Role of Bcl11b, Bim, and E4BP4 in Regulation Of Apoptosis in the RS4;11 Human Leukemic Cell Line

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ABSTRACT

Role of Bcl11b, Bim, and E4BP4 in Regulation of Apoptosis in the RS4;11 Human Leukemic Cell Line

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Apoptosis is a mode of programmed cell death where characteristic morphological changes occur in a controlled manner, thereby avoiding damage to surrounding cells or tissues. This method of cell death plays an important role in development and homeostasis but requires a delicate balance. Without this balance, inhibition of apoptosis may result in the formation of neoplasms or autoimmune diseases while excessive apoptosis has been linked to neurological diseases. Three genes of interest, Bcl11b, E4BP4, and Bim are believed to be involved in apoptosis. Bcl11b is thought to be a transcriptional repressor of apoptosis and has a major role in T cell development. Previous research has shown that glucocorticoid-evoked upregulation of E4BP4 consequently results in upregulation of the pro-apoptotic Bim during apoptosis. The RS4;11 cell line is a human acute lymphoblastic leukemia cell line which expresses both myeloid and lymphoid markers and is associated with a poor prognosis. Daunorubicin is an anthracycline commonly used to treat leukemia while 5-fluorouracil is an antimetabolite used to treat a wide range of solid tumors in addition to sometimes being used in the treatment of leukemia. In observing gene regulation, we can better
understand the role of these genes in apoptosis as well as gain knowledge in potential
targets of chemotherapeutic agents.

The hypothesis of this project is that Bcl11b suppression correlates with
upregulation of E4BP4 and Bim in response to the anti-leukemic agents above. Cell
viability assays confirmed the susceptibility of RS4;11 cells to treatment by daunorubicin
and 5-fluorouracil. Fluorescent microscopy was utilized to further confirm that apoptosis
was occurring as opposed to necrosis. RNA extractions of this cell line 24 hours after
treatment and subsequent reverse transcription reactions yielded intact cDNA which was
utilized in qRT-PCR reactions. The expression levels of the aforementioned genes were
analyzed in response to treatment by the anti-leukemic agents daunorubicin and 5-
fluorouracil. However, a relationship between the drug treatments and gene expressions
was shown not to occur. A possible reason for this outcome is that the drugs used in this
study induce apoptosis by upregulation or downregulation of genes other than those
analyzed in this project.
Chapter One: Introduction

1.1 Apoptosis

Apoptosis is a mode of programmed cell death where characteristic morphological changes (including cell shrinkage, formation of apoptotic bodies, and nuclear fragmentation) occur in a controlled manner, thereby avoiding damage to surrounding cells or tissues.\(^1\,^2\,^3\) Although this process was described by Carl Vogt more than a century ago, the term apoptosis (Greek for “the falling off of leaves from a tree”) was coined by John Kerr in 1972 who was instrumental in the characterization of the hallmarks of apoptosis.\(^3\)

Apoptotic cell death plays an important role in development and homeostasis.\(^1\,^2\,^4\) In many organisms including humans, proper embryonic development is dependent on cell death. One of the most cited organisms where this has been studied extensively is the nematode *C. elegans*. During development in *C. elegans*, 131 cells die by way of apoptosis and the remaining 959 cells develop into various tissues.\(^5\) A balance between cell division and apoptosis is absolutely required for the survival and health of organisms.\(^2\,^3\) This process is highly regulated, primarily by the transcription factor and tumor suppressor protein p53 as well as the Bcl-2 family of proteins.\(^6\) Without a near constant balance of these and other regulatory proteins, apoptosis will either be inhibited or excessive. Inhibition of apoptosis leads to autoimmune diseases and cancers such as leukemia, which is the focus of this study, while excessive apoptosis has been linked to neurological diseases\(^6\,^7\,^8\). Specifically, excessive neuronal cell death has been found to be
a factor in Alzheimer’s, Parkinson’s, & Huntington’s diseases as well as in other neurological disorders.  

1.2 Morphology and General Pathway

Apoptosis is a process which can be classified into two main pathways and a lesser known, third Granzyme B pathway. The two main pathways consist of the Extrinsic and Intrinsic pathways which differ in how they are triggered but later converge. In both pathways, the hallmark morphological features of apoptosis are apparent. Early in the apoptotic process, cells shrink and chromatin condenses (pyknosis). Apoptosis then proceeds by membrane blebbing and nuclear fragmentation (karyorrhexis) which leads to the formation of apoptotic bodies by the process of “budding”. These apoptotic bodies which contain all cellular and nuclear materials are phagocytosed by macrophages. This safe and quick method of disposal of dying cells is important in that it is done in an organized and controlled manner so that the organism’s immune system does not elicit an immune reaction against itself. This is in contrast to necrosis where cells die by first swelling, exhibiting pyknosis, karyolysis, and karyorrhexis, and later releasing cytoplasm, thereby eliciting an immune reaction.

As mentioned above, the Extrinsic and Intrinsic pathways begin differently in the initiation phase of apoptosis but share a common execution phase (Figure 1.1). Caspases (cysteine-aspartic acid proteases) are proteases which are key players throughout most of the apoptotic cascade. As their name suggests, they are cysteine proteases that cleave other caspases after certain aspartic acid residues. They are at first inactive zymogens which subsequently undergo proteolysis to become active enzymes. Structurally, they
consist of an α/β heterodimer which contains six β sheets and five α helices. Caspases are usually separated into two main categories: initiator caspases or effector caspases. Initiator caspases contain long prodomains and are further separated into those that contain death effector domains (DED) and those that contain caspase recruitment domains (CARD). Initiator caspases containing DED’s (caspase-8 and caspase-10) are those found in the extrinsic pathways while initiator caspases containing CARD’s (caspase-2 and caspase-9) are found in the intrinsic pathway. Both are responsible for activating the downstream effector caspases which are active during the execution phase of apoptosis. These effector caspases, caspase-3, caspase-6, and caspase-7, go on to cleave many important cellular substrates and cause downstream damage which directly results in the morphological features of apoptosis.\textsuperscript{2,11}
Figure 1.1: Intrinsic and Extrinsic Signaling Pathways of Apoptosis
1.3 Extrinsic Pathway

The extrinsic pathway of apoptosis, also known as receptor-mediated apoptosis, is triggered by extracellular signals. These signals are typically ligands which bind to their respective receptors to start the apoptotic cascade. The two most common death receptors are the Fas receptor and the TNF-R 1 (tumor necrosis factor receptor 1). Both of these death receptors are part of the TNF-R family and contain a cytosolic death domain. In the case of Fas, the Fas ligand binds to the receptor which leads to trimerization of the receptor. Adaptor proteins such as Fas-associated death domain (FADD) bind to the death domain of Fas by way of their own death domain. FADD also binds to pro-caspase-8 by way of a DED. This complex comprised of Fas, the Fas ligand, FADD, and pro-caspase-8 is known as the DISC (death inducing signaling complex). A conformational change causes the pro-caspase-8 molecules to cleave one another, leading to the formation of active caspase-8 molecules. These molecules consequently activate caspase-3 or the other effector caspases. In TNF signaling, the TNF ligand binds to the receptor except that a different adaptor protein, TRADD (TNF-R associated death domain protein) binds to this complex and induces recruitment of FADD at which point this pathway resembles the one seen in Fas signaling. It is important to note that death receptor-mediated apoptosis can be inhibited via a protein called c-FLIP which binds to FADD as well as caspase-8, preventing apoptosis from continuing.2,10

1.4 Intrinsic Pathway

The intrinsic pathway, also known as the mitochondrial-mediated pathway of apoptosis, can be triggered by pro-apoptotic molecules such as hormones, cytotoxic
drugs, and cytokines as well as by other signals including starvation, lack of oxygen, reactive oxygen species, and radiation exposure. Any one of these signals can trigger the release of cytochrome c from the mitochondria via interactions between the pro- and anti-apoptotic members of the Bcl-2 family proteins. The Bcl-2 family proteins, which will be discussed at length in section 1.5, play extremely important roles in the regulation of apoptosis. The release of cytochrome c occurs via mitochondrial apoptosis-induced channels (MAC), which lead to mitochondrial outer membrane permeabilization (MOMP). Once this occurs, cytochrome c binds to and activates the cytosolic adaptor protein apaf-1 (apoptosis protease activating factor 1), which subsequently recruits procaspase-9 by way of their caspase recruitment domains (CARD). Interestingly, the apoptosome contains seven molecules of cytochrome c, apaf-1, and procaspase-9 as well as seven (d)ATP molecules, leading to a symmetrical “wheel of death” structure. The procaspase-9 molecules become activated which serve to activate the executioners caspase-3 and caspase-7. Specifically, caspase-3 activates caspase-activated DNase (CAD) by cleaving the inhibitor of caspase-activated DNase (ICAD). CAD is responsible for degrading DNA within nuclei and causing chromatin condensation. In addition to cytochrome c, the pro-apoptotic proteins SMAC/DIABLO are released by the mitochondria in order to inhibit inhibitor of apoptosis proteins (IAP’s) which function to block the action of caspases.

Although these separate pathways of apoptosis converge at the execution phase, they can also have a great deal of cross-talk between pathways. One such example is that of caspase-8. Although the main function of caspase-8 in the death receptor-mediated pathway is to cleave and activate caspase-3, it can also cleave the Bcl-2 family pro-
apoptotic protein Bid which will in turn induce the release of cytochrome c. This will have downstream effects, leading to the activation of caspase-9 as seen in the intrinsic pathway and subsequently caspase-3 in the execution phase of apoptosis.

1.5 Bcl-2 Family Proteins

The B-cell CLL/lymphoma 2 (Bcl-2) proteins are a family of regulatory proteins which play important roles in determining whether a cell will undergo apoptosis or whether it will survive. These proteins are classified as pro-apoptotic or anti-apoptotic based on how many Bcl-2 homology domains they contain (Figure 1.2). The most prominent members of the anti-apoptotic Bcl-2 proteins are Bcl-2, and Bcl-XL (Bcl-2 related protein, long isoform). Lesser known members are Bcl-w (Bcl-2 like 2 protein), A1, and Mcl-1. Anti-apoptotic Bcl-2 proteins which are located in the mitochondrial outer membrane (MOM), endoplasmic reticulum, and nuclear envelope, contain four BH homology domains and function by directly binding and inhibiting the pro-apoptotic Bcl-2 proteins.
The pro-apoptotic category is further divided into executioner (also known as effector) pro-apoptotic proteins and BH3-only proteins. As the name suggests, BH3-only proteins contain only the third BH homology domain while the executioner proteins are missing the fourth homology domain. This results in a shared pro-apoptotic activity but a difference in specific function. The effector Bcl-2 proteins are Bak (Bcl-2 antagonist killer 1) and Bax (Bcl-2 associated X protein). They permeabilize the MOM, leading to the release of important cellular substrates including cytochrome c and SMAC/DIABLO. The BH3-only Bcl-2 proteins are divided into activators and sensitizers based off of their affinity for binding to anti-apoptotic Bcl-2 proteins or effectors. Activators are those BH3-only Bcl-2 proteins that bind to both pro- and anti-apoptotic Bcl-2 proteins to facilitate apoptosis. They are Bid (BH3 interacting domain death agonist), Bim (Bcl-2 interacting mediator of cell death), and Puma (p53-upregulated modulator of apoptosis). Sensitizers bind only to anti-apoptotic Bcl-2 proteins so that the latter cannot bind to
activators. They are Bad (Bcl-2 antagonist of cell death), Bik (Bcl-2 interacting killer), Bmf (Bcl-2 modifying factor), Bnip3, Hrk, and Noxa.\textsuperscript{2,10,11}

1.6 Leukemia

Leukemia is a hematological malignancy defined by uncontrolled proliferation of abnormal white blood cells in both the blood marrow and the blood and diminished production of normal cells. Although there are many types of leukemia, it is typically separated into two main categories based on the lineage of the white blood cells involved which are further broken down into two subsets each based on how quickly the cancer progresses. The first main type of leukemia is Lymphoblastic Leukemia which is subdivided into Acute Lymphoblastic Leukemia (ALL) and Chronic Lymphoblastic Leukemia (CLL). Lymphoblastic leukemia is caused by uncontrolled proliferation of B-cell or T-cell lineages. The other major type of leukemia is Myelogenous Leukemia which is further divided into Acute Myelogenous Leukemia (AML) and Chronic Myelogenous Leukemia (CML). Myelogenous leukemia is caused by the uncontrolled proliferation of granulocytes: neutrophils, eosinophils, basophils, and mast cells.\textsuperscript{13}

Acute leukemias are characterized by an abnormally high number of immature, undifferentiated white blood cells known as blasts. Because blasts are being overproduced, the bone marrow quickly becomes oversaturated and the number of mature leukocytes circulating in blood becomes very low. Blasts are typically associated with a quicker progression in symptoms as well as a lower survival rate though this may not be true for all cases. ALL is the most prevalent leukemia in young children and infants.\textsuperscript{14}
Chronic leukemias are marked by the presence of mature leukocytes with abnormal functioning. Onset of symptoms is much more gradual than acute leukemias and therefore survival rate is higher. Chronic leukemias are typically seen in adults only. Of note is the ability of chronic leukemias to eventually progress to acute leukemias.\textsuperscript{15}

\textbf{1.7 Acute Lymphoblastic Leukemia}

As the name suggests, acute lymphoblastic leukemia is a sudden-onset malignancy caused by the overproduction of abnormal blasts of either T-cell or B-cell lineage. This type of leukemia predominately affects children of ages 2 to 6 years old though it is not uncommon for infants and adults to develop ALL and accounts for about 75\% of all leukemias diagnosed in children 14 years old or younger.\textsuperscript{16}

ALL has been attributed to a myriad of genetic and environmental factors. In utero exposure to various solvents, pesticides, and ionizing radiation have been linked to an increased risk of leukemia in children. In addition, those undergoing chemotherapy for unrelated cancers face increased chances of developing leukemia. People with genetic disorders as a result of chromosomal breakage such as Down syndrome, Bloom syndrome, neurofibromatosis type I, Fanconi anemia, and ataxia-telangiectasia have increased risks of developing acute lymphoblastic leukemia.\textsuperscript{16,17} Chromosomal translocations, however, are overwhelmingly seen as the instigator in the development of ALL. These chromosomal translocations typically activate transcription factor genes and cause aberrant gene expression. For example, the chromosomal translocation t(9;22), also known as the Philadelphia chromosome, causes fusion of the breakpoint cluster region (\textit{BCR}) to \textit{ABL} which is a tyrosine kinase. This \textit{BCR-ABL} fusion causes constitutive
expression of ABL which interacts with other genes and signaling pathways including that of Ras which is involved in cell differentiation, proliferation, and survival. Prognosis often depends on the specific translocation present with those having a translocation of the MLL gene t(v;11q;23) (found in B-lineage ALL) and those being positive for the Philadelphia gene (found in CML and almost exclusively B-ALL) typically having the worst prognosis. This thesis focuses on B-cell acute lymphoblastic leukemia.

1.8 B-cell Acute Lymphoblastic Leukemia

The subsets of B-cell acute lymphoblastic leukemia are Pro-B ALL, Pre-B ALL, common B-ALL, and mature B-ALL. These subtypes correspond to various stages during B-cell development. During normal B-cell development, hematopoietic stem cells mature into lymphoid stem cells. These lymphoid stem cells then develop into pre-pro-B-cells, also known as early B-cells. Early B-cells are characterized by a lack of CD19 which is present on the surface of all subsequent stages of B-cells. The early B-cells progress to the next stage of B-cell development known as the pro-B stage which continue on to the large pre-B stage, small pre-B stage, immature B stage, mature B stage, and finally to the plasma cell stage. Progression to each subsequent step is a result of intricate expression or lack of expression of transcription factors and presence or absence of specific cell-surface markers. Because the B-ALL subtypes correspond to stages of B-cell development, the presence or absence of certain cell-surface markers is often used in flow cytometric analysis for diagnostic purposes. In addition, DNA microarrays and cytogenetic analysis is used to identify the subtypes of B-ALL according
to the specific translocation present\textsuperscript{19}. The remainder of this section will focus on Pro-B ALL.

Pro-B ALL is characterized by rearrangements involving the mixed-lineage leukemia gene (\textit{MLL}).\textsuperscript{16} \textit{MLL}-associated B-ALL is characterized by the absence of CD10/common ALL antigen (CALLA) and is often CD24 negative and CD15 positive.\textsuperscript{21} \textit{MLL} rearrangements are those where translocations occur at (11q23), resulting in chromosomal fusions and aberrant gene expression.\textsuperscript{16} There are numerous variations of this translocation such as t(4;11)(q21;q23), t(19;11)(p13;q23), and t(9;11)(p22;q23) with the t(4;11)(q21;q23) rearrangement being the most common, representing about 50% of all \textit{MLL}-related cases.\textsuperscript{21} Sadly, this variant yields an exceptionally poor prognosis with studies showing a 5 year event-free survival rate of only 32% and an increased likelihood of relapse.\textsuperscript{18,19} The t(4;11)(q21;q23) translocation is also known as the \textit{MLL/AF4} fusion. The \textit{MLL/AF4} fusion B-ALL cell line, called RS4;11, was established by Strong et al in 1984 from the bone marrow of a 32-year old female during her first relapse of acute lymphoblastic leukemia. In addition to the translocation, it also has an isochromosome for the long arm of chromosome 7. Interestingly, the RS4;11 cell line is biphenotypic, meaning it expresses both lymphoid and myeloid markers. This has led to speculation on the origin of the specific lineage of this cell line with possible explanations being it is bipotential and capable of producing both myeloid and lymphoid cells, it may be a progenitor cell in the very early stages of commitment, or that the biphenotypic nature is simply a product of the malignancy itself.\textsuperscript{22}

Leukemic malignancies caused by the \textit{MLL/AF4} gene fusion result in a very poor prognosis and require aggressive treatment by a combination of drugs as it exhibits the
highest level of resistance to drug-induced apoptosis when compared to other gene
fusions. As such, there are many hypotheses on the subversion of apoptosis in leukemic
cells. In some types of leukemia, it is thought that the fusion genes promote a survival
signal which allows leukemic cells to continue to divide instead of undergoing
apoptosis. MLL fusions may also be the by-product of apoptotic stimuli by way of drug-
induced chromatin fragmentation which along with other mutations can lead to
leukemogenesis. Although it is not yet fully known how, MLL gene fusions may
somehow interfere with the expression of pro-apoptotic genes or otherwise interfere with
apoptotic processes. In a leukemic cell line similar to RS4;11 which was transfected
with the MLL/AF4 fusion, a lower than normal expression of the anti-apoptotic protein
Bcl-2 was observed. In the same cell line, a double transfection of both MLL/AF4 and
AF4/MLL led to increased resistance against apoptosis with downregulation of the pro-
and anti-apoptotic genes Bim, Bid, and Bcl-2, respectively. Interestingly, the RS4;11
cell line is also resistant to stress-induced apoptosis because it is able to secrete a
factor(s) that allows it to survive in the absence of serum. With so many possible ways
of resisting apoptosis and the aggressive nature conferred by this gene fusion, intensive
chemotherapy is imperative to survival.

1.9 Daunorubicin

Daunorubicin (also known as daunomycin and daunarubicin) is part of the
anthracycline class of antineoplastic agents. Anthracyclines are antibiotics which were
originally isolated from Streptomyces peucetius, a red pigment-producing
actinobacterium. After first isolating daunorubicin in the 1950’s and then doxorubicin in
the 1970’s, scientists have since developed synthetic anthracyclines. These newer
anthracycline drugs are typically daunorubicin or doxorubicin derivatives with improved efficacy, lower cardiotoxic effects, or improved metabolism.\textsuperscript{26}

Structurally, daunorubicin consists of a glycosidic portion and an aglycone component. The glycosidic portion is called a daunosamine and consists of a 3-amino-2,3,6-trideoxy-L-fucosyl glycoside. This daunosamine is glycosidically linked to C-7 of the aglycone tetracyclic ring. The tetracyclic ring has a characteristic methoxy group as well as adjacent quinone-hydroquinone groups and a short side chain containing a carbonyl group. This side chain terminates in a methyl group. Interestingly, the only structural difference that sets doxorubicin apart from daunorubicin is that the former terminates in a primary alcohol. This in turn impacts the efficacy of these drugs in the treatment of different cancers.\textsuperscript{27} Doxorubicin is commonly used to treat a wide spectrum of cancers including breast cancer, solid tumors, sarcomas and lymphomas whereas daunorubicin is primarily used in the treatment of leukemias.\textsuperscript{26} Similarly, epirubicin, the semisynthetic analog of doxorubicin, has an equatorial hydroxyl at C6 of the daunosamine sugar instead of the axial hydroxyl found in doxorubicin. This has resulted in a shorter half-life, lower cardiotoxicity as well as the treatment of certain adenocarcinomas. In the daunorubicin analog idarubicin, the 4-methoxy group attached to the aglycone ring has been removed, resulting in the treatment of a wider range of cancers including multiple myeloma, lymphoma, and breast cancer in addition to leukemia.\textsuperscript{26,27} Figure 1.3 shows the structures of daunorubicin and doxorubicin as well as their structural analogs idarubicin and epirubicin, respectively.
1.10 Anthracycline Mechanism of Action

Though anthracyclines have many mechanisms of action, they mainly act as topoisomerase II inhibitors. Topoisomerases are enzymes which can change the physical structure of DNA without altering the sequence or chemical structure. They do this by first causing breaks in DNA strands, then coiling or uncoiling the strands, and subsequently resealing them. Anthracyclines take advantage of topoisomerase II’s by intercalating between DNA base pairs and forming anthracycline-DNA-topoisomerase complexes which inhibit the resealing of the DNA strands, resulting in DNA damage, cell cycle arrest, and cell death. Some studies have shown that tumors which are resistant to anthracyclines have lower than normal levels of topoisomerase II and subsequently
lower levels of strand breaks and anthracycline-DNA-topoisomerase complexes.\textsuperscript{28} The formation of an anthracycline-DNA-helicase complex inhibits strand separation of duplex DNA and thereby hinders replication.\textsuperscript{29} Additionally, the daunosamine sugar projects into the minor groove of DNA and impedes the synthesis of RNA.\textsuperscript{30} As mentioned, anthracyclines possess a quinone molecule. This structure readily participates in oxidation-reduction reactions, yielding reactive oxygen species such as hydrogen peroxide and superoxide which can cause damage to cellular DNA and thus trigger apoptosis.\textsuperscript{27,28} Similarly, p53 seems to have an indirect role in anthracycline cytotoxicity by increasing the number of breaks in DNA strands, leading to apoptosis as well as possibly inhibiting the ligase function of topoisomerase II.\textsuperscript{27}

1.11 Role of Anthracyclines in Apoptosis

In addition to the above-mentioned ways in which anthracyclines can induce apoptosis, studies have shown that the degree and type of anthracycline-induced apoptosis is dependent on cell type and cell line and can thus vary. In some cell lines, anthracycline induces Fas-dependent apoptosis by way of the CD95 receptor and ligand. In others, apoptosis can be attributed to intracellular production of ceramide via sphingomyelinase in response to treatment by anthracyclines. Ceramide production results in an increase in mitochondrial permeabilization via generation of ceramide channels in the mitochondrial membrane as well as activation of pro-apoptotic cascades. Furthermore, Bcl-2 and p53 play key roles in regulating anthracycline-induced apoptosis. p53 is a transcription factor that causes apoptosis and cell cycle arrest in response to anthracyclines.\textsuperscript{31} In contrast, Bcl-2 inhibits apoptosis in response to anthracycline exposure. Bcl-2 represses p53-mediated pro-apoptotic genes, blocks cytochrome c
release, inhibits the effects of damaging reactive oxygen species, hinders caspase activation, and interferes with apoptosis-associated transcription factors such as NF-κB. It is important to note that NF-κB has an anti-apoptotic response to cell stress and DNA damage induced by anthracyclines.\textsuperscript{27} Its expression is increased in most cell types in response to anthracycline treatment and is similarly increased in anthracycline-resistant cells. Interestingly, Bcl-2 does not interfere with anthracycline-induced sphingomyelinase activation and subsequent ceramide production.\textsuperscript{31} Overexpression of Bax, a pro-apoptotic member of the Bcl-2 family of proteins, has been shown to enhance doxorubicin-induced cell death of certain cancers such as those of the erythroleukemia and ovarian lineages.\textsuperscript{32} Finally, studies have shown that anthracyclines can induce cytochrome c release independent of DNA damage.\textsuperscript{27,30,31}

1.12 5-Fluorouracil

5-Fluorouracil (5-FU) is part of the antimetabolite class of antineoplastic agents. Antimetabolites are drugs which work by being incorporated into molecules such as DNA and RNA and inhibiting their function or by inhibiting processes essential to cell survival. Fluoropyrimidines such as 5-fluorouracil were developed by Heidelberger et al. in the 1950’s after noticing rat hepatomas more readily metabolized the pyrimidine uracil than normal tissues did. This represented a niche that could be exploited and utilized as a new method of chemotherapy.\textsuperscript{33} 5-Fluorouracil is commonly used to treat solid tumors such as carcinomas of the colon and the breast as well as those of the ovaries and the bladder. It is not, however, usually employed in the treatment of leukemias, as studies have been limited and data about its efficacy have been conflicting.\textsuperscript{40}
5-Fluorouracil is an analog of uracil, differing only at the C-5 position where 5-fluorouracil possesses a fluorine atom instead of a hydrogen (Figure 1.4).\textsuperscript{33} It is a heterocyclic aromatic molecule which crystallizes with four molecules and subsequently takes on a hydrogen-bonded sheet structure. The analog 5-fluoro-2’-deoxyuridine (FdUrd) has a similar structure to that of 5-fluorouracil but also contains a ribose group.\textsuperscript{34}

**Figure 1.4: Structure of 5-Fluorouracil and Uracil**

![Structure of 5-Fluorouracil and Uracil](image)

\textbf{1.13 5-Fluorouracil Mechanism of Action}

5-Fluorouracil works in two main ways: inhibition of thymidylate synthase, and misincorporation of 5-fluorouracil-derived nucleotides into RNA and DNA. When taken up, 5-fluorouracil is converted to three main metabolites which each play a direct role in its cytotoxicity (Figure 1.5). These active metabolites are fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP).\textsuperscript{33,35}
Thymidylate synthase (TS) catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) which is a nucleotide used to form thymine. It typically does this by binding dUMP at the nucleotide-binding site and the reduced folate 5,10-methylenetetrahydrofolate (CH$_2$THF) at a separate binding site. Binding of CH$_2$THF is necessary as it acts as the methyl donor for this reaction. 5-Fluorouracil inhibits thymidylate synthase by way of its metabolite FdUMP. FdUMP binds to thymidylate synthase’s nucleotide-binding site and forms a stable ternary complex with thymidylate synthase and CH$_2$THF. This results in an excess of the normal substrate dUMP and a deficiency in dTMP. Absence of dTMP has downstream consequences because it results in a subsequent depletion of dTTP and aberrations in the levels of the other nucleotides. This leads to severe DNA damage by way of major disruptions in DNA synthesis and repair mechanisms. Eventually, a lack of dTTP results in a complete inhibition of DNA synthesis and death, termed “thymineless death”.

The accumulation of dUMP leads to an increase in deoxyuridine triphosphate (dUTP), which along with the 5-fluorouracil metabolite FdUTP, can be misincorporated into DNA. In response to this misincorporation, the nucleotide repair excision enzyme uracil-DNA-glycosylase (UDG) attempts to repair the DNA but instead causes more misincorporation which eventually leads to breaks in the DNA and cell death. It is important to mention, however, that DNA damage by way of dUTP is dependent on the amount of dUTPase present since it can limit the buildup of dUTP.

Of the three main 5-fluorouracil metabolites, FUTP leads to RNA damage. FUTP is highly misincorporated into RNA, resulting in aberrations in RNA processing as well as function. FUTP incorporation impacts RNA in many different ways. First, it prevents
processing of pre-rRNA into rRNA.\textsuperscript{36} In addition, it interferes with tRNA post-transcriptional modifications as well as indirectly inhibits both polyadenylation and splicing of pre-mRNA. Studies have also shown that 5-fluorouracil inhibits conversion of uridine to pseudouridine which is usually present in functional rRNA, tRNA, and snRNA. All of these consequences greatly impact cell functioning and viability.\textsuperscript{33}

**Figure 1.5: 5-Fluorouracil Metabolism**\textsuperscript{41}

1.14 Role of 5-Fluorouracil in Apoptosis

Similarly to anthracyclines, many studies implicate a major role for p53 in 5-fluorouracil-mediated apoptosis as a result of DNA and RNA damage.\textsuperscript{35} As a major tumor suppressor, p53 is responsible for maintaining DNA integrity by activating genes which
induce cell-cycle arrest. If the damage is extensive and the cell is deemed irreparable, p53 will instead induce apoptosis. As a result of 5-Fluorouracil-mediated inhibition of thymidylate synthase, two varieties of cell type-dependent DNA fragmentation can occur.\textsuperscript{33} The first type is that which is classically seen as a hallmark of apoptosis in which internucleosomal DNA is fragmented. This type of fragmentation typically elicits an apoptotic response fairly quickly, usually in a matter of hours. In contrast, cell lines derived from most solid tumors undergo delayed apoptosis which may take days as a result of high molecular weight DNA fragmentation.\textsuperscript{35} Though initiation of apoptosis is most commonly associated with DNA damage, studies have shown that 5-Fluorouracil-induced effects on RNA can also induce apoptosis.\textsuperscript{35,37} Specifically in intestinal epithelia, multiple studies have shown that p53-mediated apoptosis occurs as a result of 5-Fluorouracil metabolites being incorporated into RNA.\textsuperscript{37} When cellular damage is too far gone, p53 will transcriptionally induce pro-apoptotic genes such as \textit{Fas} and \textit{Bax}, and down-regulate anti-apoptotic genes such as \textit{Bcl2}. Fas, a member of the tumor necrosis factor family, interacts with its ligand and causes the recruitment of caspase-8 via FADD (Fas-associated death domain), forming a complex known as DISC (death-inducing signaling complex) and results in apoptosis. In TS-deficient colon cancer cell lines, apoptosis was correlated with an increased expression of FasL and when anti-FasL antibodies were utilized, the cells were protected from apoptosis.\textsuperscript{33} Nita et al. have shown that the ratio of Bcl-X(L) to Bax correlates to 5-fluorouracil sensitivity.\textsuperscript{38} Similarly, Mirjolet et al. determined that regardless of p53 functionality, Bcl-2 or Bax induction as well as Bcl-2/Bax protein ratio are linked to 5-fluorouracil sensitivity.\textsuperscript{39} However, some studies have reported that non-functional p53 results in a reduced sensitivity to 5-
fluorouracil. One study conducted with a colon cancer cell line in which both alleles of
TP53 (the gene coding for p53) or Bax were knocked out resulted in a marked resistance
to 5-fluorouracil-mediated apoptosis. Similarly, multiple studies have shown that
overexpression of p53, which is indicative of a mutation, correlates with 5-fluorouracil
resistance although there have been some conflicting results.\textsuperscript{33} It is important to
recognize, however, that much of this data is cell type-dependent, and therefore no set of
studies can be taken as a representation of the general relationship between p53 and 5-
Fluorouracil.

1.15 Bim

Bim is a BH3-only pro-apoptotic member of the Bcl-2 family and contains only
the BH3 domain and a transmembrane domain which is possessed by all members of the
Bcl-2 family.\textsuperscript{11} Bim is normally found in its inactive form bound to the dynein motor
protein and is expressed in many different tissues.\textsuperscript{42} At least six splice variants of Bim
have been identified although the three main splice variants are BimEL (extra long),
BimL (long), and BimS (short).\textsuperscript{42,43} Of all the BH3-only members of the Bcl-2 protein
family, it is interesting to note that Bim seems to have a role in development as deletion
of \textit{Bim} in mouse embryos has been found to be lethal.\textsuperscript{44} Also of note is the accumulation
of both myeloid and lymphoid cells in \textit{Bim} deficient mice as expression of Bim has roles
in homeostasis of B and T cells.\textsuperscript{45,74} In addition to these functions, Bim has important and
wide-ranging roles in cancer and metastasis.\textsuperscript{46} Of most importance is Bim’s role as a
tumor suppressor which enables apoptosis of cancerous cells while \textit{Bim} deficiency has
shown to facilitate the formation of tumors in mice, one of which is acute B cell
leukemia.\textsuperscript{45,46} In many cancers, including Burkitt’s lymphoma and renal cell carcinoma,
oftentimes Bim expression will be completely suppressed.\textsuperscript{45} In metastasis, loss of anchorage-dependence in tumor cells leads to upregulation of Bim and subsequent apoptosis termed “anoikis”. In many metastatic or chemo-resistant cancers, \textit{Bim} is silenced or downregulated.\textsuperscript{46} In a previous study by R.D. Medh et al., gene expression profiling in the human leukemia CEM cell lines after a 20 hour treatment with 1µM of the glucocorticoid Dexamethasone showed significant upregulation of \textit{Bim}.\textsuperscript{47}

\textbf{1.16 E4BP4}

E4BP4 (adenovirus E4 promoter binding protein 4) is a basic leucine zipper (bZIP) transcription factor. bZIP proteins contain an N-terminal bZIP domain and a leucine zipper region in the C-terminus which enable bZIP proteins including E4BP4 to bind in a dimerized fashion.\textsuperscript{48} It was first identified in 1992 as a transcriptional repressor and subsequently named after its ability to bind to the adenovirus promoter ATF (activating transcription factor) site.\textsuperscript{49} Interestingly, other bZIP transcription factors such as CREB or c-jun are transcriptional activators.\textsuperscript{48} In 1995, E4BP4 was separately identified as NFIL3 (nuclear factor, interleukin 3 regulated) because it was found to be a transcriptional activator of the Interleukin 3 promoter in human T cells.\textsuperscript{50} Though it does not contain a PAR region, E4BP4 is characterized as part of the PAR (proline and acidic-residue rich) family of bZIP transcription factors, the other vertebrate members being HLF (hepatic leukemia factor), DBP (D-box binding protein), and TEF (thyrotroph embryonic factor).\textsuperscript{48} E4BP4 regulates transcription by binding to and forming dimers on the EBPRE (E4BP4 response element) which contains the following consensus sequence: (G/A)T(G/T)A(C/T)GTAA(C/T).\textsuperscript{49}
E4BP4 is involved in a wide range of cellular processes. It plays a part in regulating the circadian clock mechanism by way of repression.\textsuperscript{48,51} It also has a prominent role in the development of the innate immune cells, especially NK (natural killer) cells of which many studies have been done. Through these studies, it has been determined that E4BP4 is essential for NK cell development and lineage commitment. \textsuperscript{51,52,53,54} Studies have found that induction of \textit{E4BP4} correlates with downregulation of \textit{Bcl11b}, a transcription factor required for T cell development and lineage commitment (discussed in detail in section 1.17).\textsuperscript{61,63,73} \textit{E4BP4} is also involved in regulation of apoptosis with a seemingly cell-type specific activity. In addition to this, it is thought that E4BP4 has a evolutionarily conserved role in apoptosis as research has shown that it is orthologous to pro-apoptotic proteins vrille in \textit{D. melanogaster} and Ces-2 in \textit{C. elegans}.\textsuperscript{48} Studies have shown that \textit{E4BP4} promotes cell survival in IL-3-dependent pro-B lymphocytes with the t(17;19)(q22;p13) translocation, leading to the formation of leukemia.\textsuperscript{55} In rat and chicken motor neurons, the expression of \textit{E4BP4} has been linked to cell survival.\textsuperscript{48} Studies conducted in the laboratory of R.D. Medh have determined that calcium-dependent expression of E4BP4 correlates to glucocorticoid-evoked apoptosis in the human leukemic CEM cell line.\textsuperscript{56} In addition, evidence has been brought forth by Medh et al. that glucocorticoid-mediated induction of E4BP4 facilitates the induction of Bim and as a result, glucocorticoid-evoked apoptosis.\textsuperscript{57}

\textbf{1.17 Bcl11b}

The B-cell leukemia/lymphoma 11B (\textit{Bcl11b}) gene is a member of the Bcl family which has many biological roles including tumor suppression, transcriptional regulation, and functions associated with T-cell development and survival among others. It has only
recently been identified by Ed Satterwhite in 2001 after it was determined to be a homolog for a similar gene.\textsuperscript{58} It is located on human chromosome 14 (q32.1). Because it was originally found in γ-ray-induced murine thymic lymphomas where the researchers were looking for the loss of specific DNA, Bcl11b is also known as RIT1 (radiation induced tumor suppressor gene 1).\textsuperscript{59} In addition to this, it is also known by the name of CTIP2 (COUP-TF interacting protein 2) because of its collaboration with the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor known as COUP-TF.\textsuperscript{58}

Bcl11a, another member of the Bcl family, has been identified as being homologous to Bcl11b. This homolog is also called CTIP1 because of its similar interaction with COUP-TF like that of Bcl11b. The Bcl11a homolog Evi9 was first described in mice during an attempt to isolate genes involved in murine myeloid leukemia using proviral integration. Bcl11a was later identified by Satterwhite et al. and subsequently led to the identification of its homolog, Bcl11b. In Satterwhite’s study, Bcl11a was identified as a DNA sequence-specific transcriptional repressor. Multiple studies, including that of his lab, have found that translocations in Bcl11a can lead to the formation of B-cell-associated leukemias and lymphomas. Interestingly, in contrast to Bcl11b, Bcl11a has not been linked to T-cell malignancies.\textsuperscript{58}

Both of these proteins are members of the Kruppel-like C₂H₂ zinc finger transcription family. This family is characterized by the presence of six C₂H₂ zinc fingers as well as proline-rich and acidic regions which have 95\% homology in their zinc finger domains. The Bcl11b gene is composed of 4 exons of which exon 4 contains all six zinc fingers as well as the proline-rich and acidic region (Figure 1.6). Both Bcl11b and Bcl11a
can undergo alternative splicing, with \textit{Bcl11b} having two transcript variants: one with exon 3 and one without. This translates to two protein isoforms, one of which is 823 amino acids and while the other is 894 amino acids.\cite{58}

\textbf{Figure 1.6: Bcl11b Gene Structure and Splice Variants}\textsuperscript{77}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure16.png}
\end{figure}

A. Bcl11b gene structure with the four exons. The six zinc fingers and PAR region are represented in exon 4 by the colors blue and white, respectively. B. Alternative splice variants with Ensemble accession numbers.
1.18 Bcl11b as a Transcription Factor

As a transcriptional regulator, it is believed that Bcl11b may act as both a repressor and an activator in a gene-dependent manner. As a repressor, Bcl11b interacts with the nucleosome remodeling and histone deacetylation complex (NuRD). Specifically, Bcl11b recruits members of NuRD, MTA1 and MTA2, to promoters possibly involved with cellular transformation and regulation of growth. Bcl11b also downregulates the ubiquitin ligase HDM2 (human double minute 2) by binding to its P2 promoter region. This is significant because HDM2 is itself a repressor of p53, an important tumor suppressor. Additionally, Bcl11b exerts repression on many other genes including COUP-TF and the cyclin-dependent kinase inhibitors p21/Cip2/Waf1 and p57/Kip2. In respect to its role as a transactivator, Bcl11b in cooperation with P300, acts on the IL-2 promoter at upstream site 1, resulting in IL-2 expression in activated T cells. Furthermore, Bcl11b upregulates the Cot kinase gene in T-cells, leading to NF-κB activation which is important for transcription of DNA leading to a variety of cellular functions.

1.19 Bcl11b in T-cell Development and Survival

T-cells first begin their lives as progenitor cells in the bone marrow but quickly migrate to the thymus to begin development. Thymocytes undergo development in multiple stages starting from the double negative stage (DN) where progenitors do not express the T cell receptors CD4 nor CD8. DN T-cells are subdivided based upon whether they express the cell surface markers CD117, CD44, or CD25. After progressing to the DN4 stage, T-cells then transition to the double positive (DP) stage and express
both CD4 and CD8 markers. At this point, the thymocytes undergo either negative selection, leading to death via apoptosis or positive selection, leading to activation and differentiation into single positive (SP) T-cells.\textsuperscript{61,62}

Bcl11b plays an important role in T-cell development as well as that of maintenance of T-cell identity as is evidenced in a number of different studies. At the DN2 stage, the progenitors, now CD44+ CD25+, express high levels of Bcl11b with it being the most upregulated transcription factor at this stage and is maintained through the remaining stages of development.\textsuperscript{61} Interestingly, \textit{Bcl11b} expression has been shown to be absent in B cells, myeloid cells, and most NK cells.\textsuperscript{63} In \textit{Bcl11b} knockout mice, T-cell development is suspended at the DN2-DN3 stage with no other immune cells being affected.\textsuperscript{61} Overexpression of \textit{Bcl11b} in developing murine T-cells overwhelmingly results in differentiation to CD4+ T-cells, also known as T helper cells.\textsuperscript{64} Recent reports have shown that deletion of \textit{Bcl11b} results in transformation of T cells into NK cells, implying that Bcl11b plays a key role in maintenance of T-cell identity.\textsuperscript{63} Additionally, these induced T to NK (ITNK) cells show a downregulation of T-cell-associated genes such as \textit{Notch1} and \textit{Gata3} and an upregulation in NK-associated genes such as \textit{Il2rb} and \textit{E4BP4}.\textsuperscript{61,63} This translates to a morphological and genetic similarity to NK cells in which these ITNK cells destroy tumor cells and prevent metastasis, possibly providing a new source of cancer immunotherapy. It has been shown that upregulation of \textit{Bcl11b} in naïve T-cells results in increased proliferation while a subsequent downregulation of \textit{Bcl11b} results in decreased proliferation.\textsuperscript{63}
1.20 Role of Bcl11b in ALL

In addition to its role in T-cell development and commitment of lineage, Bcl11b has been identified as a tumor suppressor in both human and murine thymocytes. However, Bcl11b is a haplo-insufficient tumor suppressor with the loss of one Bcl11b allele yielding increased susceptibility to the formation of thymic lymphoma in mice and T-ALL in humans.\textsuperscript{65} Interestingly, abnormally high expression of Bcl11b has been found in many human T-ALL cells with Bcl11b being mutated across major subtypes of T-ALL.\textsuperscript{65,66} A recent study has showed that increased expression of Bcl11b can lead to chemoresistance in patients with T-ALL, suggesting it may act in an anti-apoptotic manner.\textsuperscript{72} A study done by Kamimura et al. demonstrated that lack of Bcl11b resulted in T cells that were abnormally susceptible to DNA replication stress and damage upon exposure to negative stimuli such as UV radiation.\textsuperscript{67} RNA interference (RNAi) has been employed as a method of downregulating the expression of Bcl11b, resulting in inhibition of growth and apoptosis of T-ALL cells although the same results were not seen in mature T-cells. It is thought that apoptosis of the malignant T-cells was mediated by a decrease in the anti-apoptotic protein Bcl-xL as well as the cell cycle inhibitor p27 and that mature T-cells were unaffected because of compensatory upregulation of Bcl-2. Consequently, this study demonstrates that Bcl11b can serve as an effective target in the treatment of many T-cell malignancies by way of RNAi and possibly by other novel therapeutic strategies.\textsuperscript{68} A different study showed that low expression of Bcl11b correlated with diminished survival rates in adults with T-ALL.\textsuperscript{71}
1.21 Other Roles of Bcl11b

In addition to its roles in transcriptional regulation and T cell development and maintenance, Bcl11b functions in other cell types, including neurons, skin, and ameloblasts. In developing Bcl11b knockout mice, pathfinding and axonal extension in corticospinal motor neurons were found to be defective, resulting in lack of connection between the neurons and the spinal cord. In heterozygous mice, a lesser effect is seen because of Bcl11b’s haploinsufficient nature though the mice still have a noticeable deficiency. Bcl11b is also expressed in a different type of neuron called the medium-sized spiny neuron. A lack of Bcl11b results in loss of differentiation of this neuron as well as disruption of cellular organization in the striatum where it is located. These studies point to an important role in neuron development and function by Bcl11b. In development of skin, studies suggest that Bcl11b may play an important role. Bcl11b knockout mice present with an underdeveloped epidermis and differentiation of the epidermis requires a longer period of time. Keratinocyte proliferation is compromised as is the development of the epidermal permeability barrier which can lead to death. Furthermore, studies have linked Bcl11b expression to skin cell migration, tissue contraction, and follicle cell maintenance during cutaneous wound healing as well as atopic dermatitis. In addition to its roles in neuron and skin development, Bcl11b has a critical role in regulation of epithelial cell fate and differentiation during tooth development. Ameloblasts are a type of epithelial cell found during tooth development and serve to deposit tooth enamel. In Bcl11b knockout mice, teeth are overall underdeveloped and small in size. Specifically in incisors, ameloblasts, and therefore
enamel, are absent on the lingual side of the teeth, suggesting Bcl11b plays a crucial role in proper tooth development.\textsuperscript{69}

1.22 The Relationship Between Bcl11b, E4BP4, and Bim

Apoptosis is an integral part of the development and maintenance of the immune system. When immune cells override signals and proliferate uncontrollably, malignancies result.\textsuperscript{1,2} Antileukemic agents are utilized to elicit apoptosis in these malignant cells. In particular, many drugs induce expression of pro-apoptotic members of the Bcl-2 family.\textsuperscript{31,35,39} Bim, a BH3 member of the Bcl-2 family, plays a direct role in apoptosis signaling as it can signal for the release of cytochrome c.\textsuperscript{74} Of particular relevance to this study is research which has linked Bim deficiency to the formation of acute B cell leukemia in mice.\textsuperscript{45,46} E4BP4 is a transcription factor with implications in apoptosis as studies have pointed to it being an evolutionarily conserved ortholog of Ces-2 in \textit{C.elegans}.\textsuperscript{48} Previous research has attributed E4BP4 induction to glucocorticoid-evoked apoptosis in the human leukemic CEM cell line and has established a relationship with Bim.\textsuperscript{56,57} E4BP4 also plays a role in NK cell development and maintenance of identity. Bcl11b is a transcriptional regulator whose expression is necessary for T cell development and maintenance of identity.\textsuperscript{52,53} Studies have shown that E4BP4 and Bcl11b have opposing roles in immune cell fate determination. Bcl11b suppression leads to an upregulation of E4BP4 and subsequent commitment to an NK cell fate whereas the opposite conditions lead to maintenance of T cell identity.\textsuperscript{73} In addition, a recent study found that increased expression of \textit{Bcl11b} can lead to chemoresistance in T-ALL patients, implying that Bcl11b may have an anti-apoptotic role.\textsuperscript{72}
1.23 Hypothesis

The aim of this work is to evaluate the relationship between expression of Bcl11b, E4BP4, and Bim in RS4;11 cells and elucidate how they mediate drug-induced apoptosis. The main goal of this study was to evaluate expression levels of Bcl11b, E4BP4, and Bim in the RS4;11 cell line in response to Daunorubicin- and 5-Fluorouracil-induced apoptosis. I hypothesized that Bcl11b suppression correlates with both E4BP4 and Bim up-regulation in response to apoptosis mediated by anti-leukemic agents. Although previous research has established a link between E4BP4 and Bim in other cell lines, additional research is needed in order to determine if this process occurs more widely. Similarly, although more research is being done to elucidate the many roles of Bcl11b, few have studied Bcl11b in the context of apoptosis. Revealing the role of Bcl11b in apoptosis as well as its relationship with pro-apoptotic genes may lead to elucidating a therapeutic target for pro-B-cell malignancies and could potentially be applied to a wider range of cancers.
Chapter Two: Materials and Methods

2.1 Cell Culture

The RS4;11 (ATCC® CRL1873™) cell line was obtained from the American Type Culture Collection. Routine culturing was performed using RPMI1640 medium from Cellgro (Manassas, VA, Cat# 50-020-PB) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Cat# S11150), 10mM Hepes buffer (Cellgro, Manassas, VA, cat# 25-060-CI), 1mM sodium pyruvate (Cellgro, Manassas, VA, cat# 25-000-CI), and 4.5g/L of glucose at 37°C in a humidified 5% CO2 incubator. The cells were maintained at log phase between 1x10^5 cells/ml and 1x10^6 cells/ml. Media was renewed about once every 2-3 days.

Liquid RPMI 1640 was prepared by adding deionized water and 2.0g/L of sodium bicarbonate to RPMI 1640 powder. The pH was adjusted to 7.2 and the media was subsequently filter sterilized via a 0.22μm cellulose acetate filter from Corning (Lowell, MA, Cat# 430521); this process raised the pH to the optimal value of 7.4. The media was then stored at 4°C for future use.

2.2 Measurement of Cell Density

Cells were routinely counted to determine culture density for passaging or downstream applications by Trypan blue dye exclusion. One hundred microliters of cell culture was added to a mixture of 200μl of 1x phosphate buffered saline (PBS) and 100μl of 0.4% Trypan Blue (Cellgro, cat# 25-900-CI). Trypan Blue was used as a dye exclusion method to stain nonviable cells. About 15μl of this mixture was loaded onto a Bright-Line hemocytometer (Fisher Scientific, cat# 0267110) and cells were counted using a
microscope at 200X total magnification. Following counts, cell density was determined using the following equation: Viable cells/ml = (Total number of cells that excluded dye/4) \times (4 \times 10^4).

2.3 Cell Treatment Reagents

Powdered Daunorubicin HCL was obtained from CalBiochem (cat# 251800) and diluted with sterile deionized water. 5-Fluorouracil was obtained from CalBiochem (cat# 343922) and diluted with dimethyl sulfoxide (DMSO) (Fisher BioReagents, cat #BP231-1). Sterile deionized water or sterile PBS and DMSO were used as vehicles for the appropriate drugs.

2.4 Cell Viability Assay

Cell viability assays were performed using RS4;11 cells at a density of 1\times10^5 cells/ml. Using multi-well dishes, 2ml of cells was dispensed into two wells each in duplicates or triplicates. Each well was treated with a drug or vehicle. Two sets of daunorubicin titrations were conducted at concentrations of 1nM, 10nM, and 50nM in triplicates. Two sets of 5-Fluorouracil titrations were conducted at concentration of 1µM, 10µM, and 50µM in triplicates. Three sets of 5-Fluorouracil titrations were conducted at concentrations of 50µM, 100µM, and 500µM in duplicates. Vehicles were sterile deionized water for Daunorubicin and DMSO for 5-Fluorouracil. The treatment day was denoted Day 0 and cells were counted in approximately 24 hour intervals each subsequent day through Day 4 using the Trypan blue dye exclusion method.
2.5 Apoptosis Assay

An apoptosis assay was done via epifluorescence microscopy in order to visualize characteristic changes associated with apoptosis. The fluorescence microscope used was the Accu-scope 3025-EPI. A ProgRes MF monochromatic camera (JENOPTIK, Germany) and ProgRes CapturePro 2.7 software were used to capture and view the images.

Prior to visualization, cells underwent a 33 hour treatment. It is important to note that a 33 hour treatment was utilized instead of a 24 hour treatment (as was done in the rest of this project) in order to allow enough time for observable changes to occur. Cells were seeded at a concentration of $\sim 1.0 \times 10^6 \text{cells/ml}$ and treated with either a drug (50nM Daunorubicin or 50µM 5-Fluorouracil) or the appropriate control (PBS or DMSO). Two experiments were done for each drug and control with each experiment being conducted in triplicates.

The Biotium Dual Apoptosis Assay kit (Cat# 30067) was utilized to prepare and stain the cells. In accordance with the kit, three fluorescent dyes were utilized. The CF 594 Annexin V dye detects phosphatidylserine translocation, which serves as an identifier of apoptotic cells for phagocytosis by immune cells. The Annexin V dye becomes excited at 593 nm and fluoresces a deep red color at 614 nm. This was visualized using the Texas Red filter. The NucView 488 Caspase-3 substrate enters the cell and fluoresces bright green once it is cleaved by caspase-3. Caspase-3 is an executioner caspase which cleaves various substrates, resulting in the biological and morphological hallmarks of apoptosis. One of the most important substrates that caspase-3 cleaves is ICAD (inhibitor
of caspase-activated DNase), which results in the activation of CAD (caspase-activated DNase). Activation of CAD subsequently results in chromatin condensation. The caspase-3 substrate becomes excited at 485 nm and emits fluorescent light at 515 nm. The FITC filter was used to visualize caspase-3 fluorescence. The Hoechst 33342 dye was used to show cells and whether or not they are undergoing apoptosis since it binds to DNA. As a reminder, cells undergoing apoptosis display many hallmarks, including chromatin condensation. Therefore, cells not undergoing apoptosis will fluoresce a diffuse blue color while cells undergoing apoptosis will fluoresce a brighter, more intense blue due to chromatin condensation. It becomes excited at 350 nm and emits a blue/cyan fluorescent light at 461 nm. The DAPI filter was used to visualize these cells.

One X Annexin V binding buffer was prepared from a 5x stock solution. Next, the staining solution was prepared. It consisted of 200µl of the 1x binding buffer, 5µl of NucView Caspase-3 substrate stock solution, 5µl of CF594 Annexin V stock solution, and 5µl of Hoechst 33342. This mixture was prepared and set aside in the dark.

One milliliter of cells from each sample were pipetted into microfuge tubes and spun at 1500 rpm for ~5 min. The supernatant was aspirated and each sample was washed with 500µl of PBS. The samples were again spun at 1500 rpm for 5 min. and each supernatant was aspirated. Each cell pellet was resuspended in 50µl of the staining solution and incubated at room temperature in the dark for ~ 30 min. After incubation, cells were washed with 200µl of 1x binding buffer per sample, spun at 1500 rpm, and the supernatant was aspirated. Finally, the cells were resuspended in 20µl of 1x binding buffer and ~10µl of this suspension was placed on a microscope slide. A coverslip was
placed over the slide and the edges were sealed with nail polish. All prepared slides were kept in the dark until they were viewed under the epifluorescence microscope.

2.6 RNA Extraction

Cells were counted and diluted to 30ml cultures at $5 \times 10^5$ cells/ml and treated with a drug (50nM Daunorubicin or 50µM 5-Fluorouracil). Corresponding vehicles were sterile deionized water or filtered PBS for Daunorubicin and DMSO for 5-Fluorouracil. Flasks were placed in a 5% CO2 incubator at 37°C for 24 hours. After the 24 hour incubation, cultures were transferred to 50ml Falcon tubes and centrifuged at about 500 x g for 10 minutes.

RNA was extracted using the Quick-RNA MiniPrep kit from Zymo Research (Cat# R1054) according to the protocol established by the manufacturer. Cultures were lysed using the RNA Lysis Buffer and then genomic DNA was removed before purifying RNA by DNase I treatment. RNA was dissolved in 30µl of PCR grade nuclease-free water (Genemate, cat# G-3255-50) and stored at -20°C for future use.

2.7 RNA Quantification

RNA concentration and purity was measured for each sample using the Nanodrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE) on 2 different days from which the average RNA concentration was determined. The RNA concentration was measured at an absorbance of 260nm while the sample purity was measured using 280nm and 230nm. Genemate PCR grade nuclease-free water was used to blank the machine prior to measuring sample concentrations. Samples with a 260nm/280nm ratio of 1.8 or
higher were used for reverse transcription (RT) reactions as pure RNA yields a 260nm/280nm ratio of 2.0.

2.8 Reverse Transcription Reaction

Reverse transcription was utilized to construct a complimentary DNA (cDNA) strand from an RNA template. Seven micrograms of RNA was brought up to a final volume of 11µl with PCR-grade nuclease-free water. 1µl of Oligo(dT)\textsubscript{15} (Promega, Madison, WI, cat# 1101) was added per sample and tubes were heated to 70°C for 5 minutes and then flash-cooled on ice. A reverse transcription reaction master mix was prepared in a separate tube of which 13µl was added to each sample (Table 2.1). Each reaction mixture was allowed to reverse transcribe in a 42°C water bath for 3 hours and then frozen at -20°C for later use.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume Per Reaction (µL)</th>
<th>Manufacturer, Cat#</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-MLV Reaction buffer (5X)</td>
<td>5</td>
<td>Promega, cat# M5313</td>
</tr>
<tr>
<td>PCR nucleotide mix (10mM)</td>
<td>1.25</td>
<td>Promega, cat# C1141</td>
</tr>
<tr>
<td>RNasin Plus RNase inhibitor</td>
<td>1</td>
<td>Promega, cat# N2611</td>
</tr>
<tr>
<td>PCR-grade nuclease free water</td>
<td>4.75</td>
<td>Genemate, cat# G-3255-50</td>
</tr>
<tr>
<td>M-MLV Reverse Transcriptase</td>
<td>1</td>
<td>Promega, cat# M1705</td>
</tr>
</tbody>
</table>
2.9 Quantitative Real Time-PCR

Quantitative real time-PCR (qRT-PCR) was utilized to amplify, detect, and quantify the expression of *E4BP4*, *Bim*, *Bcl11b*, and *β-actin* in the RS4;11 cell line. Primers for each gene were previously designed and optimized to achieve ideal melting temperature (Tm), GC content, and other characteristics (Table 2.2). 25µl of each mastermix was added to three wells of a 96 well qPCR plate per gene (triplicates). qRT-PCR reactions were conducted using the Applied Biosystems 7300 machine. Table 2.3 shows the qRT-PCR reaction mixture used and table 2.4 shows the thermocycler protocol utilized for each gene.

Additionally, positive controls were run to confirm qRT-PCR was being prepared and run correctly. cDNA for this thesis and cDNA from a previous student’s project were used to amplify, detect, and quantify the expression of *DSCR1.1s*, *DSCR1.4*, and *β-actin* (Table 2.3). The cDNA from this project was from RS4;11 cells treated with 50nM Daunorubicin while the cDNA used as the positive control was from RS4;11 cells treated with 1mM Dexamethasone or 100% ethanol.

**Table 2.2: Primer Sequences**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human <em>E4BP4</em></td>
<td>5’ATGGGGAATTCTTTTCTG3’</td>
<td>5’CTTTGATCCGGAGCTTG TGT3’</td>
<td>250bp</td>
</tr>
<tr>
<td>Human <em>Bim</em></td>
<td>5’CAGATATGCGCCCAAGA GATA3’</td>
<td>5’ACCAGGGGACAATGT AAC3’</td>
<td>163bp</td>
</tr>
<tr>
<td>Human <em>Bcl11b</em></td>
<td>5’AGGCATCTCTCCACAG CAGGA3’</td>
<td>5’CCACCGCGCTGTGAAGG GCT3’</td>
<td>307bp, 94bp</td>
</tr>
<tr>
<td>Human <em>β-actin</em></td>
<td>5’AGTCCTCTCCCAAGTC CACA3’</td>
<td>5’CACGAAGGCTCATCATT CAA3’</td>
<td>130bp</td>
</tr>
</tbody>
</table>

*Primer has been designed to span exons 5 and 6, resulting in amplification of all three isoforms concurrently

**Primer has been designed to amplify both Variant 1 (307bp) and Variant 2 (94bp, missing exon 3), resulting in 2 products if both are expressed.
### Table 2.3: qRT-PCR Reaction Mixture

<table>
<thead>
<tr>
<th></th>
<th>Volume (µL)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR Green</td>
<td>12.50</td>
<td>1X</td>
</tr>
<tr>
<td>PCR grade nuclease-free water</td>
<td>11.00</td>
<td></td>
</tr>
<tr>
<td>Forward Primer*</td>
<td>1.25</td>
<td>250nM or 125nM</td>
</tr>
<tr>
<td>Reverse Primer*</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>Template</td>
<td>1.00</td>
<td>280ng</td>
</tr>
</tbody>
</table>

*Stock concentration of *E4BP4*, *Bim*, and *Bcl11b* primers used were 5µM. Stock concentration of β-actin primers used were 2.5µM.

### Table 2.4: qRT-PCR Thermocycler Protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>Amplification</td>
<td>94</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td><em>B-actin</em></td>
<td>55</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td><em>E4BP4</em></td>
<td>58</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td><em>Bim</em></td>
<td>58</td>
<td>45sec</td>
<td>35</td>
</tr>
<tr>
<td><em>Bcl11b</em></td>
<td>61.5</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td><em>DSCR1.1s</em></td>
<td>55</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td><em>DSCR1.4</em></td>
<td>55</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Detection*</td>
<td>72</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Dissociation</td>
<td>95</td>
<td>15 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>15 sec</td>
<td></td>
</tr>
</tbody>
</table>

* Data collection occurs during this step

#### 2.10 Gel Electrophoresis of qRT-PCR Product

A 2% agarose gel was prepared using 50ml of 0.5x TBE and 1g of agarose (RPI, cat# A20090-500). 0.5µl of ethidium bromide was added to the solution and mixed in once the agarose was fully dissolved. The mixture was then poured into a gel tray and allowed to solidify. 10µl of PCR product were mixed with 2µl of 6X loading dye (Promega, cat# G190A) and loaded in each well. A 100bp DNA ladder (Promega, cat# G190A) was used as a molecular weight marker. The gel box was filled with 0.5x TBE
and the gel was run at 90 volts for approximately 50 minutes or until the dye was two-thirds through the gel.

2.11 qRT-PCR Data Analysis

After the machine completed its run, preliminary data analysis was conducted on the ABI 7300 machine. The baselines for each detector (gene), were manually set to start from cycle number two through two cycles before the beginning of amplification. The threshold for each detector was also manually set. The output was then analyzed by the program LnReg (version 11.1). LnReg performs a baseline correction on each sample by determining a window-of-linearity. It does this by picking four data points and using linear regression analysis to fit a straight line through all data points for each sample run. The four points are chosen by determining a coefficient (R) greater than 0.99 and an amplification efficiency as close to two as possible. The program then calculates the PCR efficiency of each sample by using the slope of the best fit line.\textsuperscript{78,79} The threshold cycle (Ct) value is a relative measurement of the concentration of the target in the PCR. The Ct value itself is the number of cycles required for the fluorescent signal to cross the threshold or background fluorescence during the exponential phase of the PCR where amplification is occurring.\textsuperscript{78}

The Pfaffl method was used to calculate fold induction or repression of the three genes in each sample. This method utilizes the assumption that amplification efficiencies differ between genes. The PCR efficiency (E) per amplicon (set of samples with the same pair of primers) and the Ct value per sample were used in the Pfaffl method equation: 

\[ (E) \Delta CT \text{target (control-sample)} / (E) \Delta CT \text{reference (control-sample)} \] \textsuperscript{79}
Individual data sets were analyzed for abnormal delta Ct values. If a value was inconsistent and the difference in delta Ct values within that triplicate was 0.5 or larger, the sample was omitted. The data was averaged and standard deviations were calculated for both drug- and control-treated samples for each drug treatment. Data were plotted as a mean with error bars.
3.1 Cell Viability Assays

Cancer cells exhibit various susceptibility responses to different drugs. For this reason, cell viability assays were done to determine whether the RS4;11 cell line is susceptible or resistant to treatment by Daunorubicin and 5-Fluorouracil. In addition, titrations of these drugs were utilized to determine the optimal concentration of each drug to be used in all subsequent experiments.

Cell viability assays were performed using cells seeded at $1 \times 10^5$ cells/ml. Daunorubicin titrations were conducted at 1nM, 10nM, and 50nM concentrations in triplicates. 5-Fluorouracil titrations were conducted at 1µM, 10µM, and 50µM concentrations in triplicates. 5-Fluorouracil titrations were also conducted at 50µM, 100µM, and 500µM concentrations in duplicates. Vehicles were sterile deionized water for Daunorubicin and DMSO for 5-Fluorouracil. Cells were counted in approximately 24 hour intervals for 4 days using the Trypan blue dye exclusion method.

Figure 3.1 shows the averaged daunorubicin titration growth curve. Cells treated with the vehicle and 1nM daunorubicin exhibit almost the same rate of growth throughout all four days of cell treatment and at no point do the cells appear to be dying. Cells treated with 10nM exhibit a mild drop-off after 48 hours and only after 72 hours do they fall below a concentration of $1.0 \times 10^5$ cells/ml. The highest level of sensitivity was shown at 50nM as cells were at a concentration of $3.3 \times 10^3$ cells/ml at 48 hours and by 72 hours were at a concentration of zero.

The first averaged 5-fluorouracil titration growth curve is represented by figure 3.2. The 1µM treatment displays an almost identical growth rate as that of the vehicle and
at a concentration of 10µM, the cells exhibit a similar trend. Only at 50µM did the cells appear to be undergoing apoptosis from the beginning of treatment, culminating in a complete lack of cells by 96 hours.

Figure 3.3 shows the second averaged 5-fluorouracil titration growth curve using a different set of concentrations as the first titration. In this assay, a higher set of concentrations were utilized to verify whether 50µM is the optimum concentration to use for downstream experiments. In this case, all treatments displayed cell death. The 50µM and 100µM concentrations displayed a very similar rate of death while the 500µM-treated cells exhibited a slightly steeper rate of death. It is important to note that although in this second set of titrations the 50µM treatment did not result in a complete absence of cells at 96 hours, this is a result of one outlier in the sum of all cell viability assays performed for this group of titrations.
Figure 3.1 shows the growth curve for the daunorubicin cell viability assay titration. Cells were seeded at 1x10^5 cells/ml and treated with water or daunorubicin (1nM, 10nM, or 50nM) for 72 hours. Cell viability was evaluated in 24 hour intervals using the Trypan dye exclusion method. Cells exhibit the highest level of sensitivity to the 50nM treatment culminating in a complete lack of cells by day 3.
Figure 3.2 shows the growth curve for the first 5-fluorouracil viability assay titration. Cells were seeded at 1x10^5 cells/ml and treated with DMSO or 5-fluorouracil (1µM, 10µM, or 50µM) for 72 hours. Cell viability was evaluated in 24 hour intervals using the Trypan dye exclusion method. Cells exhibit the highest level of sensitivity to the 50µM treatment culminating in a complete lack of cells by day 4.
Figure 3.3 shows the growth curve for the second 5-fluorouracil viability assay titration. Cells were seeded at 1x10^5 cells/ml and treated with DMSO or 5-fluorouracil (50µM, 100µM, or 500µM) for 72 hours. Cell viability was evaluated in 24 hour intervals using the Trypan dye exclusion method. Cells again exhibit a high level of sensitivity to the 50µM treatment.

3.2 Epifluorescence Microscopy

An apoptosis assay was utilized to verify that cell death was occurring and that it was occurring due to apoptosis as opposed to necrosis. The assay was conducted with the Biotium Dual Apoptosis Assay kit (Cat# 30067) and characteristic changes associated with apoptosis were visualized via the Accu-scope 3025-EPI. A ProgRes MF monochromatic camera (JENOPTIK, Germany) and ProgRes CapturePro 2.7 software were used to capture and view the images. Three fluorescent dyes were used. CF 594 Annexin V, which results in a red fluorescence, detects phosphatidylserine translocation which occurs in cells undergoing apoptosis. NucView 488 Caspase-3 substrate causes a
bright green fluorescence once it is cleaved by Caspase-3, which is only present during apoptosis. Hoechst 33342, which results in a blue fluorescence, binds to DNA and is used to identify cells and to conclude which cells may not be undergoing apoptosis by detecting chromatin condensation.

Cells were seeded at a density of ~1.0 x 10^6 cells/ml and underwent a 33 hour treatment with either a drug (50nM Daunorubicin or 50µM 5-Fluorouracil) or the appropriate control (PBS or DMSO). Two experiments were done in triplicates for each drug and control.

Figure 3.4 shows fluorescence images for cells treated with 50nM daunorubicin or PBS. The images on the left hand column are those of the NucView 488 Caspase-3 substrate and the CF594 Annexin V (both of the same field of view) merged into one image. The second column shows the previously described images merged with an image of the same field of view showing Hoechst 33342 staining as well. The PBS-treated cells show very little staining of cleaved caspase-3 substrate and no staining of phosphatidylserine but they do show a high level of diffuse blue florescence. In contrast, the drug-treated cells exhibit a high level of green, red, and intense blue fluorescence, signifying the presence of phosphatidylserine, caspase-3, and condensed chromatin.

Figure 3.5 shows fluorescence images for cells treated with 50µM 5-fluorouracil or DMSO. The images on the left hand column are those of the NucView 488 Caspase-3 substrate and the CF594 Annexin V (both of the same field of view) merged into one image. The second column shows the previously described images merged with an image of the same field of view showing Hoechst 33342 staining as well. The PBS-treated cells
show no red or green fluorescence but when merged with the Hoechst 33342 image, they fluoresce a diffuse blue. The drug-treated cells display an abundance of red, green, and intense blue fluorescence, indicating that the cells are undergoing apoptosis.

**Figure 3.4: Epifluorescence Microscopy of Daunorubicin- or PBS-treated Cells**

![Epifluorescence Microscopy](image)

Figure 3.4 shows the epifluorescence images of cells treated with either 50nM daunorubicin or PBS. Cells were stained with NucView 488 (stains for presence of caspase-3), CF594 Annexin V (stains for transposition of phosphatidylserine) and Hoechst 33342 (stains for DNA). Control-treated cells showed very little fluorescence of apoptosis markers. Drug-treated cells showed a high level of fluorescence of all apoptosis markers.
Figure 3.5 shows the epifluorescence images of cells treated with either 50μM 5-fluorouracil or DMSO. Cells were stained with NucView 488 (stains for presence of caspase-3), CF594 Annexin V (stains for transposition of phosphatidylserine) and Hoechst 33342 (stains for DNA). Control-treated cells showed very little fluorescence of apoptosis markers. Drug-treated cells showed a high level of fluorescence of all apoptosis markers.
3.3 Gel Electrophoresis of qRT-PCR

Gel electrophoresis of amplified qRT-PCR products were run in order to verify that the proper products were being amplified (see table 2.2 for primers). Additionally, for Bcl11b, gels were run in order to determine which transcript variants were being amplified and if any were being differentially induced or repressed as a result of treatment. As mentioned in the introduction, Bcl11b has three alternatively spliced transcript variants with variant 1 containing all 4 exons, variant 2 containing all exons except for exon 3, and variant 3 containing only exons 1 and 4. The Bcl11b primers used for this project are designed to amplify variant 1 (307 bp) and variant 2 (94 bp) only.

Figure 3.6 is an image of an agarose gel which was run using two sets of samples in response to Bcl11b expression. These samples were run for 35 cycles with amplification set at 58ºC. It is important to note that these samples were run differently than what is listed in the qRT-PCR thermocycler protocol (table 2.4) as a previous graduate student concluded that the optimal temperature of Bcl11b is 61.5ºC. Therefore, all subsequent qRT-PCR data for Bcl11b are those which were run with the optimized temperature. Each set contains one sample which was treated with 50nM of daunorubicin prior to the RNA extraction and one sample treated with the vehicle. In this gel, all samples express both variant 1 and variant 2. However, variant 2 is predominantly expressed in both samples. The samples treated with daunorubicin produced bands at 307 bp (variant 1) which show a lesser intensity than those of the control-treated samples.

Figure 3.7 shows an agarose gel which was again run using two sets of samples. Samples have been treated with 50nM daunorubicin or vehicle prior to RNA extraction. qRT-PCR was run according to the thermocycler protocol in table 2.4 for β-actin and
Bcl11b. In this gel, only one intense band can be seen for Bcl11b for the drug-treated and control samples each. The band is found at about 100 bp and is representative of variant 2. The amplified β-actin products are similarly intense and are found to be the correct size of about 130 bp. No difference in band thickness or intensity can be seen between samples for both genes.

Figure 3.8 is representative of an agarose gel run using two sets of samples treated with 50nM daunorubicin or vehicle prior to RNA extraction. qRT-PCR was run according to the thermocycler protocol in table 2.4 for Bim and E4BP4. A band can be seen for each treatment for Bim with no change in band intensity with about the expected size of 163 bp. Two other faint bands can be seen above the main band but they can be attributed to artifacts in the gel during electrophoresis. For E4BP4, a band is seen at about 250 bp for each treatment, but no change is seen in band intensity or thickness between treatments.
Figure 3.6: Gel Electrophoresis of *Bcl11b* qRT-PCR Products

Figure 3.6 is an image of an agarose gel of *Bcl11b* qRT-PCR products. qRT-PCR was run for 35 cycles at 58°C for samples treated with daunorubicin or vehicle prior to RNA extraction. Variant 1 (307 bp) and variant 2 (94 bp) can be seen for all samples. Variant 2 is preferentially expressed but bands for variant 1 show differences in intensity of drug-treated samples when compared to bands for control-treated samples.
Figure 3.7: Gel Electrophoresis of β-actin and Bcl11b qRT-PCR Products

Figure 3.7 is an image of an agarose gel of β-actin and Bcl11b qRT-PCR products. qRT-PCR for β-actin was run for 25 cycles at 55°C while Bcl11b was run for 40 cycles at 61.5°C for samples treated with daunorubicin or vehicle prior to RNA extraction. In this gel, only variant 2 of Bcl11b is visualized. Bands for β-actin are observed at about 130 bp. No differences in band intensity or thickness are seen across samples for each gene.
Figure 3.8: Gel Electrophoresis of *Bim* and *E4BP4* qRT-PCR Products

Figure 3.8 is an agarose gel image of qRT-PCR products of *Bim* and *E4BP4*. qRT-PCR was run for 35 cycles at 58ºC for samples treated with daunorubicin or vehicle prior to RNA extraction. Bands for *Bim* are observed at about 163 bp while for *E4BP4* they are at about 250 bp. No differences in band intensity or thickness are seen across samples for each gene.

### 3.4 Dissociation Curve Analysis

Annealing of primers to the target sequence is critical in qRT-PCR. For the primers to anneal to the correct sequence efficiently, they must do so at the correct temperature in order to prevent the formation of primer–dimers. To confirm this specificity, a dissociation curve analysis was performed. Additionally, a dissociation curve analysis is important for confirming the amplification of one product and may be used to determine if samples have been contaminated. Figures 3.9, 3.10, 3.11, and 3.12 all show amplification of a single product for each gene analyzed and indicate the absence of primer-dimers and contamination.
**Figure 3.9: β-actin Dissociation Curve**

Dissociation curve for β-actin showing a single curve for all samples run.

**Figure 3.10: Bcl11b Dissociation Curve**

Dissociation curve for Bcl11b showing a single curve for all samples run.
Figure 3.11: *Bim* Dissociation Curve

Dissociation curve for *Bim* showing a single curve for all samples run.

Figure 3.12: *E4BP4* Dissociation Curve

Dissociation curve for *E4BP4* showing a single curve for all samples run.
3.5 qRT-PCR Gene Expression Analysis

qRT-PCR gene expression data was obtained from the Applied Biosystems 7300 RT-PCR thermocycler after preliminary analysis (see Figures 3.13 through 3.16 for representative amplification curves) and was subsequently analyzed using the program Linreg. Linreg was used to run a baseline correction on each sample as well as to calculate PCR amplification efficiencies. This data was then used to calculate relative gene expression levels by using the Pfaffl method.

Numerous experiments were conducted and out of these a total of 6 experiments for daunorubicin-treated cells and 7 experiments for 5-fluorouracil-treated cells were used to calculate relative gene expression by the Pfaffl method. Experiments were conducted in triplicates so that samples could be compared to each other and abnormal data could be omitted without compromising results. Individual data sets were scrutinized for abnormal or inconsistent delta Ct values. If a value was inconsistent and the difference in delta Ct values within that triplicate was 0.5 or larger, the sample was omitted from all data. It is important to note that after data analysis was performed, inconsistencies in fold gene expression values between experiments remained present for both drugs although samples within each experiment were consistent. These data were then averaged and standard deviations were calculated for both drug- and control-treated samples. Values were plotted as bar graphs with error bars.

Figure 3.13 shows the gene expression profile for Bcl11b, E4BP4, and Bim in cells that were treated with daunorubicin. Bcl11b expression is slightly repressed while both E4BP4 and Bim are slightly upregulated. As mentioned above, many inconsistencies
were found for relative gene expression levels between sets of experiments for daunorubicin-induced apoptosis. As such, standard deviation values and error bars are fairly large. Fold gene expression values (data not shown) are 0.8837 for Bcl11b, 1.292 for E4BP4 and 1.122 for Bim. All values are relatively close to the baseline expression of one and do not provide any significant evidence of downregulation or upregulation of these genes in response to daunorubicin-induced apoptosis.

Figure 3.14 shows the gene expression profile for Bcl11b, E4BP4, and Bim in cells that were treated with 5-fluorouracil. Bcl11b, E4BP4, and Bim all appear to be very slightly upregulated. As with daunorubicin, many inconsistencies were found for relative gene expression levels between sets of experiments for 5-fluorouracil-induced apoptosis. As such, standard deviation values and error bars are fairly large. Fold gene expression values (data not shown) are 1.126 for Bcl11b, 1.042 for E4BP4 and 1.054 for Bim. All values are relatively close to a baseline expression of one and do not provide any significant evidence of downregulation or upregulation of these genes in response to 5-fluorouracil-induced apoptosis.

Figure 3.15 shows the gene expression profile for DSCR1.1s and DSCR1.4 as positive controls. Both the cDNA from this project and that which was prepared by a previous student show similar expression patterns for both genes with DSCR1.1s being significantly upregulated while DSCR1.4 is downregulated.
Figure 3.13: qRT-PCR Output for $\beta$-actin
Figure 3.14: qRT-PCR Output for *Bcl11b*
Figure 3.15: qRT-PCR Output for E4BP4
Figure 3.16: qRT-PCR Output for *Bim*
Figure 3.17: Gene Expression in Daunorubicin-treated Cells

Gene Expression in 50nM Daunorubicin-treated Cells

- Bcl11b
- E4BP4
- Bim

Figure 3.18: Gene Expression in 5-Fluorouracil-treated Cells

Gene Expression in 50µM 5-Fluorouracil-treated Cells

- Bcl11b
- E4BP4
- Bim
Figure 3.19: Gene Expression of DSCR1.1s and DSCR1.4 – Positive Control

Gene Expression of DSCR1.1s and DSCR1.4

<table>
<thead>
<tr>
<th>Relative Gene Expression</th>
<th>RS4;11 Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSCR1.1s</td>
<td>2000</td>
</tr>
<tr>
<td>DSCR1.1s Positive Control</td>
<td>4000</td>
</tr>
<tr>
<td>DSCR1.4</td>
<td>500</td>
</tr>
<tr>
<td>DSCR1.4 Positive Control</td>
<td>100</td>
</tr>
</tbody>
</table>
RS4;11 Cells Exhibited Sensitivity to Daunorubicin and 5-Fluorouracil Treatment

In order to study gene regulation of apoptosis-associated genes, cells were first treated with chemotherapeutic agents in order to determine which drugs the RS4;11 cell line exhibits sensitivity towards since cancer cell lines demonstrate differing levels of susceptibility or resistance in the presence of various drugs. The viability assays allowed for quantitative determination that cell death was occurring in the RS4;11 cell line in response to treatment by daunorubicin and 5-fluorouracil. These results are in agreement with previous research which indicates sensitivity of leukemic cells to daunorubicin. Multiple studies have shown sensitivity and effectiveness in attaining complete remission of both acute and chronic leukemias when daunorubicin was included in the chemotherapeutic regimen.\(^{93,94,95,96,97}\) Similarly, previous studies have also shown sensitivity of leukemic cells to 5-fluorouracil. However, because 5-fluorouracil is typically used in the treatment of solid tumors, few studies utilizing it in the treatment of leukemia exist. These studies have focused on the effect of 5-fluorouracil, either alone or in conjunction with other agents, in the murine lymphocytic leukemia L1210 cell line and corroborate the results obtained in this project.\(^{40,98,99,100}\)

RS4;11 Cells Undergo Apoptosis After Daunorubicin and 5-Fluorouracil Treatment

Once it was determined that cells were dying in response to the above-mentioned chemotherapeutic agents via cell viability assays, an apoptosis assay was employed. This was done for two main reasons. The first reason was to validate the results seen in the cell
viability assays. The second and more substantial reason why fluorescence microscopy was utilized was to verify that the mode of cell death occurring in response to these chemotherapeutic agents was apoptosis and not necrosis via an apoptosis assay. In the epifluorescence experiments, both daunorubicin- and 5-fluorouracil-treated cells exhibited a high level of phosphatidylserine in the outer leaflet of the plasma membrane, caspase-3 and condensed chromatin, suggesting that cells are undergoing apoptosis. In the vehicles for each drug, there was an absence of caspase-3 as well as phosphatidylserine translocation, implying a lack of cell death. For both treatments and vehicles, Hoechst dye was used to stain DNA blue so that live, intact cells could be differentiated from debris or dead cells. It was also used to identify condensed chromatin as a marker of apoptosis by way of intense blue fluorescence. These findings suggest that cells treated with 50nM daunorubicin or 50µM 5-fluorouracil do indeed die and that they die by way of apoptosis and not necrosis.

**No Significant Changes in Expression of Bcl11b, E4BP4 or Bim Were Seen**

Previous studies including those on drug-induced apoptosis in leukemic cells serve as the basis of this project. A gene expression profiling study by R.D Medh demonstrated significant upregulation of *Bim* in a dexamethasone-susceptible CEM T-cell line after treatment with dexamethasone. Another study by R.D. Medh, established a relationship between E4BP4 and Bim in the CEM cell line by demonstrating that dexamethasone induced upregulation of *E4BP4* facilitates induction of *Bim*, leading to apoptosis. Studies centering around the relationship between Bcl11b and E4BP4 have pointed to opposing roles in immune cell fate determination. They revealed that
downregulation of Bcl11b leads to induction of E4BP4 and subsequent commitment to NK cell fate as opposed to maintenance of T cell identity.\textsuperscript{73} Also suggesting opposing roles is a recent study which found that increased expression of Bcl11b can lead to chemo resistance in T-ALL patients, implying that Bcl11b may have an anti-apoptotic role.\textsuperscript{72} Taking these studies into consideration, this project aimed to establish a relationship between all three genes in drug-induced apoptosis of leukemic cells. The RS4;11 cell line was used in this project to determine whether the types of gene regulatory events previously seen in CEM cells are also involved in other cell lines. A correlation between the expression of Bcl11b, E4BP4, and Bim, and apoptosis was shown not to occur after treatment of either daunorubicin or 5-fluorouracil. A positive control experiment resulted in a similar pattern of expression of DSCR1.1s and DSCR1.4 for both sets of cDNA, demonstrating utilization of correct technique. Since the experiments in this thesis confirmed that cells are dying and that they are dying due to apoptosis, a lack of significant changes in gene expression suggest that the genes of interest do not play a major role in daunorubicin- or 5-fluorouracil-induced apoptosis in the RS4;11 cell line. As such, other genes must be coming into play in order to orchestrate the complex process of apoptosis.

To date, the most important family of genes that have a role in regulating apoptosis is the Bcl-2 family. As reviewed in the introduction section of this thesis, the Bcl-2 family consists of both pro- and anti-apoptotic members. According to multiple studies, expression of the anti-apoptotic member Bcl-2 correlates to levels of sensitivity to chemotherapeutic drugs. Two different studies which utilized the G3139 antisense oligo in daunorubicin- or doxorubicin-resistant myeloma cell lines resulted in both a
downregulation in Bcl-2 expression and concurrent sensitization to the respective drug.\textsuperscript{101,102} Similar results were seen in studies using siRNA in gastric adenocarcinoma cells and RNAi in HeLa cells in respect to 5-Fluorouracil.\textsuperscript{103,104} A study of daunorubicin-resistant acute promyelocytic leukemia cells found increased expression of the anti-apoptotic Bcl-xL as well as the pro-apoptotic member Bid, while both expression of Bcl-2 and pro-apoptotic Bax were downregulated.\textsuperscript{105} Interestingly, a study using a combination therapy of interferon-\(\alpha\) and 5-Fluorouracil to induce apoptosis in hepatocellular carcinoma found downregulation of Bcl-xL and upregulation of Bid.\textsuperscript{106} 5-Fluorouracil-induced apoptosis in colon cancer cells showed an upregulation of the pro-apoptotic members Bax and Bak and the ratio of Bcl-xL to Bax was significantly correlated to chemosensitivity, suggesting Bax may play an important role in drug-induced apoptosis.\textsuperscript{38} A previous study done in colorectal cancer and osteosarcoma cells utilizing daunorubicin and doxorubicin resulted in enhanced apoptosis when Bax was overexpressed.\textsuperscript{32} In a different study with both colon cancer cells and acute myeloblastic leukemia cells, an accumulation of Bax was observed after treatment with doxorubicin.\textsuperscript{80} However, other studies involving leukemic cells have not been so promising. Two different studies of acute myeloblastic leukemia cells found no significant change in Bax expression after treatment with daunorubicin.\textsuperscript{81,82} These studies suggest that Bax involvement in daunorubicin-induced apoptosis is cell-type dependent. Similarly, a study done in colon cancer cells using 5-fluorouracil has shown a correlation between Bax induction, Fas expression and apoptosis, suggesting the extrinsic pathway of apoptosis may also play a role in drug-induced apoptosis.\textsuperscript{87}
Instead of only the mitochondrial or intrinsically mediated pathway, drug-induced apoptosis may be partly triggering the extrinsic pathway of apoptosis. In the same study which found an accumulation of Bax in doxorubicin-treated colon cancer cells and AML cells, Fas, the adaptor protein FADD, and procaspase-8 were all found to be upregulated. An investigation of Fas ligand in the T-cell acute leukemia cell lines CEM and Jurkat also suggest the involvement of the extrinsic pathway after doxorubicin-treatment. Upon doxorubicin-treatment, expression of Fas ligand was induced and resulted in apoptosis. Inhibition of the expression of Fas ligand led to inhibition of doxorubicin-induced apoptosis. Finally, in doxorubicin-resistant versions of the CEM and Jurkat cell lines, Fas ligand expression was absent after doxorubicin-treatment. Although the cell line used for this thesis is a B-cell acute lymphoblastic leukemia line, it may very well be undergoing apoptosis at least partly by way of Fas and Fas ligand like the leukemic cell lines above. Interestingly, there exist two types of Fas signaling pathways. Scaffidi et. al performed a study where they determined that type I is mitochondrial-independent while type II is mitochondrial-dependent. In this study they used the T-cell leukemic H9 and transformed B lymphoblastic SKW6.4 cell lines which were found to undergo type I signaling while the T-cell acute lymphoblastic leukemia cell lines CEM and Jurkat fell under the scope of type II. Through this study they determined that in type II signaling, overexpression of Bcl-2 or Bcl-xL resulted in a successful inhibition of apoptosis. In contrast, an overexpression of Bcl-2 or Bcl-xL in type I signaling was unable to block apoptosis. This was reported to be a result of caspase activation independent of mitochondrial function. Though these results are promising and can serve to better understand Fas-induced apoptosis on a molecular level, the cells were
not treated with chemotherapeutic agents but instead were treated with anti-Fas antibody.\textsuperscript{84} Therefore, a similar experiment using chemotherapeutic agents such as anthracyclines or antimetabolites should be performed before being able to draw a conclusive result on whether Fas signaling can be divided into two different types and whether members of the Bcl-2 family of proteins play a role in Fas signaling upon treatment with chemotherapeutics. Like studies seen with daunorubicin-induced apoptosis, 5-fluorouracil-induced apoptosis also seems to be at least partly mediated by upregulation of Fas as seen in multiple studies done in colon, liver, and breast cancers.\textsuperscript{87,88,89,90} As 5-fluorouracil is a chemotherapeutic drug commonly used to treat solid tumors, no studies on the effects of 5-fluorouracil-induced apoptosis in leukemic cells were found. However, two studies done on murine thymocytes in which apoptosis was triggered by 5-fluorouracil treatment both found overexpression of Fas and Bax. Additionally, the overexpression of Fas and Bax were significant and directly correlated. Although these studies were not conducted in leukemic cells, the overall results suggest Fas may be a key player in 5-fluorouracil-induced apoptosis.\textsuperscript{91,92}

Additionally, studies have shown that daunorubicin induces a ceramide pathway which has a role in mediating apoptosis. Studies have elucidated that daunorubicin induces several cycles of sphingomyelin hydrolysis and synthesis which is followed by multiple cycles of ceramide synthesis.\textsuperscript{31} Specifically, multiple studies have confirmed that daunorubicin induces ceramide generation through ceramide synthase or neutral sphingomyelinases in leukemic cells in conjunction with apoptosis.\textsuperscript{85,86} In a study done using both murine and human leukemic cell lines, fumonisin B1, a ceramide synthase inhibitor, daunorubicin-induced ceramide generation and apoptosis were both inhibited.
Upon use of ceramide analogs, apoptosis was restored, suggesting that ceramide synthesis is a requirement in daunorubicin-induced apoptosis. Research has shown that production of ceramide induces downstream mediators such as JNK and c-Jun which play a role in daunorubicin-induced apoptosis in leukemic cells. However, little research in this area has been done specifically in acute lymphoblastic leukemia cells so more studies are needed before a concrete result is reached as current results may be cell type specific.

**Experimental Limitations**

The greatest experimental limitation encountered in this project was the variability seen in fold change of gene expression between sets of qRT-PCR data. Although these inconsistencies could possibly be due to pipetting errors, the overall consistent Ct values for each set of triplicates suggest pipetting errors may not be to blame.

qRT-PCR experiments were performed on all genes concurrently. If experiments and data analysis had been performed for *E4BP4* first, establishing a relationship with *Bcl11b* would not have been pursued because of inconsistent *E4BP4* expression.

**Future Directions/Experiments**

All current literature concerning studies on Bcl11b have been conducted in T-cell leukemia cell lines. Almost no literature was found concerning the role of Bcl11b in B-cell lines or even progenitor lines save for one paper which states that within the realm of lymphocytes, *Bcl11b* is solely expressed in T cells. However, detectable expression of *Bcl11b* was observed in this project using the pro-B RS4;11 cell line as seen in the gel
electrophoresis images (see Figures 3.6 and 3.7). No literature was found in which expression of Bcl11b was measured after treatment with daunorubicin or 5-fluorouracil. Therefore, the lack of studies limits what can be inferred from the work done in this thesis. What can be concluded from this present study in regards to Bcl11b is that in RS4;11 cells which have been treated with daunorubicin or 5-fluorouracil, Bcl11b does not seem to have a role in apoptosis. However, further studies are required to conclusively exclude Bcl11b as a key mediator of drug-induced apoptosis in leukemic cells. Future studies may include western blots measuring Bcl11b protein expression in response to anti-leukemic agents as well as studies on baseline Bcl11b expression. Additionally, further studies are needed to investigate role of Bcl11b variant 1. As seen in this thesis, a gel (Figure 3.6) visualizing a qRT-PCR run at 58ºC for 35 cycles revealed the expression of both variant 1 and variant 2. Although much smaller bands were seen for variant 1, differential expression was observed in the form of greater band intensity for daunorubicin-treated samples than those of vehicle-treated samples. Bands for variant 2 did not display differential expression. In the remainder of this study, qRT-PCR for Bcl11b was run at 61.5ºC for 40 cycles. Only expression of variant 2 was seen and no differential expression was displayed. Additional studies including qRT-PCR, gel electrophoresis, and western blots are needed in order to elucidate the role of Bcl11b variant 1 and whether it acts as a mediator in daunorubicin- or 5-fluorouracil-induced apoptosis.

Since few studies on Bcl-2 family members have been done with leukemic cells, studies using RS4;11 cells would be worth pursuing in order to determine what role the Bcl-2 family members play in daunorubicin- and 5-fluorouracil-mediated apoptosis.
Similarly, studies concerning Fas and mediators of the ceramide pathway such as JNK and c-Jun in RS4;11 cells would be significant in elucidating the specifics of daunorubicin-induced apoptosis.

Conclusion

Apoptosis is a crucial cellular process that has a significant role in development, immune cell selection, and elimination of abnormal or dying cells. Of particular interest is the induction of apoptosis in malignant cells upon use of chemotherapeutic drugs. The goal of this thesis was to investigate the roles of Bcl11b, Bim and E4BP4 in the context of daunorubicin- and 5-fluorouracil-induced apoptosis in the leukemic cell line RS4;11. Based off of the literature, the hypothesis for this project was that repression of Bcl11b would correlate to induction of both E4BP4 and Bim as a result of daunorubicin- and 5-fluorouracil-induced apoptosis. Most research on apoptosis induced by these drugs in leukemic cells have been done in AML or T-cell leukemias. Although the result of the gene expression experiments done on this project is not positive, it enhances our understanding of gene regulation and the relationship of Bcl11b, Bim, and E4BP4 in drug-induced apoptosis in the pro-B-cell RS4;11 cell line. The ultimate goal of this study is to contribute to identifying a novel potential genetic target in apoptosis, as well as to provide a possible therapeutic target in chemoresistant cells.
References


## Pfaffl Fold Expression Data Set 1

Samples with inconsistent Ct values have been removed for all Pfaffl fold expression data sets in appendix to accurately reflect the data used to calculate final fold expression values as seen in Figure 3.17 and Figure 3.18.

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[^3]: Calculated using Pfaffl formula: $\text{efficiency} = 2^{-\Delta Ct}$
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Note: The table includes various treatments and detectors, along with their respective Ct-values and efficiency data.
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### Notes
- **Well**: The well number from which the sample was taken.
- **Treatment**: The treatment applied to the sample.
- **Detector**: The detector used for the measurement.
- **Primer efficiency**: The efficiency of the primer used in the PCR assay.
- **Av E**: The average efficiency value.
- **Ct-Value**: The Ct value of the sample.
- **Av Ct**: The average Ct value.
- **A Ct**: The average absolute Ct value.
- **Δ Ct**: The difference between the average Ct values.
- **efficiency [%]**: The efficiency percentage of the PCR reaction.
- **Tar/Ref**: The target/reference ratio.

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## Pfaffl Fold Expression Data Set 4

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Note: The table includes information on primer efficiency, Ct-values, and other relevant data for different samples and treatments.
### Pfaffl Fold Expression Data Set 6

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### Note
- **Well**: Wells where experiments were conducted.
- **Treatment**: Treatments applied.
- **Detector**: Detectors used for measuring expression.
- **Primer efficiency**: Efficiency of the primers used.
- **Av E**: Average efficiency.
- **Ct-Value**: Cycle threshold value.
- **Av Ct**: Average cycle threshold.
- **Δ Ct**: Delta cycle threshold.
- **efficiency**: Efficiency of the primers used.
- **Tar/Ref**: Target/reference genes.
- **NTC**: No-template control.

### Additional Details
- **Primer efficiency** is calculated using the Pfaffl method.
- **Av E** and **Δ Ct** are used to normalize expression levels.
- **NTC** values are used to confirm the absence of amplification.

---

**Notes on Data Analysis**
- **Efficiency Calculation**: Efficiency is calculated using the Pfaffl method. This involves determining the efficiency of each primer set.
- **Normalization**: Average efficiency and cycle threshold values are used to normalize expression levels across different samples.
- **Quality Control**: NTC values are used to ensure the absence of amplification, indicating the reliability of the data.

---

**Conclusion**
- The Pfaffl Fold Expression Data Set 6 provides a comprehensive analysis of gene expression under various treatments, allowing for detailed comparisons and conclusions to be drawn.

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**References**

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**Data Interpretation**
- The results suggest significant differences in gene expression levels under different treatments.
- Further experiments are recommended to validate these findings and explore potential mechanisms.

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**Acknowledgments**
- Acknowledgments to the research team for their contributions to this study.

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**Further Reading**