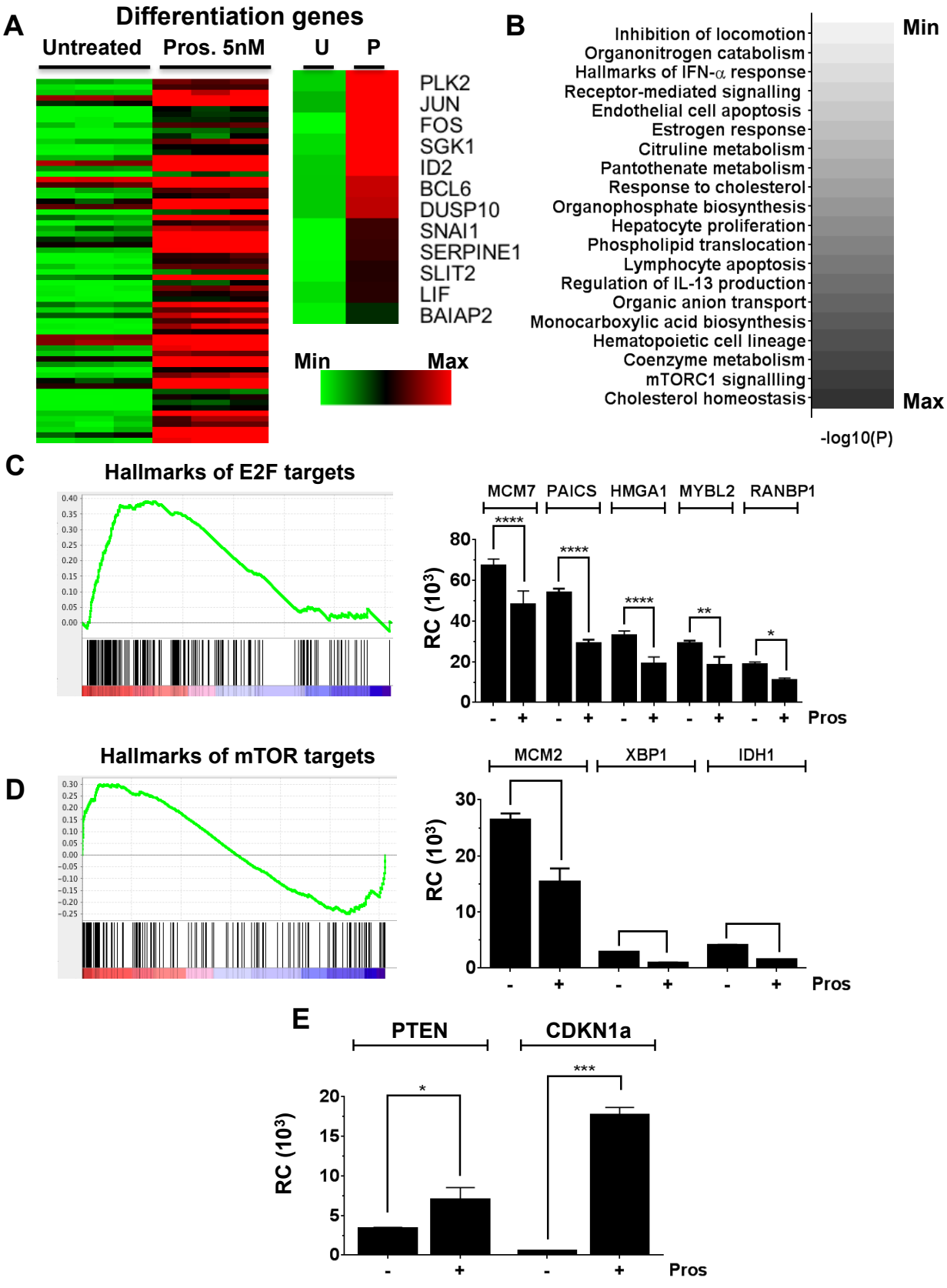

A
Differentiation genes
Untreated
Pros. 5nM
U
P
PLK2
JUN
FOS
SGK1
ID2
BCL6
DUSP10
SNAI1
SERPINE1
SLIT2
LIF
BAIAP2
Min
Max
B
Inhibition of locomotion
Organonitrogen catabolism
Hallmarks of IFN-α response
Receptor-mediated signalling
Endothelial cell apoptosis
Estrogen response
Citruline metabolism
Pantothenate metabolism
Response to cholesterol
Organophosphate biosynthesis
Hepatocyte proliferation
Phospholipid translocation
Lymphocyte apoptosis
Regulation of IL-13 production
Organic anion transport
Monocarboxylic acid biosynthesis
Hematopoietic cell lineage
Coenzyme metabolism
mTORC1 signallling
Cholesterol homeostasis
Min
Max
-log10(P)
C
Hallmarks of E2F targets
MCM7
PAICS
HMGA1
MYBL2
RANBP1
RC (10³)
Pros
D
Hallmarks of mTOR targets
MCM2
XBP1
IDH1
RC (10³)
Pros
E
PTEN
CDKN1a
RC (10³)
Pros

Figure 14: Proscillaridin A downregulates genes involved in oncogenic pathways while upregulating genes involved in cellular differentiation.

A. Left: Heat map depicting all genes involved in cellular differentiation that are upregulated after 48 hours of proscillaridin A (5 nM) treatment. A total of 67 genes were identified as being upregulated after treatment. Genes used in this analysis were obtained by taking all upregulated genes from 1 standard deviation above the mean. Right: Heat map depicting the top 12 most upregulated genes taken from the 67 genes mentioned in the right panel of figure 14A.
B. All genes downregulated by two or more standard deviations below the mean from RNA sequencing data classified according to gene function. Genes with the smallest p-value are correlated to the most expressed gene functions in our data set. A total of 612 downregulated genes were identified.
C. Left: Gene set enrichment analysis of all genes in our dataset comparing untreated control cells to 48 hours proscillaridin A (5 nM) treated cells. The E2F oncogenic pathway is shown to be downregulated in proscillaridin A treated cells compared to untreated controls. Right: Histogram depicting selected E2F target genes from RNA sequencing data which are significantly decreased after proscillaridin A treatment (5 nM) expressed as the number of read counts (RC).
D. Left: Gene set enrichment analysis of all genes in our dataset comparing untreated control cells to 48 hours proscillaridin A (5 nM) treated cells. The mTOR oncogenic pathway is shown to be downregulated in proscillaridin A treated cells compared to untreated controls. Right: Histogram depicting selected mTOR target genes from RNA

sequencing data which are significantly decreased after proscillaridin A treatment (5 nM) expressed as the number of read counts (RC).

E. RNA sequencing analysis depicting tumor suppressor genes significantly upregulated after proscillaridin A treatment expressed as the number of read counts (RC).

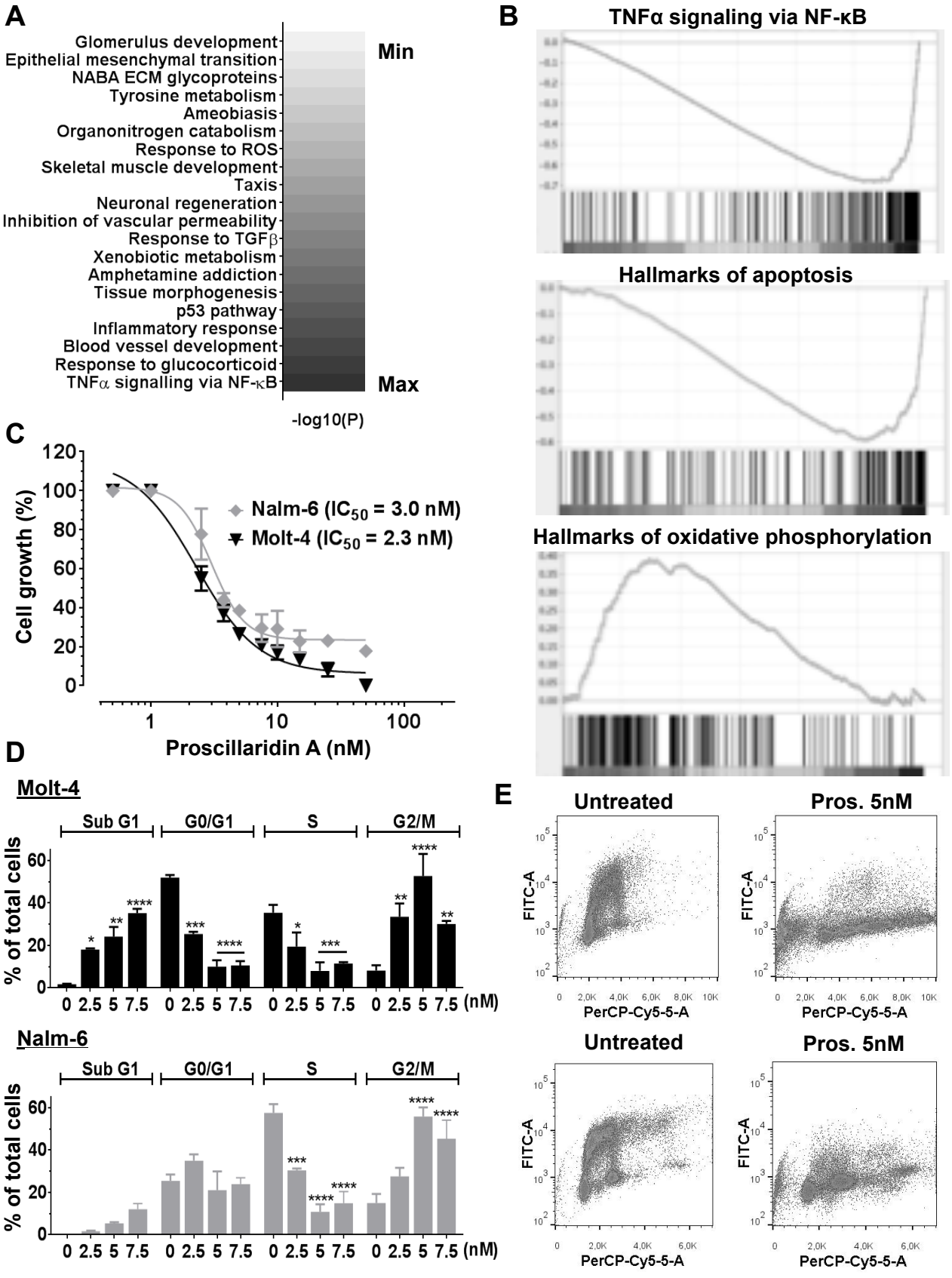
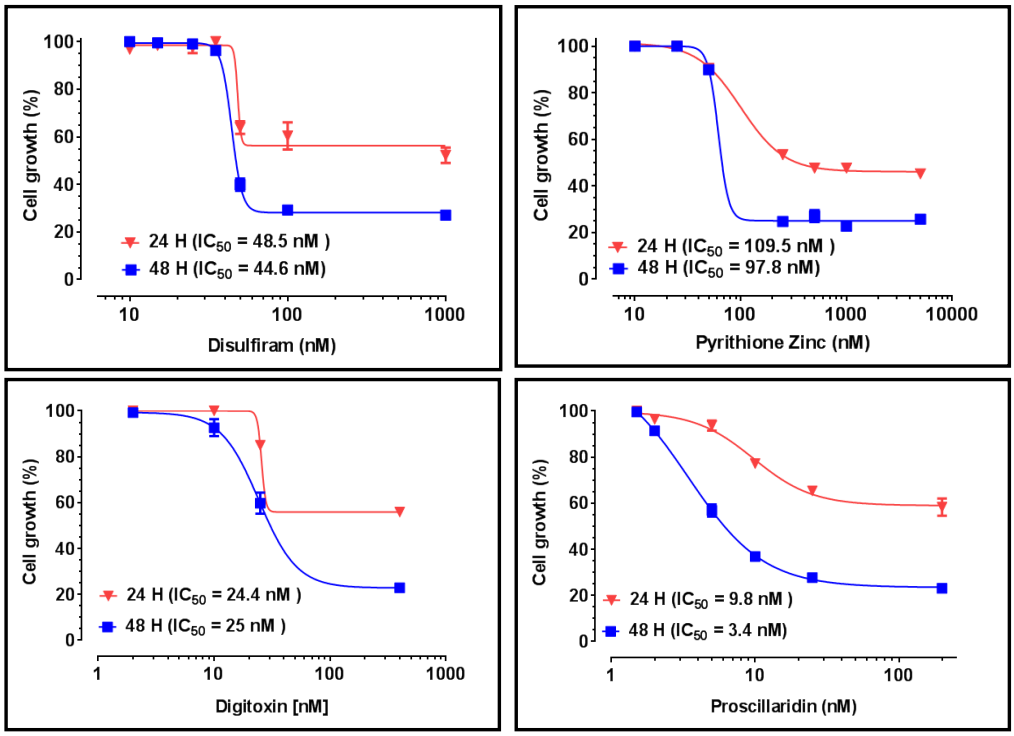

A
Glomerulus development
Epithelial mesenchymal transition
NABA ECM glycoproteins
Tyrosine metabolism
Ameobiasis
Organonitrogen catabolism
Response to ROS
Skeletal muscle development
Taxis
Neuronal regeneration
Inhibition of vascular permeability
Response to TGFβ
Xenobiotic metabolism
Amphetamine addiction
Tissue morphogenesis
p53 pathway
Inflammatory response
Blood vessel development
Response to glucocorticoid
TNFα signalling via NF-κB
Min
Max
-log10(P)
B
TNFα signaling via NF-κB
Hallmarks of apoptosis
Hallmarks of oxidative phosphorylation
C
Cell growth (%)
Nalm-6 (IC50 = 3.0 nM)
Molt-4 (IC50 = 2.3 nM)
Proscillaridin A (nM)
D
Molt-4
Nalm-6
Sub G1
G0/G1
S
G2/M
% of total cells
0 2.5 5 7.5 (nM)
E
Untreated
Pros. 5nM
FITC-A
PerCP-Cy5-5-A

Figure 15: Proscillaridin A induces growth inhibition and cell cycle arrest in Nalm-6 and Molt-4 leukemic cell lines.

A. All genes upregulated by two or more standard deviations above the mean from RNA sequencing data classified according to gene function. Genes with the smallest p-value are correlated to the most expressed gene functions in our data. A total of 189 downregulated genes were identified.
B. Gene set enrichment analysis of all genes in our data set comparing untreated control cells to 48 hours proscillaridin A (5 nM) treated cells. Genes involved in the NF-κB signaling pathway and apoptosis were shown to be significantly upregulated after treatment, whereas genes involved in oxidative phosphorylation were found to be significantly downregulated.
C. Molt-4 (black) and Nalm-6 (grey) cell lines treated for 48 hours with proscillaridin A (0.5nM – 100nM). IC_{50} values are 2.3 nM and 3.0 nM for Molt-4 and Nalm-6 and Molt-4, respectively. (n=3).
D. Quantification of cell cycle arrest in Nalm-6 (top) and Molt-4 (bottom) cell lines. Cells were treated for 48 hours with 2.5nM, 5nM, and 7.5nM of proscillaridin A Two-way ANOVA was performed to determine significant values. Statistical analysis was performed using two-way ANOVA with $p \leq 0.05$ (n=3).
E. Cell cycle analysis by flow cytometry by BrdU staining in Molt-4 (top) and Nalm-6 (bottom) cell lines. Cells were treated 48 hours with 5nM of proscillaridin A.

A

REH


Cell growth (%)
24 H (IC50 = 48.5 nM)
48 H (IC50 = 44.6 nM)
Disulfiram (nM)
24 H (IC50 = 109.5 nM)
48 H (IC50 = 97.8 nM)
Pyrithione Zinc (nM)
24 H (IC50 = 24.4 nM)
48 H (IC50 = 25 nM)
Digitoxin [nM]
24 H (IC50 = 9.8 nM)
48 H (IC50 = 3.4 nM)
Proscillaridin (nM)

B

Thp-1

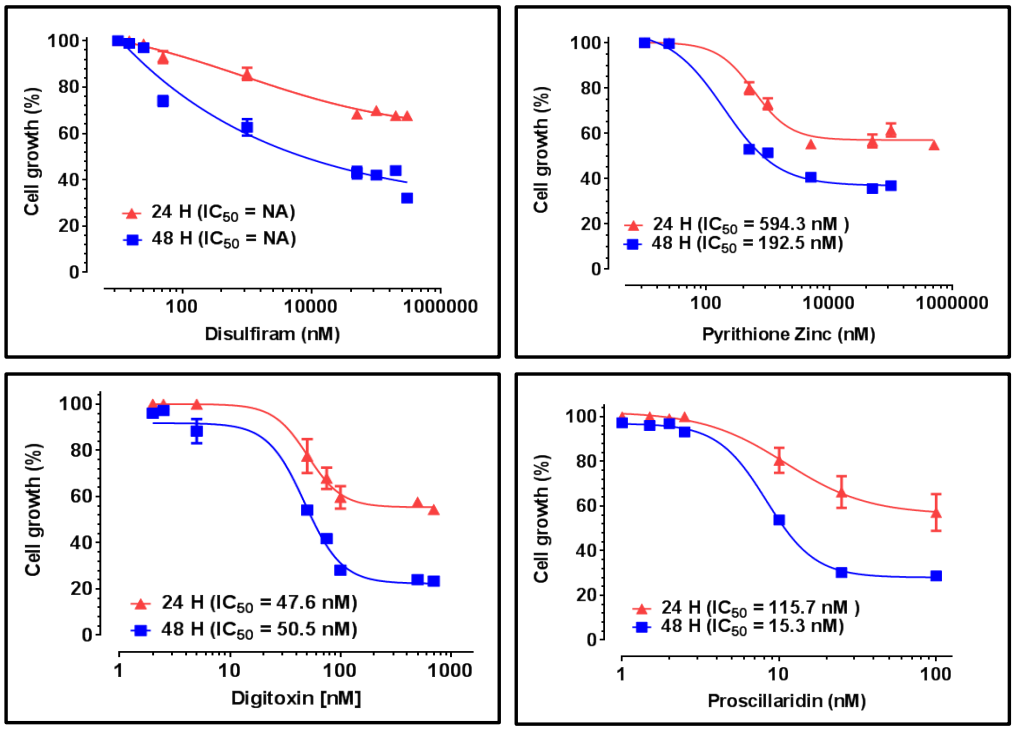

Cell growth (%)
24 H (IC50 = NA)
48 H (IC50 = NA)
Disulfiram (nM)
24 H (IC50 = 594.3 nM)
48 H (IC50 = 192.5 nM)
Pyrithione Zinc (nM)
24 H (IC50 = 47.6 nM)
48 H (IC50 = 50.5 nM)
Digitoxin [nM]
24 H (IC50 = 115.7 nM)
48 H (IC50 = 15.3 nM)
Proscillaridin (nM)

Figure 16: Secondary drug screen on ALL and AML cell lines. (Supplementary figure 1).

A. REH cells were treated for 24 (red curve) and 48 (blue curve) hours with different concentrations of disulfiram, pyrithione zinc, digitoxin, and proscillaridin A to identify an IC_{50} value for each drug. Lowest IC_{50} was identified in proscillaridin A.

B. Thp-1 cells were treated for 24 (red curve) and 48 (blue curve) hours with different concentrations of disulfiram, pyrithione zinc, digitoxin, and proscillaridin A to identify an IC_{50} value for each drug. Lowest IC_{50} was identified in proscillaridin A.

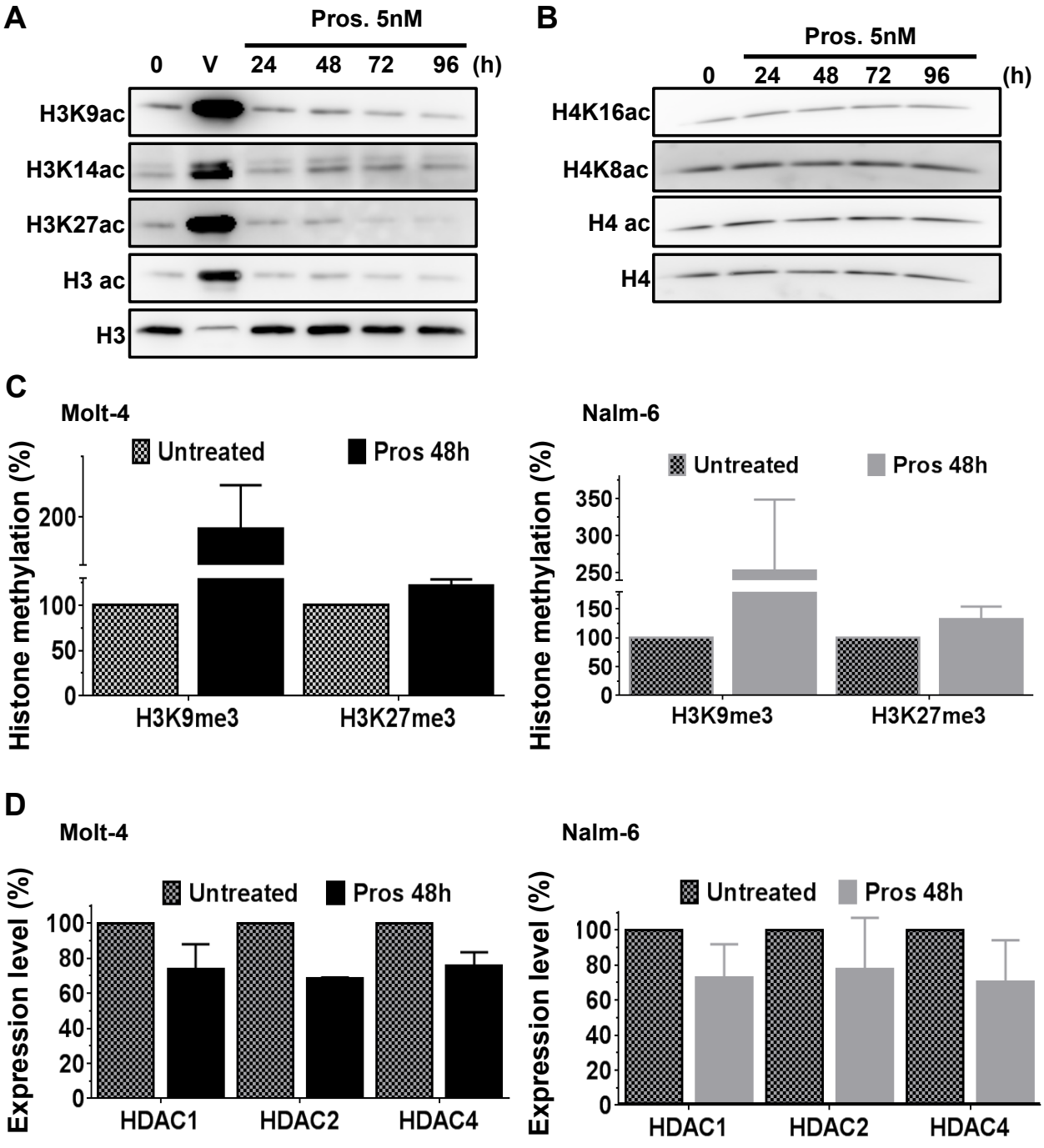

A
Pros. 5nM
0 V 24 48 72 96 (h)
H3K9ac
H3K14ac
H3K27ac
H3 ac
H3
B
Pros. 5nM
0 24 48 72 96 (h)
H4K16ac
H4K8ac
H4 ac
H4
C
Molt-4
Untreated
Pros 48h
Histone methylation (%)
200
100
50
0
H3K9me3
H3K27me3
Nalm-6
Untreated
Pros 48h
Histone methylation (%)
350
300
250
150
100
50
0
H3K9me3
H3K27me3
D
Molt-4
Untreated
Pros 48h
Expression level (%)
100
80
60
40
20
0
HDAC1
HDAC2
HDAC4
Nalm-6
Untreated
Pros 48h
Expression level (%)
100
80
60
40
20
0
HDAC1
HDAC2
HDAC4

Figure 17: Proscillaridin A induces epigenetic effects in Molt-4 and Nalm-6 cell lines. (Supplementary figure 2).

A. Histone 3 lysine residue acetylation (K9, K14, and K27) after 24, 48, 72, and 96 hours of proscillaridin A treatment in Nalm-6 cell line. Vorinostat (V) used as a positive control for acetylation.
B. Histone 4 lysine residue acetylation (K8, K16, and total acetylation) after 24, 48, 72, and 96 hours of proscillaridin A treatment in Nalm-6 cell line. Expression of H4 subunit acetylation was not significant.
C. Western blot quantification of histone 3 lysine residue methylation (9 and 27) after 24, 48, 72, and 96 hours of proscillaridin A treatment in Molt-4 (right) and Nalm-6 (left) cell lines.
D. Western blot quantification of HDAC1, HDAC2, and HDAC4 protein expression levels, expressed as a percentage, in Molt-4 (left) and Nalm-6 (right) cell lines. Changes in protein expression levels were not significant.

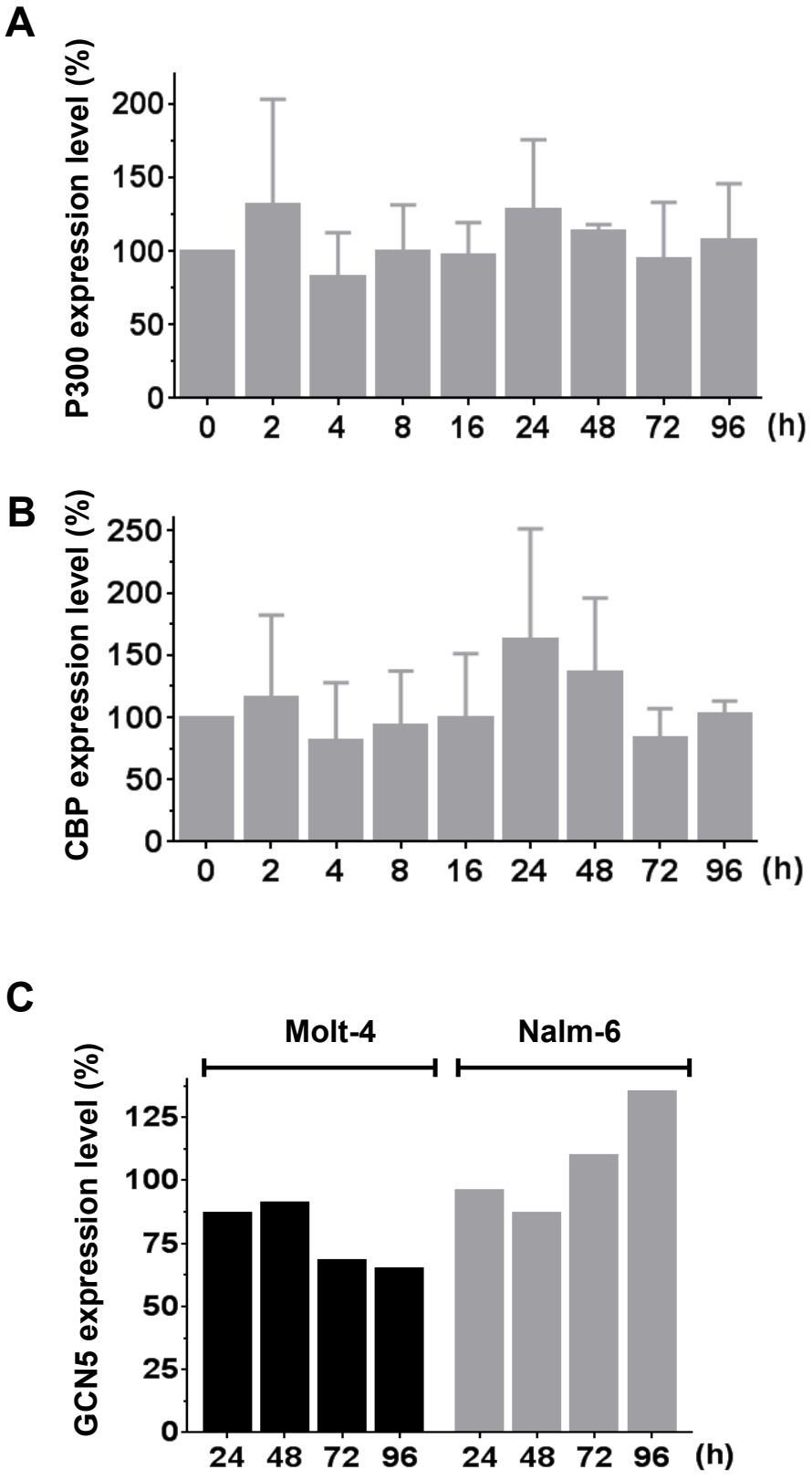
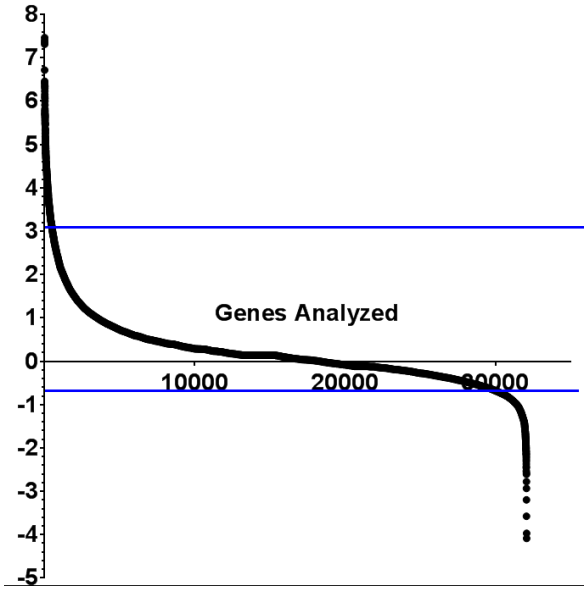

A
P300 expression level (%)
200
150
100
50
0
0 2 4 8 16 24 48 72 96 (h)
B
CBP expression level (%)
250
200
150
100
50
0
0 2 4 8 16 24 48 72 96 (h)
C
Molt-4
Nalm-6
GCN5 expression level (%)
125
100
75
50
25
0
24 48 72 96 24 48 72 96 (h)

Figure 18: Expression of P300, CBP, and GCN5 HATs remain stable after proscillaridin A treatmentin Nalm-6 and Molt-4 cell lines. (Supplementary figure 3).

A. Western blot quantification of P300 levels after 2h – 96h treatment with proscillaridin A (5 nM) in Nalm-6 cell line.
B. Western blot quantification of CBP levels after 2h – 96h treatment with proscillaridin A (5 nM) in Nalm-6 cell line.
C. Western blot quantification of GCN5 levels after 24h – 96h treatment with proscillaridin A (5 nM) in Molt-4 (black) and Nalm-6 (grey) cell lines.

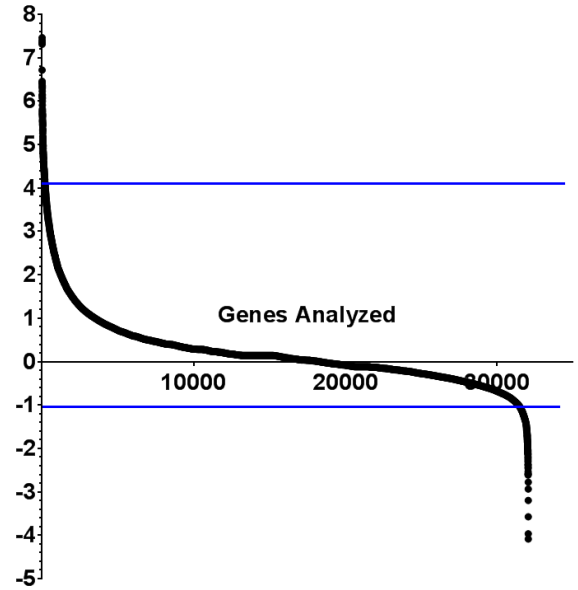
A



Upregulated genes (1 Standard deviations from mean): 491 genes

Downregulated genes (1 Standard deviations from mean: 1963 genes

B



Upregulated genes (2 Standard deviations from mean): 482 genes

Downregulated genes (2 Standard deviations from mean: 612 genes

Figure 19: Proscillaridin A treatment induces changes in gene expression in Molt-4 cell line. (Supplemental figure 4).

A. Graph depicting changes in gene expression analyzed by RNA sequencing. Blue lines indicate one standard deviation above the mean for upregulated genes and one standard deviation below the mean for downregulated genes.
B. Graph depicting changes in gene expression analyzed by RNA sequencing. Blue lines indicate two standard deviation above the mean for upregulated genes and two standard deviations below the mean for downregulated genes.

4. Discussion

Despite recent treatment advances and generally high cure rates, ALL remains the leading cause of death by disease in children. In order to treat pediatric leukemia, patients must receive extremely high doses of chemotherapy drugs to attain remission before the eventual cure. These toxic compounds produce many short and long-term side effects that are detrimental for the patient such as toxicity, fertility issues, secondary cancers, and neurocognitive disorders [11, 36, 458, 459]. Hence, the need to develop less toxic drugs is evident. In addition, patients experiencing relapsed or resistant ALL are characterized as having a very poor prognosis. Epigenetic deregulation, including DNA methylation and post-translational histone modifications, is an important player in development of relapsed or resistant leukemia. These epigenetic modifications are reversible chemical reactions; hence, by targeting these deregulated epigenetic marks, we can induce cancer cell differentiation and ultimately cell death.

Here, we demonstrate that repositioning the cardiac glycoside proscillaridin A has substantial epigenetic and anticancerous properties in pediatric leukemia cell lines. We observed a significant decrease in histone acetylation correlated with decreases of three HATs (TIP60, CBP, and P300). In addition, we observed a significant decrease in C-MYC oncogene protein levels and its target genes. Interestingly, we determined a decrease in C-MYC acetylation, a post-translational modification involved in stabilizing the C-MYC oncogene and preventing its degradation. In addition to targeting the C-MYC oncogenic pathway, proscillaridin A also affects mTOR and E2F signaling, two additional pathways involved in development and progression of leukemia. Moreover, proscillaridin A upregulates genes involved in T-cell differentiation and activation. When testing effects of clinically relevant

concentrations of proscillaridin A on cell viability, we observed an upregulation of genes involved in apoptosis as well as cell cycle block at the G2/M phase.

One of the most frequently upregulated oncogenes in pediatric ALL, and human cancers in general, is C-MYC. In normal cells, C-MYC expression is very tightly controlled; in tumor cells, however, regulatory mechanisms are dysfunctional [211, 218]. Furthermore, changes in either post-translational modifications favoring C-MYC protein stability or increases in protein expression levels enabling cells to proliferate at very high rates [201]. In other cases, constitutive C-MYC expression is due to activation mutations, genetic amplifications, or chromosomal translocations [201, 218-221]. In order for C-MYC to upregulate expression of its target genes, it is recruited to acetylated histone tail residues by the bromodomain protein family, dimerizes with MAX, and forms a complex to initiate transcription. In normal cells, expression of the C-MYC master regulator is controlled mainly by post-translational modifications like ubiquitination, acetylation, and phosphorylation of specific residues on the protein. Some of these modifications induce low protein half-life or stability, while others have an opposite effect. In cancer cells, these regulatory mechanisms are deregulated, ultimately leading to increases in C-MYC expression and stability [205, 206]. For example, acetylation of seven different lysine residues (K143, K157, K275, K317, K323, K371, and K417) directed by HATs such as TIP60, CBP, P300, and GCN5, are known to stabilize the protein and prevent its degradation in cancer cells. Two of these residues, lysines K323 and K417, are ubiquitination sites as well that, when present, direct C-MYC to proteasomal degradation. When these residues are acetylated, they prevent ubiquitin attachment. Hence, the observed decrease of lysine K323 acetylation induced by proscillaridin A can potentially liberate this specific site so a ubiquitin moiety can be linked.

In recent years, many researchers have tried to target C-MYC therapeutically. However, the lack of an active site or ligand-binding domain has prevented the development of novel therapeutic inhibitors. Hence, some groups have tried targeting the bromodomain proteins involved in C-MYC recruitment as a means to decrease C-MYC target gene effects [237, 238]. There are three members of the bromodomain and extra-terminal (BET) family that associate with C-MYC recruitment, termed BRD2, BRD3, and BRD4, with the latter being the most pertinent in terms of C-MYC association and activation [207, 239]. The link between BET proteins and C-MYC recruitment prompted the development of JQ1, the first selective BET inhibitor (BETi), which blocks C-MYC recruitment onto BRD4 and ultimately blocks both C-MYC and C-MYC target gene transcription [240]. In fact, numerous completed or ongoing clinical trials are studying the effects of these BETi on a variety of human cancers, including acute leukemia (NCT02543879, NCT01949883, NCT02157636, and NCT01987362).

Despite all these promising results, there has been proof that BETi are not as efficient as initially thought out to be. Recently, studies have shown that some cells either do not respond to JQ1 or develop resistance to treatment [106, 242, 247]. For instance, Ott and colleagues demonstrated that mice treated with JQ1 eventually die of leukemia and that upon analysis of these mice, there is an upregulation of the C-MYC protein [239]. In another study, Rathert and colleagues determined that upon JQ1 treatment, AML cell lines and patient samples overexpress WNT signaling genes that confer resistance to treatment.

One way to develop new drugs for treating pediatric ALL is by drug repositioning FDA-approved molecules. In order to avoid the lengthy and costly process, drug repositioning – also known as drug repurposing – can be conducted to develop new anticancer therapies

[395, 396]. This process consists of using drugs that are already approved by the FDA for one indication in order to treat other disease types, such as cancer [395, 396]. Because drug repositioning uses approved FDA drugs that have a long history of preclinical and toxicological studies, they can quickly be developed or incorporated into treating cancer without passing through rigorous testing [395, 396]. The primary benefits in screening and repositioning these FDA-approved molecules include much lower cost and reduced side-effects compared to cytotoxic therapies currently used [395].

Cardiac glycosides are naturally occurring compounds that can be separated into two main groups: bufadienolides and cardenolides. Both cardiac glycoside families share a similar molecular structure and function as sodium-potassium (Na^+/K^+) channel blockers, leading to increases in intracellular sodium levels. These variations in intracellular sodium levels decrease activity of the sodium-calcium (Na^+/Ca^{2+}) pump, thereby leading to increases in calcium levels and cardiac contractility in patients suffering from arrhythmias and congestive heart failure [400, 401, 403]. Interestingly, cardiac glycoside intake has been previously correlated to anti-cancerous properties. It has been revealed that patients taking cardiac glycosides during or prior to chemotherapy not only have less invasive cancers, but also present a decreased mortality risk after induction phase therapy is completed [404-407]. In fact, epidemiological studies have demonstrated a decreased risk in developing leukemia in patients who are treated with cardiac glycosides, while other studies demonstrate a synergy between cardiac glycosides and some cytotoxic chemotherapy drugs [408, 409, 412]. Although the exact anticancerous mechanism elicited by cardiac glycosides is unknown, a few potential speculated mechanisms are currently being investigated. For instance, some groups believe that cardiac glycosides upregulate important tumor suppressor genes and genes

involved in apoptosis, while others believe that energy-producing pathways like oxidative phosphorylation and glycolysis are targeted [423-430]. Regardless, the overall beneficial effects on cancer treatment has encouraged some research groups to investigate cardiac glycosides repositioning in treating cancer patients (NCT00837239, NCT01887288, NCT02106845).

All in all, we demonstrate that proscilaridin A acts as an epigenetic drug on ALL cell lines inducing potent gene expression reprogramming. Proscillaridin A targets both C-MYC protein levels and histone acetylation levels, which may in part decrease binding of C-MYC to acetylated DNA residues. More studies are required to fully study the mechanism of action of proscillaridin A and to discover drug combinations to produce synergistic responses for further clinical trials.

Chapter 3: Discussion and Future Considerations

In order to treat pediatric leukemia, patients must receive extremely high doses of chemotherapy drugs to attain remission before the eventual cure. These toxic compounds produce many short and long-term side effects that are detrimental for the patient such as toxicity, fertility issues, secondary cancers, and neurocognitive disorders [11, 36, 458, 459]. Hence, the necessity to develop new cancer medication is evident. The best means to attain higher remission rates, counter treatment resistance, and decrease long-term side effects, is by combining different types of therapies together.

By introducing epigenetic drugs into chemotherapy regimens, we hope to accomplish two very important things. First, the addition of epigenetic drugs can potentially sensitize cancer cells to chemotherapy drugs by decreasing the apoptotic threshold [248]. Subsequently, by inducing epigenetic reprogramming, epigenetic drugs are able to alter gene expression in cancer cells making them more sensitive to lower doses of cytotoxic therapies [248]. Moreover, these consequences may also decrease severe side effects of treatment. Second, it has been widely proven that cancer cells are addicted to epigenetic marks; they become dependent on these epigenetic modifications for their survival, drug resistance, and maintenance of a malignant phenotype [357, 358, 460]. Since epigenetics governs gene expression, cancer cells rely on their epigenomes to express certain proteins while repressing others [357, 358, 460]. By treating patients with drugs inducing epigenetic changes, we are able to target proteins involved in proliferation and cell survival, while simultaneously activating differentiation and apoptosis.

By screening FDA-approved drugs, we can identify possible candidates in order to reposition them to treat many types of cancers. As demonstrated by Raynal and colleagues in their drug screen, many FDA-approved compounds have unsuspected epigenetic and anticancerous properties [399]. In our study, we tested four of these FDA-approved drugs identified (disulfiram, pyrithione zinc, digitoxin, and proscillaridin A) in pediatric ALL and AML cell lines. We discovered that proscillaridin A, a cardiac glycoside that has been administered to patients for over 40 years, induced the most prominent epigenetic and anticancerous characteristics and decided to pursue our research project with this drug. Drug repositioning has many positive points. For instance, previously acquired knowledge on side effects, clinically administered doses, and plasma levels of the given medication are all known due to a large amount of previous preclinical and clinical tests. Additionally, studies on toxicology, pharmacokinetics, and pharmacodynamics have already been thoroughly reviewed. These aspects of repositioning enable drugs to pass rapidly into preclinical and clinical testing.

Here, we explain the efficacy of repositioning the cardiac glycoside proscillaridin A in B- and T-ALL cell lines. We demonstrate that proscillaridin A targets the C-MYC oncogene in pediatric ALL cell lines and decreases histone acetylation levels that are required for C-MYC recruitment and transcriptional activation of its target genes. The fact that proscillaridin A treatment induces downregulation in HAT enzymes TIP60, P300, and CBP is important for two main reasons. First, it explains the decreases in H3 subunit acetylation levels. Second and most importantly, it explains decreases in C-MYC acetylation levels that occur prior to C-MYC degradation. All three aforementioned HAT enzymes are involved in post-translational acetylation of the C-MYC oncogene, consequently stabilizing it and preventing its

degradation. Hence, decreases of C-MYC acetylation levels correlated to decreases in TIP60, P300, and CBP enzymes induce formation of an unstable protein with a very low half-life. By targeting both histone and C-MYC acetylation levels, proscillaridin A is able to directly and indirectly inhibit C-MYC functions by an epigenetic mechanism. For these reasons, we believe that proscillaridin A can potentially be developed to treat pediatric cancer patients in combination with either low dose of cytotoxic therapy or an FDA-approved epigenetic DNA demethylating agent like decitabine. Interestingly, our RNA sequencing data on the T-ALL cell line Molt-4 demonstrate the ability for proscillaridin A to target multiple oncogenic pathways oftentimes upregulated in T-ALL, as demonstrated by a decrease in C-MYC, mTOR, and E2F signaling. Finally, induction of selected T-cell differentiation genes occurs after 48 hours treatment, further demonstrating the positive role of proscillaridin A on T-ALL cells.

The mechanism of action of proscillaridin A on pediatric B- and T-ALL cell lines has not yet been fully characterized. Raynal and colleagues demonstrated that proscillaridin A and other members of the cardiac glycoside family modulate changes of intracellular calcium levels in their colon cancer cell model [399]. We believe that these increases of intracellular calcium levels also occur in pediatric ALL cell lines, which in turn induce downstream effects on histone acetylation levels, expression of HATs, and the C-MYC oncogene. In their study, Raynal and colleagues demonstrated the importance and necessity of calcium/calmodulin-dependent protein kinase (CamK) activation after proscillaridin A treatment. Their study also showed the importance of the Orai calcium channel in triggering the downstream calcium pathway affecting CamK [399]. When calcium levels in the endoplasmic reticulum are low, an interaction between stromal interaction molecule 1 and 2 (STIM1/2) and the selective calcium

channels Orai1/2/3 is formed, causing an influx of calcium ions from the outside of the cell into the cytoplasm [399, 461]. This phenomenon is termed store-operated calcium entry (SOCE). Based on their results, we believe a relationship between the Orai channels and activation of downstream calcium signaling pathways mediates the proscillaridin A mechanism. In order to test our hypothesis, we would first need to knock out CamK or calmodulin by shRNA or CRISPR/Cas9 and determine if decreases in cell viability, histone acetylation, HATs, and C-MYC are still observed. If no changes are observed compared to untreated controls (phenotype rescue), then proscillaridin A requires calcium signaling in order to accomplish its anticancerous and epigenetic properties. Additionally, we plan on conducting either an shRNA or CRISPR/Cas9 knock out of CBP and/or TIP60 to determine whether decreases of cell viability and C-MYC levels are results of those two enzymes.

In addition, although our RNA sequencing data demonstrate upregulation of certain T-cell differentiation genes after 48 hours, we believe that additional genes involved in cell differentiation and activation will be upregulated as a consequence following long-term treatment of 96 hours or more. To test for long-term differentiation marks, we believe treating for 96 hours, followed by 48 hours incubation in drug-free medium, will upregulate genes involved in B- or T-cell receptor formation and cell surface markers like CD24 and CD34 for T-cells and CD19 and CD25 for B-cells [462, 463].

Finally, we believe that C-MYC decreases are a consequence of ubiquitin mediated proteasomal degradation. Poly-ubiquitination is an important post-translational modification that regulates expression of C-MYC in normal cells. We believe a link exists between increases of intracellular calcium levels and ubiquitination of the C-MYC protein; however, we have yet to obtain concrete evidence linking the two pathways. To test this hypothesis, we

can perform two experiments. First, we can conduct an immunoprecipitation of C-MYC to probe for ubiquitination. Another way to test whether C-MYC is ubiquitinated and sent to the proteasome for degradation is by treating cells with proscillaridin A in combination with the proteasomal inhibitor MG-132. If cell growth and C-MYC protein levels are rescued after combination treatment, then C-MYC is degraded by the proteasome in proscillaridin A treated cells. Furthermore, in order to confirm the observed decreases of C-MYC acetylation after treatment, we plan on conducting a mass spectrometry for post-translational acetylation on lysines K143, K157, K275, K317, K323, K371, and K417 shown to stabilize the C-MYC protein [206, 279].

The ability for proscillaridin A to induce epigenetic and anticancerous effects on acute leukemia cell lines raises the question as to whether it will have similar effects on other cancer cell lines as well. Thus, investigating effects of proscillaridin A in drug repositioning studies can potentially aid us in evaluating its effects that are either specific for leukemia cell lines, or might be a general mechanism in which it can induce epigenetic and anticancerous effects. So far, we have obtained similar results in pediatric sarcoma, but not pediatric neuroblastoma, cell lines tested thus far, suggesting that proscillaridin A treatments might only work on selected pediatric cancers. Unfortunately, the reason as to why proscillaridin A has greater effects on certain cancers compared to others has not yet been determined. Further tests on additional pediatric cancer cell lines should be conducted in future studies in order to test its potency in other liquid or solid malignancies.

Using any drug as a monotherapy is not the right approach when treating any types of cancers. Hence, we would like to study effects of combining proscillaridin A, a histone-targeting drug, to DNA demethylating agents like decitabine for the following two reasons.

Firstly, targeting both histones and DNA methylation levels can potentially lead to synergistic drug interactions that might involve additional upregulation of tumor suppressor genes and differentiation pathways compared to decitabine or proscillaridin A monotherapies. Secondly, because a cancer cell is addicted to its epigenetic modifications, targeting both histone and DNA epigenetics can potentially reverse a cancerous phenotype by triggering growth arrest and cell differentiation. Many people believe administration of drugs inducing epigenetic changes cause a systemic effect, but that is not necessarily true; cancer cells depend on their epigenomes for their survival whereas normal cells do not. Hence, when a drug induces epigenetic reprogramming, a normal cell is able to adapt to these changes whereas a cancerous cell cannot. This event forces the cancerous cell to change it behavior, which can potentially trigger apoptosis, senescence, or even differentiation. After completing these necessary experiments, our next step is to validate these results *in vivo*. We would need to inject non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice with either Nalm-6 or Molt-4 cells, wait for leukemic engraftment, and treat these mice with vehicle, proscillaridin A alone, decitabine alone, or proscillaridin A in combination with decitabine. We would then analyze overall mouse survival, spleen morphology, and blast counts in the bone marrows and blood streams. All in all, results that we have obtained to date are very promising in terms of anticancerous properties of proscillaridin A. We will continue to investigate this candidate drug in the future in hopes of eventually administering proscillaridin A in clinical trials.

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