THE EPIGENETIC REGULATION OF RIZ1 IN HUMAN LEUKEMIA

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ABSTRACT

Cancer has been thought of as a mostly genetic phenomenon, however recent research into epigenetic causes of cancer emphasizes that these causes of cancer are also important. RIZ1 is a tumor suppressor which is silenced in many human leukemias, such as human Acute Myeloid Leukemia and Chronic Myelogenous Leukemia. It was the goal of this thesis to re-express RIZ1 using three epigenetic drugs: decitabine, a DNA methylation inhibitor, Trichostatin A, a histone deacetylase inhibitor and chaetocin, an inhibitor of SUV39h1. Cells were treated with these drugs and analyzed for toxicity, methylation status, and RIZ1 expression levels. The synergy between the drugs was also determined. It was found that cells treated with decitabine and chaetocin had an induction of RIZ1 expression. Chaetocin induced RIZ1 expression without affecting the methylation status of the cell. Also, cells which were treated with decitabine paired with either Trichostatin A or chaetocin showed the highest amount of RIZ1 expression. Cells treated with all three drugs together had a higher amount of RIZ1 expression than cells treated with either drug alone, however had less expression than cells which had been treated with decitabine paired with either Trichostatin A or chaetocin. Using these data a model was developed in which H3K9 methylation is the dominant epigenetic event in transcriptional silencing.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation

AML	Acute Myelogenous Leukemia
CML	Chronic Myeloid Leukemia
DNMT	DNA Methyltransferase
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
H3K9	Histone 3 Lysine 9
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDACI	Histone Deacetylase Inhibitor
HMT	Histone Methyltransferase
IL-3	Interleukin-3
IMDM	Iscove's Modified Dulbecco's Medium
MBD	Methyl Binding Domain
MSP	Methylation Specific PCR
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PCR	Polymerase Chain Reaction
RTPCR	Reverse Transcription PCR
TSA	Trichostatin A

1. REVIEW OF THE LITERATURE

1.1 Introduction

Cancer is a genetic disease, and so determining the genetic causes of cancer is currently a common topic of research. Chromosomal translocations such as the translocation which creates the BCR-ABL oncogene are at the forefront of this research. Genetic causes of cancer are not the only source of cancer that is being investigated; epigenetic mechanisms also play an important role in the appearance and progression of cancers (Iacobuzio-Donahue, 2008).

Epigenetics involves the study of heritable changes in gene function that do not involve changes to the DNA code; epigenetics literally means "above" the genes. Two important developments in cancer biology occurred as the result of the discovery of epigenetic gene regulation. The first discovery is that gene regulation is a means by which cancer appears and progresses, and the second is that new treatments can be developed based on epigenetic mechanisms. An example of this is that the epigenetic drugs decitabine and vorinostat, a DNA methylation inhibitor and histone deacetylase inhibitor respectively, have been approved for treatment of specific tumors (Esteller, 2008).

The general goal of this thesis was to explore the epigenetic gene regulation of tumor suppressor genes involved in human Chronic Myelogenous Leukemia (CML) and Acute Myeloid Leukemia (AML), and to explore the relationship between DNA methylation, histone acetylation and histone methylation using small molecule inhibitors. The literature review aims to familiarize the reader with CML and AML, RIZ1, epigenetic mechanisms in general, and some pharmaceuticals that are showing great promise in the field of cancer. Emphasis will be placed on aberrant epigenetic events as well as the prospect of treating cancer with epigenetic pharmaceuticals.

1.2 Acute Myeloid Leukemia

Normal mammalian hematopoiesis involves the differentiation of blood cells from a single pluripotent stem cell (Figure 1.1). AML is a hematological malignancy associated with a defect in the maturation process whereby myeloid precursors are no longer converted into white

blood cells, but remain immature. This malignancy usually occurs in the bone marrow. The disease primarily occurs in adults, having equal frequency in males and females, with the incidence rising as age increases. (Alderson, 1980). The incidence rates of AML are relatively stable over time, however there is a slight increase in incidence in the older population. Although AML survival rates in the young have almost doubled, there is still a low survival rate in both the younger and eldery population (Xie *et al*, 2003).

The exact etiology of AML is unknown, however environmental factors and chemical exposure are associated with the disease. Benzene is one of the chemicals with the strongest link to AML, having its own subclassification of benzene-induced AML (Natelson, 2007). Down's syndrome is also a known risk factor for AML, as children with Down's syndrome have a 20-fold increased risk of developing leukemia. The mechanism of this increased risk is unknown, however several hypotheses including chromosomal instability and gene expression dysregulation due to the trisonomy 21 have been proposed (Robison, 1992; Fong and Brodeur, 1987). Like other leukemias, the true etiology of AML most likely involves a combination of genetic and environmental factors.

1.3 Chronic Myeloid Leukemia

Chronic Myeloid Leukemia is a myeloproliferative disorder which affects all lineages of hematopoiesis. This disease is defined by the presence of the BCR-ABL oncogene. This oncogene is formed when a translocation occurs between chromosomes 9 and 22, to form the Philadelphia chromosome (Sawyers, 1999).

The incidence of CML is 1-1.5 per 100,000 people and accounts for 15-20% of all adult leukemias. CML can be diagnosed at any age, however most people are in their 50's or 60's at diagnosis. The death rates of people affected with CML is relatively low, with only 490 deaths predicted in the coming year in the United States. This is mostly due to the efficacy of kinase inhibitors, which specifically target the kinase activity of the BCR-ABL oncogene (Sessions, 2007).

Patients are generally asymptomatic at presentation, and generally report to a physician for an unrelated matter. Fatigue is a common symptom, however it is an elevated white blood cell count which leads to the eventual diagnosis of CML (Sessions, 2007).

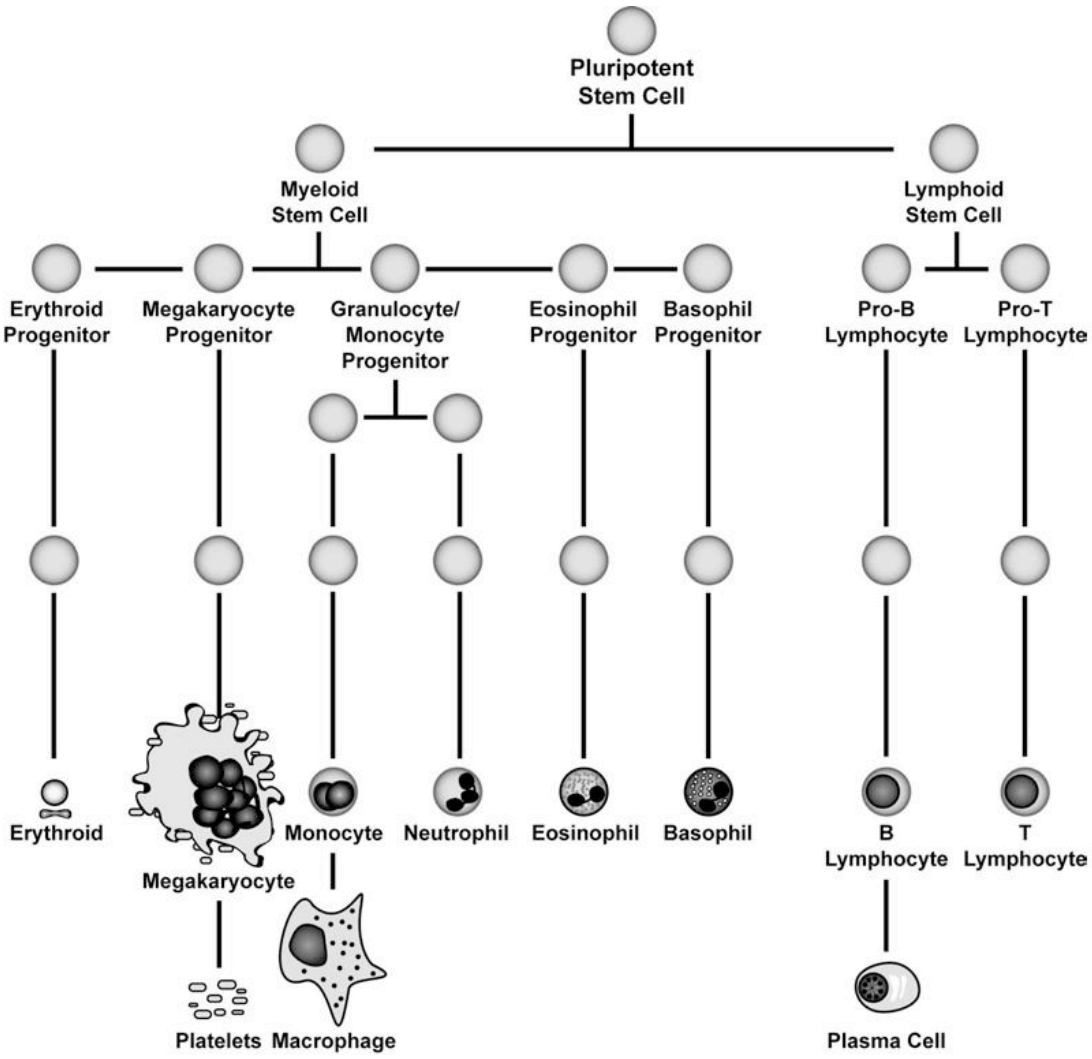


Figure 1.1. Mammalian Hematopoiesis. Illustrated are the various differentiation pathways of the major types of blood cells. Normal hematopoiesis involves the differentiation of all cells from a single pluripotent stem cell (Scott, 2005).

CML can be divided into two phases, a chronic phase and a blast phase. Untreated patients remain in the chronic phase for two to five years, and survive upon entering blast phase for three to six months. The definition of each phase varies slightly, however chronic phase is defined as the patient having less than 15% blasts, less than 20% basophils and less than 30% of blasts and promyelocytes in both peripheral blood and bone marrow (Druker *et al.*, 2006; O'Brien *et al.*, 2003). Once CML progresses to blast crisis, the symptoms closely resemble an acute leukemia. The World Health Organization defines blast phase as being when blasts have exceeded 20% of all cells in the bone marrow and periphery, extramedullary blast proliferation and large clusters of blast cells in bone marrow biopsies (Jaffe *et al*., 2001).

Imatinib, a kinase inhibitor, is very effective for treatment of CML, however patients often develop resistance to the drug quickly, creating the need for new treatments to be developed (Lee *et al*., 2008).

1.4 The Molecular Basis of Cancer

There are six characteristics which define a cell as malignant: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion, and metastasis (Hanahan and Weinberg, 2000). Studies have revealed that these characteristics are obtained through the activation of oncogenes or the silencing of tumor suppressor genes. Proto-oncogenes are genes which normally have no detrimental effect, however, mutations and deregulation can cause proto-oncogenes to become oncogenes. An oncogene is a protein encoding gene which causes the onset of cancer (Todd and Wong, 1999). Tumor suppressor genes are genes which normally perform a repressive role in the cell. Aberrant silencing of tumor suppressor genes causes uncontrolled growth and can lead to cancer. The irregularities that occur in oncogenes and tumor suppressor genes provide a foundation for diagnosis and treatment (Weinberg, 1994).

1.4.1 Tumor Suppressor Genes

The protein products of tumor suppressor genes inhibit the proliferation of cells, and thus are very important to both normal cell growth and transformation of cells. When a cell loses its ability to produce a tumor suppressor, it no longer processes certain growth antagonizing signals from outside the cell and growth becomes uncontrolled (Massague, 1990).

One of the most famous and most studied tumor suppressor gene is the Retinoblastoma gene. If one copy of the gene is lost through deletion or translocation, a functional protein is still created from the remaining allele. If the second allele is also lost or silenced, then no functional protein can be made and cancer develops. This concept forms the "two hit" hypothesis postulated by Alfred Knudsen in 1971 (Knudsen, 1971). If the absence of the first allele is caused by a deletion, offspring have the potential to inherit this defect, putting them at an increased risk, as only one additional event is necessary to induce cancer. It is also possible for a dominant negative mutation to occur. In this case a mutation arises in the first allele which creates a non functional protein with the ability to interfere with the function of the remaining intact proteins function (Blagosklonny, 2000).

1.4.2 Regulation of Apoptosis

Apoptosis is a cascade of events which leads to the programmed death of a cell. When properly regulated, apoptosis protects the body from the effects of such occurrences as DNA damage, oxidative stress, and viral infections by sacrificing affected cells to prevent adjacent healthy cells from acquiring the defects of the damaged cells (Miller, 1997). Apoptosis also plays an important role in embryological development, tissue homeostasis and immune cell education (Vaux and Korsmeyer, 1999). The importance of apoptosis is suggested by the large amount of genes involved, approximately 200 genes, or 0.6% of the entire genome, though this is thought to be an underestimation (Reed *et al.*, 2003).

Cellular transformation can result from the activation of oncogenes or the disruption of tumor suppressor genes that regulate apoptosis. Often tumorigenesis occurs because of an inhibition of apoptosis rather than an increase in proliferation rates. Inhibition of apoptosis

creates excess cellular growth even though proliferation rates do not increase (Hanahan and Weinberg, 2000). Deregulation of apoptosis is not only implicated in cancer but also in various other conditions, such as neurodegenerative disease (Yu and Zhang, 2004).

1.4.3 Multistep Molecular Carcinogenesis

Carcinogenesis does not occur from one event; it is the accumulation of many abnormalities occurring over time. There are at least three steps which can be defined in the process of carcinogenesis: initiation, promotion, and progression. It is estimated that most cancers require at least five or six genetic mutations for carcinogenesis to occur (Fearon and Vogelstein, 1990).

A model was proposed in 1990 for cancer, which suggested that as a cell moves through the various stages of malignancy (hyperplasia, metaplasia, etc.), various genetic "hits" are acquired, which affect multiple genes in multiple pathways (Fearon and Vogelstein, 1990). Similarly, Knudsen's previously mentioned "two hit" hypothesis postulates that there is more than one event necessary to perpetuate carcinogenesis (Knudsen, 1971). For instance, in the first hit one allele is lost due to a deletion, and in the second hit the other allele becomes hypermethylated at the promoter region. Multistep molecular carcinogenesis also refers to an accumulation of mutations, which together form the basis for tumor formation. The model for multistep molecular carcinogenesis is illustrated in Figure 1.2. As the neoplasm evolves into a malignant cancer, multiple mutations occur combined with a decrease in overall DNA methylation, an increase in promoter region DNA methylation, and a change in histone modifications (Esteller, 2008). These different mechanisms are discussed in subsequent sections. Understanding the order that genetic "hits" occur is essential to the understanding of tumor formation and progression.

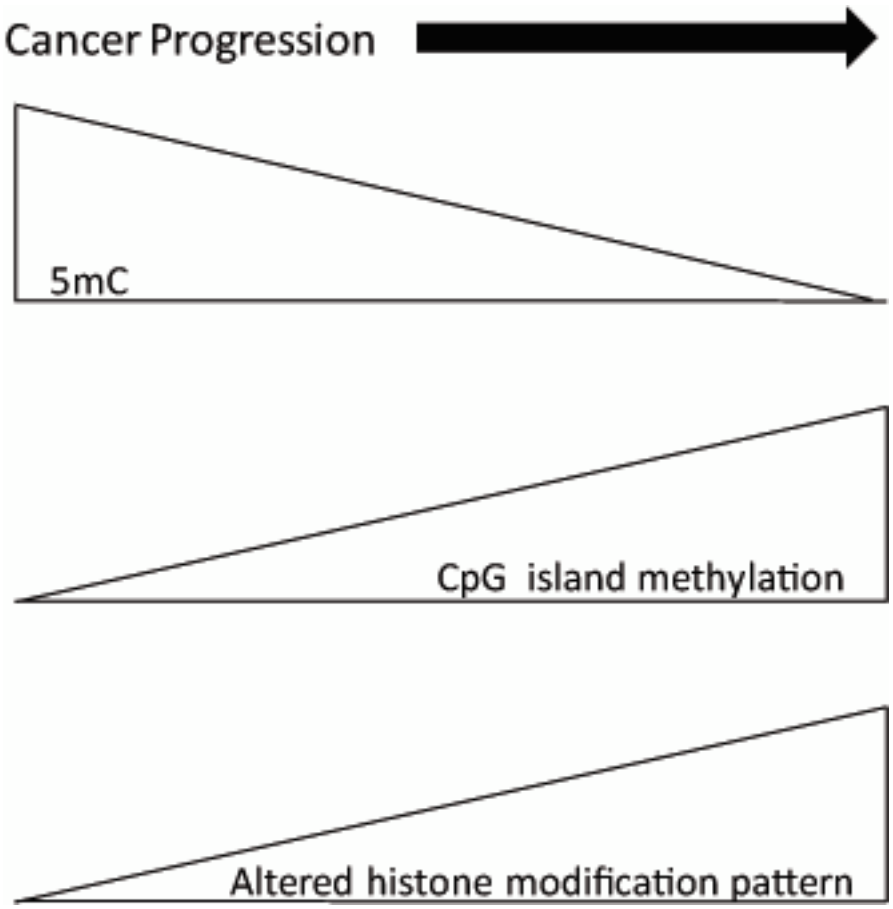


Figure 1.2. Multistep Molecular Carcinogenesis. As cancer progresses there is a decrease in overall DNA methylation, an increase in promoter region CpG island DNA methylation, and an altered histone modification pattern. 5mC denotes 5-methyl cytosine.

1.5 Epigenetics

The term "epigenetics" was coined in the 1940's by Conrad Waddington, and literally translates to "above the genome" (Slack, 2002). It involves two concepts: 1) the study of heritable developmental processes in an organism and 2) the study of heritable changes in expression that occur without any change in the genomic DNA sequence. Both concepts are concerned with the study of heritable changes that are not coded for within the DNA sequence.

1.5.1 DNA Methylation

DNA methylation occurs when the C5 position of a cytosine gains a methyl group (Figure 1.3). DNA methylation occurs in the promoter region in areas called CpG islands. CpG

islands are cytosine-guanine dinucleotide rich areas. CpG islands are usually not methylated in normal DNA, as active transcription appears to protect this area from methylation (Clark and Melki, 2002; Herman and Baylin, 2003; Weber *et al.,* 2007). CpG islands comprise 1-2% of the genome, existing in approximately 40-50% of the promoter regions of genes and were originally arbitrarily defined to be areas with 50% CG content and approximately five times the normal occurrence of the CG dinucleotide (Gardiner-Garden and Frommer, 1987). In order to avoid including such CG-rich areas as Alu-repetitive elements and intragenomic parasites, the criteria has recently been modified to be more stringent. More recent criteria for CpG islands include regions of DNA of greater than 500 bp with a G+C content equal to or greater than 55% and observed CpG/expected CpG ratio of 0.65 (Takai and Jones, 2002).

In cancer, the genome undergoes an overall hypomethylation (Feinberg and Vogelstein, 1983) however promoter regions of tumor suppressor genes are often hypermethylated (Greger *et al.*, 1989; Sakai *et al.,* 1991). DNA methylation at the promoter region of genes is associated with gene repression. DNA methylation is also responsible for genomic imprinting (Feinberg *et al*, 2002) and the formation of Barr bodies in females (Reik and Lewis, 2005).

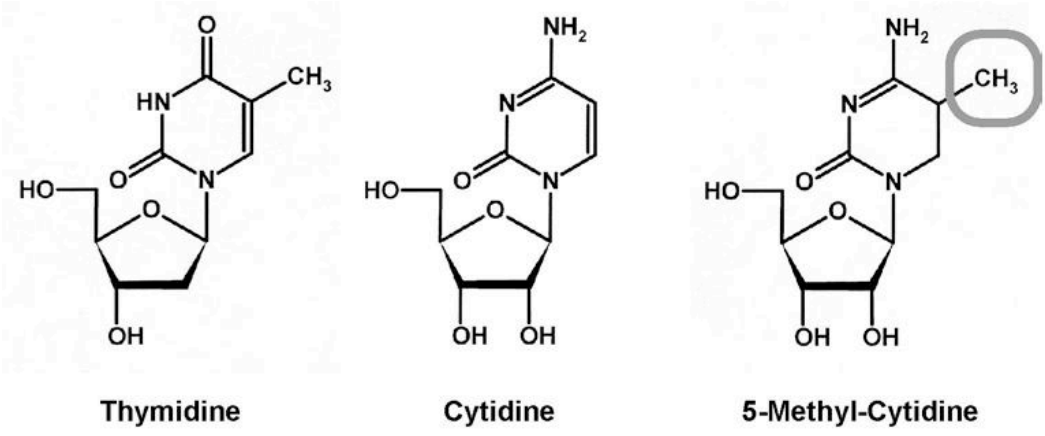


Figure 1.3. Chemical Structures of Pyrimidine Nucleosides and 5-Methyl-Cytidine. Illustrated are the chemical structures and names of the common pyrimidine nucleosides, thymidine and cytidine, and 5-methyl-cytidine. The grey box highlights the methyl group attached to the carbon-5 of cytidine. Note: Thymidine is depicted as deoxy in this figure

1.5.1.1 DNA Global Hypomethylation

The first epigenetic phenomenon discovered in relation to cancer was the low level of methylation in tumor cells compared to their normal counterparts (Feinberg and Vogelstein, 1983). The overall decrease in DNA methylation is due to the demethylation of introns and exons, which allows alternate versions of mRNA to be transcribed (Feinberg and Tycko, 2004). As a neoplasm develops, the degree of hypomethylation increases as the neoplasm progresses from benign to invasive. This increase in hypomethylation is coupled with an increase in promoter region DNA methylation (Figure 1.2).

1.5.1.2 CpG Island Hypermethylation

Site specific DNA methylation was first discovered in 1986 with studies done on the calcitonin gene (Baylin *et al,* 1986). This study determined that 5' promoter region methylation of certain residues is linked to a tumor state in lung cancer and lymphoma. Aberrant hypermethylation of tumor suppressor genes was also observed in many other carcinomas such as renal (Herman *et al.,* 1994), AML (Herman *et al.*, 1996), breast (Ottaviano *et al.*, 1994), ovarian (Esteller *et al.*, 2000), prostate (Lee *et al.*, 1994), and brain (Bachman *et al.*, 1999).
It is still not clear whether DNA methylation is a spontaneous event or whether events predispose an individual to aberrant hypermethylation. One event that appears to predispose people to hypermethylation is age. In young people, the promoter regions of certain tumor suppressor genes are slightly methylated, however, as a person ages, DNA methylation increases, with the highest amount of DNA methylation observed in cancer development (Toyota *et al.*, 1999).

Currently DNA hypermethylation markers are being investigated as prognostic factors, diagnostic tools, and treatment response predictors (Esteller, 2008). DNA hypermethylation also has a role in cancer treatment. Like genetic mutations, DNA hypermethylation allows the tumor to thrive in its environment better, however unlike genetic mutations, DNA hypermethylation is reversible. This makes it a target for new theurapeutic drugs. Currently DNA methylation inhibitors 5-aza-cytidine (Vidaza) and 5-aza-deoxycytidine (decitabine) have been approved for clinical use in leukemia and myelodysplasic syndrome (Mack, 2006; Muller *et al,* 2006; Oki *et al,* 2007).

1.5.1.3 Mammalian DNA Methyltransferases

The major enzymes involved in DNA methylation are DNA methyltransferases (DNMT). When DNA methylation occurs, the cytosine is everted from the DNA helix and placed into the active site of the DNMT. DNMTs use the methyl donor S-adenosyl-L-methionine to methylate the DNA (Bestor, 2000). In mammals, three families of DNA cytosine-5 methytransferase enzymes have been discovered: DNMT1, DNMT2, and DNMT3a and DNMT3b.

DNMT1 is the largest methyltransferase, with a molecular mass of 184 kDa (Smith *et al.*, 1992). DNMT1 has a regulatory domain in the amino-terminal two thirds of the protein and the catalytic domain in the carboxy-terminal region (Yen *et al.*, 1992). The catalytic region is similar among all known methyltransferases (Kumar *et al.,* 1994). In proliferating cells, DNMT1 is involved in a process known as maintenance methylation. This process ensures reciprocal methylation of the newly synthesized daughter strand during replication (Leonhardt *et al.,* 1992). Disruption of DNMT1 in mice results in abnormal imprinting (Li *et al.*, 1993), embryonic lethality, greatly reduced levels of DNA methylation (Li *et al.,* 1992), and activation of endogenous retroviruses (Walsh *et al.,* 1998). DNMT1 abnormalities are found in colon cancer (Rhee *et al.,* 2000), lymphoma (Lee *et al.,* 2001), and pancreatic cancer (Peng *et al.,*2005). DNMT1 is able to bind to the histone methyltransferase SUV39H1, and HP1, which suggests a direct link between DNA and histone methylation (Fuks *et al*., 2003a).

In comparison to DNMT1, DNMT2 is much smaller, with a predicted molecular weight of 45 kDa. DNMT2 lacks the large amino terminal domain but contains all of the conserved methyltransferase motifs. Until recently the function of DNMT2 has been largely unknown, and it still remains controversial. DNMT2 knockout mice show no defects or reduction in global methylation levels (Okano *et al.,* 1998). A recent study showed that DNMT2 is involved in methylation of tRNA, and suggested that it is also important in embryo development in zebrafish (Rai *et al.,*2007).

D*e novo* DNA methylation was confirmed by the discovery of DNMT3a and DNMT3b. Both of these methyltransferases are crucial for embryonic development and for the methylation during embryogenesis, which establishes the somatic methylation pattern of the organism (Okano *et al.,* 1999). DNMT3a and 3b are intermediate in size (100-130 kDa) compared to DNMT1 and DNMT2 and possess a smaller amino terminal region. DNMT3a associates with

histone 3 lysine 9 (H3K9) methylation activity (Fuks *et al*., 2003a). This complex most likely contains the histone methlytransferase SUV39H1, as DNMT3a binds to SUV39H1 *in vivo* (Fuks *et al*., 2003a). DNMT3b abnormalities are found in bladder cancer, breast cancer, colon cancer, lung cancer (Beaulieu *et al.,* 2002), and hepatocellular carcinoma (Saito *et al.,* 2002).

1.5.1.4 Mammalian Methyl-CpG Binding Proteins

DNA methylation is not believed to be the primary cause of carcinogenesis in tumor cells. Instead it is believed that DNA methylation allows the chromatin to become "locked in" a state of transcriptional repression, which involves other mechanisms along with DNA methylation. Many of the complexes required to initiate transcription are not present at the promoter when methylation is present, which results in a state of gene repression. There are factors which bind methylated DNA called methyl binding proteins that provide the basis for the DNA to transition into an inactive form (Bird, 2002).

A family of five methyl CpG binding proteins has been characterized, each of which has a methyl CpG binding domain similar to that of MeCP2 (Nan *et al*, 1993; Cross *et al*, 1997; Nan *et al*., 1997; Hendrich and Bird, 1998). Four members of this family, MB1, MB2, MB3 and MeCP2 are implicated in methylation dependent repression of transcription. MB3 shares a 70% amino acid sequence similarity to MB2 and contains a methyl binding domain (MBD) motif but is unable to specifically recognize methylated DNA (Hendrich and Bird, 1998). There is a fifth MBD protein, Kaiso, which targets methylated DNA and brings about transcriptional repression, but it differs in that it binds DNA through a zinc finger motif (Prokhortchouk *et al*, 2001). Instead of being involved in transcriptional silencing, MB4 is involved in DNA repair. MB4 has a preference for binding 5-methyl-CpG-to-TpG mismatches, which indicates a role in the minimization of mutations at 5-methyl-CpG (Hendrich *et al*, 1999). Also, MB4 knockout mice have a significantly increased rate of CpG mutations and tumorigenesis (Millar *et al*, 2002).

MeCP2 was the first MBD to be cloned and the second MBD to be discovered (Lewis *et al*, 1992). It is also the protein from which the MBD motif is defined (Nan *et al*, 1993). MeCP2 is located at Xq28 and is highly abundant (Nan *et al*., 1997). The colocalization of MeCP2 to the nucleus with methylated DNA indicates the involvement of MeCP2 in DNA methylation mediated repression. MeCP2 associates with and facilitates H3K9 methylation by bringing

histone methyltransferase activity to the hypermethylated promoter of a DNA methylated gene that it regulates (Fuks *et al*., 2003b).

MBD1 is unique among MBD proteins as it represses transcription in both methylated and unmethylated promoter regions (Fujita *et al*., 1999). MBD1 is similar to MeCP2 in that it is an abundant chromosomal protein (Ng *et al*., 2000), which contains a transcriptional repression domain (Fujita *et al*, 1999; Ng *et al*., 2000). However, a proportion of its repression activity relies on recruitment of HDAC activity (Patra *et al*, 2003).

MBD2 is part of the methyl-CpG binding protein 1 complex (Ng *et al*., 1999), which represses transcription in a methylation density dependent fashion (Bird and Wolffe, 1999). MBD3 does not have methyl-CpG binding capacity, however, it exists in an abundant nucleosome remodelling and histone deacetylation corepressor complex in humans (Zhang *et al*., 1999). This complex can be recruited to DNA by several different repressor proteins and is essential to embryogenesis (Ahringer, 2000).

MBD proteins are present at the hypermethylated promoter regions of genes in hepatocellular carcinoma (Bakker *et al*., 2002), colon cancer (Magdinier and Wolffe, 2001), bladder cancer (Nguyen *et al*., 2002) and T-cell leukemia cell lines (El-Osta *et al*., 2002). Upon treatment with Decitabine, a DNA methylation inhibitor, promoter demethylation occurs at the p16 locus, which is accompanied by the release of MBD proteins (Magdinier and Wolffe, 2001; Nguyen *et al*., 2002). This is also associated with a local enrichment of acetylation of histones H3 and H4, suggesting that the recruitment of MBD proteins to the hypermethylated promoter regions of tumour suppressor genes also involves the deacetylation (or the inhibition of acetylation) of histones. This role is also supported by the binding of MBD1 to a histone methyltransferase SUV39H1 and HP1, a methyl lysine binding protein. This complex is believed to contain HDAC1 or HDAC2 as well. MBD1 tethers the MBD1-SUV39h1-HP1 complex to methylated DNA, which causes MBD1 dependent transcriptional repression (Fujita *et al.,* 2003). This finding is important as it creates a direct link between DNA methylation, histone methylation, and histone acetylation.

1.5.1.5 Decitabine

Inhibitors of DNA methylation rapidly reactivate expression of genes silenced by DNA hypermethylation. 5-azacytidine and its analog 5-azadeoxycytidine (decitabine) were the first

DNA methylation inhibitors to be characterized (Sorm *et al*., 1964) (Figure 1.4). They were initially developed as chemotoxic reagents, however it was soon discovered that they have the ability to inhibit DNA methylation and to induce gene expression and differentiation in cells (Constantinides *et al.,* 1977; Jones and Taylor, 1980). In AML and MDS, the p15 promoter region is hypermethylated; treatment with Decitabine results in demethylation of the promoter region and re-expression of the gene (Daskalakis *et al*., 2002).

Upon entering the cell, both analogs are changed into the deoxynucleotide form and are incorporated into newly synthesized DNA upon replication. They are therefore most active in the S phase of cells. DNMTs become covalently linked to the modified bases and are unable to methylate DNA any further (Jones and Taylor, 1980; Zhou *et al*., 2002). This covalent attachment is responsible for the toxicity of decitabine (Michalowsky and Jones, 1987).
Decitabine has been approved for clinical use in myelodysplastic syndrome and leukemia (Mack, 2006; Muller *et al*., 2006; Oki *et al*., 2007), however decitabine is highly toxic to patients (Stresemann and Lyko, 2008).

1.5.2 Post Translational Histone Modification

Eukaryotic chromosomes exist as a DNA:protein complex. There are two types of chromatin: heterochromatin, a highly condensed compact form of chromatin linked to transcriptional repression, and euchromatin, a more open form of chromatin linked to transcriptional activation (Wolffe and Kurumizaka, 1998).

1.5.2.1 Nucleosomes and Chromatin Organization

The histone is the most massive component of chromatin. This protein component of the DNA:protein complex consists of 5 families: H1, H2A, H2B, H3 and H4. The DNA is wrapped around a nucleosome core, which is then packed into an octet, consisting of two copies each of H2A, H2B, H3 and H4 (Kornberg, 1974). The DNA forms two complete left hand turns around the histone by binding to the positively charged residues on the amino tails of the histones.

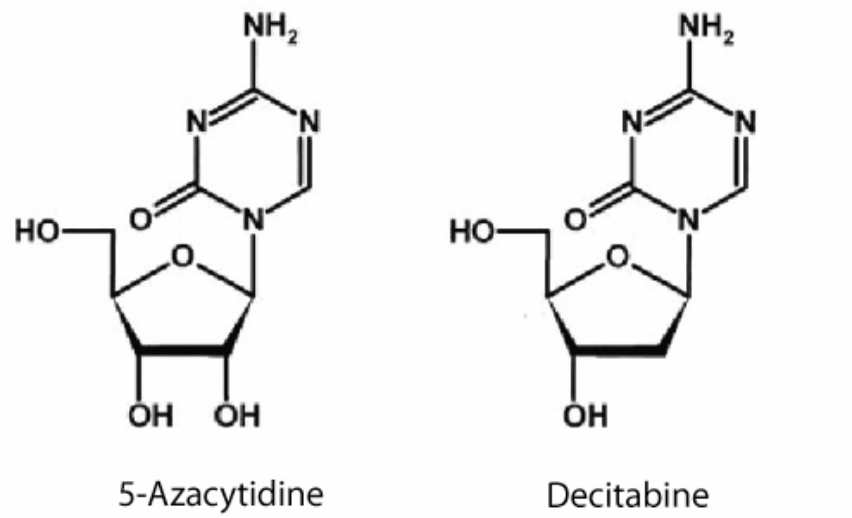


Figure 1.4. Chemical Structures of Common DNMT Inhibitors. Illustrated are the common chemical structures and names of cytidine analog inhibitors of DNA methylation.

The H1 histone is the linker histone and is responsible for binding the DNA in a cavity of the core particle where DNA both enters and exits the nucleosome (Allan *et al.,* 1980).

In order to successfully regulate transcription, several multisubunit and protein complexes act upon the chromatin. Chromosomal segregation and repair require chromatin manipulation and it is evident that covalent modifications of regional histones play a part in this process. The evidence for this stems from the reports correlating chromatin modifications to specific post-translational modifications of histone tails (Luo and Dean, 1999; Strahl *et al.,* 1999).

Histone tails provide additional gene regulatory information that contributes to chromatin conformation (Strahl and Allis, 2000; Jenuwein and Allis, 2001). The "histone code" hypothesis theorizes that specific modifications to histone tails act sequentially or in combination to form a code that is read by other proteins to bring about downstream events such as changes to transcription levels. This thesis deals with two of these modifications, histone acetylation and histone methylation.

1.5.2.2 Histone Acetylation

Acetylation of the lysines on histone tails was first discovered in the 1960's (Allfrey *et al.,* 1964). This led to the discovery of the link between the acetylation state of the histone and transcriptional activation. Histone acetylation is linked to transcriptional activation while histone deacetylation is linked to transcriptional silencing. Acetylation of the lysines within the histone tail neutralizes the tails' positive charge, allowing the chromatin to relax and providing space for the transcriptional machinery to access the DNA. The acetylation state of the histone tail is reversibly regulated by two classes of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Archer and Hodin, 1999).

The first acetyltransferase to be discovered was HAT1 (Kleff *et al.,* 1995). Although this enzyme was first found to localize to the cytoplasm, recent studies have found that HAT1 can also exist in the nucleus and participate in transcriptional regulation (Kelly *et al.,* 2000). There are three main families of histone acetyltransferases, which can be grouped according to their sequence similarity. These families are Gcn5/PCAF, p300/CPB and MYST (Gray and Ekstrom, 2001; Khochbin *et al*., 2001; Grozinger and Schreiber, 2002). All three of these families can acetylate both histone tails and other proteins (Roth *et al*., 2001; Nakatani, 2001; Carrozza *et al*., 2003).

Histone deacetylases catalyze the removal of acetyl groups from the lysine on the tails of histones, which leads to chromatin condensation and transcriptional repression. The first mammalian histone deacetylase was identified in 1996 by Taunton and colleagues (Taunton *et al*., 1996). HDACs are divided into three classes based on their homology to HDACs in *Saccharomyces cerevisiae* in terms of size, cellular expression, and enzymatic domains. Class I HDACs are expressed ubiquitously in various human tissues, are homologous to yeast Rpd3p, and can be found in the nucleus of cells. Class II HDACs share homology with the yeast Hda1p and are shuttled between the nucleus and the cytoplasm. Class III HDACs are homologous to Sir2 and are structurally unrelated to the other two classes of HDAC. Class III HDACs have a unique enzymatic mechanism that requires NAD^+ for activity (Mottet and Castronovo, 2008).

There are a lot of data supporting the link between aberrant histone acetylation and carcinogenesis. Histone hypoacetylation has been directly linked to the initiation and/or progression of various cancers such as acute promyelocytic leukemia (Lin *et al*., 1998).

1.5.2.3 Trichostatin A

Trichostatin A (TSA) is a histone deacetylase inhibitor (HDACI) (Figure 1.5). It inhibits histone deacetylation at nanomolar levels, and also induces arrest in the G1 phase of the cell cycle (Yoshida *et al*., 1995). Transcriptomic analysis reveals that only 2-17% of expressed genes are affected by HDACIs, which suggests that HDACIs affect only a specific subset of genes (Glaser *et a*l., 2003; Della *et al*., 2001; Van Lint *et al*., 1996). Genes involved in apoptosis, transcriptional regulation, cell cycle and growth, differentiation, cell migration, and angiogenesis are within this subset. The cell cycle kinase inhibitor p21 is the most studied gene affected by HDACI. In cells with silenced p21, HDACIs can induce p21 expression, arrest cells in G1 and increase promoter histone acetylation. Although growth arrest is seen in almost all non-malignant cells, the response to HDACI in malignant cells is apoptosis (Mottet and Castronovo, 2008). The exact mechanism by which HDACIs invoke this response is unknown, however HDACIs up regulate pro-apoptotic genes such as Bax, Bad, (Gillespie *et al*., 2006), and APAF-1 (Maiso *et al*, 2006).

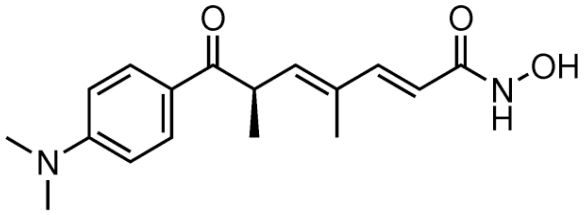


Figure 1.5. Chemical Structure of Trichostatin A.

1.5.2.4 Histone Methylation

Histone methylation is a common epigenetic modification found in all eukaryotes. Lysine residues on histone tails can be mono-, di-, or trimethylated. Histone methylation is catalyzed by histone methyltransferases (HMTs), which introduce methyl groups onto lysine or

arginine groups present on the histone tail. Mutational studies have shown that each HMT is functionally distinct (Tachibana *et al*., 2005). The first HMT discovered was SUV39h1, which is responsible for trimethylation of lysine 9 of histone 3 (H3K9) (Rea *et al*, 2000). Methylation of H3K9 is associated with transcriptional repression, while methylation of H3K4 is linked to transcriptional activation (Jones and Baylin, 2007).

SUV39h1 uses monomethylated H3K9 as a substrate for trimethylation of the same residue (Peters *et al*., 2003). The SU(VAR)3-9 Enhancer of zest Trithorax (SET) domain of SUV39h1 is responsible for the H3K9 methyltransferase activity. There are two loci which code for SUV39h genes: SUV39h1 and SUV39h2. Although single SUV39h1 and SUV39h2 mice are viable, double null mice are born at only about 20-25% of the expected mendelian ratio, are growth retarded, have a predisposition to tumors, and show very unstable genomes due to a lack of histone methylation (Peters *et al.*, 2001). The ability of one locus to compensate for the loss of the other suggests the two loci have redundant functions (Rea *et al*., 2000).

SUV39h1 normally regulates the trimethylation of histones in pericentric chromatin, however SUV39h1 localization is not limited to this area of chromatin. The retinoblastoma protein is able to recruit SUV39h1 and HP1 to the promoter regions of genes and cause transcriptional repression, which could include aberrant silencing of tumor suppressor genes (Nielsen *et al*., 2001).

SUV39h1 is able to form a complex with HP1. HP1 is a transcriptional repressor which recognizes and binds to areas of chromatin where SUV39h1-mediated methylation occurs. HP1 then recruits other transcriptional repression machinery such as HDACs and DNMTs (Wang *et al*, 2000). It is reported that DNMT3a and DNMT3b co-localize with HDAC activity (Bachman *et al.,* 2001), and that H3K9 methylation is required in order for DNA methylation to occur (Tamaru and Selker, 2001). This suggests a model whereby H3K9 methylation by SUV39h1 would be the first event in transcriptional silencing, followed by recruitment of HDACs and DNMT by HP1 to further silence the gene. This complex may also contain a DNA methylation binding protein as MBD1 is able to form a complex with SUV39h1 (Fujita *et al*., 2003).

Another important HMT is G9a. Unlike SUV39h1, which regulates trimethylation of histones in pericentric heterochromatin, G9a dominantly regulates mono- and dimethylation in euchromatic regions (Peters *et al.,* 2003; Rice *et al*., 2003). G9a is essential for genome-wide dimethylation levels of H3K9, which is crucial for the silencing of many genes (Tachibana *et al*.,

2002). Mutations in G9a are sufficient to reduce levels of mono- and dimethylated H3K9 in euchromatic regions. Localization of HP1 to pericentric heterochromatic regions is regulated by SUV39h1 methylation, however, there is also localization of HP1 proteins to euchromatic regions. Upon mutation of G9a, there is a gross relocalization of HP1 proteins away from euchromatic regions, suggesting that G9a influences the recruitment of HP1 proteins to euchromatic regions (Tachibana *et al*., 2005). G9a also stimulates DNMT1 activity (Esteve *et al*., 2006).

1.5.2.5 Chaetocin

Chaetocin is a specific inhibitor of SUV39h1 and the first specific inhibitor of an H3K9 methyltransferase (Figure 1.6) (Greiner *et al*., 2005). It was first isolated from the fermentation broth of *Chaetomium minutum* and belongs to a class of molecules called 3-6 epidithio-diketopiperazines. Cells treated with chaetocin show a marked decrease in H3K9 dimethylation and trimethylation, due to decreased SUV39h1 activity. Chaetocin provides for a unique tool to study the effects of inhibition of SUV39h1 to the transcriptional regulation of a cell.

Chaetocin has also shown promising anti-myeloma effects. Chaetocin largely spared normal bone marrow, B-cells, and neoplastic B-chronic lymphocytic leukemia cells, while having a potent effect on myeloma cells. This indicates a large degree of selectivity of the drug (Isham *et al*., 2007).

1.5.2.6 Dominance of Epigenetic Events

Determining the order in which epigenetic events occur is vital to developing our understanding of transcription. Currently it is known that treatment with decitabine is sufficient to induce expression of genes with heavily methylated promoter regions, while TSA alone is not able to induce expression. This suggests that DNA methylation is dominant over histone acetylation (Kawamoto *et al*., 2008). There have also been studies done suggesting that H3K9 methylation must occur before DNA methylation, which would make H3K9 methylation the dominant transcriptional event (Tamaru and Selker, 2001). TSA is able to potentiate the induction seen by decitabine, suggesting that histone acetylation is playing a role in gene silencing (Kawamoto *et al*., 2008).

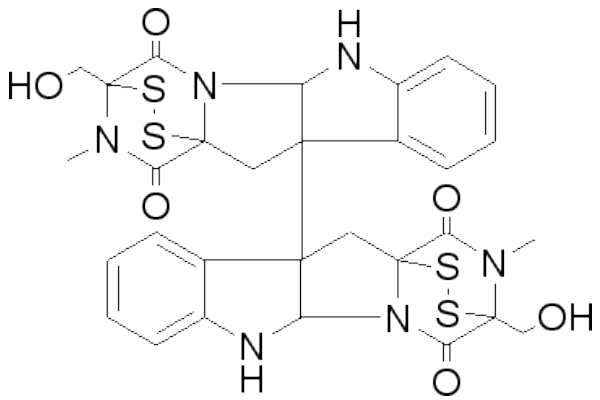


Figure 1.6. Chemical Structure of Chaetocin.

1.6 RIZ1

RIZ1 was first discovered in a functional screening for Retinoblastoma binding proteins (Buyse *et al.*, 1995), then later as a GATA-3 binding protein (Shapiro *et al.*, 1995) and as a DNA binding protein (Muraosa *et al.*, 1996). It belongs to a superfamily of proteins called nuclear protein methyltransferases (Xiao *et al.*, 2003). It contains eight zinc finger DNA binding motifs, a PR binding domain, an RB binding motif, a hormone receptor binding domain, an SH3 domain, a GTPase domain, an acidic region, a proline-rich domain, and a PR domain (Figure 1.7). It contains 1710 amino acids, with a molecular mass of 280 kDa.

The PR domain of RIZ1 is a ~100 amino acid region of the protein, present at the N-terminus (Buyse *et al*, 1995). This domain is homologous to the SET domain, a motif found in chromosomal proteins that modulate gene activities in yeast and mammals (Tschiersch *et al.*, 1994; Stassen *et al.*, 1995). PR domain-containing proteins have two products: one which contains the PR domain, and one which does not (Liu *et al.*, 1997). The RIZ1 gene has an alternate product, RIZ2, which is produced from an internal promoter (Figure 1.7). The PR domain has H3K9 methyltransferase activity, which is linked to gene repression (Kim *et al.*, 2003).

RIZ1 can act as both a repressor and activator of gene expression. When estradiol is absent, RIZ1 represses expression of estrogen sensitive genes and increases the amount of H3K9 methylation present, however, in the presence of estradiol, RIZ1 is able to activate gene expression by binding to the estrogen receptor. When this occurs, H3K9 methylation is decreased and H3K9 acetylation is increased at estrogen receptor targets, likely through the interaction of RIZ1 with HAT co-activators (Carling *et al.*, 2004). RIZ1 has the ability to induce G2/M cell cycle arrest and/or apoptosis. Interestingly, this ability is not dependent on p53 or Retinoblastoma, which suggests RIZ1 is acting on an alternate pathway to induce apoptosis (He *et al.*, 1997). There is a correlation between the differentiation of myeloid cell lines and an increase in RIZ1 expression (Gazzerro *et al.*, 2001).

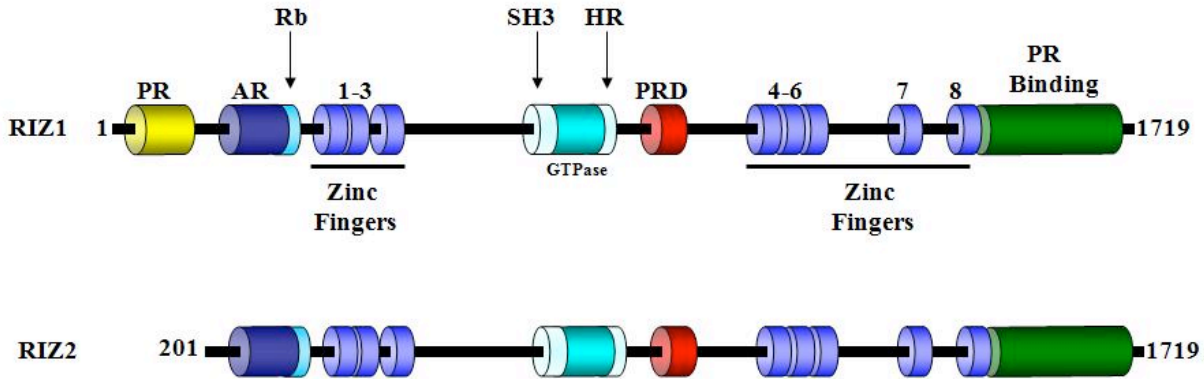


Figure 1.7. Domain Structure of RIZ1 and RIZ2. AR is the acidic region, RB is the RB binding motif, HR is the hormone receptor binding domain, PRD is the proline rich domain.

1.6.1 RIZ1 as a Tumor Suppressor

The RIZ1 gene is located at the distal arm of chromosome 1 at 1p36, a region frequently deleted in numerous carcinomas (Buyse *et al.*, 1996; Muraosa *et al.*, 1996). RIZ1 is silenced in a number of cancers (Table 1.1). Epigenetic silencing is the most common method of RIZ1 silencing, (Du *et al.*, 2001; Carling *et al.*, 2003; Chang *et al.*, 2003; Oshimo *et al.*, 2004) however deletion (Carling *et al.*, 2003), frameshift mutations (Piao *et al.*, 2000; Sakurada *et al.*, 2001; Tokumaru *et al.*, 2003) and missense mutations (Steele-Perkins *et al.*, 2001; Kim *et al.*, 2003) also occur. This loss of expression is confined solely to RIZ1, as RIZ2 expression is unchanged (He *et al.*, 1998; Jiang *et al.*, 1999; Chadwick *et al.*, 2000; Sasaki *et al.*, 2002). The

fact that RIZ1 but not RIZ2 is silenced in these carcinomas suggests that the silencing of RIZ1 is not a randomly occurring event, and that there may be selection of RIZ1 silencing over RIZ2 in tumor tissues (Huang, 1999). Animal models where RIZ1 has been selectively knocked out show a wide variety of tumor development such as diffuse large B cell lymphoma (Steele-Perkins *et al.*, 2001).

Loss of heterozygosity often occurs in the 1p36 region, where RIZ1 is located (Hofmann *et al.,* 2001). Loss of heterozygosity is the first "hit" of the two hit hypothesis; the second "hit" is the second allele being silenced by epigenetic means.

Table 1.1. Mechanisms of RIZ1 silencing

Carcinoma	**Mechanism of Silencing**	**Reference**
Acute Myeloid Leukemia	DNA Methylation	Sasaki *et al*., 2002
Breast	DNA Methylation	Du *et al.*, 2001
Colon	DNA Methylation	Du *et al.*, 2001
Colorectum	Frameshift Mutation	Sakurada *et al.*, 2001
DLBL	Missense Mutation	Steele-Perkins *et al.*, 2001
Endometrium	Frameshift Mutation	Piao *et al.*, 2000
Hepatoma	DNA Methylation	Du *et al.*, 2001
Liver	DNA Methylation	Du *et al.*, 2001
Lung	DNA Methylation	Du *et al.*, 2001
Nasopharyngeal	DNA Methylation	Chang *et al.*, 2003
Neuroblastoma	Missense Mutations	Kim *et al.*, 2003
Osteosarcoma	Missense Mutations	Steele-Perkins *et al.*, 2001
Pancreas	Frameshift Mutations	Sakurada *et al.*, 2001
Parathyroid Tumors	Deletion	Carling *et al.*, 2003
Parathyroid Tumors	DNA Methylation	Carling *et al.*, 2003
Pheochromocytomas	Deletion	Carling *et al.*, 2003
Pheochromocytomas	DNA Methylation	Carling *et al.*, 2003
Stomach	Frameshift Mutations	Sakurada *et al.*, 2001
Stomach	DNA Methylation	Oshimo *et al.*, 2004

2. HYPOTHESIS AND SPECIFIC AIMS

The aim of this study was to re-express a tumor suppressor gene, RIZ1, in human AML and CML cell lines. In the process of doing so, it was a goal to elucidate a model by which transcriptional silencing can be reversed. Three epigenetic drugs decitabine, TSA and chaetocin were used to investigate the dominance of three epigenetic events: DNA methylation, histone acetylation and histone methylation respectively. The specific aims of my study are to 1) re-express RIZ1 in two model cell lines 2) determine any phenotypic effects the three epigenetic drugs have on the cells 3) determine any epigenetic effects the drugs have on the cell and 4) determine any synergistic or antagonistic effects the drugs have on the cells. There is evidence that histone methylation is the dominant event in transcriptional silencing, as it has been found that histone methylation is necessary for DNA methylation to occur (Tamaru and Selker, 2001). It was my hypothesis that histone 3 methylation at lysine 9 is the dominant event in transcriptional silencing, and that treatment with decitabine, TSA and chaetocin will induce RIZ1 expression.

3. MATERIALS AND METHODS

3.1 Reagents and Suppliers

The reagents used for experiments in this thesis were all molecular biology or reagent grade and are listed in Table 3.1. Several of the procedures used in this study were performed using commercially available kits, which are listed in Table 3.2. Table 3.3 lists the companies from which all reagents and kits were obtained.

3.2 Oligonucleotides

Table 3.4 lists all primers used in this study, along with their optimal annealing temperature. All primers were purchased from IDT DNA.

3.3 Cell Lines and Tissue Culture

3.3.1 Cell Lines and Standard Culture Conditions

The human AML cell line AML-193 and human CML-BP cell line K562 were purchased from the German Collection of Organisms and Cell Cultures (DSMZ). AML-193 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) with 20% (v/v) fetal bovine serum (FBS, Invitrogen) supplemented with 2 ng/mL granulocyte macrophage colony stimulating factor (GM-CSF) and 3 units/mL Interleukin-3 (IL-3) (R&D Systems). K562 cells were cultured in IMDM medium with 10% (v/v) FBS. All cultures contained 1% (v/v) penicillin/streptomycin solution (Gibco) and were maintained at 37°C and 5% CO_2. All cell culture media was supplied from Invitrogen. Cells were incubated with decitabine, TSA or chaetocin for 72 hours. An

equivalent volume of 50% (v/v) acetic acid, ethanol or DMSO was used as vehicle controls respectively.

Table 3.1. Reagents and Suppliers Used in This Study

Reagent	Supplier Name
100 bp DNA ladder	Fermentas
5-aza-2'deoxycytidine	Sigma-Aldrich
acetic acid	EMD Chemicals
agarose	Invitrogen Life Technologies
boric acid	EMD Chemicals
Chaetocin	Sigma-Aldrich
dATP	Fermentas
dCTP	Fermentas
dGTP	Fermentas
DMSO	Sigma-Aldrich
dTTP	Fermentas
ethanol	EMD Chemicals
ethidium bromide	Invitrogen Life Technologies
fetal bovine serum	Invitrogen Life Technologies
GM-CSF	R&D Systems
HCl	EMD Chemicals
HotStar*Taq* Polymerase	Qiagen
IL-3	R&D Systems
IMDM	Invitrogen Life Technologies
isopropanol	EMD Chemicals
loading dye 6X solution	Fermentas
methanol	BDH
$MgCl_2$	Qiagen
MTT reagent	Invitrogen Life Technologies
NaOH	BDH
nuclease free water	Ambion
PCR buffer 10X	Qiagen
penicillin/streptomycin 100X mix	Invitrogen Life Technologies
propidium iodide	Sigma-Aldrich
RNAse	Worthington
RNasin	Promega
SDS	EMD Chemicals
sodium bicarbonate	BDH
sodium borate	EMD Chemicals
Trichostatin A	Sigma-Aldrich
Trypan Blue	Invitrogen Life Technologies
iQ SYBR Green Supermix	Bio-Rad

Table 3.2. Commercially Available Kits Used in This Study

Commercially Used Kits	Company
DNeasy Blood and Tissue Kit	Qiagen
EZ DNA Methylation Kit	Cedarlane
iScript cDNA Synthesis Kit	Bio-Rad
RNeasy Mini Kit	Qiagen

Table 3.3. Names and Addresses of Suppliers

Supplier	**Address**
Ambion	Austin, Texas, USA
BDH	Chicoutimi, Quebec, Canada
Bio-Rad	Mississauga, Ontario, Canada
Biosoft	Box 1013, Great Shelford, Cambridge, GB, CB22 5WQ
EMD Chemicals	San Diego, California, USA
Fermentas	Canada Inc., Burlington, Ontario, Canada
Invitrogen Life Technologies	Burlington, Ontario, Canada
Molecular Devices	Sunnyvale, California, USA
Promega	Nepean, Ontario, Canada
Qiagen	Mississauga, Ontario, Canada
R&D Systems	Minneapolis, Minnesota, USA
Sigma-Aldrich	Oakville, Ontario, Canada
Tree Star Inc.	Ashland, Oregon, USA
Worthington	Freehold, New Jersey, USA

Table 3.4. Sequences and Optimal Annealing Temperatures of Primers Used in This Study

Name	**Sequence**	**Temp**
RIZ1-RT-F	5-ATTGATGCCACTGATCCAGAGA-3	56.0°C
RIZ1-RT-R	5-GCTCTGTTGATTTCCAGTGGGA-3	56.0°C
RIZ1-MSP-U-F	5-TGGTGGTTATTGGGTGATGGT-3	60.0°C
RIZ1-MSP-U-R	5-ACTATTTCACCAACCCCAAGA-3	60.0°C
RIZ1-MSP-M-F	5-GTGGTGGTTATTGGGCGACGG-3	68.0°C
RIZ1-MSP-M-R	5-GCTATTTCGCCGACCCCGACG-3	68.0°C
RIZ1-PYRO-F	5-TTTGGGATAGTGGGGAGA-3	64.0°C
RIZ1-PYRO-R	5-GATTGGAGTTAAGATG-3	64.0°C
B-actin-F	5-GCCCCGCGAGCACAGAGC-3	59.0°C
B-actin-R	5-GCGGTTGGCCTTGGGGTTCAG-3	59.0°C

3.4 General Molecular Techniques

3.4.1 Isolation of Total RNA From Eukaryotic Cells

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) which typically yielded 1-3 mg of RNA per $5x10^6$ starting cells. RNA pellets were dissolved in nuclease free water (Ambion). The concentration and purity of samples were determined by standard A260/A280 spectrophotometric reading as well as by agarose gel electrophoresis. Samples were stored at -80°C until needed.

3.4.2 Isolation of DNA from Eukaryotic Cells

Genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen). The procedure was followed according to manufacturer's instructions and yielded approximately 20 μg of DNA per 5 x 10^6 starting cells. The concentration and purity was determined by standard A260/A280 spectrophotometric reading and stored at -20°C until needed.

3.5 Reverse Transcription PCR (RTPCR)

Total RNA was used as a template for the synthesis of cDNA using the iScript cDNA Synthesis kit (Bio-Rad) according to manufacturer's specifications. Briefly, 1 μg of total RNA was added to a mix containing 4 μL 5X iScript Reaction Mix, 1 μL iScript Reverse Transcriptase and 1 μg RNA in a final volume of 20 μL. Synthesis of cDNA was completed by incubation at 25°C for 5 minutes followed by 30 minutes at 42°C and 5 minutes at 85°C. Samples were used immediately or stored at -20°C.

Polymerase chain reaction (PCR) was performed in a final volume of 50 μL containing 1μL of cDNA, 1X PCR Buffer (containing 1.5 mM $MgCl_2$) (Qiagen), 1.5 mM $MgCl_2$, 0.4 mM dNTP, 0.2 μM of each primer and 1 unit of HotStar *Taq* (Qiagen). The amplification consisted of an initial *Taq* activation step of 95°C for 15 minutes followed by 35 cycles (30 for β-actin, 45 for pyrosequencing) of (95°C for 30 seconds, annealing temperature for 30 seconds, 72°C for

one minute) and a final incubation of 72°C for 6 minutes. Following amplification, 10 μL of PCR products were visualized using standard agarose gel electrophoresis. Primers and annealing temperatures are listed in Table 3.4.

3.6 Standard Agarose Gel Electrophoresis

Standard DNA fragment gel electrophoresis was typically performed in 2% (w/v) agarose gels in 1X sodium borate buffer containing 0.5 μg/mL ethidium bromide. The gel was run at 150 volts for the appropriate amount of time required to obtain optimal resolution. Gels were visualized under ultraviolet light and digitally captured using a gel documentation system (Bio-Rad).

3.7 Real Time PCR

PCR was performed in a final volume of 20 μL containing 0.3 μL cDNA, 66 ng of each primer, and 12.5 μL of iQ SYBR Green Supermix (Bio-rad). The amplification consisted of 5 minutes at 95°C followed by 40 cycles (95°C for 30 seconds, annealing temperature for 30 seconds, 72°C for one minute). RIZ1 transcript expression levels were compared to the amplification of a housekeeping gene hypoxanthine-guanine phosphoribosyltransferase. Data was analyzed by the ΔΔCt method (Livak and Schmittgen, 2001).

3.8 DNA Methylation Analysis

3.8.1 Sodium Bisulfite Modification

Genomic DNA was treated with sodium bisulfite reagent for downstream methylation analysis essentially as described elsewhere (Herman *et al*., 1996; Tao *et al*., 2002). This procedure was performed using the EZ DNA Methylation kit (Cedarlane). 2 μg of genomic DNA was modified per sample according to the manufacturer's instructions. Samples were stored at -20°C until needed.

3.8.2 Methylation Specific PCR (MSP)

DNA methylation within promoter associated CpG islands was determined by MSP following sodium bisulfite treatment of genomic DNA. PCR was performed in a final volume of 50 µl containing 100 - 200 ng of bisulfite-treated DNA, 1x Qiagen PCR Buffer, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 µM of each primer set, and 1 unit of HotStar *Taq* (Qiagen). The amplification consisted of a *Taq* activation step at 95°C for 15 min followed by 35 amplification cycles (94°C for 1 min, annealing temperature for 1 min, and 72°C for 1 min) and a final incubation at 72°C for 10 min. The 5' CpG islands were identified using the online 'CpG Island Searcher' tool (Takai and Jones, 2003) and appropriate primers were designed near the major transcriptional start site of target genes using the online 'MethPrimer' tool (Li and Dahiya, 2002). The primer sequences and optimal annealing temperatures are listed in Table 3.4. Modified AML-193 DNA served as the methylated MSP positive control while modified THP-1 DNA served as the unmethylated MSP positive control. Following amplification, 10 µL of PCR products were visualized using standard agarose gel electrophoresis.

3.9 MTT Assay

Cell proliferation/cytotoxicity was measured by the [3-(4,5-dimentylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. Cells were plated in triplicate at 2 x 10^4 cells per well in a 96 well plate, cultured as described in section 3.3, and treated with a vehicle control, 4 µM Decitabine, and/or indicated concentrations of TSA and Chaetocin for 72 hours. Following the treatment period, 1/10 culture volume of 5 mg/mL MTT labelling reagent (Sigma-Aldrich) was added to each well and incubated for 4 hours at 37°C. The resulting crystals were solubilized by adding 100 µL of solublization solution (10% (v/v) SDS, 0.01M HCl) to each well and incubating overnight at 37°C. Spectrophotometric absorbance readings were then taken (570 nm with 650 nm background subtraction) using a Spectramax 340 PC plate reader (Molecular Devices).

3.10 Cell Cycle Analysis

Cell cycle analysis was performed by propidium iodide (PI) staining of DNA content. Cells were cultured as described in section 3.3, and treated with a vehicle control, 4 μM decitabine, and/or indicated concentrations of TSA and Chaetocin. Briefly, 1 x 10^6 cells were washed in ice cold 1X PBA [1X PBS, 0.1% (w/v) bovine serum albumin, 0.02% (w/v) sodium azide] fixed in ice cold ethanol and incubated overnight at 4°C. Cell pellets were reconstituted in Triton-PBA [0.1% (v/v) Triton X-100, 1X PBA] for 3 min, pelleted by centrifugation, and incubated in 500 units/mL RNase working solution (Worthington) at 37°C for 45 min. Samples were stained 15 minutes in 0.6 mL PI working solution (0.05 mg/mL in PBA; Sigma) at room temperature and filtered through 35 μm nylon mesh into glass tubes for flow cytometry analysis. Ten thousand cells were gated and sorted according to DNA content, then analyzed the using FloJo software (Tree Star, Inc.)

3.11 Trypan Blue Staining

Cell viability was determined by Trypan Blue Staining. Briefly, cells were cultured as above and treated with a vehicle control, 4 μM Decitabine, and/or indicated concentrations of TSA and Chaetocin. 10 μLs of the sample were then diluted 2X in Trypan Blue staining reagent (Invitrogen), and then counted using a hemocytometer (Spencer).

3.12 Calcusyn

Synergy calculations were performed via Calcusyn software (Biosoft). MTT data were inputted into the software and values generated using the software.

4. RESULTS

4.1 Expression and Methylation Analysis of Leukemia Cell Lines in Study

RIZ1 is silenced in both CML and AML cell patient material (Figure 4.1, 4.2) (Geyer *et al.,* unpublished). Reverse transcription PCR and methylation specific PCR were conducted to confirm these results. Three model cell lines were investigated: K562 (CML blast crisis), THP-1 (acute monocytic leukemia) and AML-193 (AML). RIZ1 expression was shown to be absent in both K562 and AML-193, whereas RIZ1 expression was observed in THP-1 (Figure 4.3a). Two assays, methylation-specific PCR (MSP) and pyrosequencing, were used to determine the methylation status of the RIZ1 promoter region. For both assays, DNA was extracted from the cells and subjected to bisulfite modification, which changes all unmethylated cytosines to uracil, and subsequently to thymidine after PCR amplification. Bisulfite-modified DNA is used as a template for PCR with two sets of primers, one for unmethylated DNA and one for methylated DNA. The primers for unmethylated DNA have all of the cytosines substituted with thymidine while the primers for methylated DNA retain all cytosine bases. MSP analysis revealed that in K562 the promoter is hemi-methylated, in THP-1 the promoter is unmethylated, and in AML-193 the promoter is predominantly methylated (Figure 4.3b). Based on these initial results, K562 and AML-193 were chosen as model cell lines in this study as they both had silenced RIZ1.

4.2. Toxicity of Three Epigenetic Drugs in Human AML and CML Cell Lines

Prior to analyzing the affects of decitabine, TSA, and chaetocin on RIZ1 expression and promoter methylation, the toxicity of these drugs was measured using trypan blue staining and MTT assays to determine acceptable dose ranges for this study. Trypan blue dye exclusion assay measures cell viability based on the ability of non-viable cells to absorb blue dye once their cell membrane is breached.

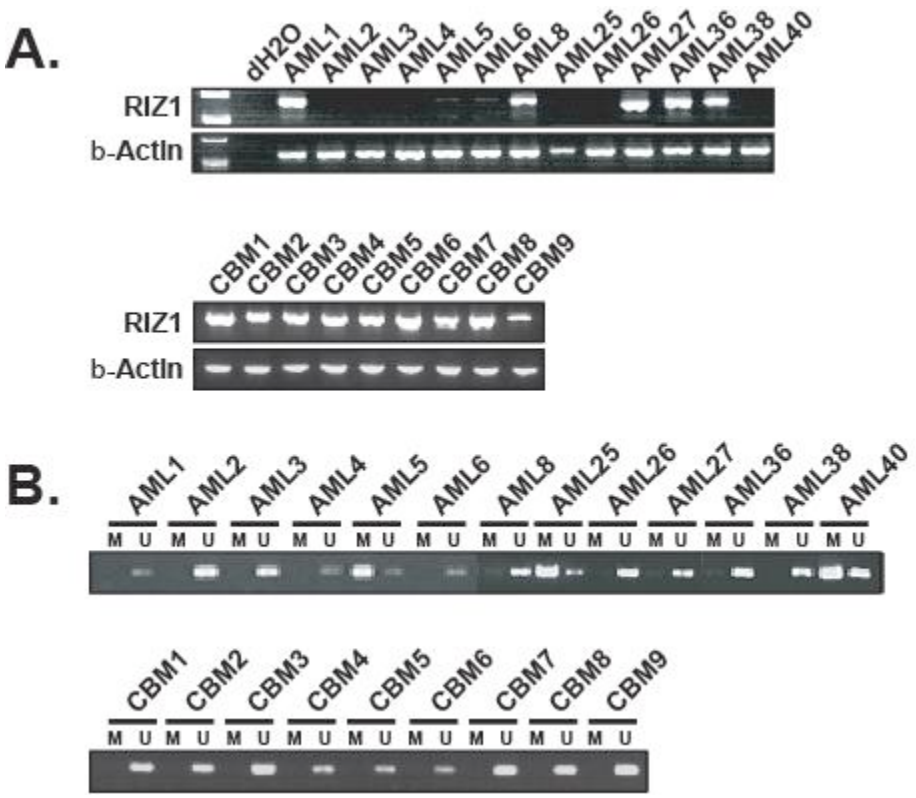


Figure 4.1. Methylation Analysis of AML Patient and Normal Bone Marrow Samples. A) RT-PCR shows that RIZ1 mRNA transcripts are decreased in samples 2, 3, 4, 5, 6, 25 and 40 (top panel). All cases showed similar β-actin mRNA transcript levels (bottom panel). B) Methylation specific PCR of the RIZ1 promoter region in AML patient samples shows methylated (M) DNA in samples 5, 25 and 40, all of which have markedly decreased RIZ1 mRNA transcript levels in the RT-PCR. In contrast, all normal bone marrow samples show unmethylated (U) DNA in the promoter region of RIZ1 (Geyer *et al.,* unpublished).

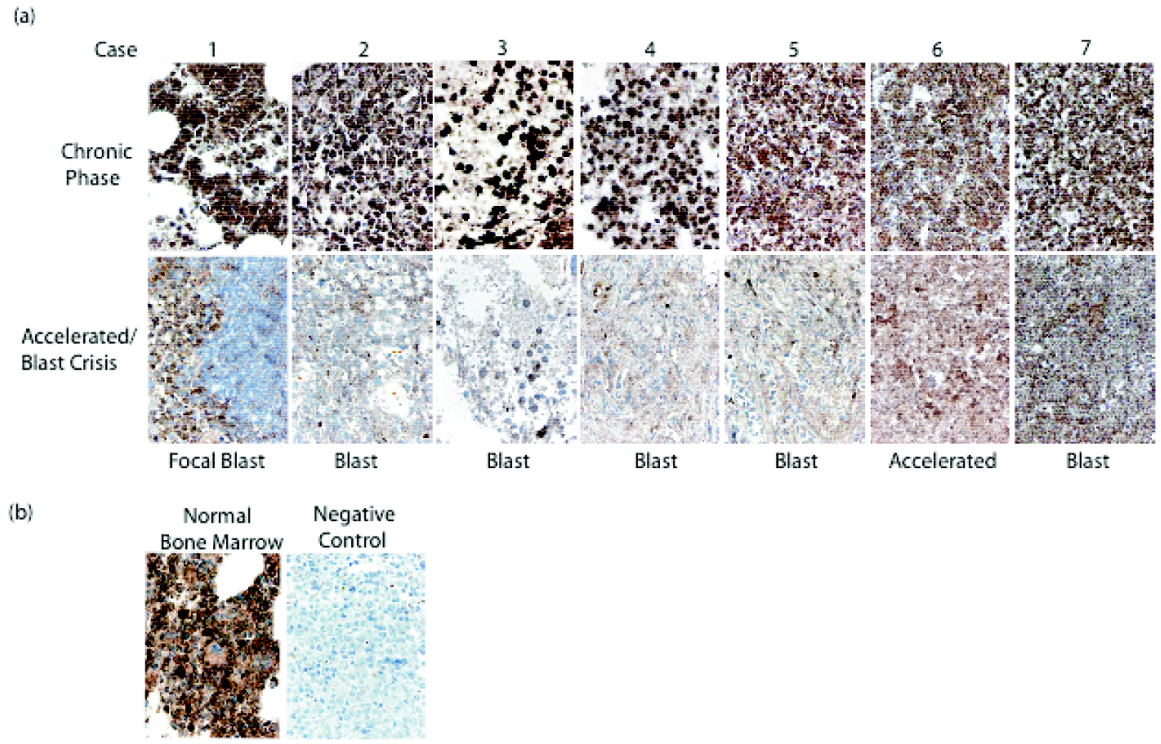


Figure 4.2. RIZ1 Expression in Bone Marrow of CML Patients. a) Immunohistochemical analysis of RIZ1 expression in matched bone marrow biopsies from patients in chronic phase or accelerated/blast crisis. Brown staining indicates presence of RIZ1 b) RIZ1 expression in normal bone marrow and normal bone marrow staining in the absence of RIZ1 primary antibody (negative control). (Geyer *et al.,* unpublished).

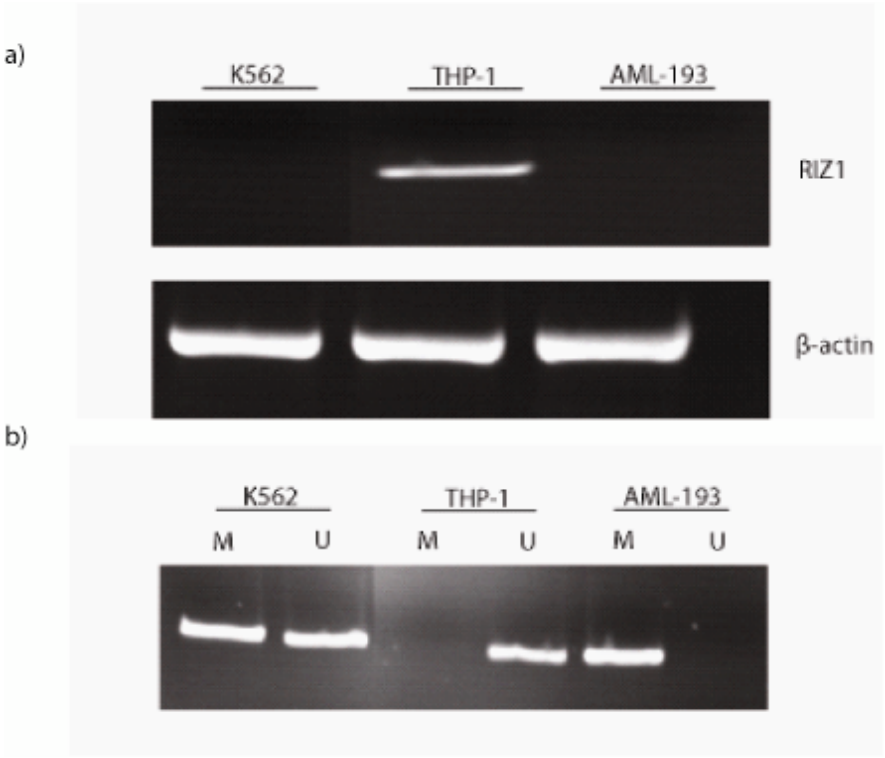


Figure 4.3. Analysis of RIZ1 Expression and Promoter Methylation. a) cDNA of cell lines was subject to RT-PCR analysis for RIZ1 expression. β –actin was used as a loading control. b) MSP analysis of modified DNA of three cell lines. M is an amplicon generated from primers specific to methylated RIZ1. U is an amplicon generated from primers specific to unmethylated RIZ1.

Viability is determined by counting the number of blue cells versus total cell number. An MTT assay is a colorimetric assay that determines the activity of enzymes that reduce MTT to formazan. Reduction of MTT to formazan is a colorimetric process, with the dye changing from a yellow colour to a purple colour, which allows the results to be measured using a spectrophotometer. Enzymes that catalyze the reaction are present in the mitochondria and are only active when a cell is viable, allowing a correlation between spectrophotometric readings and viability (Carmichael *et al*., 1987). The MTT assay is the more sensitive of the two assays, as it measures the activity of enzymes within the cells, whereas a loss of viability will not be detected in the trypan blue assay until the cell membrane has been breached. The two model cell lines, K562 and AML-193, were treated for 72 hours with varying doses of decitabine, TSA, and chaetocin and then subjected to trypan blue exclusion and MTT assays.

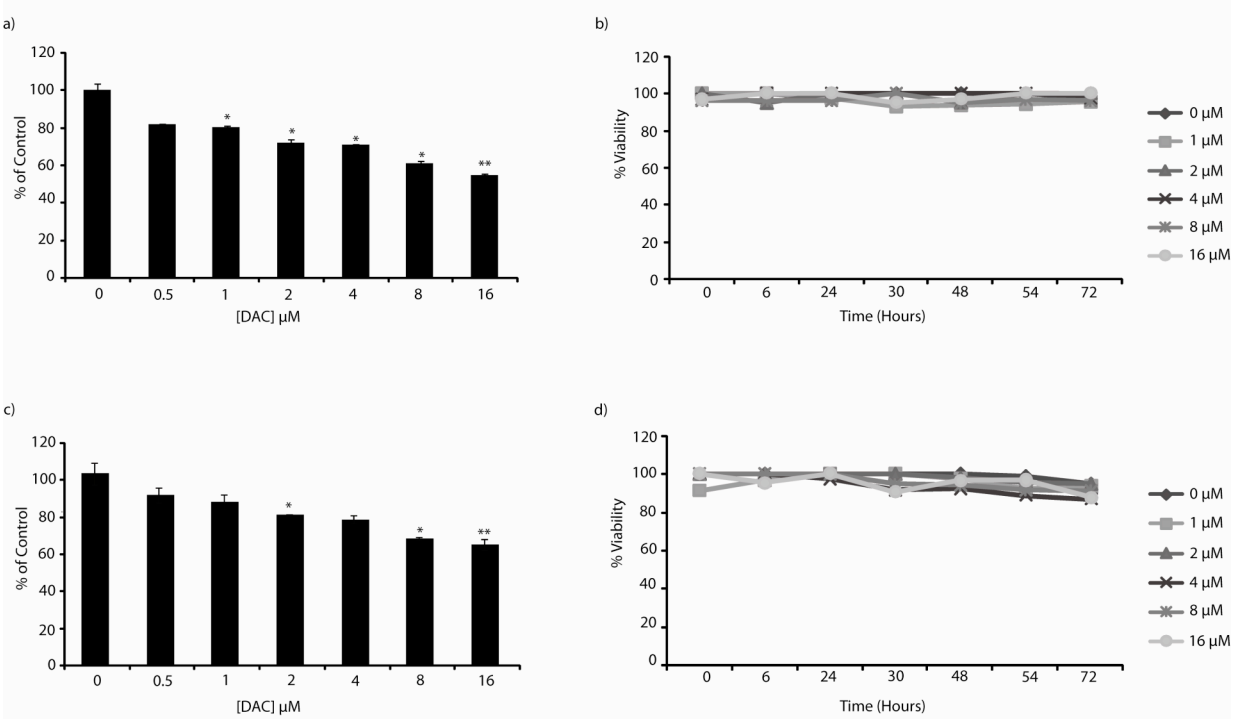


Figure 4.4. Viability of AML-193 and K562 Cell Lines Treated with Decitabine. (top) AML-193 and (bottom) K562 cells were treated with indicated concentrations of decitabine for 72 hours and then analyzed by the MTT assay (a, c). Absorbanes shown were normalized to the 0 µM condition. Samples were taken at indicated times and then analyzed by trypan blue assay (b, d). Error bars represent standard deviation from three independent experiments. ** Indicates $p<0.005$, * indicates $p<0.05$ relative to the 0 µM condition.

Treatment of cells with 1 µM decitabine induced a statistically significant loss of viability in AML-193 as measured in the MTT assay (Figure 4.4a), while treatment of K562 cells with 2 µM decitabine was required to induce a statistically significant loss of viability in K562 (Figure 4.4c). This trend was not seen in the trypan blue exclusion assay (Figure 4.4b, Figure 4.4d) which suggested that decitabine was toxic enough to reduce cell proliferation in the MTT assay, but not enough to cause the cell wall to rupture. To confirm that the doses of decitabine used in the viability assays are high enough to induce cell cycle arrest, I used propidium iodide staining to analyze the affect of decitabine on the cell cycle (Figure 4.5). Treatment of K562 and AML-193 cells with 4 µM decitabine caused an arrest in the G2 phase of the cell cycle. This is accompanied by an increase in the percentage of cells in S phase and subG1 population and a decrease in the percentage of cells in the G1 phase.

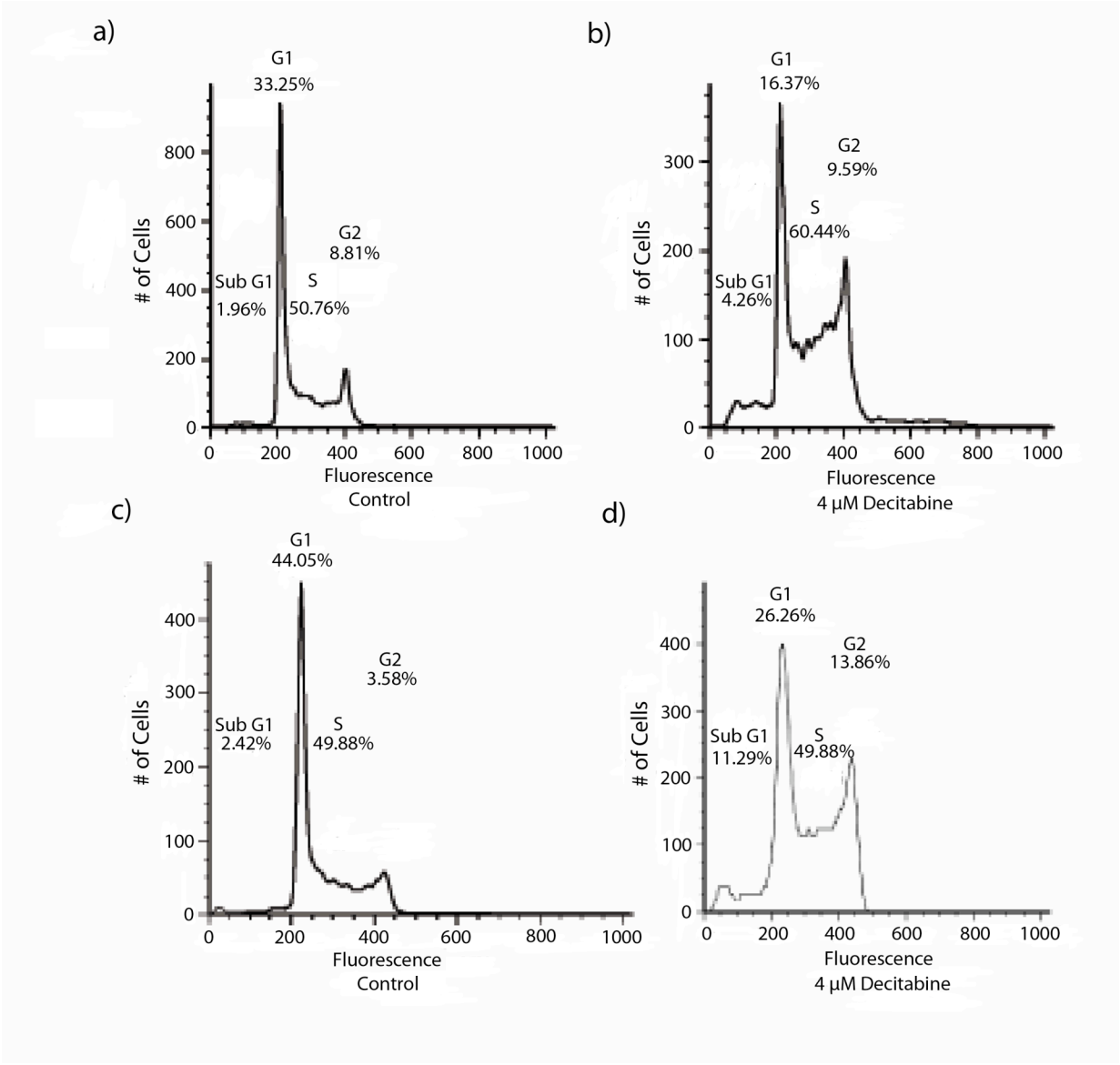


Figure 4.5. Cell Cycle of AML-193 and K562 Cell Line Treated with Decitabine. (top) AML-193 and (bottom) K562 cells were treated for 72 hours with either (b, d) 4 µM decitabine or a (a,c) vehicle control. Cells were then stained with propidium iodide and cell cycle analysis performed. Shown is the percentage of cells in each phase of the cell cycle. Cell cycle analysis was performed using the Watson algorithm of the FloJo software.

TSA showed significantly higher toxicity than decitabine in AML-193 and K562 cells. In AML-193, treatment with 31 nM TSA caused a statistically significant loss of viability. The viability continued to decrease until treatment with 125 nM TSA, where viability was reduced to 0% of the control (Figure 4.6a). This trend was similar in the trypan blue assay, where treatment with 62.5 nM caused the viability to decrease to 30% of control (Figure 4.6b). Treatment with 250 nM over 72 hours caused complete cell death. TSA was less toxic in K562 cells. Treatment of K562 cells with 31 nM of TSA induced a statistically significant loss in viability. Treatment with 1 μM TSA caused complete cell death (Figure 4.6c). This trend is also seen in the trypan blue assay. Treatment with 250 nM TSA decreased viability to 90% of control cells while treatment with 1 μM TSA caused viability to decrease to 18% of control cells (Figure 4.6d).

Chaetocin was more toxic than either decitabine or TSA. Treatment of AML-193 cells with 10 nM chaetocin induced a statistically significant loss of viability (Figure 4.7a). Viability continued to decrease until a dose of 20 nM chaetocin, where complete cell death occured. This trend was observed in the trypan blue analysis with a slight loss of viability occurring at 10 nM chaetocin and 0% viability at 50 nM chaetocin (Figure 4.7b). K562 was less sensitive to chaetocin than AML-193. K562 cells required a dose of 40 nM chaetocin to induce a statistically significant decrease in viability (Figure 4.7c). The decrease in viability continued until treatment with 80 nM chaetocin when complete cell death occurred. Similar results are obtained using the trypan blue assay, where treatment with 50 nM chaetocin decreased viability by 30%. This decrease in viability continued until treatment with 100 nM chaetocin where a minimum viability of 55% was obtained (Figure 4.7d).

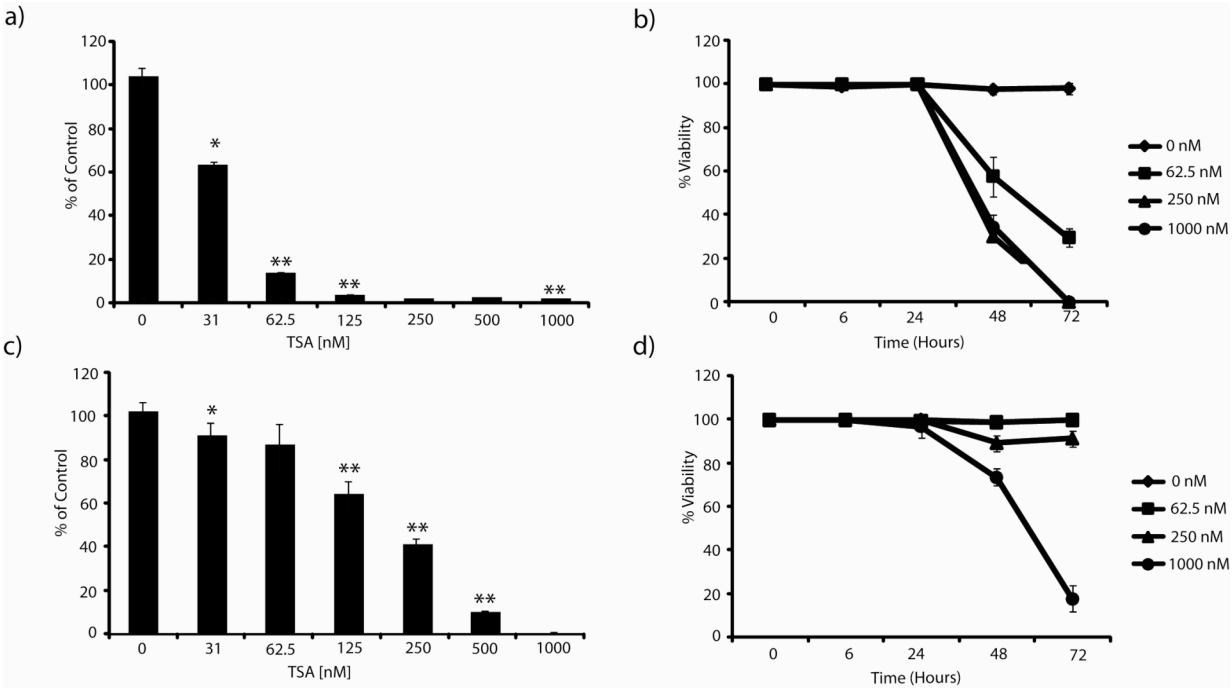


Figure 4.6. Viability of AML-193 and K562 Cell Lines Treated with TSA. AML-193 (a,b) and K562 (c,d) cells were treated for 72 hours with indicated concentrations of TSA and analyzed by the MTT assay. Indicated is the absorbance observed normalized to the control (a, c). Samples were taken at indicated time points and analyzed by trypan blue analysis (b, d). Error bars represent the standard deviation from three independent experiments. ** Indicates $p<0.005$, * indicates $p<0.05$ relative to the 0 nM condition.

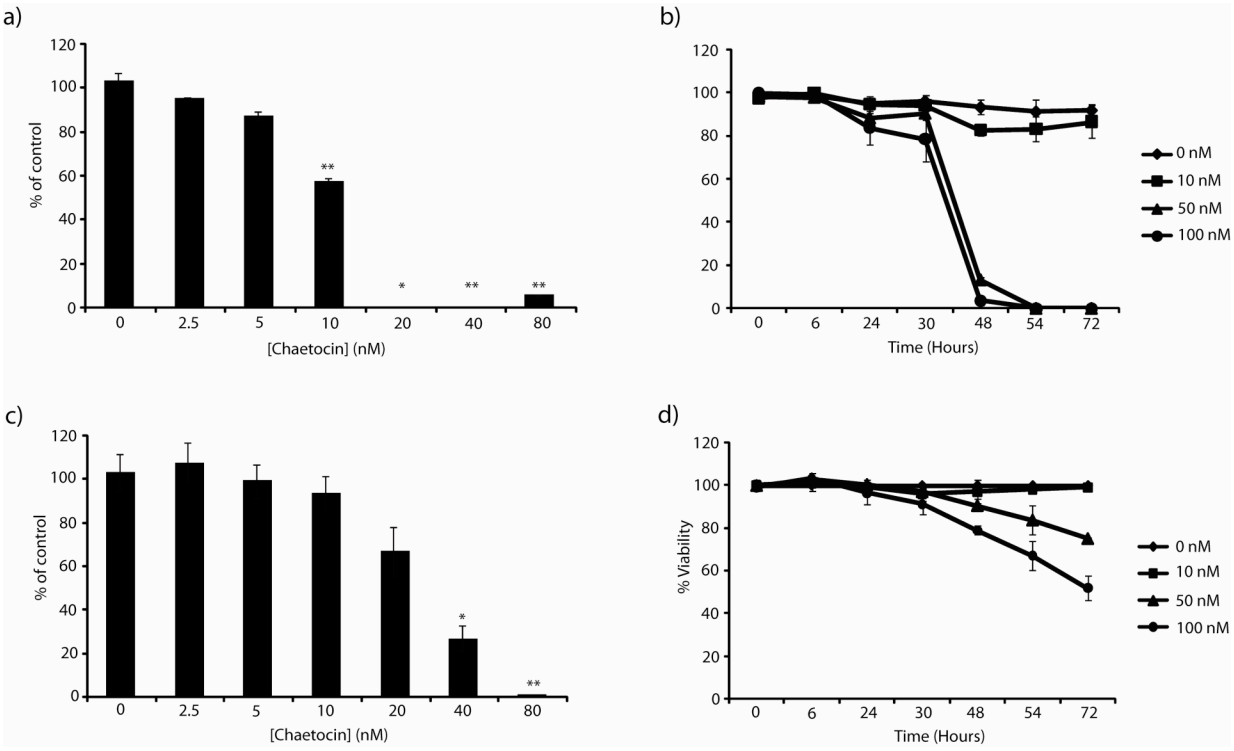


Figure 4.7. Viability of AML-193 and K562 Cell Lines Treated with Chaetocin. AML-193 (a,b) and K562 (c,d) cells were treated for 72 hours with indicated concentrations of Chaetocin and then analyzed by MTT. Shown is the percentage absorbance of the control (a, c). Samples were taken at indicated time points and analyzed by trypan blue analysis (b, d). Error bars represent the standard deviation from three independent experiments. ** Indicates $p<0.005$, * indicates $p<0.05$ relative to the 0 nM condition.

4.3. Determination of the Median Dose of TSA and Chaetocin in MTT assays

In order to determine the median doses for TSA and Chaetocin, MTT assays were carried out, and the fraction affected was calculated from Eq.1.

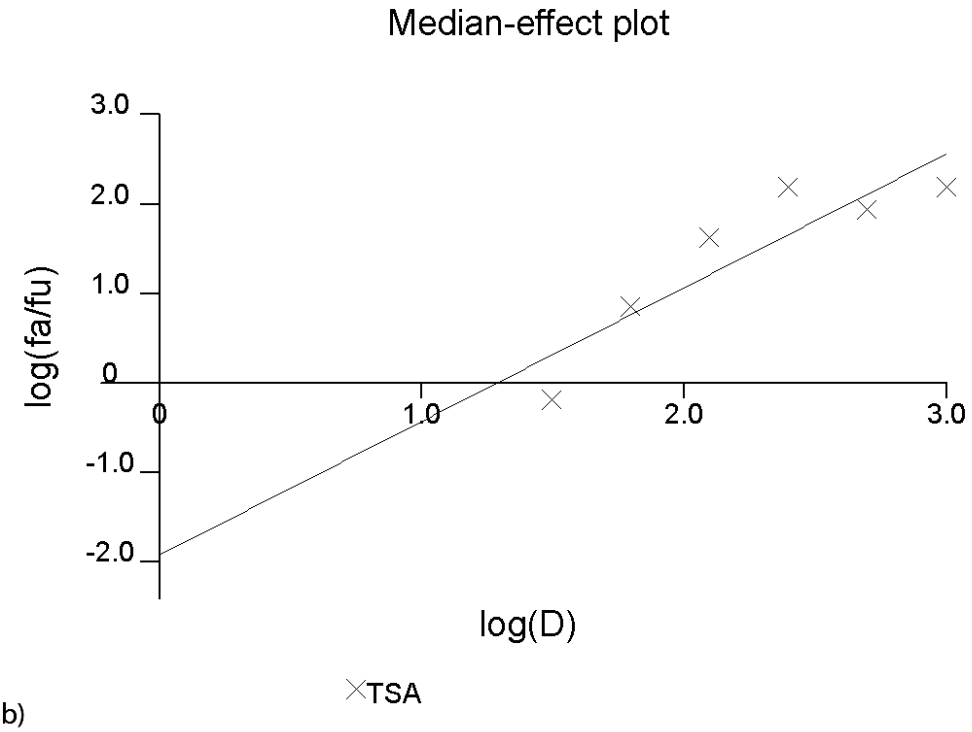
$$\text{Fraction Affected} = \frac{\text{(absorbance of control-absorbance of sample)}}{\text{(absorbance of control-absorbance of highest dose)}} \quad \text{(Eq. 1)}$$

This effect is then plotted versus the dose to create a dose effect curve. The median dose is found by taking the log of this curve, and applying equation 2. The median dose is the antilog of the x intercept of the median effect plot.

$$\text{Log (fa/fu)} = m \log (D) - m \log (D_m) \quad \text{(Eq. 2)}$$

In equation 2, fa is the fraction affected, fu is fraction unaffected, D is the drug dose, D_m is the median dose of the drug, and m is the slope of the line when plotting Log(fa/fu) versus LogD. The median effect plots for TSA and chaetocin are shown in Figures 4.8 and 4.9, respectively. A relevant median dose could not be determined for decitabine because with the doses used in this study viability is not reduced enough to model a dose response curve. The median dose for TSA in AML-193 and K562 cells was 33 nM and 128 nM, respectively (Figure 4.8). The median dose for chaetocin in AML-193 and K562 cells was 10.3 nM and 23.8 nM, respectively (Figure 4.9).

a)



b)

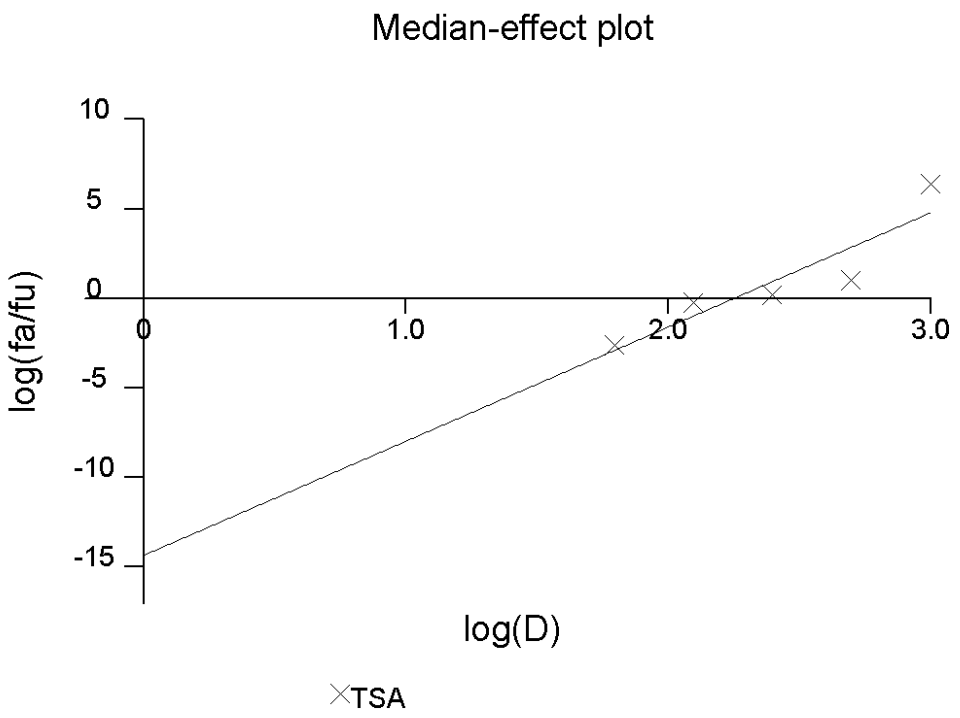
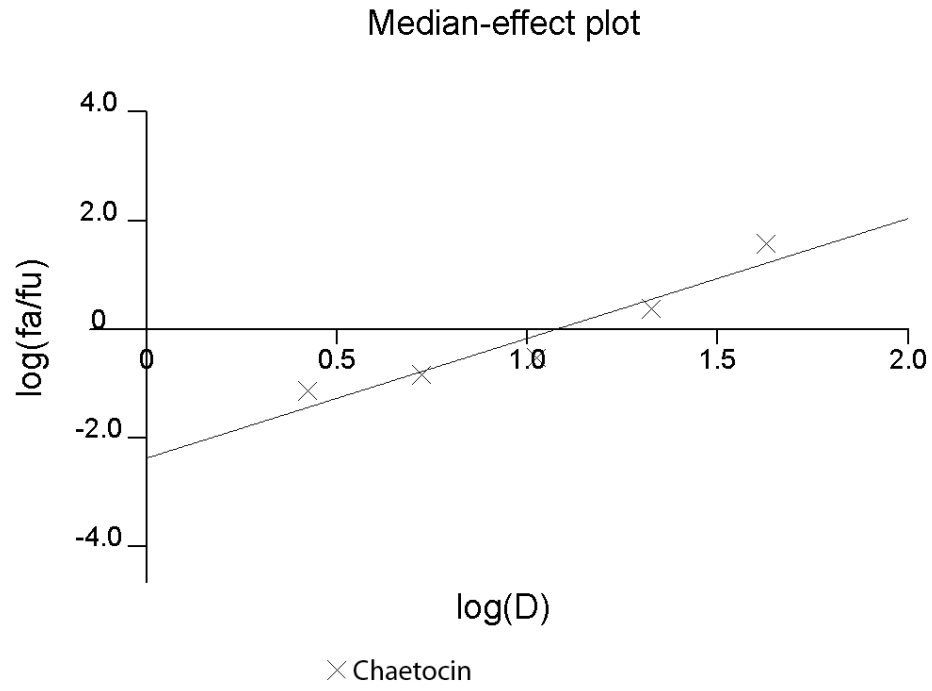


Figure 4.8. Median Effect Plot of AML-193 and K562 cells Treated with TSA. AML-193 (a) and K562 (b) cells were treated with TSA for 72 hours and a median dose curve was created. (fa) Indicates fraction affected, (fu) indicates fraction unaffected, and (D) indicates dose.

a)



b)

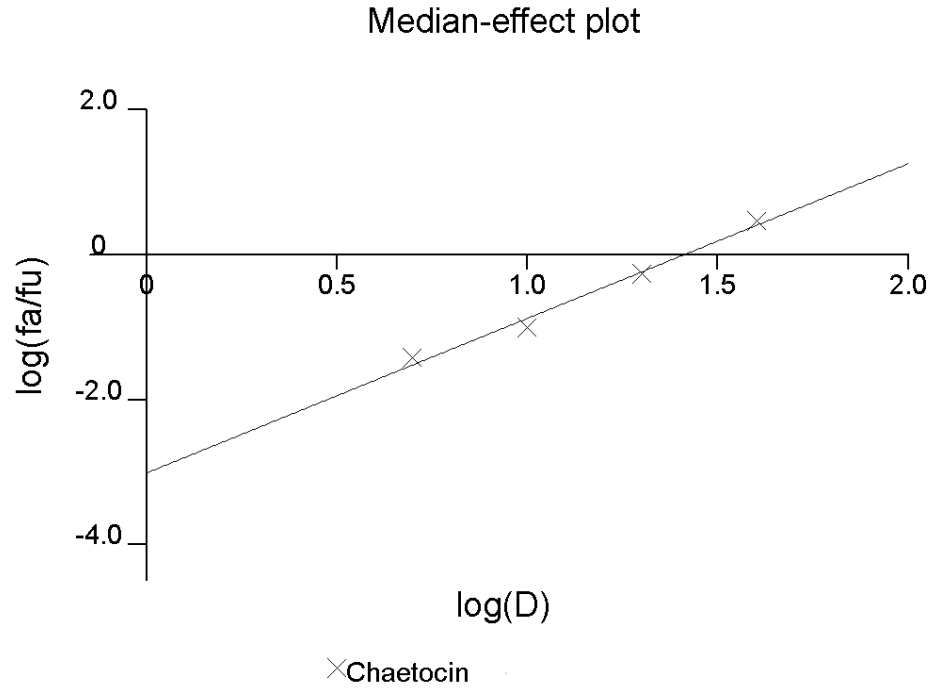


Figure 4.9. Median Effect Plot of AML-193 and K562 Cells Treated with Chaetocin. AML-193 (a) and K562 (b) cells were treated with chaetocin for 72 hours and a median dose curve was created. (fa) Indicates fraction affected, (fu) indicates fraction unaffected, and (D) indicates dose.

4.4 Methylation Status of the RIZ1 Promoter Region

Previously it was determined that the RIZ1 promoter region is methylated in both AML and CML cell lines (Figure 4.3). The RIZ1 5'-promoter region as well as CpG island and primer positions are illustrated in Figure 4.10. If there are differences in the bands between control and treatment samples, those samples are sent for pyrosequencing. Pyrosequencing allows for a quantitative analysis of the methylation present in the promoter region. The DNA to be pyrosequenced is incubated with DNA polymerase, luciferase, ATP sulfurylase and substrates such as luciferan and adenosine phosphosulfate. Upon incorporation of a nucleotide, a pyrophosphate is released, and is converted to ATP by ATP sulfurylase. This ATP acts as the fuel for the luciferase mediated conversion of luciferan to oxyluciferan, which can be measured as this reaction releases light. In quantifying methylation, the modified DNA is sequenced, and the percentage of methylated DNA is determined by the chemically-induced C/T differences (White *et al*, 2006).

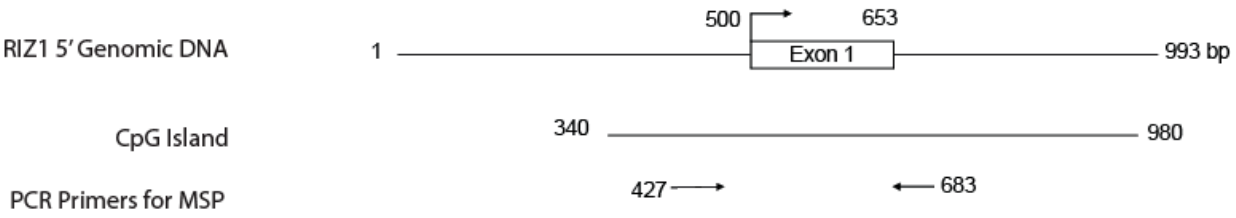


Figure 4.10. Schematic of RIZ1 genomic DNA and primer binding sites. The 5' end of RIZ1 genomic DNA is shown on top and exon 1 and CpG island locations are shown. PCR primer locations for MSP analysis are indicated by arrows.

Treatment with decitabine caused a decrease in overall RIZ1 promoter methylation in both cell lines. This was most pronounced in the AML-193 cell line where treatment of cells with 16 μM decitabine caused a decrease in average promoter region CpG methylation from 94% to 74% (Figure 4.11a). Interestingly, upon analyzing the pyrosequencing results, the 8th CpG in Figure 4.12 was not affected by the decitabine treatment and remains 100% methylated even when treated with 16 μM decitabine (Figure 4.12g). Other than this CpG, no other discernable pattern

was evident. All CpGs showed a similar loss of methylation within a few percentage points across the various treatments.

Treatment with decitabine lowered the amount of methylation in the RIZ1 promoter region from 39% to 30% in K562 cells. Treatment of cells with decitabine caused a reduction in the amount of RIZ1 promoter DNA methylation (Figure 4.11). Pyrosequencing analysis revealed that the 8th CpG which was resistant to demethylation in AML-193 was susceptible to demethylation in K562 (Figure 4.13). Overall, AML-193 cells displayed a more gradual decline in DNA promoter region methylation upon treatment with decitabine, while K562 cells showed a large drop off upon treatment with 0.5 μM decitabine, and then slight changes from then on.

TSA had no effect on the methylation status of the promoter region in either AML-193 or K562 (Figure 4.14) as shown by MSP. Since there was no change in methylation observed by MSP, pyrosequencing was not performed. Treatment of AML-193 cells with chaetocin had no effect on the methylation status of the RIZ1 promoter region as determined by MSP (Figure 4.15a). Treatment of K562 cells with 2.5 nM chaetocin caused a small decrease in methylation, however the methylation began to increase with increasing dosage until treatment with 80 nM chaetocin, where the methylation increased from 39% to 65% (Figure 4.15b). Treatment with 80 nM chaetocin caused the most dramatic effects.

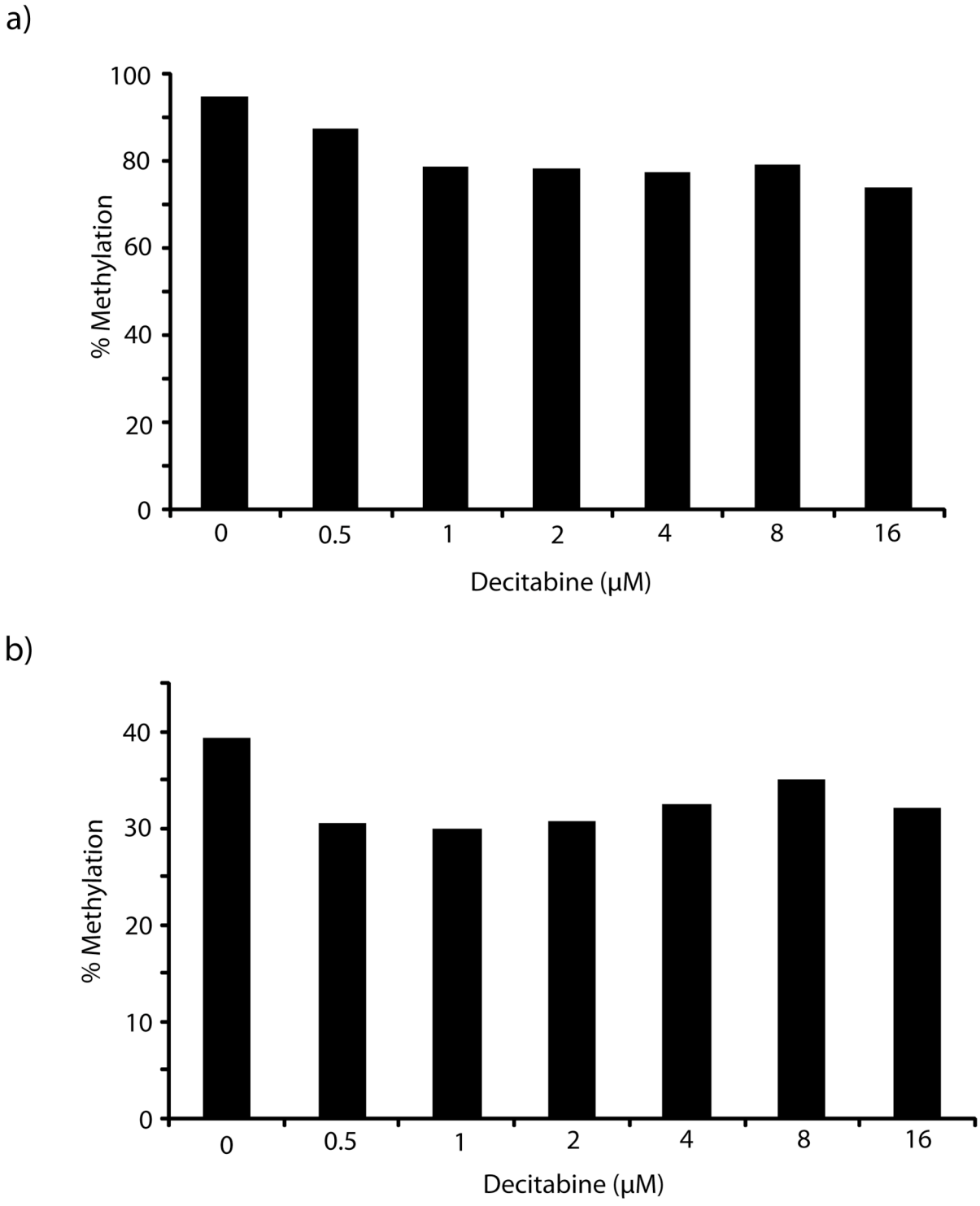
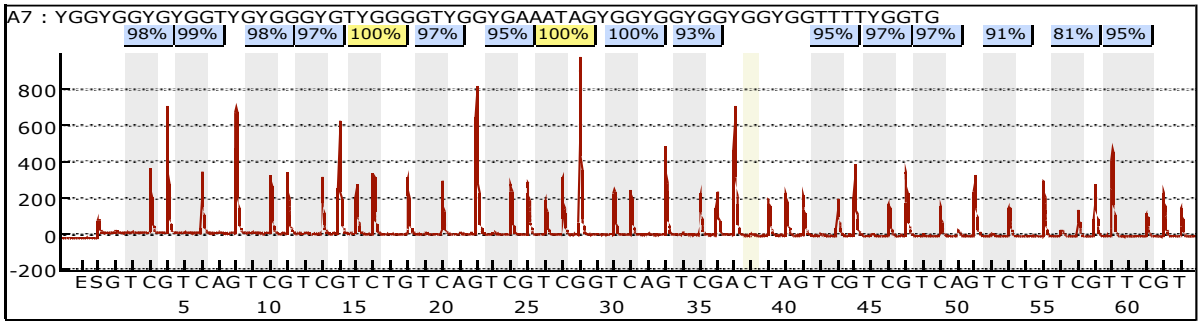


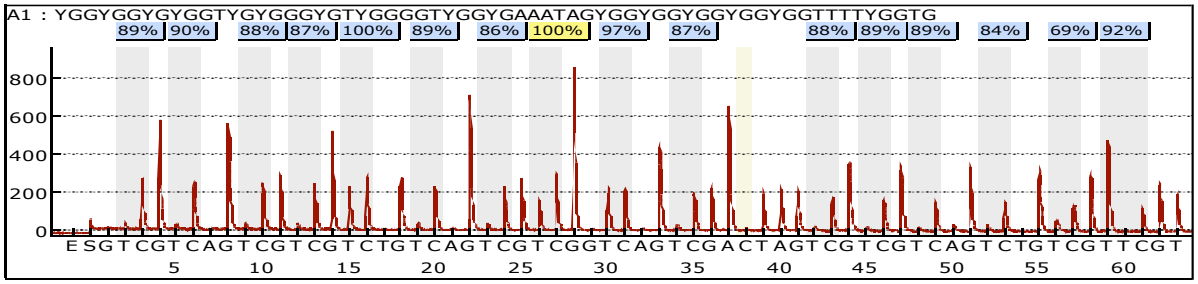
Figure 4.11. Mean Methylation Status of the RIZ1 Promoter Region DNA of AML-193 and K562 cells upon Treatment with Decitabine. AML-193 (a) and K562 (b) cells were treated for 72 hours with indicated concentrations of decitabine, DNA was extracted, modified by bisulfite and pyrosequenced. Shown is a histogram of the mean methylation percentages over entire region examined.

Figure 4.12. Pyrosequencing Analysis of 17 CpGs in RIZ1 Promoter Region of AML-193 Cells Treated with Decitabine.

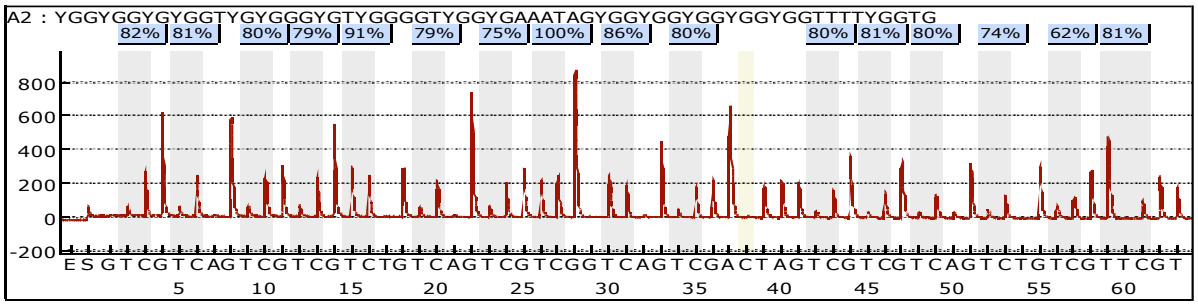
a)
Control



b)
0.5 µM



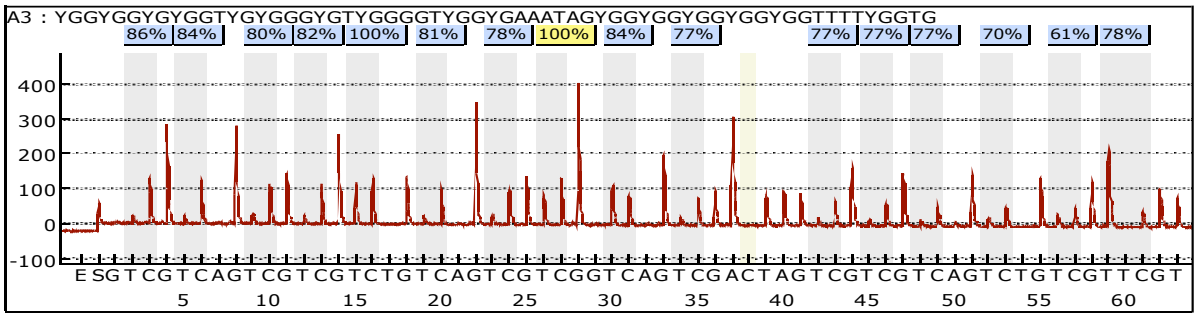
c)
1 µM



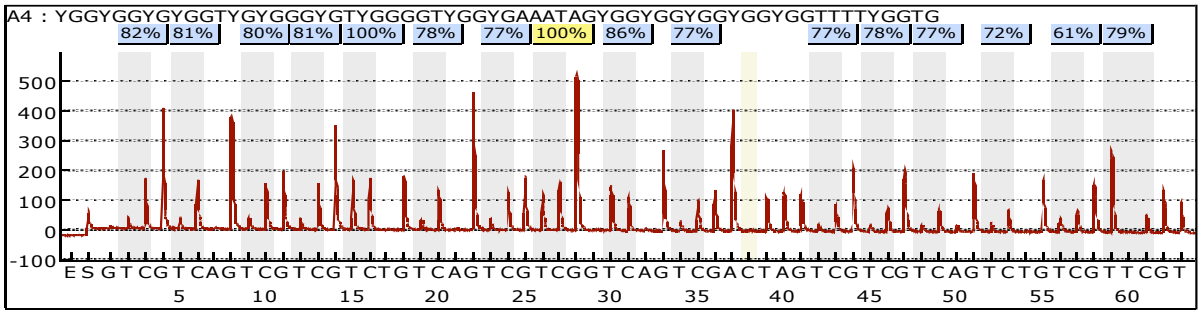
(cont.)

(Figure 4.12. cont)

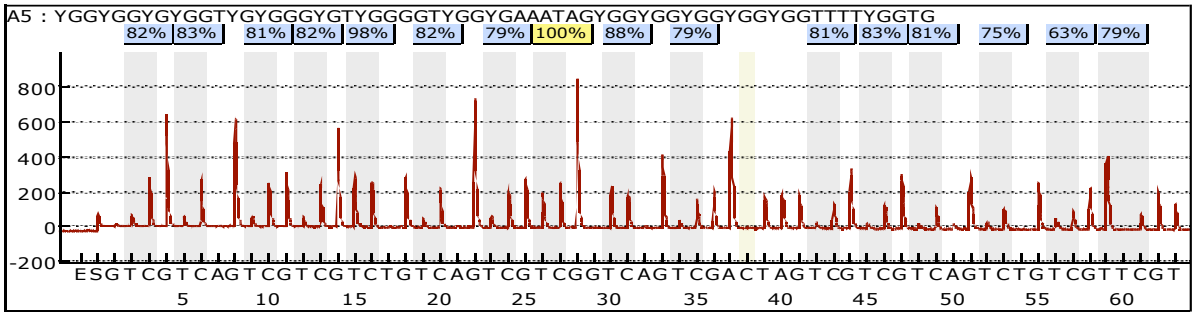
d)
2 µM



e)
4 µM



f)
8 µM



(cont)

g)
16 µM

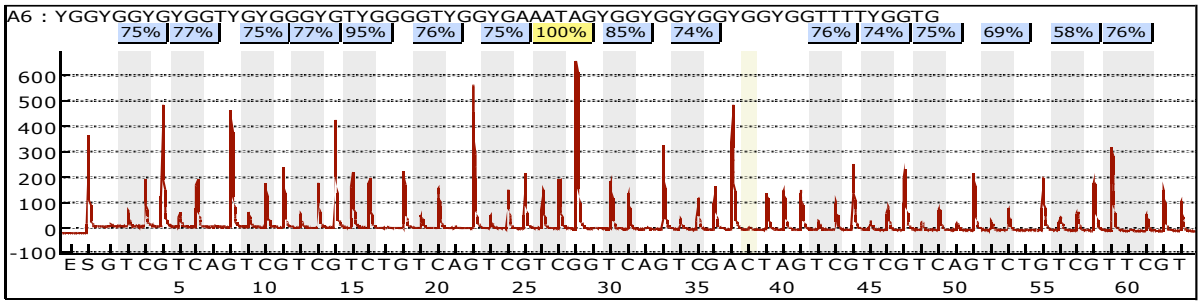
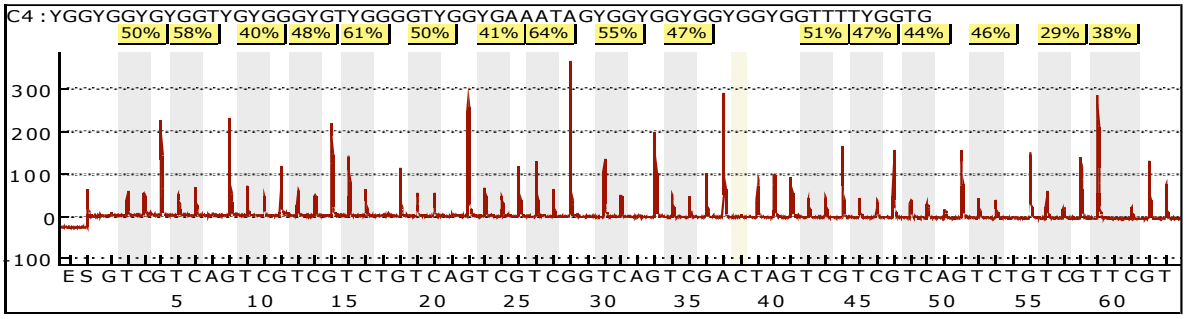


Figure 4.12. Pyrosequencing Analysis of 17 CpGs in RIZ1 Promoter Region of AML-193 Cells Treated with Decitabine. Cells were treated for 72 hours with the indicated concentrations of decitabine, DNA was extracted, modified by bisulfite and analysed by pyrosequencing. Pyrosequencing output indicates methylation percentage at various CpGs

Figure 4.13. Pyrosequencing Analysis of 17 CpGs in RIZ1 Promoter Region of K562 Cells Treated with Decitabine.

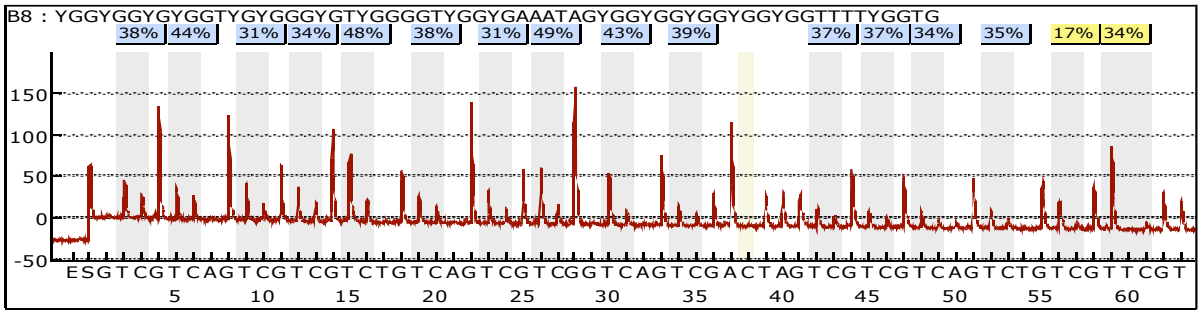
a)

Control



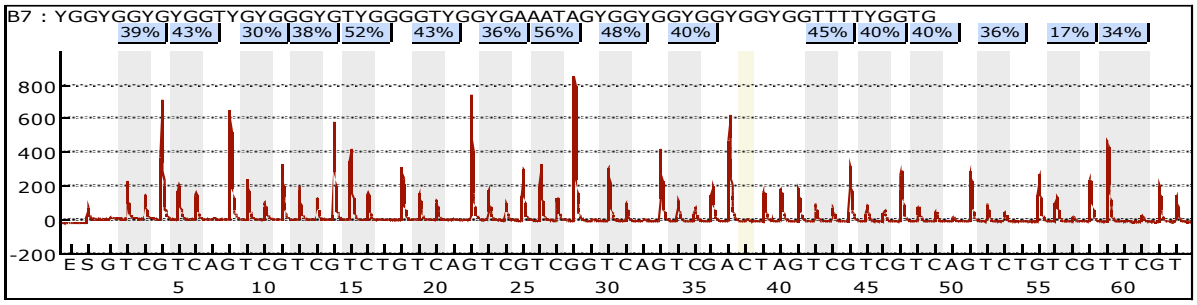
b)

0.5 µM



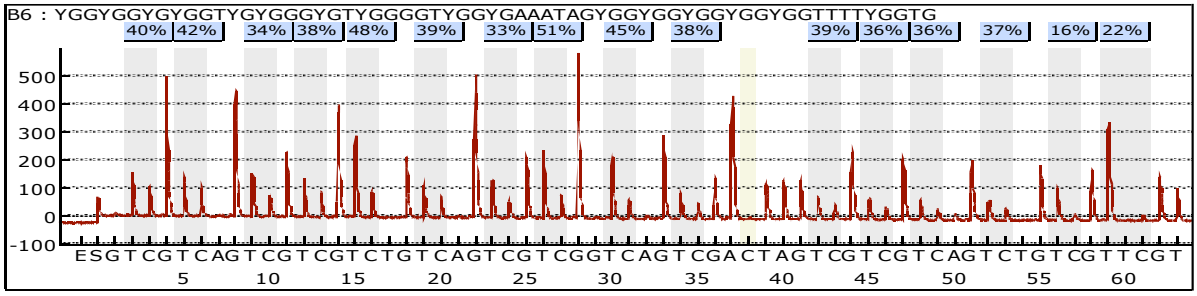
c)

1 µM

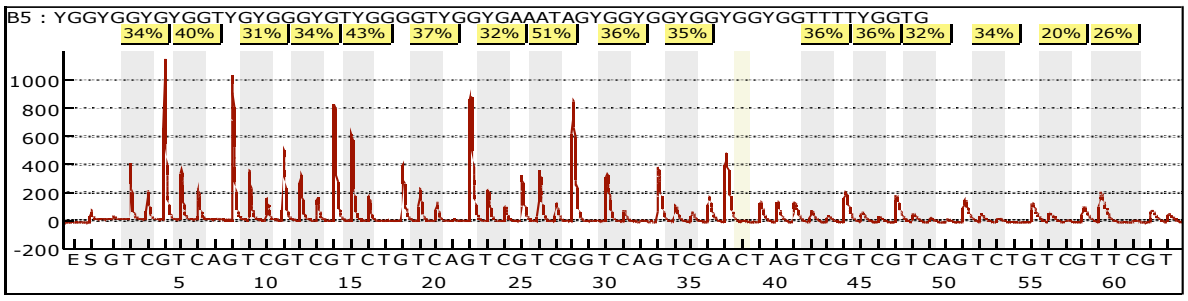


(Cont)

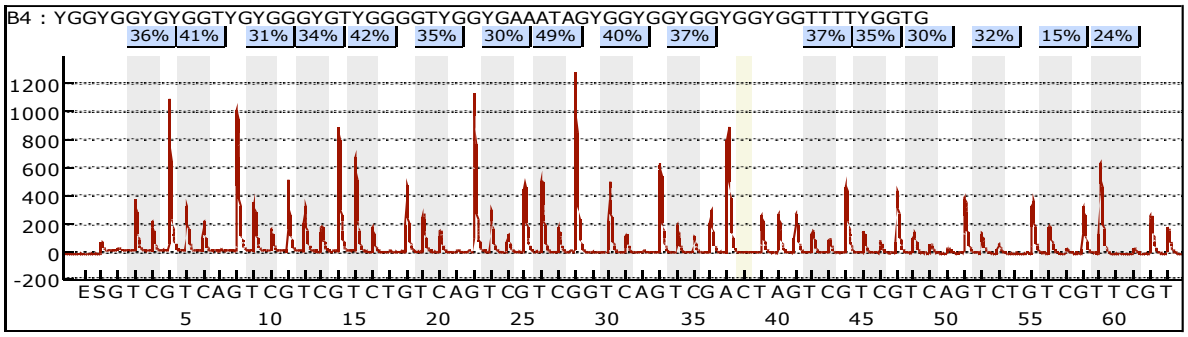
(Figure 4.13 cont.)
d)
2 µM



e)
4 µM



f)
8 µM



(cont)

g)
16 µM

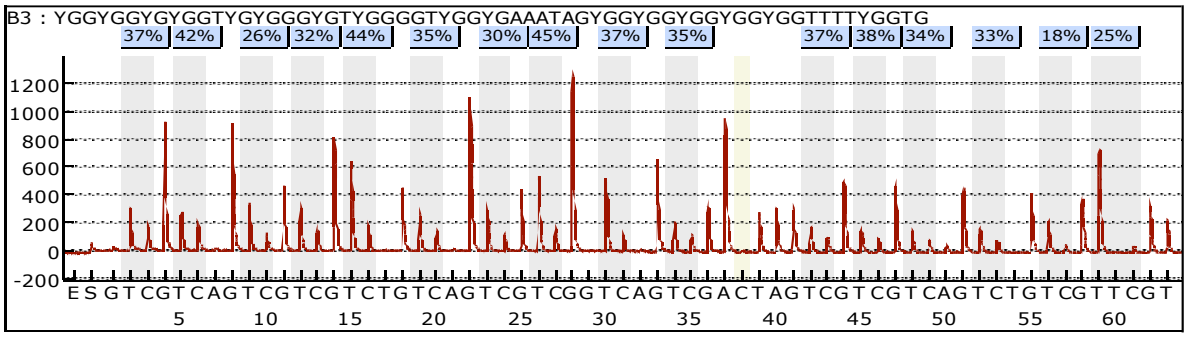


Figure 4.13. Pyrosequencing Analysis of 17 CpGs in RIZ1 Promoter Region of K562 Cells Treated with Decitabine. Cells were treated for 72 hours with indicated concentrations of decitabine, DNA was extracted, modified by bisulfite and sent for pyrosequencing. Pyrosequencing output indicates methylation percentage at various CpGs

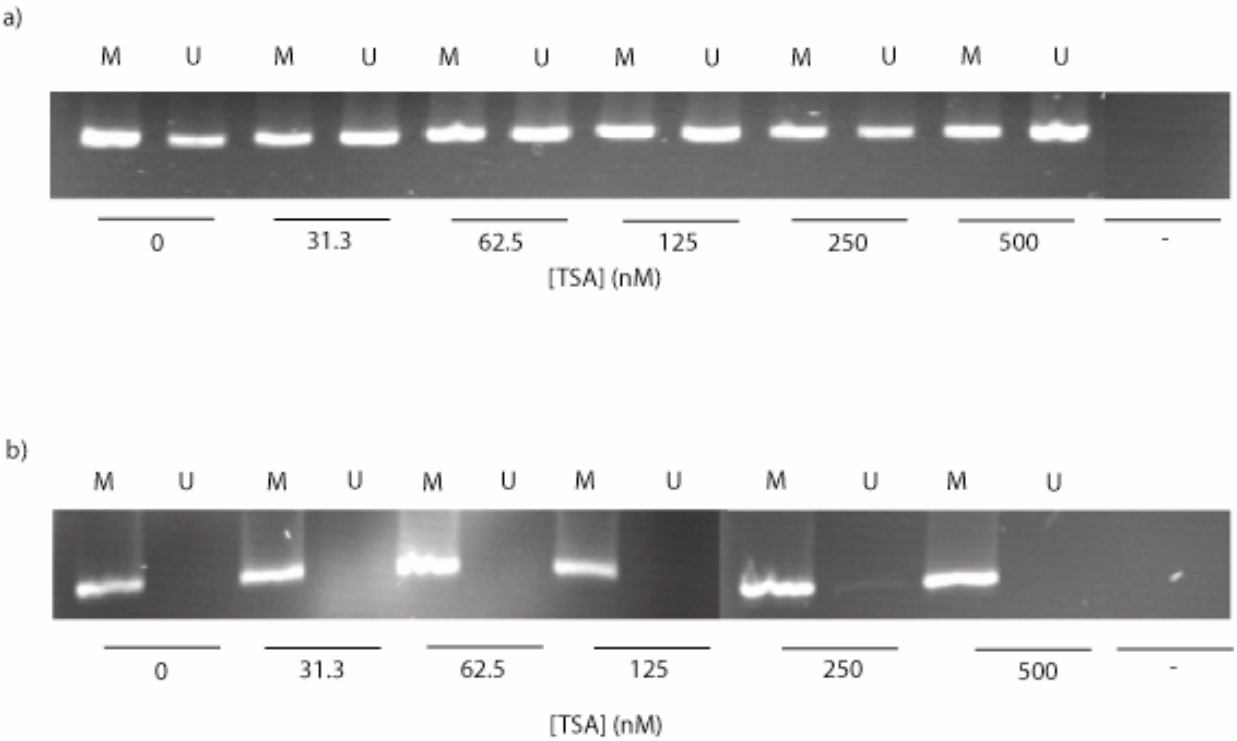
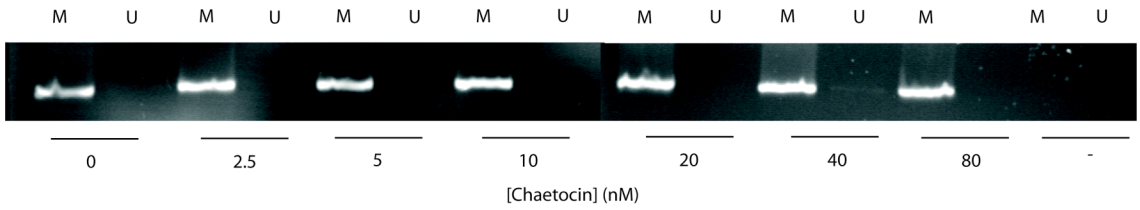


Figure 4.14. Methylation status of the RIZ1 promoter region DNA of K562 and AML-193 cells upon treatment with TSA. M is an amplicon generated from a primer specific for methylated RIZ1. U is an amplicon generated from a primer specific for unmethylated RIZ1. (–) is PCR water negative control.

a)



b)

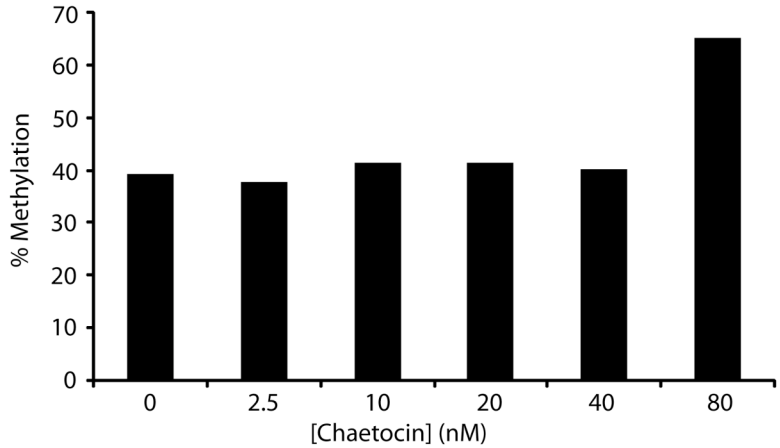
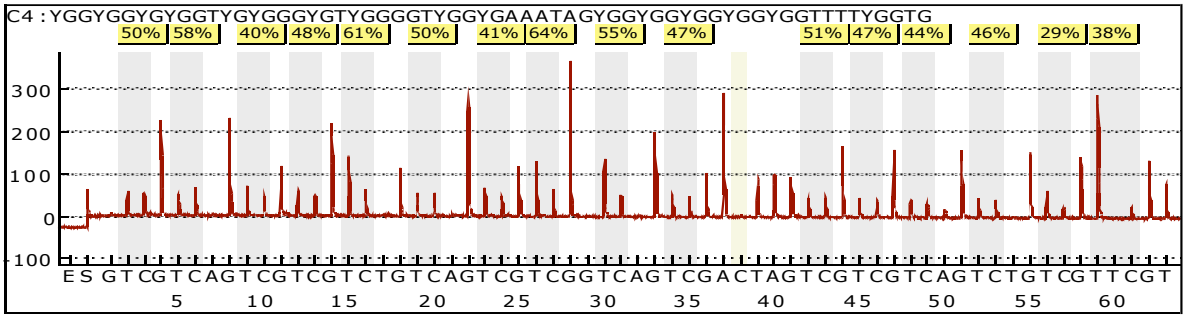


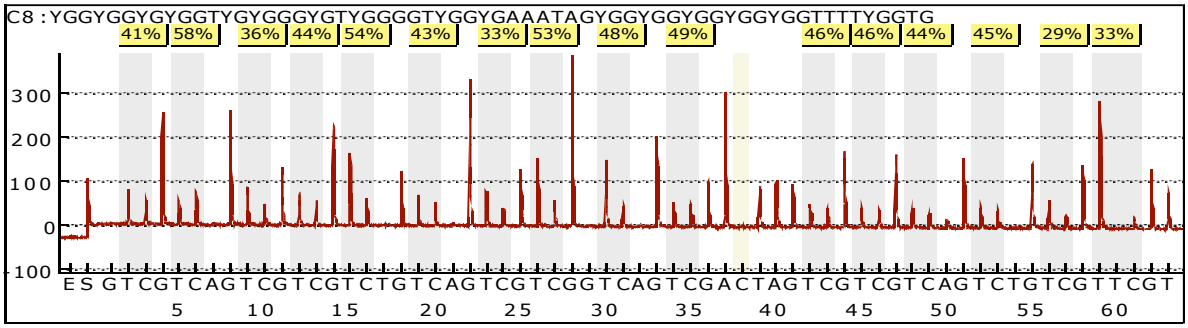
Figure 4.15. Methylation Status of the RIZ1 Promoter Region DNA of AML-193 and Mean Methlation Status of K562 Cells upon Treatment with Chaetocin. a) AML-193 DNA was subjected to methylation specific PCR. M is the amplicon generated from a primer specific for methylated RIZ1. U is the amplicon generated from a primer specific for unmethylated RIZ1. Water was used as a negative control for the two PCRs. (b) Pyrosequencing analysis of K562 DNA samples. Displayed is the mean methylation percentage over the entire promoter region.

Figure 4.16. Pyrosequencing Analysis of 17 CpGs in RIZ1 Promoter Region of K562 Cells Treated with Chaetocin.

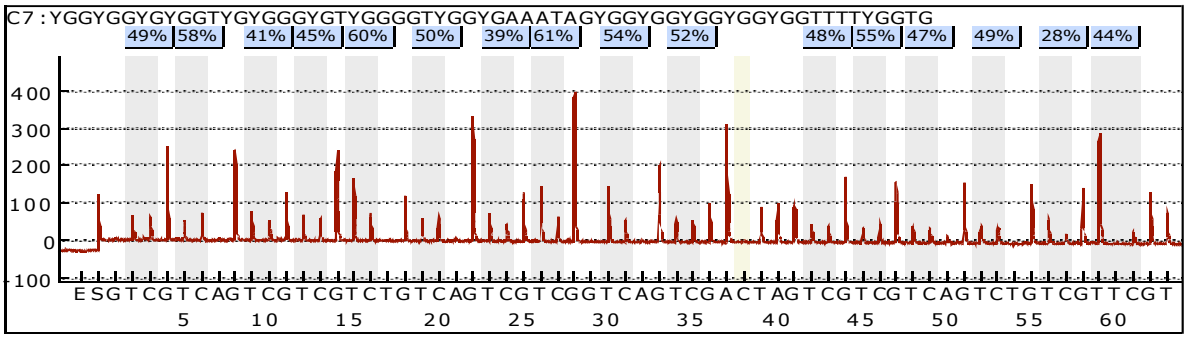
a)
Control



b)
2.5 nM

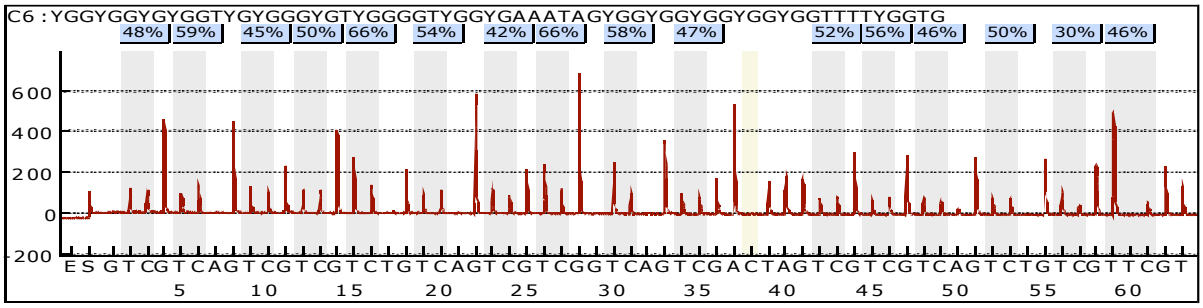


c)
10 nM

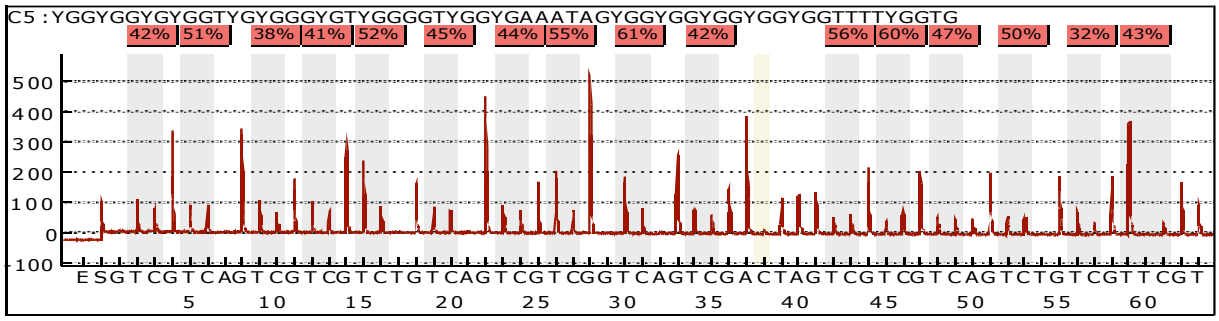


(cont)

d)
20 nM



e)
40 nM



f)
80 nM

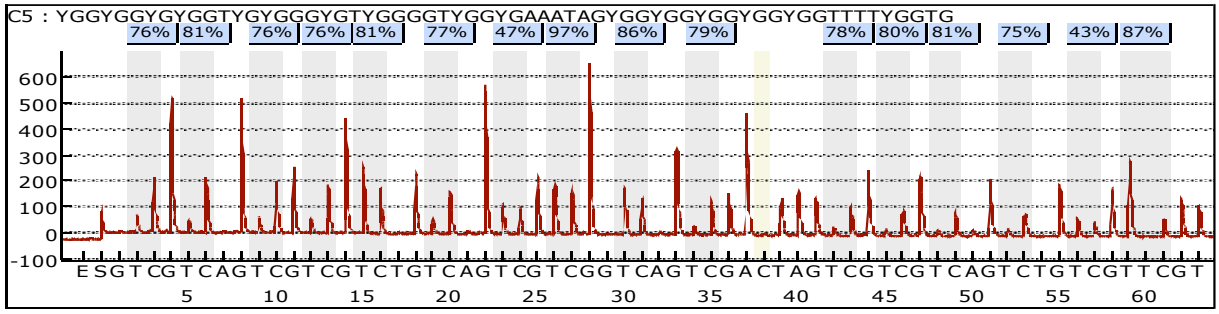


Figure 4.16. Pyrosequencing Analysis of 17 CpGs in RIZ1 Promoter Region of K562 Cells Treated with Chaetocin. Cells were treated for 72 hours with the indicated concentrations of chaetocin, DNA was extracted, modified by bisulfite and sent for pyrosequencing. Pyrosequencing output indicates methylation percentage at various CpGs

4.5 Induction of RIZ1 Expression using Epigenetic Drugs

RIZ1 is silenced in both AML-193 and K562 cell lines (Figure 4.3). Real time PCR was used to determine if decitabine, TSA or chaetocin have an effect on RIZ1 expression in these two cell lines. Treatment of cells with 0.5 μM decitabine induced a statistically significant amount of RIZ1 expression in AML-193 cells. RIZ1 expression increased in a dose dependant manner until treatment with 8 μM decitabine, where a dramatic 36-fold increase in RIZ1 expression was observed (Figure 4.17a). In K562 cells, treatment with 4 μM decitabine induced a statistically significant increase in RIZ1 expression, with a maximum increase of 9 fold after treatment with 8 μM decitabine (Figure 4.17b).

Treatment with TSA had no effect on the amount of RIZ1 expression in either cell line. Treatment with up to 750 nM TSA was unable to induce any RIZ1 expression (Figure 4.18). Treatment with 80 nM chaetocin induced a statistically significant increase of RIZ1 expression in AML-193, which increased upon treatment with 100 nM chaetocin to 5-fold higher than untreated cells (Figure 4.19a). In K562, treatment with 100 nM chaetocin induced a statistically significant difference (Figure 4.19b).

4.6 Potentiation of TSA and Chaetocin with Decitabine on Cell Proliferation and Viability

Previously, we had shown that at the concentrations used in this study, no median dose for decitabine could be established, and thus no claims of synergy or antagonism can be made for decitabine (Chou, 2006). However, whether decitabine potentiates the effect of TSA and/or chaetocin on cell proliferation and viability can be determined.

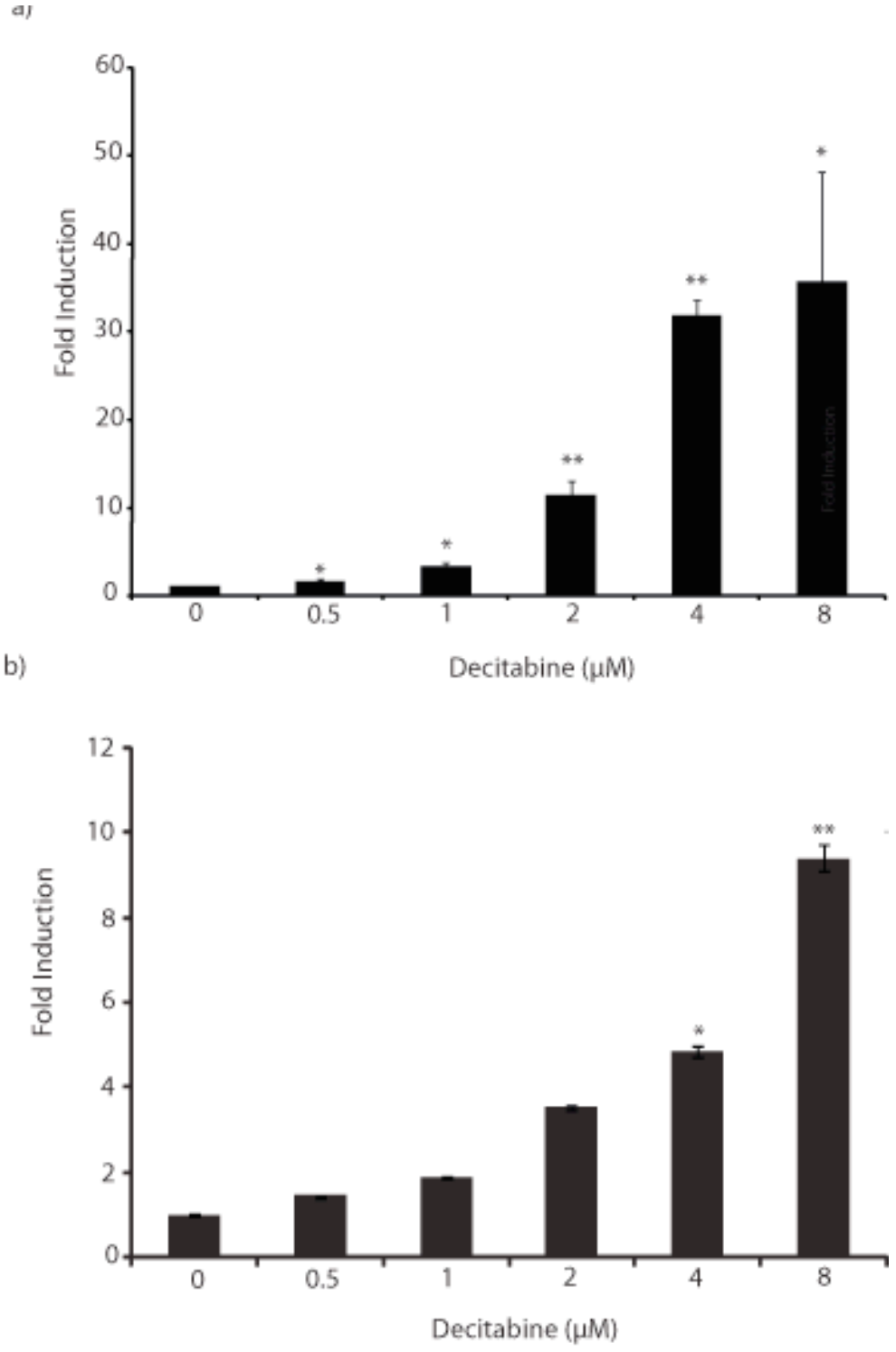


Figure 4.17. Effect of Decitabine treatment of AML-193 and K562 cells on RIZ1 expression AML-193 (a) and K562 (b) cells were treated with the indicated concentrations of decitabine for 72 hours, RNA was extracted and real time PCR was performed. Error bars represent the standard deviation of three independent experiments. * Indicates $p < 0.05$, ** indicates $p < 0.005$ relative to the 0 μM condition.

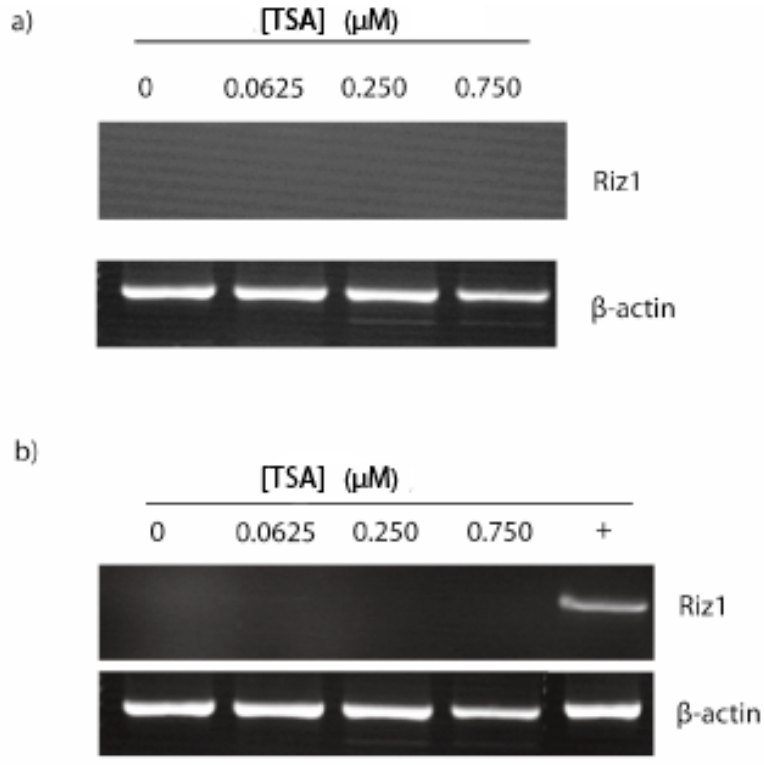


Figure 4.18. Effect of TSA Treatment of AML-193 and K562 cells on RIZ1 expression. AML-193 (a) and K562 (b) cells were treated for 72 hours with the indicated concentrations of TSA, RNA was extracted and RTPCR was performed (μM). (+) is THP-1 cell line, which is used as a positive control for RIZ1 expression. β-actin was used as a loading control.

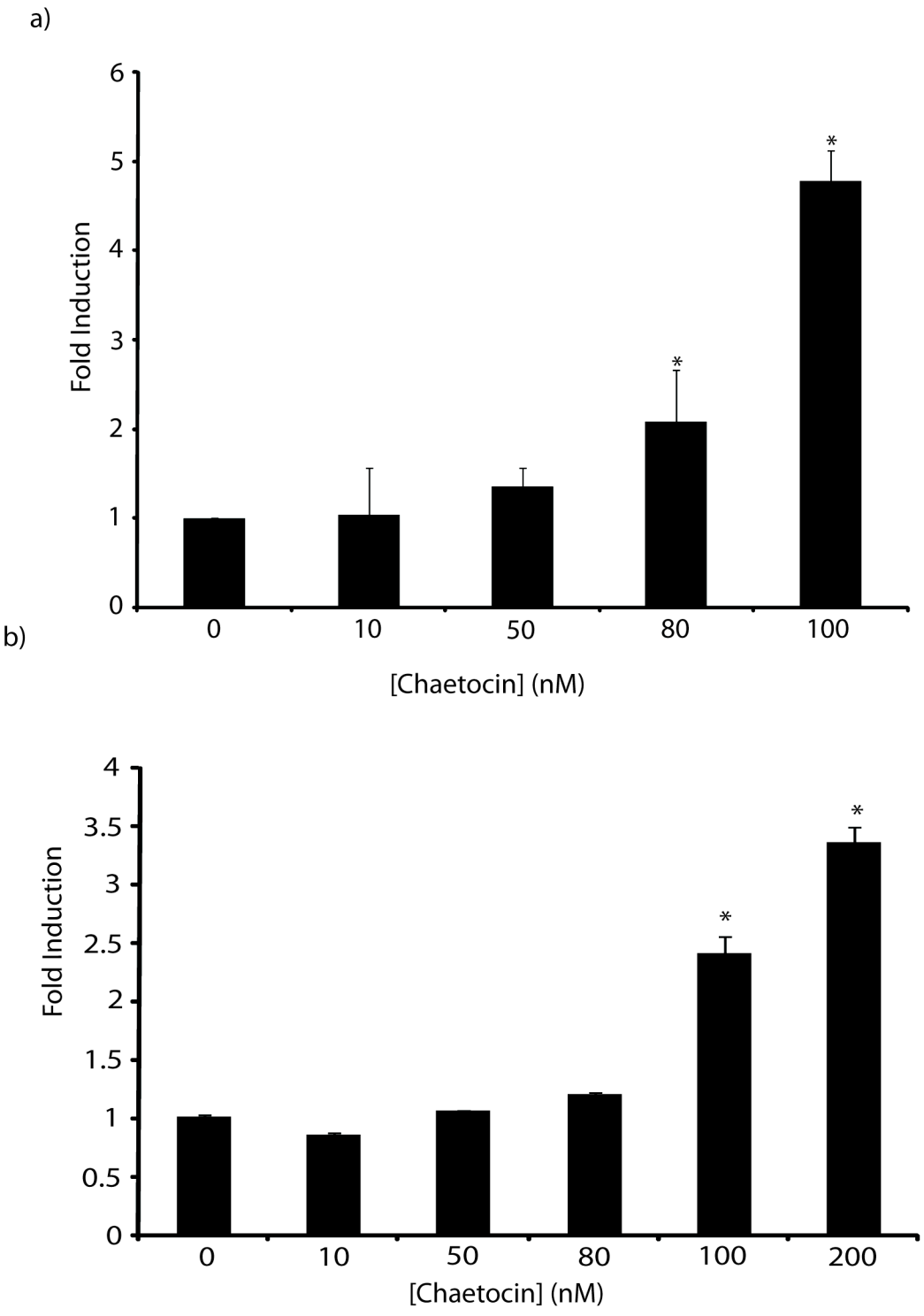


Figure 4.19. Effect of Chaetocin Treatment of AML-193 and K562 cells on RIZ1 expression. AML-193 (a) and K562 (b) Cells were treated for 72 hours with indicated concentrations of chaetocin, RNA was extracted and real time PCR performed. Error bars indicate the standard deviation from three separate experiments. *indicates $p<0.05$ relative to the 0 nM condition.

To investigate if decitabine potentiated the effect of TSA and/or chaetocin on cell proliferation and viability, MTT assays were performed. A plus decitabine and minus decitabine condition in each case was used. The data was plotted on a median effect plot with the median effect being the antilog of the X intercept. The median doses from the MTT assay of the drugs with and without decitabine were compared. In AML-193, the median doses dropped for both TSA and chaetocin, suggesting that decitabine potentiated the effects of both drugs. The median dose for TSA dropped from 33 nM to 21 nM, (Figure 4.20) (Table 4.1) while the median dose for chaetocin dropped from 10 nM to 5 nM (Figure 4.21) (Table 4.1). In K562 the median dose for TSA was much higher than previously determined and therefore this should be investigated further. (Figure 4.22). Although the median dose for chaetocin was higher than previously determined, a trend was still seen. The median dose for chaetocin dropped from 35 nM to 29 nM, which indicates that decitabine potentiated chaetocin (Figure 4.23) (Table 4.1). Overall, decitabine potentiated chaetocin in both AML-193 and K562 cell line, and potentiated TSA in AML-193 cell line, with no potentiation of TSA observed in K562.

Table 4.1. Median Doses for AML-193 and K562 Cell Lines Treated with TSA and Chaetocin in the Presence and Absence of Decitabine. Cells were treated with TSA or chaetocin, in increasing concentrations in the presence or absence of decitabine for 72 hours. Cells were analyzed by MTT and median doses generated.

Cell Line	+/- Decitabine	Drug	Median Dose (nM)	Upper 95% Confidence Interval (nM)	Lower 95% Confidence Interval (nM)
AML-193	-	TSA	33	41.1	21.89
AML-193	+	TSA	21	39.3	10.79
AML-193	-	Chaetocin	10	16.6	8.37
AML-193	+	Chaetocin	5	9.4	2.48
K562	-	TSA	436	461.2	413.7
K562	+	TSA	436	867.5	219.6
K562	-	Chaetocin	35	116.7	16.57
K562	+	Chaetocin	29	39.2	21.19

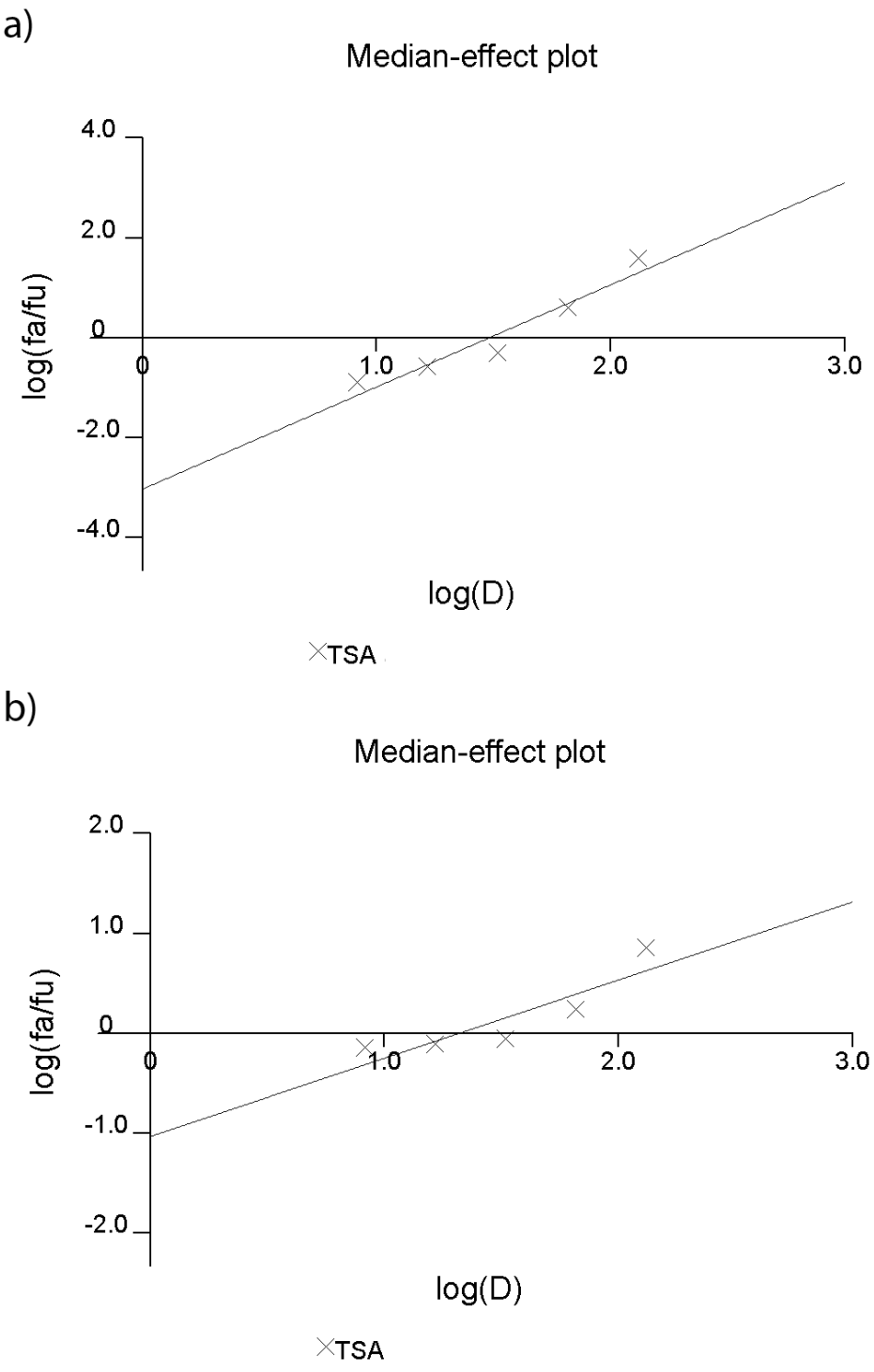


Figure 4.20. Median Effect Plot of AML-193 cell line upon Treatment with TSA in the Presence or Absence of Decitabine. AML-193 cells were treated in the absence (a) or presence (b) of decitabine and a median effect plot was generated. (fa) Indicates fraction affected, (fu) indicates fraction unaffected, and (D) indicates dose.

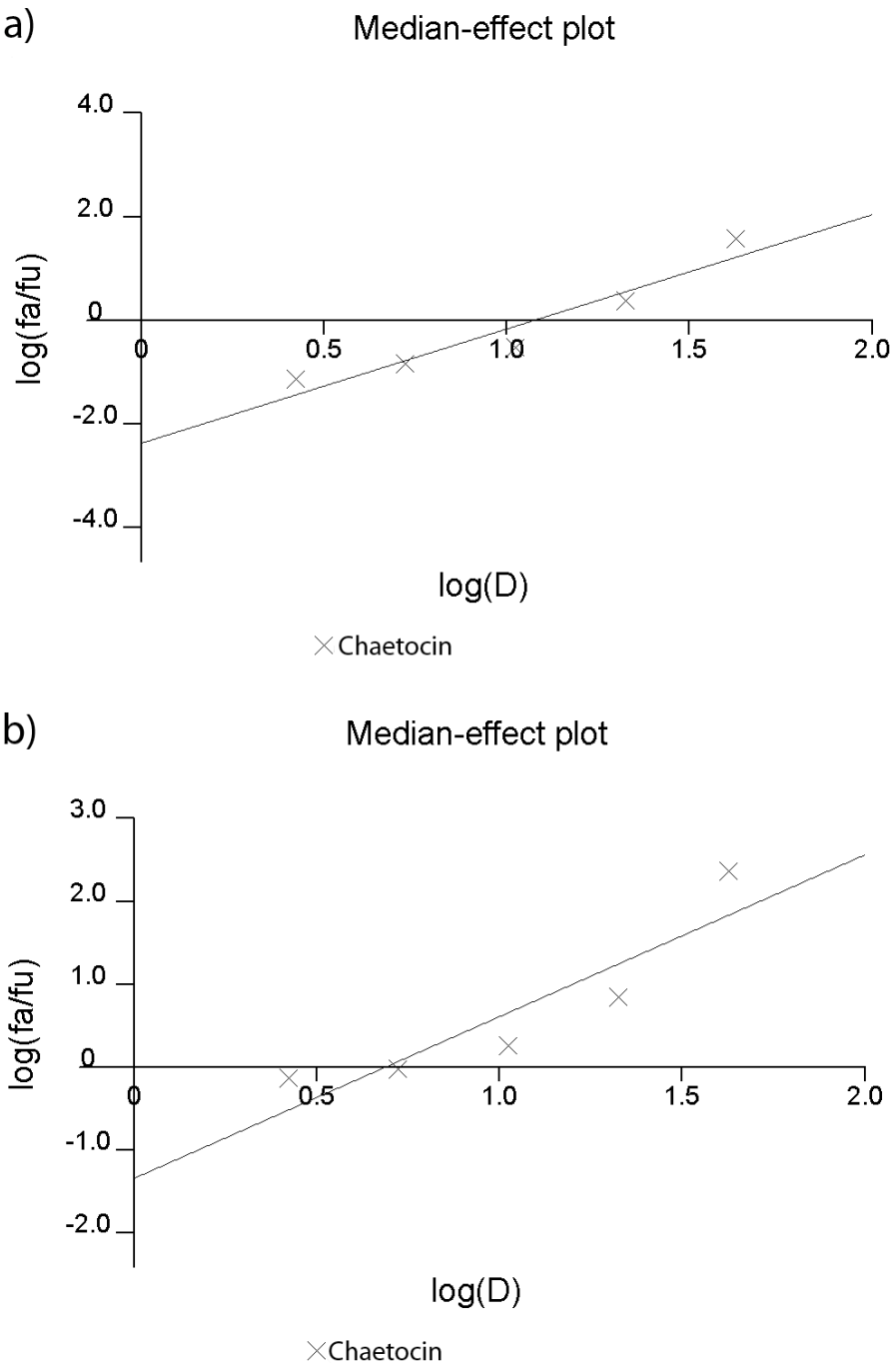


Figure 4.21. Median Effect Plot of AML-193 cell line upon Treatment with Chaetocin in the Presence or Absence of Decitabine. AML-193 cells were treated in the absence (a) or presence (b) of decitabine and a median effect plot was generated (fa) Indicates fraction affected, (fu) indicates fraction unaffected, and (D) indicates dose.

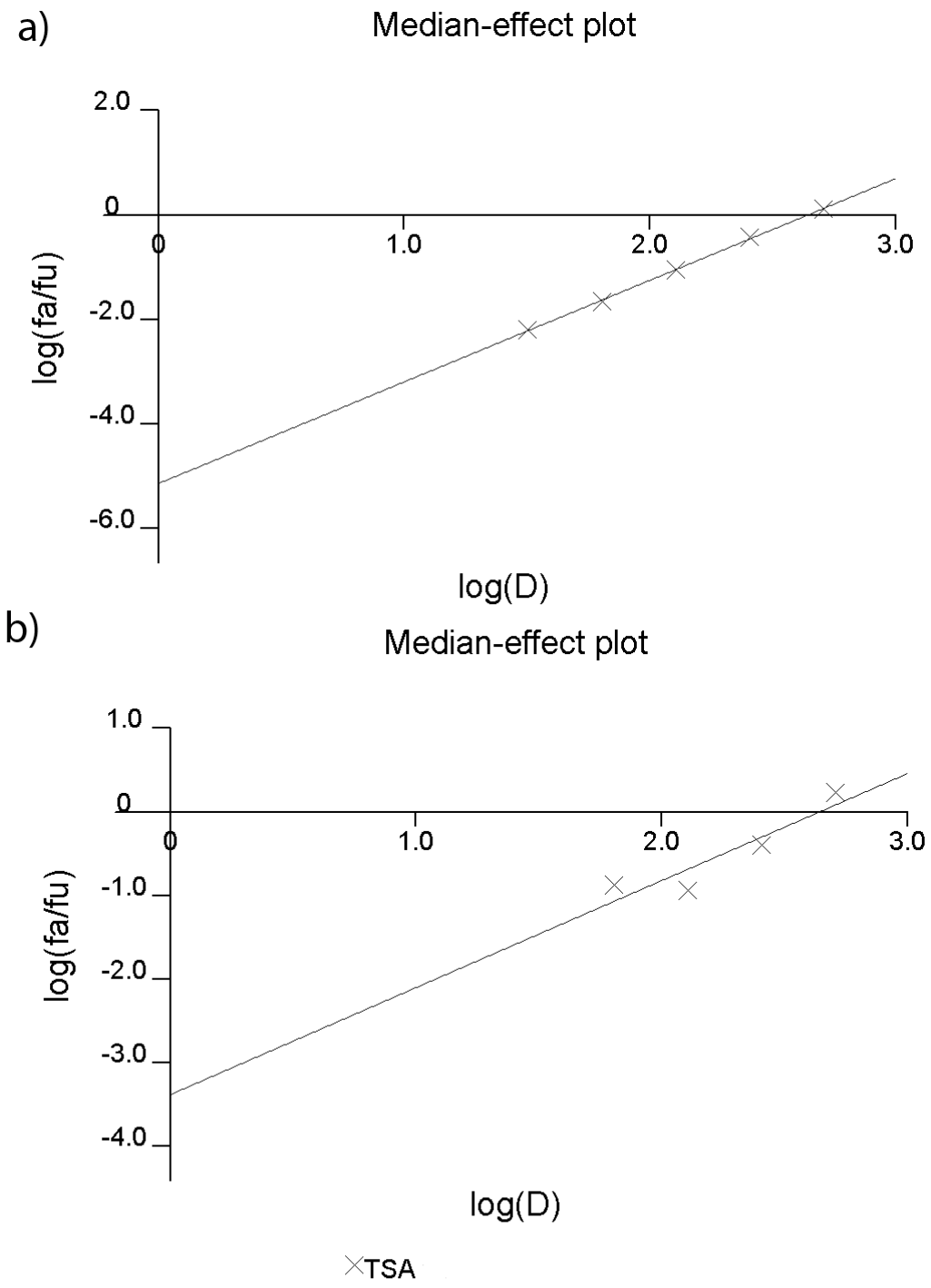


Figure 4.22. Median Effect Plot of K562 cell line upon Treatment with TSA in the Presence or Absence of Decitabine. K562 cells were treated in the absence (a) or presence (b) of decitabine and a median effect plot was generated. (fa) Indicates fraction affected, (fu) indicates fraction unaffected, and (D) indicates dose.

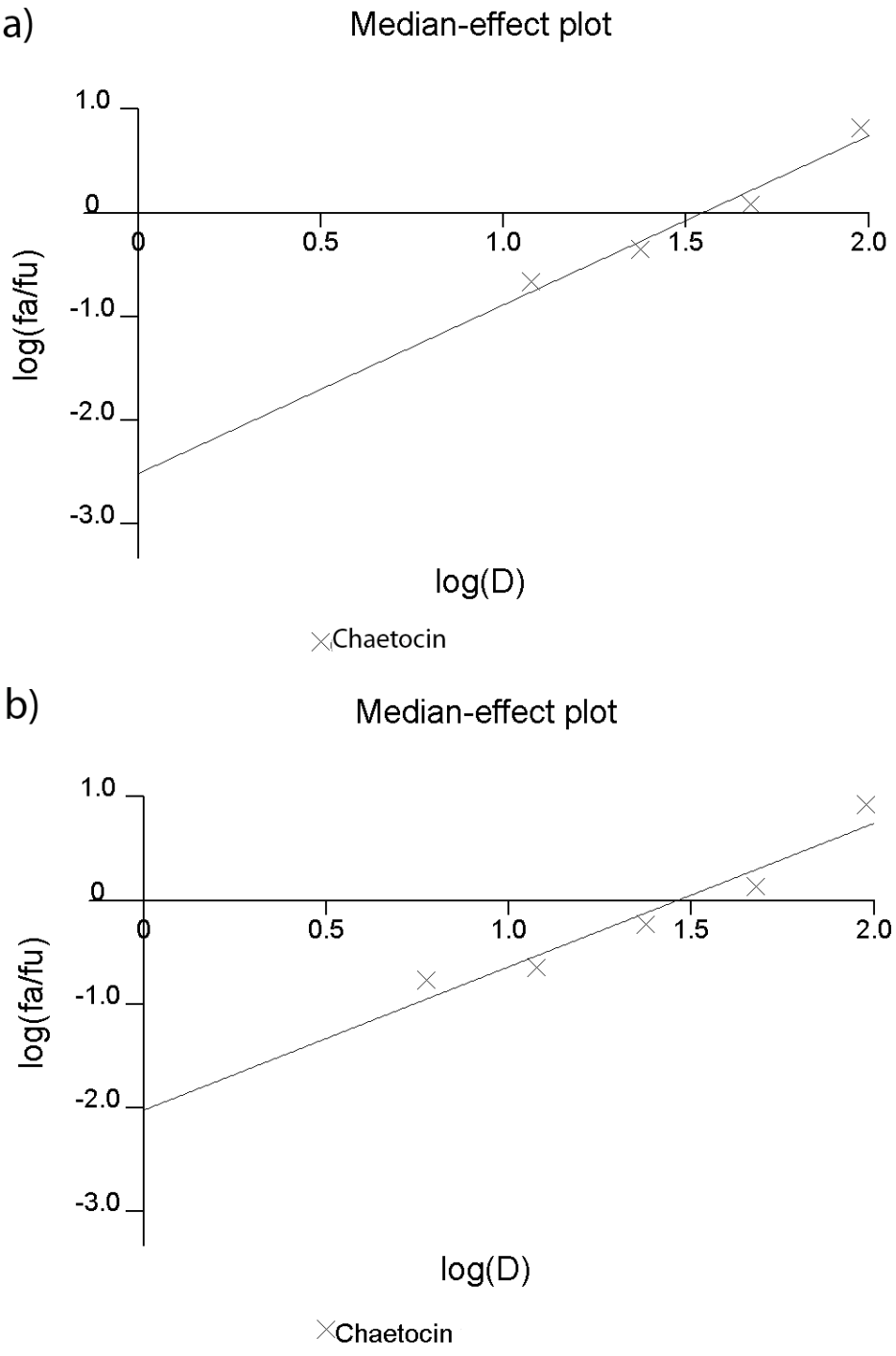


Figure 4.23. Median Effect Plot of K562 cell line upon Treatment with Chaetocin in the Presence or Absence of Decitabine. K562 cells were treated in the absence (a) or presence (b) of decitabine and a median effect plot was generated. (fa) Indicates fraction affected, (fu) indicates fraction unaffected, and (D) indicates dose.

4.7 Synergy/ Antagonism of TSA and Chaetocin

Synergy or antagonism was determined using Calcusyn software (Chou, 2006). Synergy is defined as an effect that is more than additive of the effect of the individual drugs and antagonism is defined as an effect that is less than additive of the effect of the individual drugs.

The equations used in Calcusyn software follow the Mass Action Law, which deals with the kinetics of chemical reactions (Chou, 2006). MTT assays were conducted which contained a range of doses of both TSA and Chaetocin as indicated in Figure 4.24. The fraction affected was calculated as shown in section 4.3, and the CI value was calculated using Equation 3.

$$CI = \frac{(D)_1}{(D)_{m\,1}[fa/(1\text{-}fa)]^{1/m1}} + \frac{(D)_2}{(D)_{m\,2}[fa/(1\text{-}fa)]^{1/m2}} \qquad \text{(Eq.3)}$$

In Equation 3, CI is the combination index, D_1 is the dose of the first drug, D_2 is the dose of the second drug, D_{m1} is the median dose of the first drug alone, D_{m2} is the median dose of the second drug alone, fa is fraction affected when both drugs are used, m1 is the slope of the median effect plotline of the first drug and m2 is the slope of the median effect plotline of the second drug. The CI value denotes the extent of the synergism or antagonism, with a value of 1 indicating an additive effect, a value < 1 indicating synergy and a value > 1 indicating antagonism.

Also, the CI value was calculated at different effect levels, denoted as ED values. For example, ED75 is the CI value at the 75% fraction affected level. For infectious diseases or cancer therapies, synergism at high effect levels such as ED90 is much more therapeutically relevant, and thus these values are reported here (Chou, 2006). The CI value for chaetocin and TSA at ED90 for AML-193 was 0.816, indicating synergy between the two drugs. The CI value for chaetocin and TSA at ED90 for K562 was 0.392 (Table 4.2). The CI value was also determined in the presence and absence of decitabine. In both cell lines the CI increased upon addition of decitabine. This indicates that decitabine interfered with the synergy between TSA and chaetocin (Table 4.2).

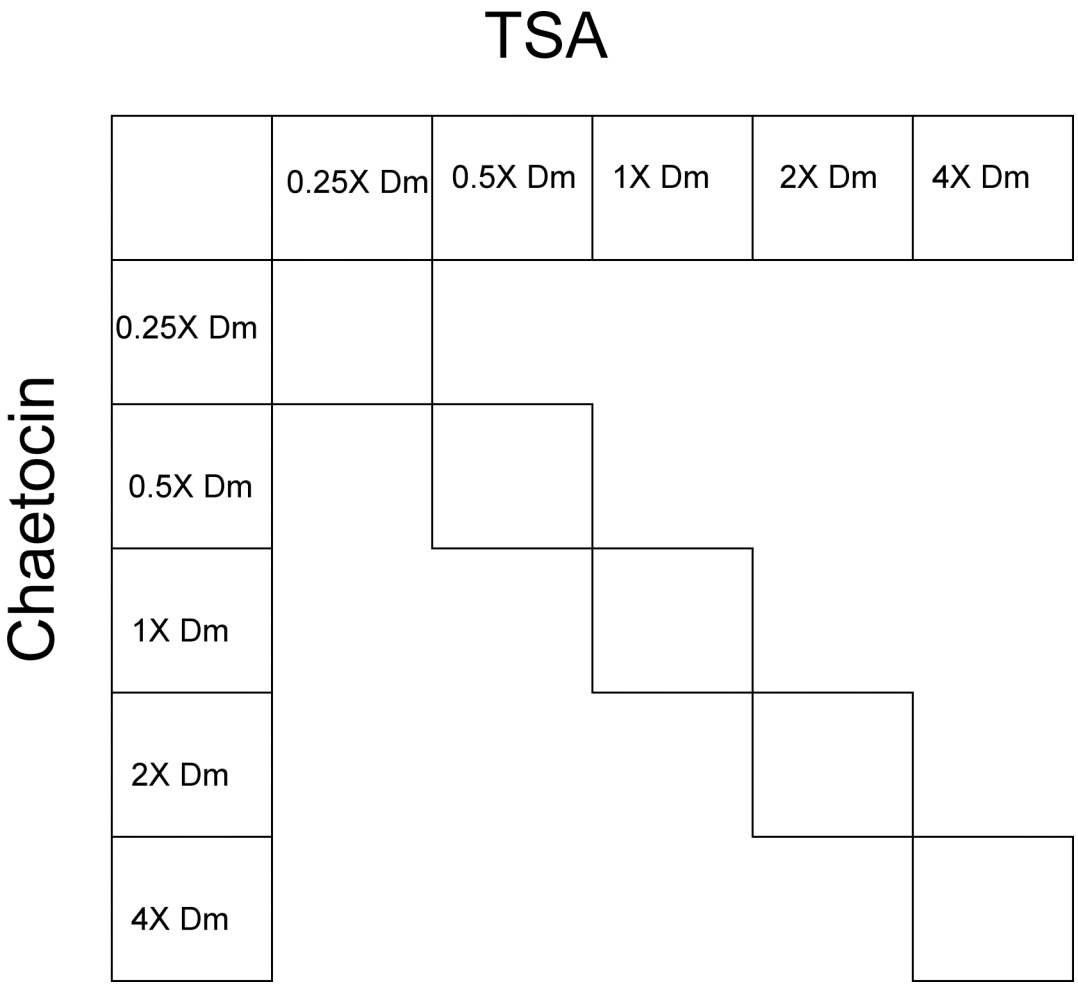


Figure 4.24. Experimental Setup of Combination MTT Assays. Boxes indicate doses which were used when TSA and Chaetocin were used in combination. Dm indicates median dose.

Table 4.2. CI Values for TSA and Chaetocin in AML-193 and K562 Cell Lines. Cells were treated for 72 hours with increasing concentrations of TSA plus chaetocin in the presence and absence of decitabine. Cells were then analyzed by MTT. Shown are the CI values for ED90. CI values <1 indicate synergism, >1 indicate antagonism

Cell Line	+/- Decitabine	CI Value
AML-193	-	0.816
AML-193	+	2.200
K562	-	0.392
K562	+	0.655

4.8 Methylation Status of the RIZ1 Promoter Region Upon Treatment with Epigenetic Drugs in Combination

AML-193 and K562 cell lines were treated with the drugs in combination to determine what affects the drugs have on the methylation status of the RIZ1 promoter region. AML-193 cells were treated with 4 μM decitabine, and/or 33 nM TSA and/or 10.3 nM chaetocin, which are the median doses for TSA and chaetocin. Median doses were chosen in order to ensure appropriate viability would be obtained. Treatment with decitabine decreased the amount of DNA methylation present, while treatment with TSA or chaetocin had no effect on the methylation status of the promoter region (Figure 4.25a). This trend was seen in the pyrosquencing analysis as well, where treatment with decitabine decreased the amount of methylation by 15-20% across various CpGs, where very little difference was seen across the various CpGs upon treatment with TSA or chaetocin (Figure 4.26c, Figure 4.26d). Treatment with decitabine and TSA together decreased the amount of methylation present similar to that of decitabine treatment alone, with 71.6% methylation from a control value of 94% (Figure 4.25a).

Examining the individual CpGs, this appears to be a universal occurrence. Interestingly in all treatments there is no change in the methylation levels of the 5^{th} CpG except for the decitabine + TSA treatment (Figure 4.26e). Treatment with decitabine plus chaetocin caused methylation levels to return to nearly control levels (92.1%) (Figure 4.25a). Again, this effect occured across all CpGs except the 5^{th} and 8^{th} (Figure 4.26f). Treatment with chaetocin and TSA together caused a slight drop in methylation to 90.9% (Figure 4.25a).

Also, there was very little difference between treating cells with chaetocin plus TSA and treating cells with decitabine plus chaetocin either in overall methylation levels (Figure 4.25a) or pattern of methylation among the CpGs (Figure 4.26f, Figure 4.26g). Treating cells with all three drugs together caused methylation to drop slightly from control levels to 88.9% methylation (Figure 4.25a).

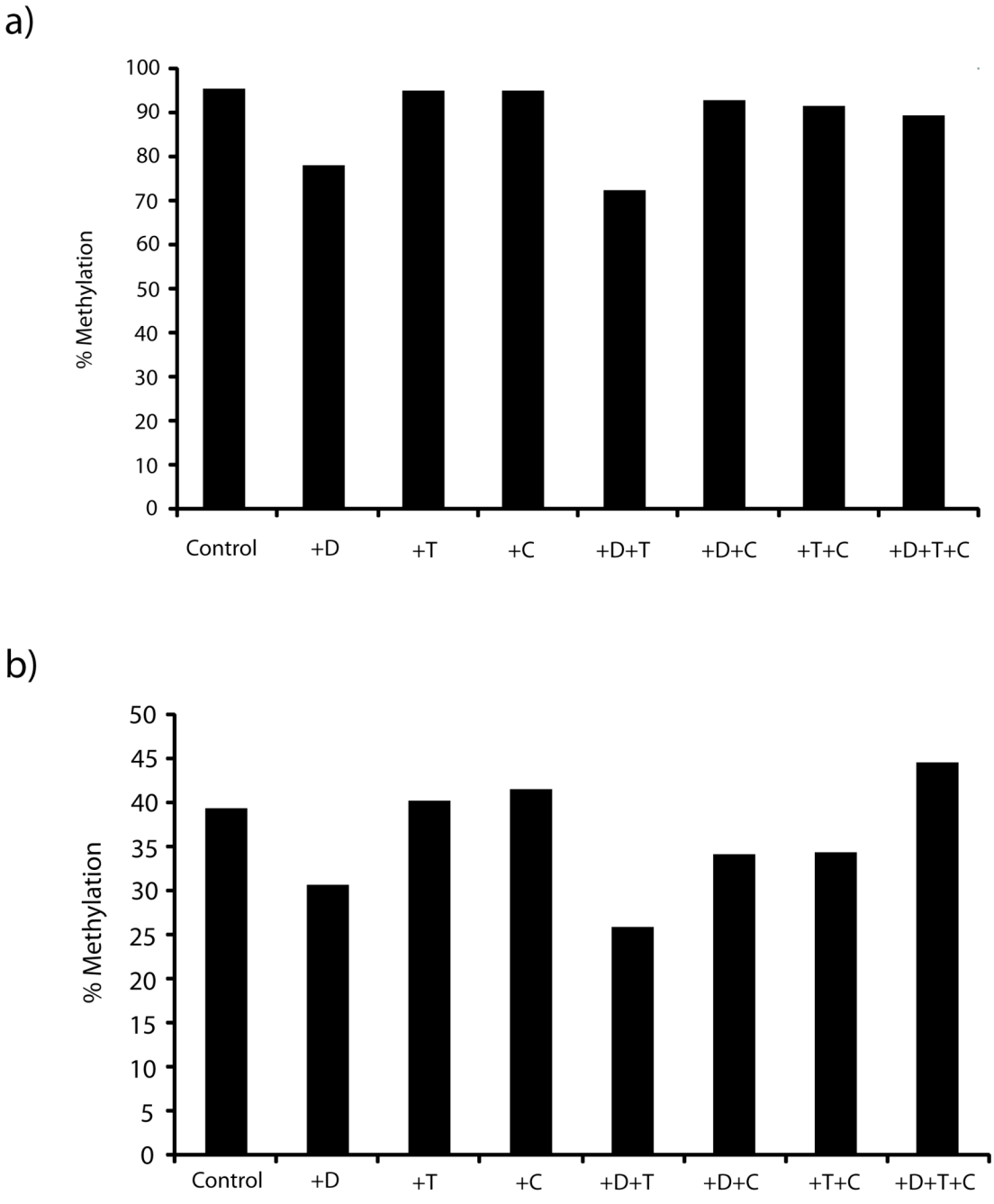
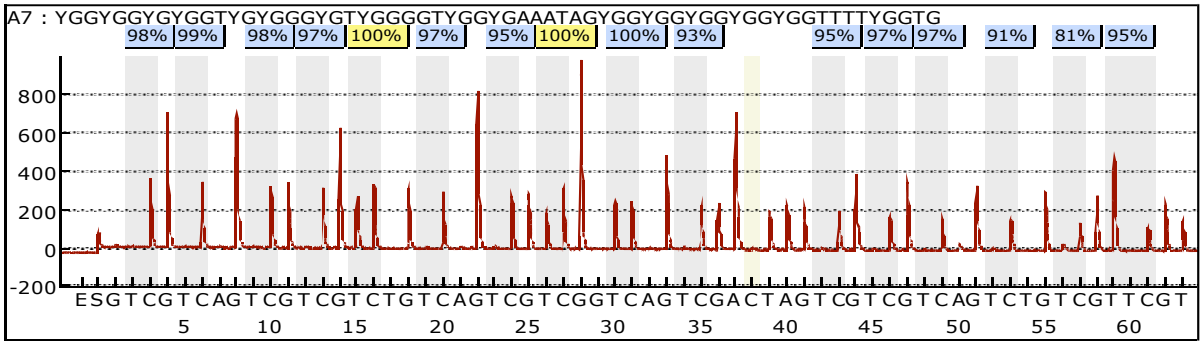


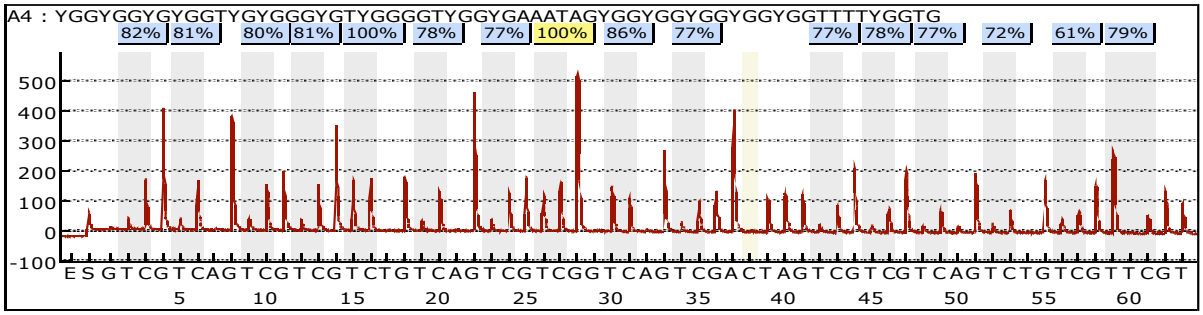
Figure 4.25. Mean Methylation Status of RIZ1 Promoter Region DNA of AML-193 and K562 cells upon Treatment with Decitabine, TSA and Chaetocin in combination. AML-193 (a) and K562 (b) cells were treated with decitabine and/ TSA and/or for 72 hours. DNA was extracted and pyrosequenced. Shown is the mean percentage methylation over the entire promoter region. D indicates decitabine, T indicates TSA and C indicates chaetocin.

Figure 4.26. Pyrosequencing Analysis of 17 CpGs in RIZ1 Promoter Region of AML-193 Cells Treated with Decitabine and/or TSA and/or Chaetocin

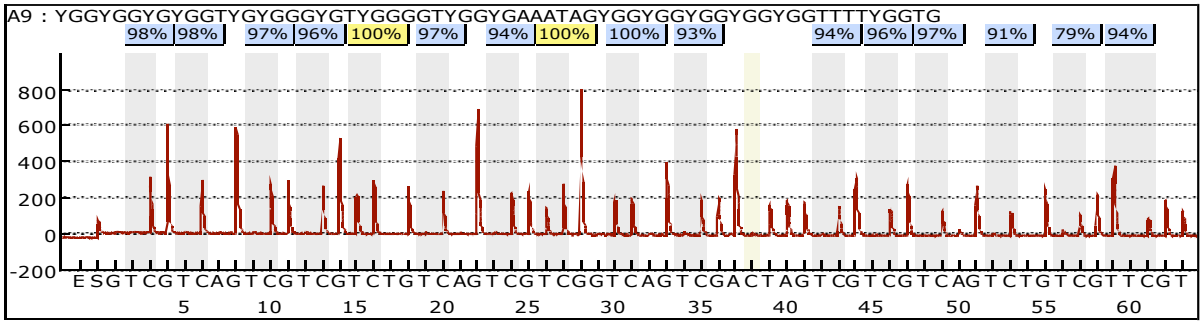
a)
Control



b) Decitabine



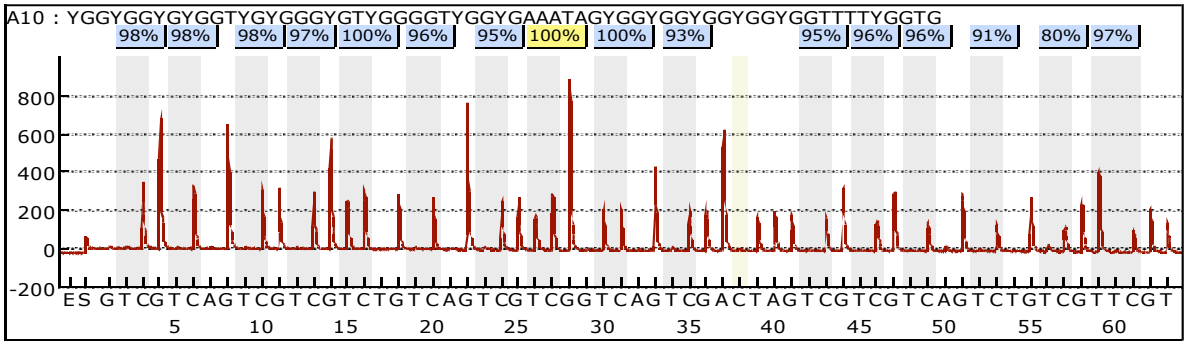
c) TSA



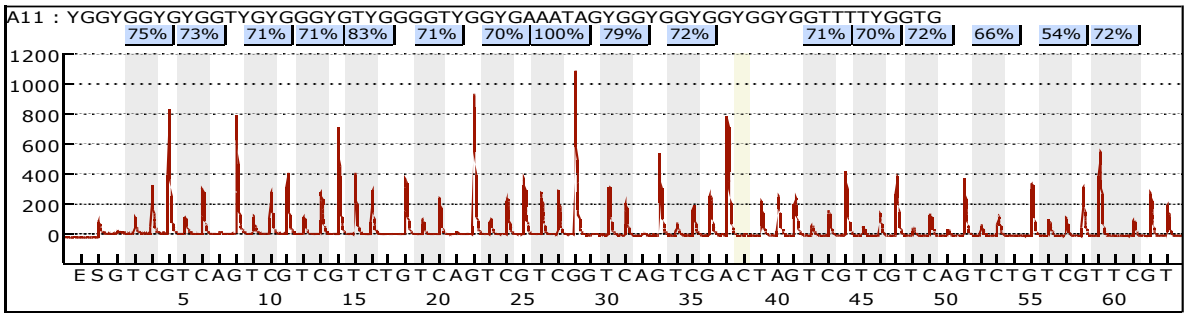
(cont)

Figure 4.26 cont

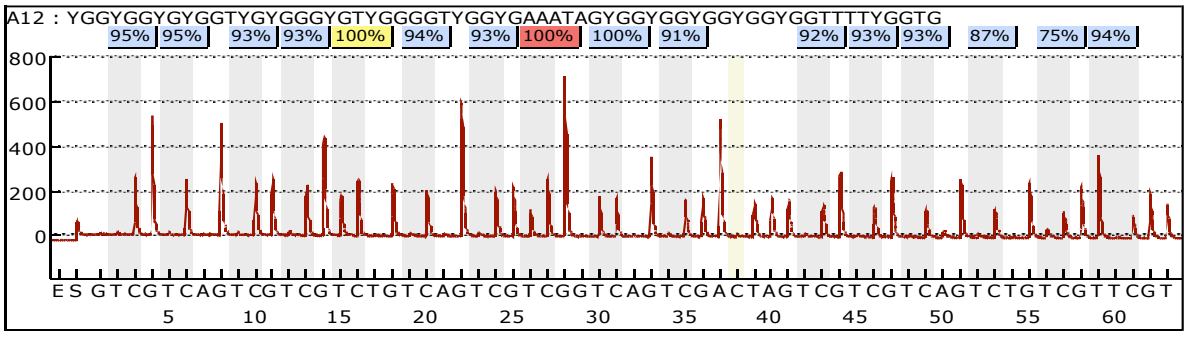
d)
Chaetocin



e)
Decitabine+TSA

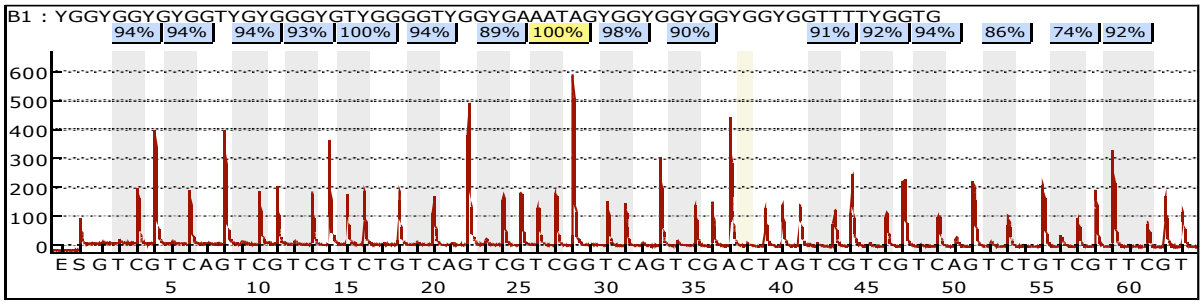


f)
Decitabine+Chaetocin



(cont)

g)
TSA+Chaetocin



h)
Decitabine+TSA+Chaetocin

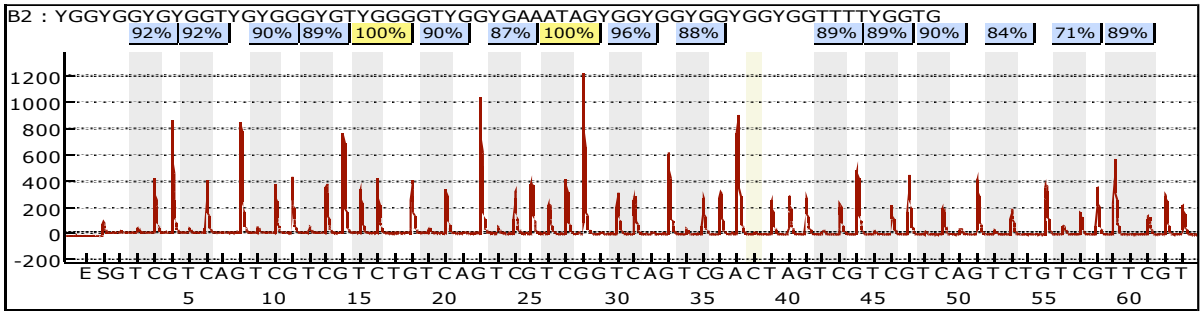


Figure 4.26. Pyrosequencing Analysis of 17 CpGs in RIZ1 Promoter Region of AML-193 Cells Treated with Decitabine and/or TSA and/or Chaetocin. DNA was extracted, modified by bisulfite and sent for pyrosequencing. Pyrosequencing output indicates methylation percentage at various CpGs.

As seen previously, there were two CpGs which did not respond to the demethylation effects at all (Figure 4.26, 5th and 8th CpG). The increase in methylation seen from treatment with decitabine to treatment with decitabine plus chaetocin suggested that chaetocin is able to increase the amount of DNA methylation present, and that this effect was simply masked by the already high percentage of methylation present in AML-193.Decitabine is not able to overcome the effects of chaetocin in the presence of TSA as the methylation level when there is treatment with all three drugs is similar to that of TSA plus chaetocin (Figure 4.25a). Except for the 5th and 8th CpG these effects were fairly homogenous across the CpGs examined.

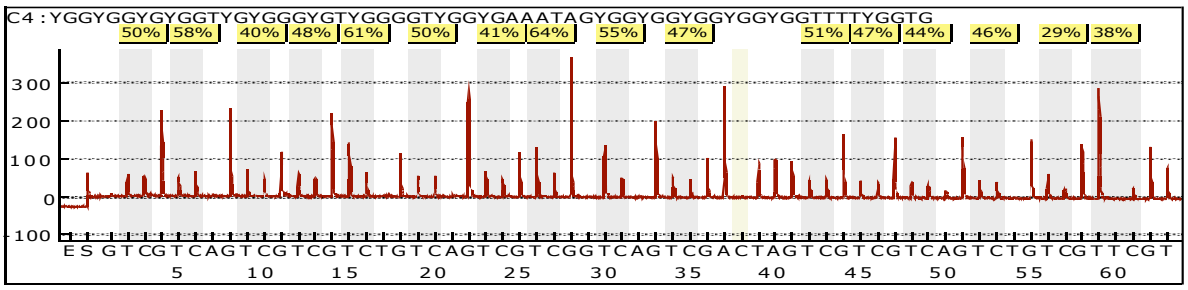
K562 cells were treated with 4 μM decitabine, and/or 128 nM TSA and/or 23.8 nM chaetocin. Treatment with decitabine decreased RIZ1 promoter DNA methylation, TSA had no effect on methylation and chaetocin slightly increased the DNA methylation as previously seen with AML-193 cells (Figure 4.25b). Treating cells with decitabine plus TSA decreased the DNA methylation levels to 25.8%, lower than decitabine treatment alone (Figure 4.25b). Treating cells with decitabine plus chaetocin caused an increase of methylation over that of decitabine treatment alone to 34.2% (Figure 4.25b). Treating cells with TSA plus chaetocin showed similar effects with 34.4% methylation while treating cells with all three drugs caused an increase in methylation to 44.6% (Figure 4.25b). Pyrosequencing analysis reveals that although the majority of the CpGs react with a degree of uniformity, CpG 8 often has a much more dramatic effect on it than the others.

4.9 Treatment with Small Molecule Inhibitors in Combination Induces RIZ1 Expression

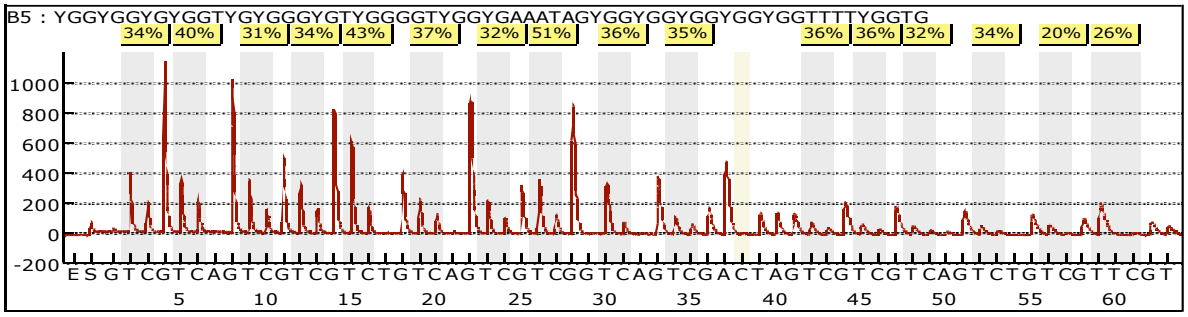
RIZ1 was silenced in both AML-193 and K562 cell lines (Figure 4.3a). In order to induce RIZ1 expression, cells were treated with the three small molecule inhibitors in combinations. Doses were chosen to ensure appropriate viability of the cells for the assay. AML-193 cells were treated for 72 hours with combinations of 0.5 μM decitabine, 100 nM TSA, and/or 10 nM chaetocin.. Treating cells with decitabine plus TSA in AML-193 showed an increased amount of induction over both decitabine or TSA alone (Figure 4.28a). Treating cells with decitabine alone increased RIZ1 expression by 2 fold where treatment with decitabine plus TSA increased RIZ1 expression by 9 fold. Induction upon treating cells with decitabine plus chaetocin was also higher than when either drug is treated alone (Figure 4.28a), with induction increasing from 1.6 fold when decitabine is administered alone to 8.8 fold when decitabine and chaetocin were treated together. Treating cells with TSA plus chaetocin increased RIZ1 expression slightly, with a 2.5 fold induction (Figure 4.28). Treatment with all three drugs increased the amount of RIZ1 induction more than the drugs alone, but not as much as the when used in pairs (Figure 4.28a).

Figure 4.27. Pyrosequencing Analysis of 17 CpGs in RIZ1 Promoter Region of K562 Cells Treated with Decitabine and/or TSA and/or Chaetocin.

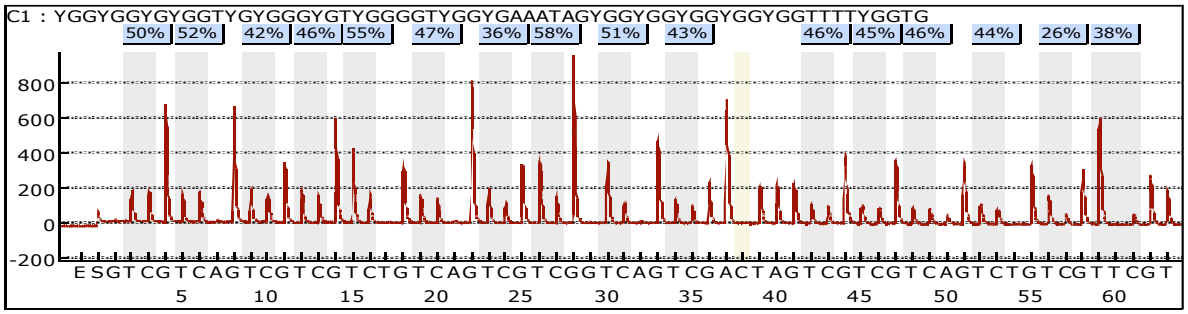
a)
Control



b)
Decitabine

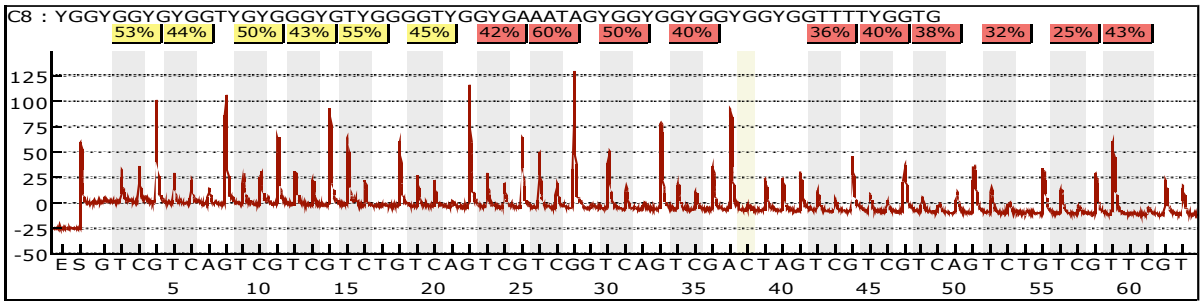


c) TSA

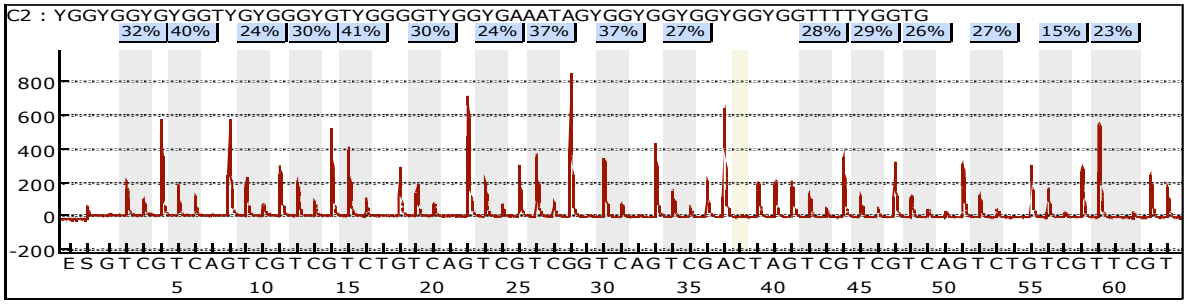


(cont)

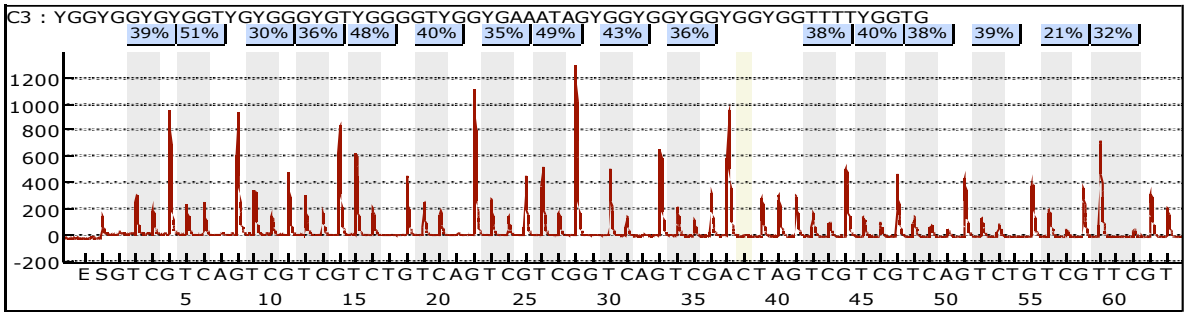
(Figure 4.27 cont.)
d)
Chaetocin



e)
Decitabine+TSA

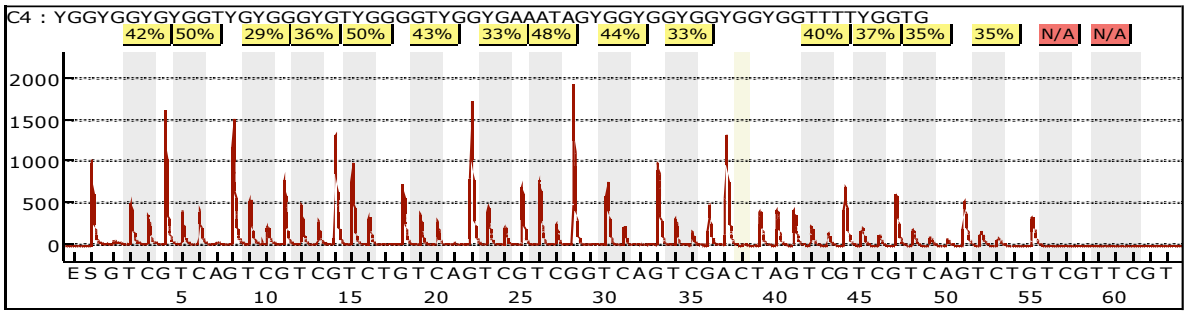


f)
Decitabine+Chaetocin



(cont)

g)
TSA+Chaetocin



h)
Decitabine+TSA+Chaetocin

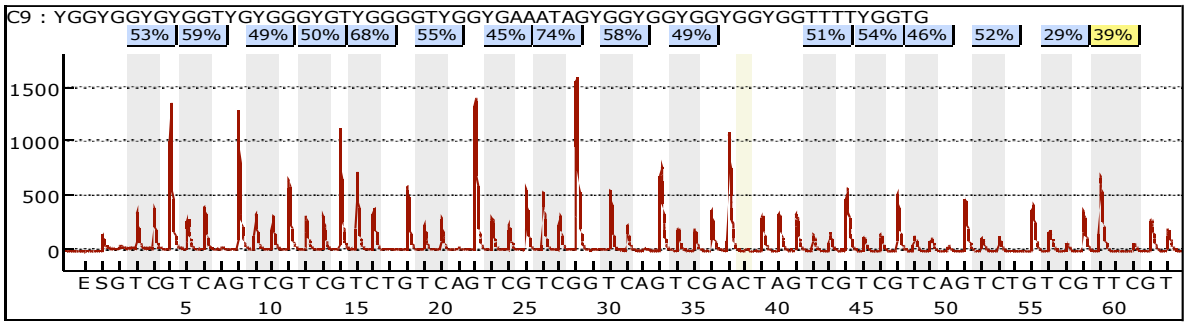


Figure 4.27. Pyrosequencing Analysis of 17 CpGs in RIZ1 Promoter Region of K562 Cells Treated with Decitabine and/or TSA and/or Chaetocin. Cells were treated for 72 hours with decitabine, and/or TSA, and/or chaetocin. DNA was extracted, modified by bisulfite and sent for pyrosequencing. Pyrosequencing output indicates methylation percentage at various CpGs.

The largest amount of induction is seen when decitabine and TSA are treated together. This correlated with the synergy data obtained from the MTT assays, where the CI values increased when all three drugs were present. This suggested that the drugs did not function best when all three were present (Table 4.2).

Many of the same trends were seen in K562. K562 cells were treated for 72 hours with combinations of 1 μM decitabine, 500 nM TSA and/or 50 nM chaetocin. Treatment with decitabine plus TSA showed a larger induction that of each drug alone. Decitabine plus TSA induced a 4 fold induction while decitabine alone only induces a 2 fold induction (Figure 4.28b). Treating cells with decitabine plus chaetocin also showed a larger induction than that of each

drug alone, having induced a 5 fold induction of RIZ1 expression (Figure 4.28b). Treating cells with TSA plus chaetocin also showed increased induction over each drug alone, inducing a 2 fold increase of RIZ1 expression (Figure 4.28b). Treating cells with all three drugs together induces RIZ1 expression more than each drug alone, however not as well as any of the drugs in pairs (Figure 4.28b).

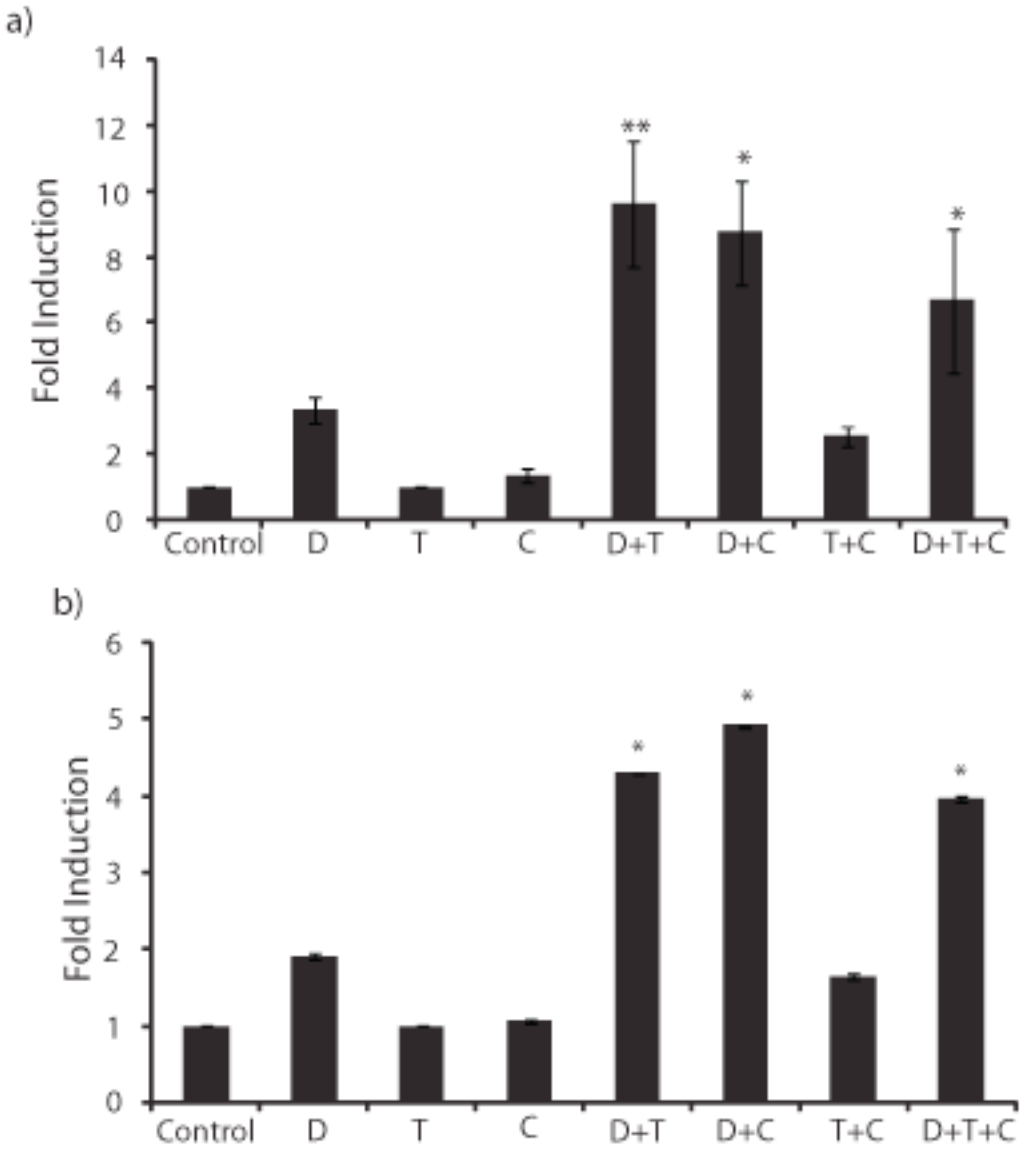


Figure 4.28. Effect of Decitabine, TSA and/or Chaetocin Treatment of AML-193 and K562 cells on RIZ1 expression. (a) AML-193 cells were treated for 72 hours with 0.5 μM decitabine, and/or 100 nM TSA and/or 10 nM chaetocin (b) K562 cells were treated for 72 hours with 1 μM decitabine, and/or 500 nM TSA, and/or 50 nM chaetocin. Error bars represent standard deviation from three independent experiments. D indicates Decitabine, T indicates TSA, C indicates Chaetocin. * indicates $p < 0.05$, ** indicates $p < 0.005$ relative to the control.

5. DISCUSSION

The aim of this study was to induce re-expression of a tumor suppressor gene, RIZ1, using epigenetic drugs. Two model cell lines were used in the study, AML-193 (AML) and K562 (CML blast crisis). RIZ1 was silenced in both of these cell lines, however they differed in methylation status, with AML-193 being completely methylated (94%) and K562 being hemi-methylated (39%) (Figure 4.3). Three epigenetic drugs were used: a DNA methylation inhibitor, decitabine, a histone deacetylation inhibitor, TSA, and an H3K9 methylation inhibitor, chaetocin. In order to effectively use these three drugs it was necessary to first determine their toxicity. Trypan blue and MTT assays were used to determine the toxicity of decitabine, TSA and chaetocin. Treatment with decitabine caused a significant loss of viability, however this loss of viability was not enough to determine a reasonable median dose (Figure 4.4). Clinical trials of decitabine are now concentrating on low dose schedules of the drug and it appears that lower doses enhance the ability of the drug to inhibit methylation of the DNA in patients as patients have shown a better response to lower doses of decitabine (Yang *et al.*, 2006). Therefore it seemed unreasonable to increase the dose further when lower doses have been shown to be more effective. Cell cycle analysis was conducted on cells treated with decitabine and a G2 arrest was seen in both cell lines, along with an increase in the number of cells in the S phase (Figure 4.5). This indicated that although toxicity was low, there was a significant effect on the cell. Both TSA and chaetocin caused significant loss of viability, enough for a median dose value to be determined (Figure 4.6, 4.7).

DNA methylation is a common mechanism for silencing of tumor suppressor genes (Greger *et al.*, 1989; Sakai *et al.,* 1991). DNA methylation in the RIZ1 promoter region has been observed in both AML and CML cell lines (Figure 4.3b). Treatment with decitabine decreased the RIZ1 promoter region DNA methylation in both cell lines (Figure 4.11). Interestingly, in AML-193 there were three CpGs which remained at 100% methylation, even upon treatment with 16 μM decitabine, suggesting that these CpGs have some protection from demethylation (Figure 4.12g). Currently the cause for this protection is unknown. Most CpGs have similar losses of methylation within a few percentage points in AML-193 (Figure 4.12). In K562 cells the effects of decitabine were not universal, but instead certain CpGs experienced increases in methylation while others experienced decreases. Treatment with 2 μM decitabine caused CpGs

1, 5, 9 and 15 to decrease in methylation by 4-9% while the 3' most CpGs increased in methylation by 4% (Figure 4.13d). The reasons for the different reactions to the treatments across the area analyzed and the difference between K562 and AML-193 are not yet known. A possible reason may be that the two cell lines have a different reaction to decitabine treatment due to the differing levels of methylation between the two cell lines; K562 is only 39% methylated whereas AML-193 is 94% methylated. Treatment with TSA had no effect on the methylation status of the RIZ1 promoter region in either cell line (Figure 4.14).

Treatment with chaetocin caused an increase in promoter region DNA methylation in K562 (Figure 4.15b), and there was a slight pattern to the methylation changes seen (Figure 4.16). Treatment with 20, 40, and 80 nM chaetocin had a global effect with the exception of a few CpGs outlined in the results section. Interestingly, although treatment with chaetocin increased the amount of DNA methylation present, it was still able to induce RIZ1 expression (Figure 4.19a). This suggested that DNA methylation is not the dominant event in transcriptional silencing. Treatment with chaetocin had no effect on the methylation status of the RIZ1 promoter region in AML-193 (Figure 4.15a), yet RIZ1 expression was still induced. It is possible that because of the already high methylation levels in AML-193 that any increase in methylation would not be seen. Evidence for this is seen when AML-193 is treated with decitabine and chaetocin together. Cells treated with decitabine or decitabine plus TSA experienced a decrease in DNA methylation levels, however, treatment with chaetocin brought the methylation levels back up to near control levels (Figure 4.25a). This was mirrored in K562 where cells treated with decitabine or decitabine plus TSA experienced a drop in methylation and treatment with chaetocin increased the amount of methylation (Figure 4.25b). One possibility as to why DNA methylation increased upon treatment with chaetocin is that the inhibition of SUV39h1 is increasing the levels or activity of the H3K9 dimethyltransferase G9a. G9a stimulates DNMT1 activity, and so the increased presence of G9a would increase DNMT1 activity and lead to higher RIZ1 promoter region DNA methylation levels (Esteve *et al.,* 2006)

Once the methylation profile had been established, the ability of these three drugs to induce RIZ1 expression was explored. Both decitabine and chaetocin were able to induce RIZ1 expression on their own (Figure 4.17, 4.19). Decitabine is able to induce expression of tumor suppressor genes which have heavy promoter region DNA methylation (Constantinides *et al.,* 1977; Jones and Taylor, 1980), so it was expected that some induction would be seen by

decitabine. Induction of a tumor suppressor by chaetocin is a novel finding, and the significance is increased as chaetocin did not reduce the amount of RIZ1 DNA promoter methylation. This shows that demethylation of the RIZ1 promoter region DNA is not necessary to induce expression of RIZ1. Although treatment with TSA alone had no effect on RIZ1 expression levels, TSA was able to potentiate the amount of induction seen by both decitabine and chaetocin (Figure 4.25). Decitabine and chaetocin were also able to induce more RIZ1 expression together than each one alone (Figure 4.28). This correlated with what was found when synergy and antagonism were investigated, whereby decitabine potentiated the effect of chaetocin in both cell lines and TSA in AML-193 (Table 4.1). Treatment with all three drugs together induced RIZ1 expression more than any drug treated alone, however there was less induction than treatment with any two drugs together. In order to determine if there was any synergy between the drugs, a value called a combination index (CI) was determined. Treatment with TSA and chaetocin induced a higher level of expression than each drug alone, however not as much as either decitabine plus TSA or decitabine plus chaetocin together (Figure 4.28). This suggested that although demethylation of H3K9 is sufficient to induce expression, in order to achieve a high level of expression, DNA demethylation must occur. Treatment with all three drugs must affect the way the drugs are being taken up in the cell, distributed or secreted in some way that is different than when just two drugs are present, as there is similar induction when all three are treated together as treatment with decitabine plus TSA and decitabine plus chaetocin (Figure 4.28) (Hartshorn, 2006).

Previous studies indicated that decitabine and TSA work synergistically (Kawamoto *et al*., 2008), however whether there was synergy between chaetocin and decitabine, and between chaetocin and TSA, were unknown. In order to determine if there was any synergy between the drugs, a value called a combination index (CI) was determined. The CI value is a value that measures the degree of synergism or antagonism between drugs. In both cell lines at relevant ED values the CI value was less than 1. This indicated that TSA and chaetocin acted synergistically. This was confirmed upon examining the amount of expression induced upon treatment of the cells with TSA and chaetocin (Figure 4.28). In both cell lines there was a larger amount of RIZ1 expression when cells were treated with TSA and chaetocin together than when cells were treated with the drugs individually.

Since a median dose was not determined for decitabine, no claims of synergy or antagonism could be made. However, potentiation of the effects of TSA and/or chaetocin by decitabine could be investigated. In order to do this, the median doses of TSA and Chaetocin in the absence or presence of decitabine was examined. In the presence of decitabine a drop in median dose values for both TSA and chaetocin was seen in AML-193. This indicated that decitabine was potentiating the effect of TSA and chaetocin as it takes less of TSA or chaetocin in order to see the same effect (Table 4.1). When examining RIZ1 expression, cells treated with decitabine plus TSA and decitabine plus chaetocin showed a larger induction of RIZ1 expression than cells treated with any drug alone (Figure 4.28).

In all conditions examined, the addition of decitabine caused the CI values to increase, which indicated that decitabine interfered with the synergy between TSA and chaetocin. This again correlates with the real time PCR data. Although there was an increase in the amount of expression seen upon the addition of all three drugs together, it was less than any pair containing decitabine (Figure 4-28).

From my data I conclude that through the use of three epigenetic drugs, it is possible to induce expression of RIZ1 in human CML and AML cell lines, and that treating cells with two drugs together worked better than one alone or when all three were present. From the data presented here, a model was developed to explain what is occurring at the promoter during these events.

Upon silencing, SUV39h1 is recruited to the histone, possibly by the Retinoblastoma protein, where it methylates the H3K9 residue (Nielsen *et al*., 2001). The methylation of the H3K9 residue recruits HP1 to the site, which then recruits DNMT and HDACs to the silenced region, creating a complex that is able to effectively silence the gene (Wang *et al*, 2000). This complex also likely contains MBD1 as it forms a complex with SUV39h1 (Fujita *et al*., 2003) (Figure 5.1). Treatment with chaetocin induced RIZ1 expression even though the DNA methylation status of the promoter region remained unchanged, or the methylation increased. This suggested that H3K9 methylation is the dominant event in transcriptional silencing. Therefore, once SUV39h1 is inhibited by chaetocin, there is no methylation of the histone, and gene transcription can progress (Figure 5.1f, Figure 5.1g). Treatment with decitabine also induces RIZ1 expression. The demethylation of the DNA by RIZ1 caused MBD1 to release from the DNA, and the complex disassociated (Figure 5.1b, Figure 5.1c). Once the complex is

disassociated, SUV39h1 can no longer methylate the H3K9 residue and transcription is able to start (Figure 5.1d).

This model is important as it suggests how transcription begins and is aberrantly stopped. This model also allows for new pharmaceutical development into the targeting of this silencing complex. Chaetocin was able to induce RIZ1 expression without affecting the methylation status of the RIZ1 promoter region DNA. This suggests that DNA methylation is not the dominant event in transcriptional silencing, and that H3K9 methylation is the dominant event. From this data a model was developed to explain how the drugs are affecting the silencing complex present at the promoter region, and how treatment with either decitabine or chaetocin is able to induce expression. Also, I determined that when cells are treated with decitabine, TSA and/or chaetocin in pairs there is a higher level of induction than any other treatment course studied. Determining the dominance of epigenetic events in transcriptional silencing is important as pharmaceuticals can be targeted to the dominant events, thus increasing the effectiveness of cancer therapy. Also, by determining whether pharmaceuticals targeted to certain epigenetic events are synergistic or antagonistic, it is possible to design treatment schedules to be more effective.

5.1 Future Directions

In order to test this model, chromatin immunoprecipitation (CHIP) experiments could be done to confirm the disassociation of SUV39h1 from the promoter region upon treatment of cells with decitabine and to assess the methylation status of the H3K9 residue upon this dissassociation. Other tumor suppressor genes such as p15 or p21 could be studied to determine if this is a model specific to RIZ1 or if it is applicable to a wider set of genes. The experiments conducted in this study could also be performed in patient material to confirm the results that were seen in cell lines are seen in actual cases of AML and CML.

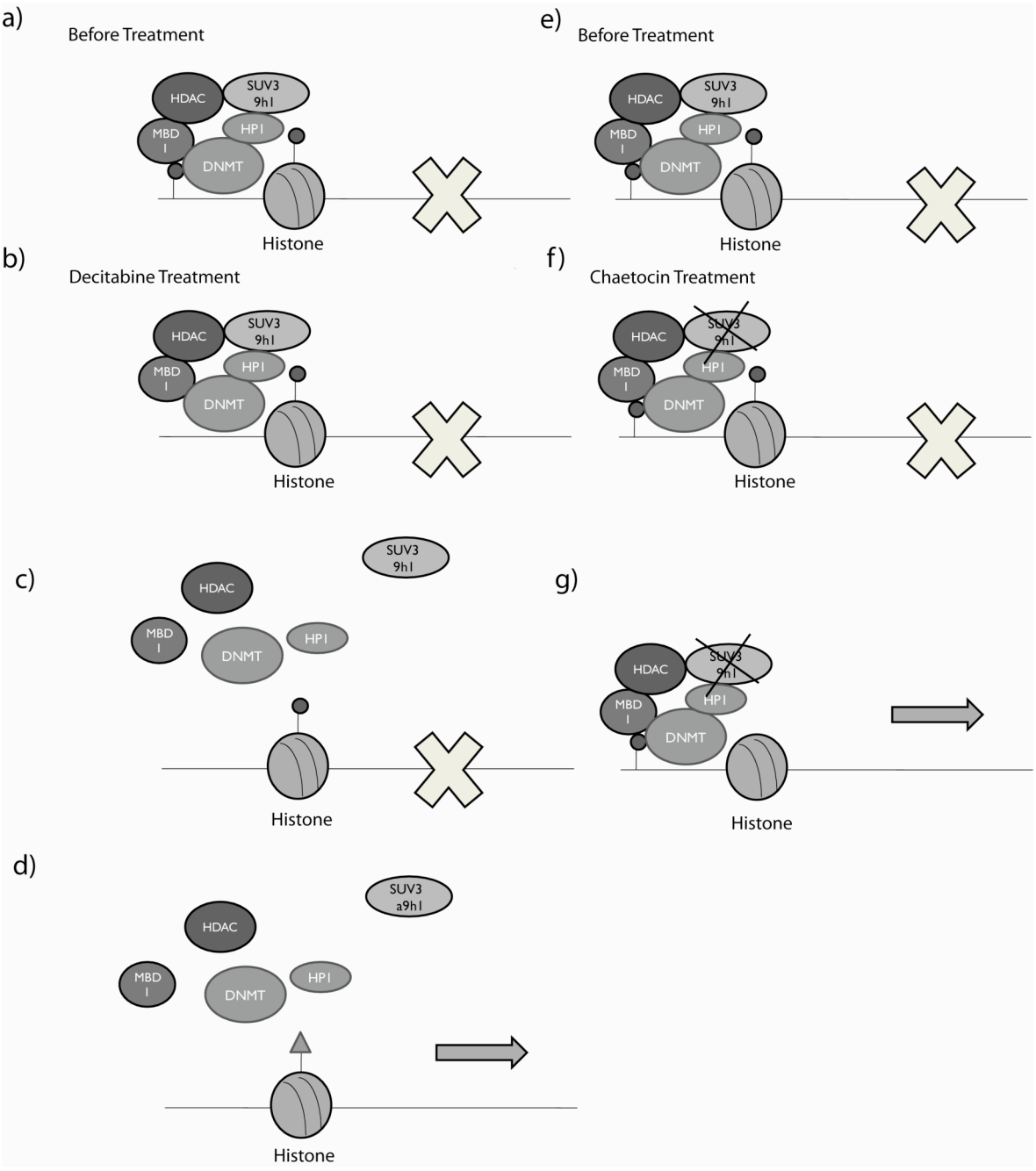


Figure 5.1. Model of Chromatin Remodelling During Drug Treatment. (a,e) Before treatment the histone is methylated on H3K9, promoter region DNA is methylated (circle on stick) and the complex is fully formed. The gene is silenced (a) after decitabine treatment, (b) the DNA becomes demethylated, (c) the complex disassociates and, (d) upon SUV39h1 disassociating, the histone is no longer methylated and transcription can proceed. After chaetocin treatment (f) the histone becomes demethylated and (g) transcription can proceed.

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