EFFICACY AND MECHANISTIC STUDIES OF A NOVEL SMALL MOLECULE THERAPEUTIC AGENT “XB05” IN ACUTE MYELOID LEUKEMIA (AML)

By

Jaspreet Singh Grewal

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Clinical Laboratory Sciences—Master of Science

2014

ABSTRACT

EFFICACY AND MECHANISTIC STUDIES OF A NOVEL SMALL MOLECULE THERAPEUTIC AGENT “XB05” IN ACUTE MYELOID LEUKEMIA (AML)

By

Jaspreet Singh Grewal

Acute myeloid leukemia (AML) is the most common acute leukemia in adults characterized by a clonal proliferation of myeloid precursors with a reduced capacity to differentiate into more mature cellular elements resulting in pancytopenia. Current treatment options include induction chemotherapy followed by consolidation and hematopoietic stem cell transplantation (HSCT). DNA hypomethylating agents such as decytabine have been used to treat AML, especially in older patients. The drug has shown significant clinical benefit in a recent Phase III trial in older patients demonstrating the proof of concept of inhibiting DNA methylation as a therapeutic strategy in AML. We have discovered a novel synthetic small molecule, named XB05 that has potent activity against myeloid leukemia cell lines. Its precise molecular mechanism is not yet fully understood; however, we believe that it induces leukemia cell death through multiple mechanisms, including reactivation of tumor suppressor genes via inhibition of DNA methylation. In this study, mechanistic studies were performed with this novel agent in previously established leukemia cell lines.

ACKNOWLEDGEMENTS

I am using this opportunity to express my sincere gratitude to Dr. Tariq Malik who supported me throughout the course of this Masters project. I am thankful for his aspiring guidance, invaluably constructive criticism and friendly advice during the project work.

I would also like to thank Dr. David Thorne and Dr. John Gerlach at the Michigan State University for their guidance and direction in the completion of my thesis project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

5'AZA	5'azacytadine
AFF1	AF4/FMR2 family, member 1
AML	Acute myeloid leukemia
ARE	Antioxidant response elements
ASXL1	Additional Sex Combs protein 1
ATCC	American Type Culture Collection
BHA	Butylated hydroxyanisole
CALGB	Cancer and Leukemia Group B
CBFB	Core-binding factor, beta subunit
CD	Cluster differentiation
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CEBPA	CCAAT-enhancer binding protein – alpha
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta
CR	Complete remission
CSF1R	Colony stimulating factor 1 receptor
CTNNA1	Catenin (cadherin-associated protein), alpha 1
DAPK	Death-associated protein kinase
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferases
DRD2	Dopamine receptor D2

ER-α	Estrogen receptor α
ESI-MS	Electrospray ionization mass spectrometry
ETO	Eight-Twenty-One
EXT1	Exostosin glycosyltransferase 1
FANCC	Fanconi anemia, complementation group C
FANCL	Fanconi anemia, complementation group L
FBS	Fetal bovine serum
FGFR	Fibroblast growth factor receptor
FLT-3	Fms-like tyrosine kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSH	Cysteine and glutathione
HDAC	Histone deacetylase
HIC-1	Hypermethylated in cancer – 1
HMT	Histone methyltransferase
HOXA7	Homeobox A7
HOXB5	Homeobox B5
HRP	Horseradish peroxidase
HSCT	Hematopoietic stem cell transplantation
IDH	Isocitrate dehydrogenase
IHC	Immunohistochemistry

IL	Interleukin
JUNB	Jun B proto-oncogene
Keap1	Kelch-like ECH-associated protein 1
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LMNA	Lamin A/C
MAFB	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B
MDS	Myelodysplastic syndrome
MEN1	Multiple endocrine neoplasia I
MGMT	O-6-methylguanine-DNA methyltransferase
MLL	Myeloid/lymphoid, or mixed-lineage, leukemia
MTG8	Myeloid translocation gene on 8
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYH1	Myosin, heavy chain 1
NCI	National Cancer Institute
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
NMR	Nuclear magnetic resonance
NPM1	Nucleophosmin -1
Nrf2	Nuclear factor, erythroid 2-like 2
O.D.	Optical density
OS	Overall survival
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor receptor

PER2	Period circadian clock 2
PML	Pro-myelocytic leukemia
PS	Performance status
qRT-PCR	Quantitative real-time PCR
RARα	Retinoic acid receptor – alpha
RAR-β	Retinoic acid receptor – beta
RAS	Rat sarcoma viral oncogene homolog
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse Transcription PCR
RUNX1	Runt-related transcription factor 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLC4A8	Solute carrier family 4, sodium bicarbonate cotransporter, member 8
SOX9	SRY (sex determining region Y)-box 9
SPOCK2	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2
TET2	Ten eleven translocation protein 2
TLE1	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)
TP53	Tumor protein p53
UPR	Unfolded protein responses
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

Acute Myeloid Leukemia – Brief Review

Acute myeloid leukemia (AML) is the most common acute leukemia in adults especially in patients older than 65 years. It accounts for approximately 50 percent of all cases of leukemia in this age group [1], with five year survival rate <10% [2]. It is a heterogeneous disease characterized by a clonal proliferation of myeloid precursors with a reduced capacity to differentiate into more mature cellular elements resulting in accumulation of leukemic blasts or immature cells in the bone marrow and peripheral blood.[1] This leads to pancytopenia (anemia, neutropenia and thrombocytopenia), a reduction in the number of mature blood cells, which accounts for the symptoms in AML—anemia, infections, and bleeding [1].

Underlying etiology of AML cannot be determined in most cases; however, it has been hypothesized that exposure to environmental carcinogens may play a role [1]. Association of AML to chemotherapy has clearly been demonstrated. Two distinct subtypes of chemotherapy – related AML have been described. First subtype is the result of exposure to topoisomerase II inhibitors (etoposide, mitoxantrone and teniposide), anthracyclines (doxorubicin) and epipodophyllotoxins (VP-16 and VM-26) which can present within months to two years following treatment with these agents and is associated with MLL (myeloid/lymphoid, or mixed-lineage, leukemia) gene locus (11q23) abnormalities [1]. Second subtype presents five to seven years post exposure

to alkylator agents (cyclophosphamide, procarbazine and melphalan) which is preceded by myelodysplastic syndrome (MDS) and is associated with complex karyotypic abnormalities [1]. It is thought that various inherited polymorphisms may be associated with differential metabolism of these chemotherapeutic agents resulting in exposure to toxic by-products and DNA damage [1]. Also, various mutations, translocations and epigenetic changes have been described in AML. Common mutations involve Fms-like tyrosine kinase (FLT-3), CCAAT-enhancer binding protein – alpha (CEBPA), nucleophosmin -1 (NPM1), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) and rat sarcoma viral oncogene homolog (RAS) pathways [1, 3, 4]. And, common chromosomal abnormalities involve RUNX1/ETO [t(8;21)] (Runt-related transcription factor 1/ Eight-Twenty-One), CBFB/MYH11 [inv(16)] (core-binding factor, beta subunit/ myosin, heavy chain 1), and PML/RARα [t(15;17)] (Pro-myelocytic leukemia/ Retinoic acid receptor – alpha) translocations which result in constitutively active fusion gene products [1, 5]. Epigenetic silencing of genes related to DNA-hypermethylation from up-regulation of DNA methyltransferases 1, 3A and 3B (DNMT3A) and other epigenetic regulators such as ten eleven translocation protein 2 (TET2) and Additional Sex Combs protein 1 (ASXL1) has been described resulting in silencing of numerous genes including, isocitrate dehydrogenase 1 and 2 (IDH 1 and 2), cyclin-dependent kinase inhibitor 2A (CDKN2A), hypermethylated in cancer – 1 (HIC-1), retinoic acid receptor – beta (RAR-β), death-associated protein kinase (DAPK), O-6-methylguanine-DNA methyltransferase (MGMT), and estrogen receptor α (ER-α). [1, 6-9]

Response to treatment and overall survival (OS) of patients with AML is heterogeneous; patients with poor performance status (PS), complex karyotypes, and older age have worse prognosis . Current treatment options include induction chemotherapy with combination of daunorubicin and cytarabine (7+3 regimen) followed by consolidation and hematopoietic stem cell transplantation (HSCT) . Induction chemotherapy is given with the aim to achieve complete remission (CR) while consolidation chemotherapy which includes intermediate- or high-dose cytarabine alone is given to eliminate residual leukemic cells [1]. Patients with unfavorable risk disease do poorly with 5-year OS of 15 to 40 percent with current chemotherapy regimens and HSCT; thus, improved treatments are urgently needed [10]. Outcomes are even worse in patients over 65 years with five-year survival rate of less than 10 percent with treatment with intensive regimens (Figure. 1) [1, 7]. Although reduced intensity conditioning regimens have been used in older patients to help them undergo allo-HSCT, the results have been similar to myeloablative regimens. Older patients in these studies who underwent induction chemotherapy had good performance status and only 5 percent of all the patients recruited could undergo allo-HSCT [1, 11] Current modality of induction regimen has been in place for over 30 years. Numerous trials evaluating the substitution of daunorubicin with other anthracyclines or addition of a third agent have failed to demonstrate improvements in outcomes [1, 11, 12].

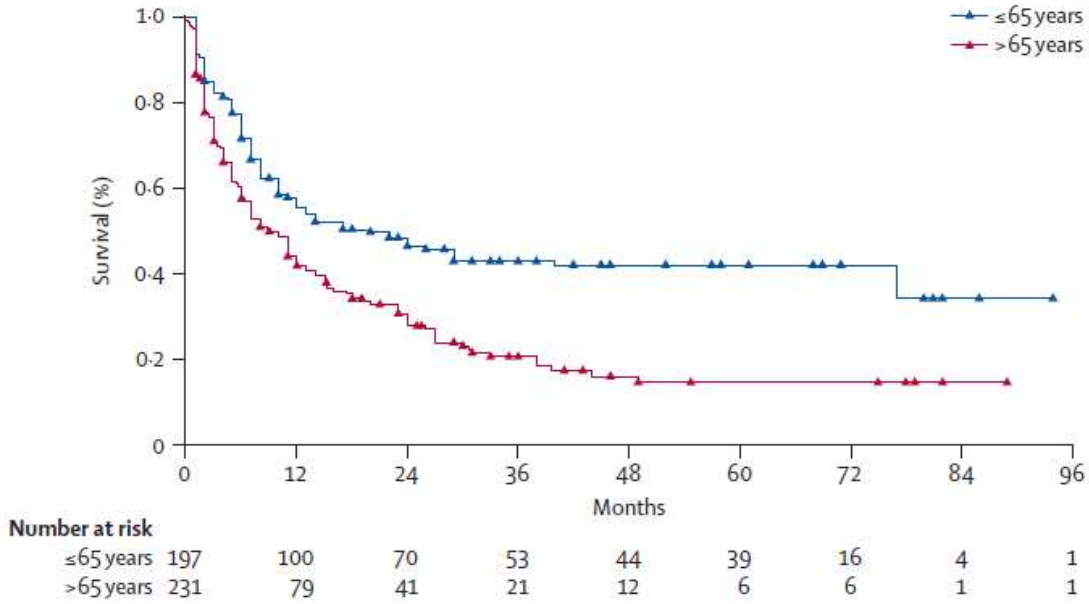


Figure. 1. **Survival curve of 428 consecutive patients with AML treated at Cardarelli Hospital in Naples, Italy** [1]. Patients older than 65 years had significantly poor overall survival than younger patients.

Over the years, investigators have tried to optimize the dose of daunorubicin. Trials conducted so far have demonstrated that the 90mg/m2 dose of daunorubicin is more effective than 45 or 60mg/m^2 dose with similar toxicities in patients with intermediate risk cytogenetics and CBFB-AML [1]. In younger patients, a second induction is given to help achieve durable disease control. However, without further treatment or consolidation majority of the patients relapse. Trial done by the Cancer and Leukemia Group B (CALGB) has demonstrated that consolidation with high-dose cytarabine at the dose of 3gm/m^2 resulted in long-term relapse-free survival of 45% in patients up to 60 years with favorable cytogenetics [1]. Optimal number of cycles of cytarabine have not been determined but can vary from three to four cycles depending

on patient tolerability and adverse effects. In older patients, studies using low-dose cytarabine have found survival advantage in patients with favorable cytogenetics [1, 11].

With further understanding of molecular mechanisms involved in pathogenesis of AML, new therapies are being developed. In order to achieve meaningful results with new targeted therapies, patients will need to be stratified based on their underpinning molecular abnormalities. A recent trial from the United Kingdom – UK Medical Research Council AML group showed that the addition of gemtuzumab ozogamicin (anti-CD33 monoclonal antibody conjugated with cytotoxic antibiotic, calicheamicin) to chemotherapy resulted in survival advantage in patients with core binding factor AML [1]. This was further verified by two other studies that demonstrated survival advantage in patients with intermediate risk karyotypes [1]. However, there was no survival benefit seen in patients with unfavorable cytogenetics. Gemtuzumab ozogamicin has been withdrawn from market because of increased mortality seen in treated patients. Trials are ongoing to test the efficacy of FLT-3 inhibitors [1, 13]. In addition, Phase I and Phase II studies are ongoing evaluating the efficacy and safety profile of numerous other targeted agents shown in (Table.1) [13].

Drug	Mechanism of Action
Sorafenib	Small-molecule inhibitor of Raf, c-kit, RET, FLT3, VEGF, PDGFR and FGFR
Midostaurin	Small-molecule inhibitor of protein kinase C, VEGFR-2, PDGFR-α, PDGFR-β, c-kit and FLT3
Quizartinib	Bis-aryl urea inhibitor of FLT3, KIT, PDGFR-α, PDGFR-β, RET and CSF1R
Vosaroxin	Quinolone derivative which inhibits topoisomerase II
Elacytarabine	Nucleoside analog which inhibits DNA polymerase
Sapacitabine	Cytosine nucleoside analogue which leads to DNA strand breaks and apoptosis
Table 1 (cont'd)	
Flavopiridol	Synthetic flavone derivative which inhibits multiple serine-threonine cyclin dependent kinases
Volasertib	Polo-like kinase inhibitor which leads to cell cycle arrest and apoptosis
CPX-315	Liposomal cytarabine and daunorubicin
Lintuzumab	Humanized monoclonal antibody against CD33
SGN33a	Monoclonal CD33 antibody-drug conjugate with pyrrolobenzodiazepine which causes DNA crosslinking and cell death

Table 1. Targeted agents in development in AML.

DNA hypomethylating agents such as decytabine have been used to treat AML, especially in older patients who cannot tolerate the side effects of standard chemotherapies [1, 10]. The intent is to reduce DNA methylation (a mechanism that "silences" the expression of many tumor suppressor genes in leukemia) and thereby reactivate genes that will inhibit leukemic cell survival and progression [1, 10, 14]. These drug has shown significant clinical benefit in a recent Phase III trial in older

patients with newly diagnosed AML, with 15.7 percent complete remissions (CR) and a clinically meaningful improvement in OS, and was generally well tolerated [10, 15]. This proves the value of inhibiting DNA methylation as a therapeutic strategy in AML, but clearly there remains a need for developing new hypomethylating drugs with better activity and fewer side effects.[10, 14]

Epigenetic Mechanisms in AML – DNA Methylation

Epigenetic mechanisms are gene regulatory mechanisms which result in altered gene expression without changes in the gene sequence. These mechanisms include DNA methylation and histone deacetylation. Epigenetic changes are a normal part of physiologic development and regulation of gene expression, which at times can results in an abnormal phenotypes such as cancer, leading to silenced important critical genes due to hypermethylated promoters regions [6, 16-18]. DNA methylation results in altered gene expression via covalent addition of a methyl group to 5'-position of cytosine in the CpG dinucleotide (regions of DNA which are GC rich or are concentrated in large clusters called the CpG islands) resulting in epigenetic silencing of genes [6, 19, 20]. Methylation in mammalian cells is the function of three DNA methyltransferase (DNMTs) – DNMT1a, DNMT3a and DNMT3b [6-9, 16, 18, 20]. Mice studies have shown that DNMT1 helps to maintain pre-existing methylation patterns during DNA replication, while DNMT3a and DNMT3b initiate de novo methylation [7, 16, 20]. Expression of DNMT3a is ubiquitous. DNMT3b is present in the cells at low levels except in testes, thyroid, and bone marrow [16].

DNA methylation once established in CpG islands is maintained after cell division through the activity of DNMTs, which localize to the replication foci to work on newly synthesized hemi-methylated DNA [6]. Methylation of cytosine residues in CpG islands with resultant binding of methyl-binding proteins (MBP) to DNA [8, 9, 18]. This further attracts histone deacetylase (HDAC) and histone methyltransferase (HMT) resulting in condensation of chromatin which prevents specific transcription factors or DNA-dependent RNA polymerase from accessing the promoter regions to cause gene silencing [8, 9, 18] (Figure. 2). DNA methylation can be reversed physiologically and resets in early embryogenesis.

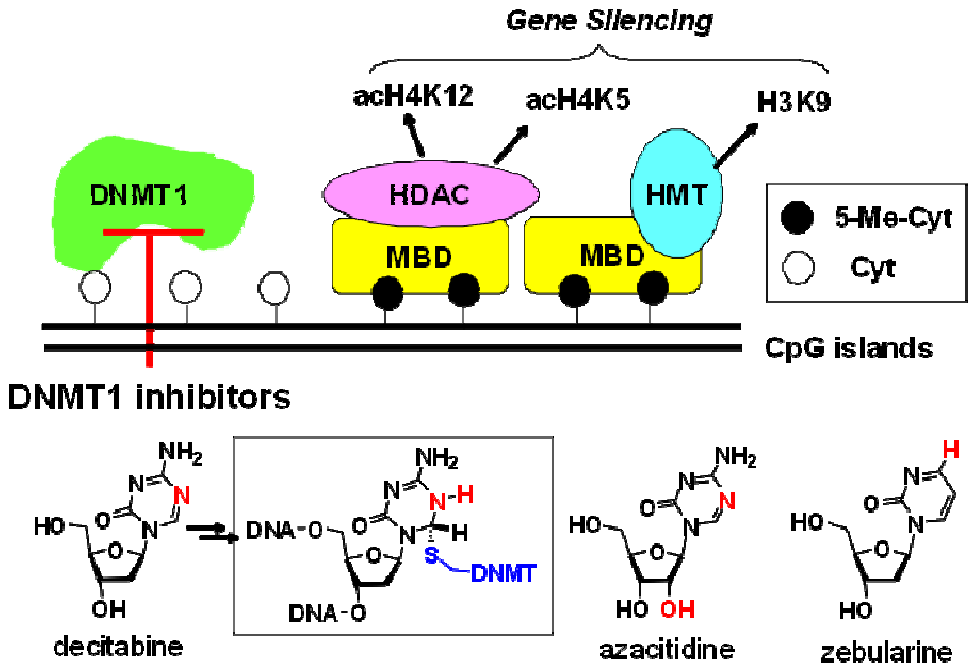


Figure 2. Gene silencing that occurs in AML as a result of DNMT, HDAC and HMT, and specific inhibitors of these enzymes.[6]

Hypermethylation of the promoter regions of important tumor suppressor genes by the DNA methyltransferase results in gene silencing and hence cancer development. DNA methylation not only results in alterations in promoter and repetitive sequences of DNA but can also alter the expression and regulation of noncoding RNAs such as micro-RNAs (mi-RNA) [19]. The CpG islands hypermethylated in AML span the promoters of house-keeping genes and tumor suppressor genes. Multiple genes are concurrently methylated including genes involved in cell cycle regulation, DNA repair, drug resistance, detoxification, differentiation, apoptosis, angiogenesis and metastasis.[6, 16-18, 21]

Methylated promoter regions of tumor suppressor genes are important targets in cancer treatment as these epigenetic changes are reversible, this has been demonstrated in MDS and AML especially in the elderly [19]. Currently, two hypomethylating agents are approved for the treatment of MDS; azacytidine and decytabine. Both agents have been demonstrated to be equally effective in the treatment of MDS; however, azacytidine has a better safety profile than decytabine [22]. Azacytidine or 5'Azacytidine (Vidaza®) was approved by the FDA for the treatment of MDS in 2004 [7, 23]. It is a chemical analogue of cytosine nucleoside. This drug causes DNA hypomethylation resulting in reactivation of tumor suppressor genes, and direct cytotoxicity through incorporation into the tumor DNA and RNA. Decytabine or 5-Aza-2'-deoxycytadine (Dacogen®) was approved by the FDA in 2006 for the treatment of MDS and was recently approved for the treatment of AML in elderly patients [7]. It is an analogue of the nucleoside 2'-deoxycytidine. It exerts its antineoplastic effects after phosphorylation and direct incorporation into the tumor DNA, and inhibition of the DNA

methyltransferases, causing hypomethylation and cellular differentiation or apoptosis. In clinical trials both of these agents were compared with best supportive care or low-dose cytarabine in patients with low-, intermediate- or high-risk AML, and showed better OS compared to both modalities [20]. Even though the epigenetic changes and gene silencing is reversible with the use of these agents; however, aberrant methylation of genes returns as soon as they are stopped [17].

CHAPTER 2

XB05 – A Novel Synthetic Small Molecule

XB05 (1-bromo-1,1-difluoro-non-2-yn-4-ol) is a novel synthetic small molecule originally developed to introduce fluorine containing groups in to organic molecules [24]. This molecule was found to be selectively cytotoxic to certain malignant cells compared to non-malignant cells, and had particularly potent activity against myeloid leukemia cell lines [24]. XB05 was not originally designed as an anti-cancer agent—a serendipitous observation led to the discovery of its effects on cancer cells—so its precise molecular mechanism is not yet fully understood; nonetheless, our studies have identified several activities that contribute to its selective anti-cancer effects . Based on the data collected so far, we propose that XB05 induces leukemia cell death through multiple mechanisms, including reactivation of tumor suppressor genes via inhibition of DNA methylation, induction of lethal oxidative and proteotoxic stress through down regulation of the transcription factor nuclear factor, erythroid 2-like 2 (Nrf2) [24-27]. This last activity is particularly significant because Nrf2 is frequently over expressed in AML cells and is known to contribute to the resistance of AML against a variety of chemotherapies.[24, 26-28] In fact, based on our preliminary data, we predict that XB05 will sensitize AML cells to the effects of chemotherapy, while simultaneously protecting healthy cells from side effects [29].

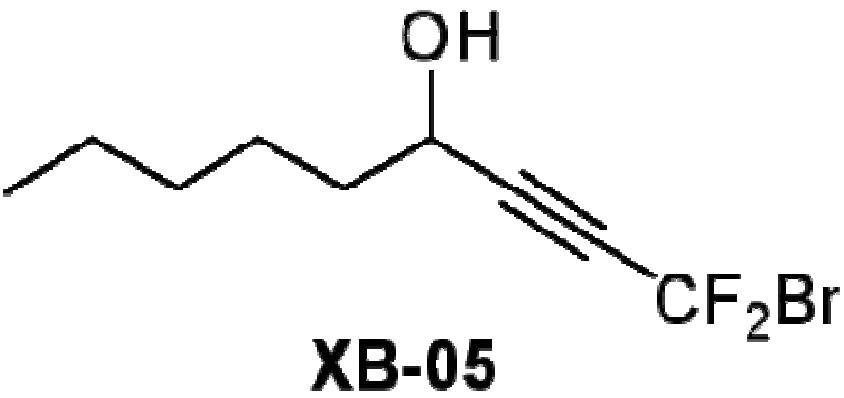


Figure 3. Molecular structure of XB05[29]

XB05 has a novel chemical structure which prompted initial anti-cancer studies showing cancer selectivity [24]. Following this, the agent was sent to the National Cancer Institute (NCI) for further testing. NCI-60 screen was performed where the activity of the agent is tested on 60 human cancer cell lines. XB05 was shown to be cytotoxic in some cell lines while not in others. The specific activity of XB05 can be compared to 40,000 other compounds screened by NCI in terms of mechanism of action. On further analysis and comparative studies, we came across number of interesting findings. XB05 was selectively cytotoxic to malignant cells and had no cytotoxicity against non-malignant human cells such as fibroblast cell line “Hs27”, mammary cell line “MCF-10A and lung fibroblast cell line “IMR-90”.[29] Mechanism of action of XB05 was unique and not related to any existing chemotherapeutic agents. XB05 was closely related to a naturally occurring marine product called “halomon” which inhibits the activity of DNMTs.

One of the major concerns while studying XB05 was that a molecule like it will be highly reactive and non-specific [24]. However, studies done using fluorine-19 nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry (ESI-MS) showed that XB05 was not reactive with single stranded DNA oligonucleotides or with basic amino acids. These studies did reveal that XB05 was slowly reactive with thiol-containing amino acids such as cysteine and glutathione (GSH) [24]. Further studies showed that addition of glutathione and N-acetyl cysteine inhibited the anti-proliferative activity of XB05 but addition of non-thiol antioxidants such as ascorbic acid or butylated hydroxyanisole (BHA) had no effect [24].

One of the important determinants that can help guide therapy and response to XB05 is the transcription factor SRY (sex determining region Y)-box 9 (SOX9) [24]. SOX9 gene is located on chromosome 17q23 and encodes for a high mobility group box transcription factor that plays a critical role in embryogenesis and is required for development, differentiation, and lineage commitment in various tissues especially skeletal and reproductive systems [30]. SOX9 is over expressed in many cancer cells which correlates with malignant potential of the tumor and possibly its response to anti-cancer agents [30].

XB05 has also been shown to increase the sensitivity of cancer cells to oxidative stress. Cancer cells have increased levels of reactive oxygen species (ROS) and are constantly under increased oxidative stress secondary to elevated metabolism, increased expression of oncogenic pathways, dysfunctional mitochondrial machinery and loss of tumor suppressor genes [25-27]. In order to counteract the increased levels

of oxidative stress, cancer cells up regulate anti-oxidative mechanisms [26, 27]. One of the major pathways that is involved in oxidative stress response is Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response elements (ARE) or Keap1-Nrf2-ARE pathway and protects cancer cells from apoptosis [26-28]. When cells are exposed to oxidative and electrophilic stress, Nrf2 induces transcriptional activation of cytoprotective and detoxification genes preventing damage to DNA, proteins and lipids [26-28]. In AML, Nrf2 is present in the nucleus where it is primed to up-regulate transcription of genes involved in detoxification [26-28].

In non-malignant cells, the inhibitor of Nrf2 or Keap1 or iNrf2 mediates ubiquitination and proteasomal degradation of Nrf2 [26-28]. In-vitro when cells are exposed to increased oxidative stress such as exposure to chemotherapeutic agents, Keap-1 mediated proteasomal degradation of Nrf2 is blocked [26-28]. The activated Nrf2 then translocate to the nucleus and forms a complex with Maf proteins which facilitates its binding to the ARE and stimulate transcription of cytoprotective and detoxification genes. Nrf2 is regulated by another control element called the Bach1 which is a transcriptional repressor and binds to ARE enhancers blocking its activity until cells are exposed oxidative stress [26-28]. In normal cells, this pathway helps to protect cells from oxidative stress and damage to cellular machinery. On the contrary, this pathway is also beneficial to the cancer cells protecting them from increased oxidative stress in the presence of chemotherapeutic agents [26-28]. Up-regulation of this pathway in the cancer cells helps them acquire growth advantage and chemo-resistance [26-28]. Also, it has been recently shown that up-regulation of Nrf2 in AML

cells is directly related to higher nuclear levels of NF-κB indicating that it regulates the transcription of Nrf2 m-RNA [26-28].

Cancer cells are prone to increased cellular and oxidative stress through increase in metabolism and increased oncogene activation which results in the production of ROS [25]. ROS can damage DNA, proteins and lipids inducing oxidative and proteotoxic stress and causing cell death by both apoptotic and non-apoptotic pathways. In order to survive this stress, cancer cells upregulate anti-stress mechanisms such as GSH, heat shock proteins and unfolded protein responses (UPR) [25]. Studies have demonstrated that inducing cellular stress and inhibiting stress response pathways can selectively induce cancer cell death.

In addition to the above described mechanisms, we believe that XB05 suppresses cancer proliferation by suppressing epigenetic modulation by DNMTs. This mechanism of action of XB05 is likely related to the naturally occurring marine compound called “halomon”. Preliminary in vitro studies done in our laboratory have confirmed that XB05 inhibits DNMT1 activity. Detailed epigenetic modulation via DNMTs is described in the previous chapter (chapter 1). However, studies looking at the re-expression of tumor suppressor genes silenced by methylation are lacking. In this study, we performed studies attempting to elucidate the mechanism of action of XB05 from the perspective of inhibition of DNMT activity.

We believe that anti-cancer effects of XB05 are associated with its ability to induce cellular stress and down-regulation of DNMTs. Based on preliminary studies, we have determined that XB05 is highly selective and can induce cell death via both

apoptotic and non-apoptotic mechanisms in cancer cells but has no activity in benign cells [24]. XB05 can generate high levels of ROS in cancer cells increasing cellular stress and inducing DNA damage . XB05 can reduce the level of glutathione and Nrf2 in malignant cells exposing them to increased oxidative stress and inhibiting cellular cytoprotective mechanisms [24]. Also, it inhibits the activity of DNMTs resulting in re-expression of tumor suppressor genes silenced in various cancers including AML.

CHAPTER 3

SPECIFIC AIMS

Aim 1: Determination of the inhibitory concentration or IC_{50} for XB05 in two AML cell lines.

Rationale: XB05 was studied in the NCI-60 screen where it was found to be cytotoxic in leukemia cell lines. Expanding on the existing data and preliminary studies done in our laboratory, MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-assays were planned to determine the IC_{50} for XB05 in two AML cell lines (Kasumi-1 and U937). Comparative MTT-assays with 5'azacytidine, a known hypomethylating agent, and combination drug (XB05 and 5'azacytidine) MTT-assays were also planned to evaluate the synergistic activity of XB05.

Aim 2: Assess the expression of DNA-methyltransferases and tumor suppressor genes in AML following treatment with XB05 and 5'-azacytidine at different time intervals.

Rationale: Preliminary in vitro studies done in our laboratory had suggested that XB05 is a DNA-hypomethylating agent. We plan to perform polymerase chain reaction (PCR) to assess the expression of DNMTs and commonly methylated tumor suppressor genes in AML following treatment with XB05 and 5'AZA at 48- and 96-hour intervals. Treated and non-treated specimens will also be assessed for percent promoter methylated of many tumor suppressor genes using Methyl PCR-assay. Furthermore, Western Blots

will be performed on nuclear and cytoplasmic extracts and immunohistochemistry (IHC) will be performed on whole cells following treatment to assess for protein expression.

Materials and Methods

AML Cell lines

Following cell lines were chosen because of our past experience working with these cell lines and they are easy to culture with rapid turnover. DNMT (especially DNMT1) expression has been reported to be high in Kasumi-1 and U937 cell lines [31-34].

1. Kasumi-1 – Established cell line obtained from the American Type Culture Collection (ATCC-CRL-2724) *(http://atcc.org/Products/All/CRL-2724.aspx).*

This cell line was established from the peripheral blood of a seven year old juvenile Japanese patient with AML. The leukemic cells have t(8;21) chromosomal translocation which juxtaposes the AML1 gene on chromosome 21 with ETO (eight twenty one) or MTG8 (myeloid translocation gene on 8) gene on chromosome eight resulting in a fusion gene AML1-ETO (also known as AML1-MTG or RUNX1-CBF2T1), and thus producing a chimeric AML1-ETO protein. This protein down-regulates CEBPA mRNA, protein and DNA binding activity, which is crucial for the differentiation of granulocytes.

The cells are positive for myeloperoxidase and in proliferation assay the cells in culture show response to interleukin-3 (IL-3), IL-6, granulocyte colony-stimulating factor (G-CSF), and granulocyte macrophage CSF (GM-CSF), but not to IL-1 or IL-5 [35, 36].

The base media for these cells is RPMI supplemented with 10% FBS and 1% penicillin and streptomycin. Cells are seeded at the density of 1 to 300,000 cells/mL.

2. U937 – Established cell line obtained from the ATCC (ATCC-CRL-1593.2) (http://atcc.org/Products/All/CRL-1593.2.aspx)

The U-937 cell line was established in 1974 from malignant cells obtained from the pleural effusion of a 37 year old Caucasian male patient with histiocytic lymphoma. These cells can be induced to terminal monocytic differentiation by supernatants from human mixed lymphocyte cultures. These cells are negative for immunoglobulin production and Epstein Barr virus expression. These cells express lysozyme, beta-2-microglobulin (beta 2 microglobulin) and tumor necrosis factor alpha (TNF-alpha), after stimulation with phorbol myristic acid (PMA). The cells also express the Fas antigen, and are sensitive to TNF and anti-Fas antibodies [37].

The base media for these cells is RPMI supplemented with 10% FBS and 1% penicillin and streptomycin. Cells are seeded at the density of 1 to 300,000 cells/mL.

Culturing Media

RPMI (Roswell Park Memorial Institute medium) supplemented with 10% fetal bovine serum (FBS) and 1% PenStrep (Penicillin-Streptomycin) containing 5000 units of penicillin and 5000 µg of streptomycin per ml.

Culture Methods

Each cell line was grown in RPMI (supplemented with 10% FBS and 1% Pen-Strep) for three days in AutoFlow NU-4750 water jacket CO_2 incubator before splitting. Cells were seeded in the media at recommended concentration of 1 to 300,000 cells/mL.

Drugs

1. XB05: Stock concentration of 15.7mM in 100% DMSO.

2. 5'Azacytidine (5'AZA) (Sigma Aldrich: A1287) Stock concentration of 500µM in RPMI.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) Assay

Principle: MTT is a colorimetric assay that measures the reduction of the oxidized substrate MTT which is yellow in color by the mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble formazan product which is dark purple in color. The cells are then solubilized with cell lysis buffer and the released, solubilized formazan product is measured by spectrophotometer. Since the reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of cell viability. This assay is broadly used to measure in vitro cytotoxic effects of drugs on cell lines or primary patient cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve.

Assay Design: ***Refer to appendix for experimental details and design.***

Initial studies involved optimizing conditions for the experiment. Cells from both cell lines were plated at 1000, 1500, 2000, 2500 and 3000 cells per well per in 100µl of

culturing media. Results from these experiments suggested that Kasumi-1 cells should be plated at 1500 cells per well and should be treated on the same day. However, U937 cells should be plated at 2000 cells per well and treated on the next day. Cells were counted using an automatic cell counter (TC20 Automated Cell Counter System. BioRad) according to manufacturer instruction. Viable cell count was more than 90% at plating. Different XB05 and 5'azacytidine concentrations were added in quadruplicates to determine inhibitory concentration (IC_{50}) for each drug. Concentration of each drug added to wells was three times. Volume of drug added was 50uL which resulted in the total volume of 150uL and originally planned final concentration in each well. Also, drug concentrations were determined based on pilot MTT-assays. Based on the pilot assays, different serial concentrations for both XB05 and 5'AZA were determined. Control for XB05 was dimethyl sulfoxide (DMSO) and for 5'AZA was RPMI. Cells were incubated for 72 hours. At 96 hours, MTT reagent at 10% of the well volume or 15uL was added and allowed to incubate for four hours. Then cell lysis buffer or sodium dodecyl sulfate (SDS) at 50% of the well volume or 75uL was added and allowed to incubate overnight. The following day, absorbance is read with spectrophotometer at wavelength of 570nm.

In addition, MTT-assays for combination treatment with XB05 and azacytidine were performed. Cells were treated with constant concentration of XB05 along with serial concentrations of 5'azacytidine.

Polymerase Chain Reaction (PCR) – Reverse Transcription PCR and Real-time PCR

Principle: PCR is a method DNA amplification developed by Kary Mullis in the 1980s. It is based on the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, a primer is needed which attaches to the complimentary sequence on the DNA and provides the polymerase with a starting point where the first nucleotide is added. This requirement helps the researcher to identify and amplify a specific sequence of the template DNA segment. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies or amplicons. (Figure. 1)

Reverse Transcription PCR (RT-PCR): This method of PCR is used to amplify DNA from mRNA. Reverse transcriptase transcribes RNA to cDNA and is then amplified by PCR. It is used for expression profiling and determine the expression of a gene.

Quantitative real-time PCR (qRT-PCR): This method is used to determine the level of expression of a gene in real-time. It can quantitate the starting amounts of DNA, cDNA (obtained from RT-PCR), or RNA. qPCR has a very high degree of precision. It uses fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time.

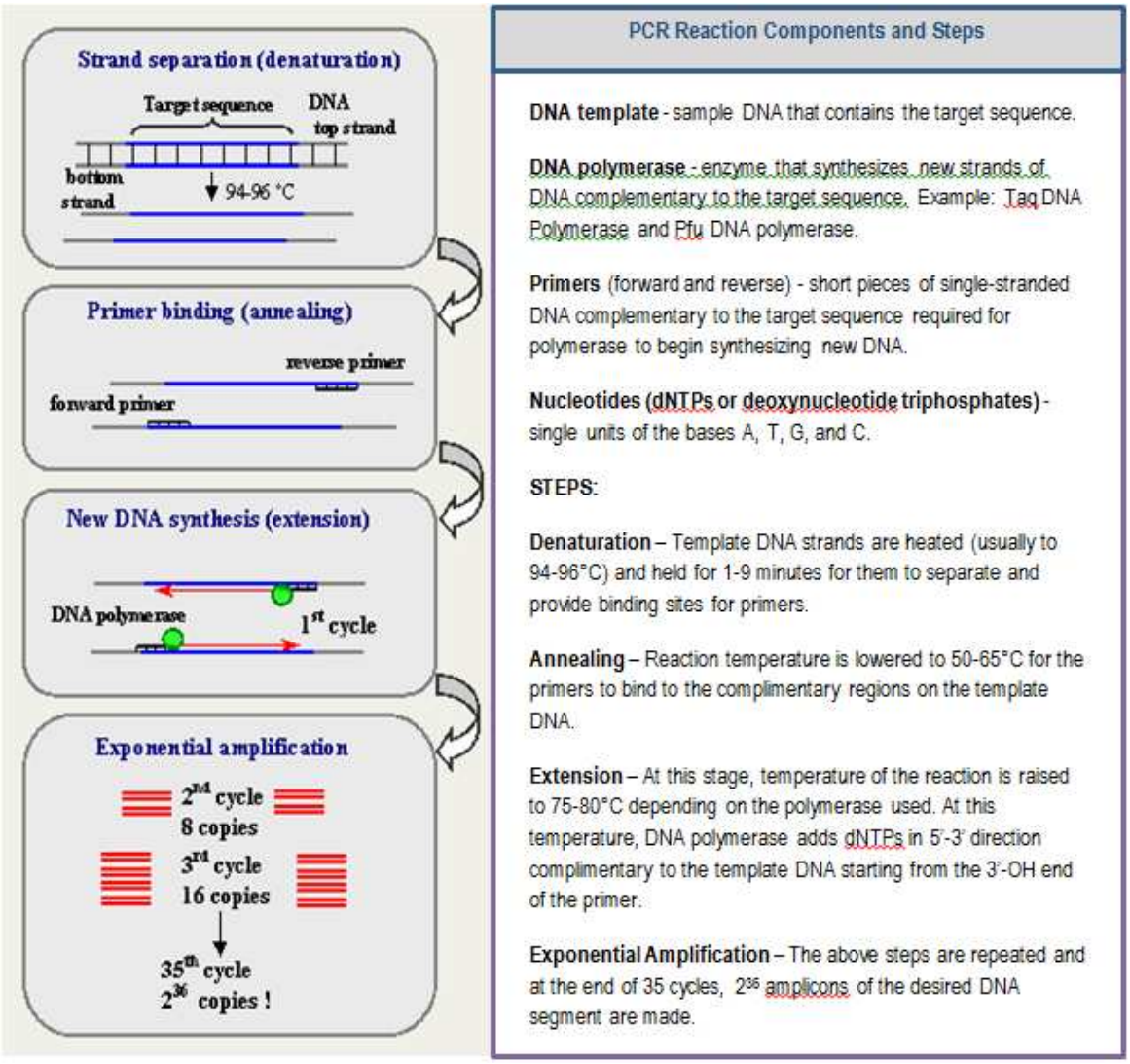


Figure 4. Outline of the principle of PCR. (Adapted from NCBI. http://www.ncbi.nlm.nih.gov/genome/probe/doc/TechPCR.shtml)

Assay Design: Refer to appendix for experimental design and details

After determining the IC_{50} of XB05 and 5'AZA for both cell lines, they were treated in flasks to collect post-treatment RNA. Cells were plated in flasks at the recommended count of 1×10^5 cells/ml. Both the cell lines were treated with both XB05 and 5'AZA at their respective IC_{50}. RNA from cells was collected at different time intervals – 48- and

96-hours. Cells collected were homogenized with Trizol reagent. RNA was extracted as per manufacturer's protocol. (Outlined in Appendix) Extracted RNA was quantified using NanoDrop. Using RT-PCR, 1ug/uL of RNA was converted to complimentary DNA (cDNA). Protocol as per manufacturer – outlined in appendix. cDNA obtained with this method was then quantified using qRT-PCR to determine the expression of various genes of interest or the genes which are highly methylated in AML. Also, levels of the three DNA methyltransferases was also determined to evaluate if the expression of these genes decreases after treatment with XB05 and 5'AZA. We quantified the expression of the following genes; Hypermethylated in Cancer – 1 (HIC-1), estrogen receptor – alpha (ERα), p16, Methylguanine-DNA Methyltransferase (MGMT), Death-Associated Protein Kinase 1 (DAPK1), DNMT1, DNMT3a, DNMT3b and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR experiments were performed to elucidate that at what time interval, does XB05 result in down-regulation of DNMTs and in turn result in re-expression of the hypermethylated genes. qRT-PCR protocol as described by the manufacturer and outlined in appendix.

EpiTect Methyl II PCR Array (Qiagen®): *Refer to appendix for experimental details*

This method is based on detection of remaining input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme. The restriction enzymes digest unmethylated and methylated DNA. Following digestion, the remaining DNA in each individual enzyme reaction is quantified by RT-PCR using primers that flank a promoter for the gene region of interest. The relative fractions of methylated and unmethylated DNA are subsequently determined by comparing the

amount in each digest with that of a mock (no enzymes added) digest using a ΔCT method. We used a 22 gene panel array to evaluate the methylation pattern of promoter sequences of commonly methylated genes in leukemia at 48 & 96 hour intervals in non-treated cells, & following treatment with XB05 & azacytidine.

AFF1 (Oncogene)	AF4/FMR2 family, member 1
CD9 (TS)	Cluster differentiation 9
CEBPD (TS)	CCAAT/enhancer binding protein (C/EBP), delta
CTNNA1 (TS)	Catenin (cadherin-associated protein), alpha 1
DRD2 (TS)	Dopamine receptor D2
EXT1 (TS)	Exostosin glycosyltransferase 1
FANCC (Oncogene)	Fanconi anemia, complementation group C
FANCL (Oncogene)	Fanconi anemia, complementation group L
HCK (Oncogene)	HCK proto-oncogene, Src family tyrosine kinase
HOXA7 (Oncogene)	Homeobox A7
HOXB5 (Oncogene)	Homeobox B5
JUNB (Oncogene)	Jun B proto-oncogene
LMNA (TS)	Lamin A/C
MAFB (Oncogene)	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B
MEN1 (TS)	Multiple endocrine neoplasia I
NFATC1 (Oncogene)	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1

Table 2. Oncogenes and tumor suppressor (TS) genes in 22-panel EpiTect Methyl II PCR Panel.

Refer to http://www.ncbi.nlm.nih.gov/gene for detailed gene functions

Table 2 (cont'd)

NPM1 (Oncogene)	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)
PER2 (TS)	Period circadian clock 2
SLC4A8 (Oncogene)	Solute carrier family 4, sodium bicarbonate cotransporter, member 8
SPOCK2 (TS)	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2
TLE1 (Oncogene)	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)
TP53 (TS)	Tumor protein p53

Western Blot

Principle: Primarily used to detect specific proteins expressed in a tissue or cell lysate. Cytoplasmic or nuclear proteins extracted with different protocols are separated using gel electrophoresis based on size and electrical conductivity. The separated proteins are then transferred to a nitrocellulose or PVDF membrane where they are stained with antibodies specific to that target protein. Then secondary antibody tagged with a HRP-chemiluminesence agent is added which attaches to the primary antibody and intensity of the chemiluminesence can be measured using photographic film.

Assay Design: Refer to appendix for experimental design and details

Western Blot was performed on the protein samples obtained after treatment with the two drugs at the time interval which showed maximal DNMT inhibition and re-expression of methylated genes based on the results of PCR. Proteins selected for Western Blot assay were based on the results of PCR and available antibodies. Western blot was performed for the following proteins – DNMT1, DNMT3a, DNMT3b, HIC1, ER-α, MGMT, p16, p21, alpha-tubulin, beta-actin and SOX9. Cytoplasmic and nuclear proteins were extracted from both treated and untreated cells and quantified using Coomassie Blue (BioRad) or Bradford protein assay (BioRad). Using commercially available Bio-Rad SDS-PAGE gels, the nuclear and cytoplasmic protein were separated. After separation, proteins were transferred to PVDF membranes using wet transfer method. Membranes were incubated with primary antibody overnight at 4ºC. After appropriate washings, membranes were incubated with secondary antibody tagged with horseradish peroxidase (HRP). Using enhanced chemiluminescence (ECL, GE Healthcare Life Science) substrate, amount of protein expression was quantified on X-ray films. GAPDH was used as a control for cytoplasmic proteins and histone H3 was used as a control for nuclear proteins.

Protein Quantitation by Coomassie (Bradford) protein assay

Coomassie (Bradford) protein assay was used for colorimetric detection and quantitation of total protein. Protocol as per manufacturer's recommendations – outlined in appendix.

Immunohistochemistry

Principle: Immunohistochemistry or IHC involves detecting an expression of a protein in a cell or a tissue specimen by targeting the antigen with primary antibody followed by incubation with fluorescence or peroxidase tagged secondary antibody to grade the protein's expression in the tissue.

Assay Design: Refer to appendix for experimental design and details

Using cytospin machine, treated and untreated cells from both cell lines were plated on the slides and fixed with ice cold methanol for 15 minutes. Permeabilize cells with 0.5% TritonX. Using the Pierce peroxidase detection kit (Thermo Scientific®), slides were stained. DNMT1 and HIC1 expression was probed in non-treated and cells treated at 48- and 96-hour intervals. Mouse and goat serum was used as negative control. Primary antibodies were used at 1:25 and secondary antibodies were used at 1:50 dilution. Protocol outlined in appendix.

RESULTS

MTT-ASSAYS

MTT-assay results for Kasumi-1 cell line reported in both tabulated and graphical illustrations.

nM	0.00	50.00	100.00	200.00	400.00	800.00
XBO5	0.57	0.56	0.57	0.49	0.45	0.30
XBO5	0.63	0.60	0.61	0.56	0.50	0.38
XBO5	0.59	0.60	0.63	0.56	0.50	0.43
XBO5	0.62	0.65	0.57	0.58	0.57	0.47
AVG	0.60	0.60	0.59	0.55	0.50	0.40
% Cell Proliferation	100.00	99.79	98.46	90.73	83.79	65.79
nM	0.00	50.00	100.00	200.00	400.00	800.00
AZA	0.58	0.56	0.47	0.43	0.36	0.34
AZA	0.65	0.60	0.52	0.42	0.38	0.35
AZA	0.61	0.58	0.53	0.43	0.40	0.32
AZA	0.70	0.55	0.47	0.39	0.35	0.33
AVG	0.64	0.57	0.50	0.42	0.37	0.33
% Cell Proliferation	100.00	89.78	77.98	65.28	58.83	52.38

Table 3. First MTT-assay performed on Kasumi-1 cells with serial concentrations of XB05 and 5'azacytidine. Percent proliferation is reported with serial concentration of XB05 and 5'azacytidine.

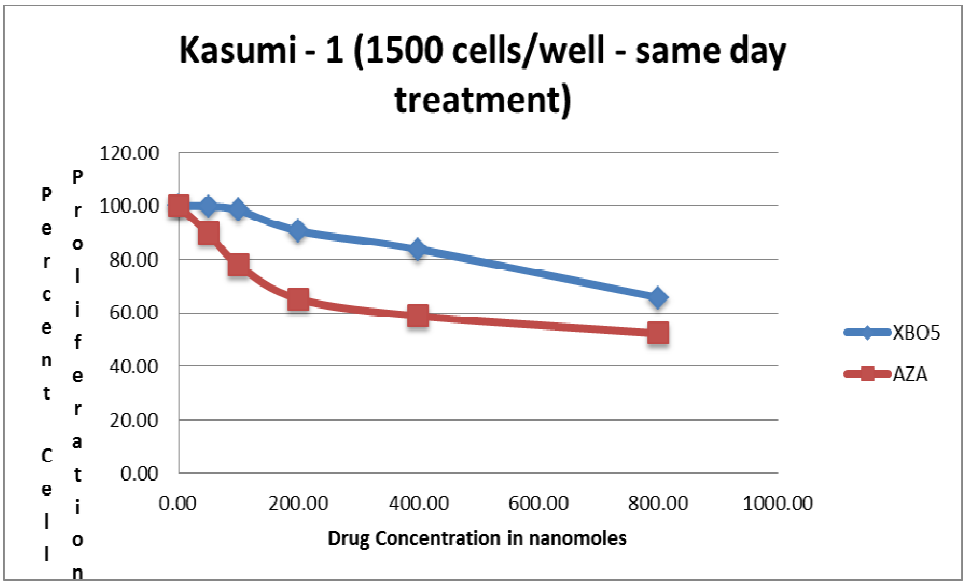


Figure 5. Percent cell proliferation in Kasumi-1 cell line is represented on graph showing decreased proliferation with increasing concentration.

nM	0.00	100.00	200.00	400.00	600.00	800.00	1000.00	1200.00	1600.00
XBO5	0.48	0.47	0.45	0.38	0.35	0.37	0.33	0.29	0.31
XBO5	0.50	0.48	0.47	0.41	0.42	0.35	0.31	0.29	0.29
XBO5	0.50	0.48	0.45	0.43	0.36	0.34	0.33	0.28	0.28
XBO5	0.48	0.51	0.45	0.41	0.37	0.33	0.33	0.29	0.31
Avg	0.49	0.48	0.46	0.41	0.38	0.35	0.32	0.29	0.30
% Cell Proliferation	100.00	98.88	92.81	82.61	76.70	70.47	66.04	58.80	60.63
nM	0.00	100.00	200.00	400.00	600.00	800.00	1000.00	1200.00	1600.00
AZA	0.49	0.40	0.33	0.28	0.28	0.27	0.25	0.24	0.25
AZA	0.59	0.41	0.34	0.29	0.29	0.30	0.28	0.27	0.25
AZA	0.57	0.45	0.36	0.30	0.29	0.31	0.27	0.26	0.24
AZA	0.53	0.45	0.32	0.31	0.31	0.28	0.28	0.26	0.25
Avg	0.54	0.43	0.34	0.30	0.29	0.29	0.27	0.26	0.25
% Cell Proliferation	100.00	78.78	62.29	54.34	53.70	52.92	49.61	47.36	45.38

Table 4. Second MTT-assay performed on Kasumi-1 cells with serial concentrations of XB05 and 5'azacytidine. Percent proliferation is reported with serial concentration of XB05 and 5'azacytidine.

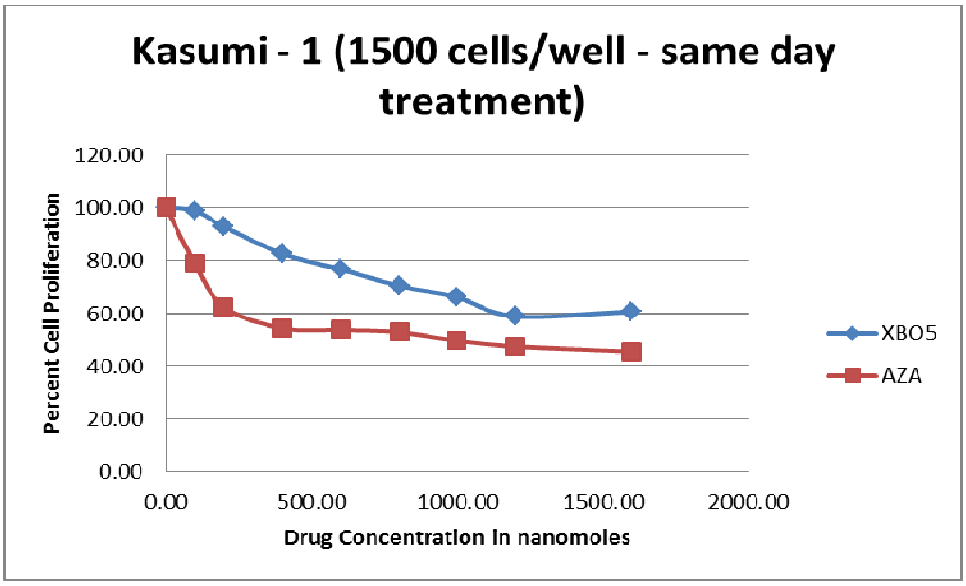


Figure 6. Percent cell proliferation in Kasumi-1 cell line is represented on graph showing decreased proliferation with increasing concentration.

nM	0.00	100.00	200.00	400.00	600.00	800.00	1000.00	1200.00	1600.00
XBO5	0.51	0.45	0.47	0.41	0.36	0.35	0.31	0.28	0.27
XBO5	0.49	0.46	0.47	0.43	0.34	0.31	0.29	0.25	0.24
XBO5	0.50	0.48	0.42	0.41	0.38	0.35	0.30	0.28	0.28
XBO5	0.50	0.41	0.45	0.39	0.38	0.38	0.31	0.27	0.25
Avg	0.50	0.45	0.45	0.41	0.37	0.35	0.30	0.27	0.26
% Cell Proliferation	100.00	90.18	90.68	82.26	73.18	69.67	60.05	54.39	51.83
nM	0.00	100.00	200.00	400.00	600.00	800.00	1000.00	1200.00	1600.00
AZA	0.52	0.39	0.32	0.27	0.26	0.28	0.26	0.25	0.24
AZA	0.52	0.38	0.33	0.31	0.29	0.29	0.27	0.26	0.24
AZA	0.52	0.39	0.34	0.30	0.29	0.31	0.28	0.27	0.25
AZA	0.53	0.40	0.33	0.31	0.32	0.31	0.32	0.27	0.23
Avg	0.52	0.39	0.33	0.30	0.29	0.30	0.28	0.26	0.24
% Cell Proliferation	100.00	75.12	63.25	56.60	54.98	56.70	53.16	50.19	45.89

Table 5. Third MTT-assay performed on Kasumi-1 cells with serial concentrations of XB05 and 5'azacytidine. Percent proliferation is reported with serial concentration of XB05 and 5'azacytidine.

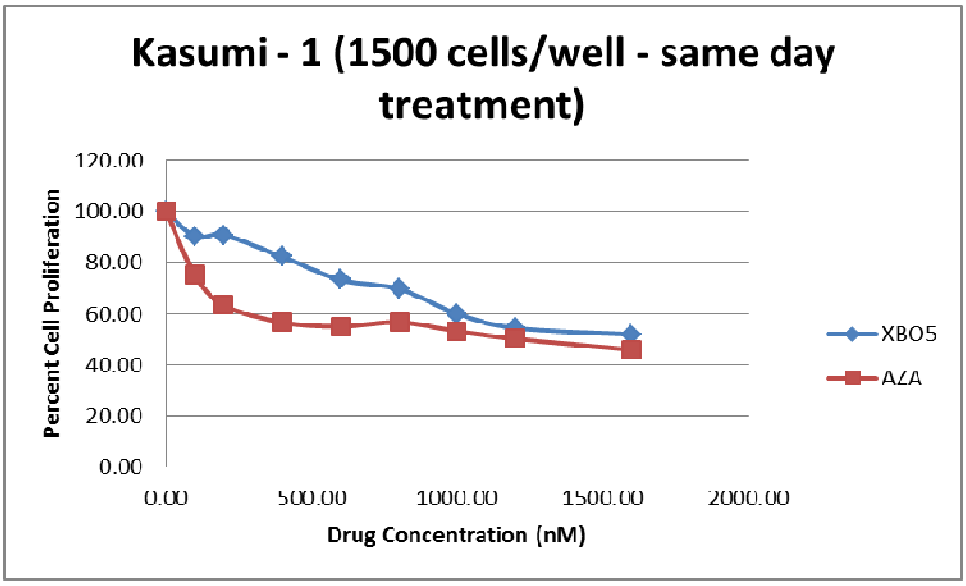


Figure 7. Percent cell proliferation in Kasumi-1 cell line is represented on graph showing decreased proliferation with increasing concentration.

XBO5 (nM)	0.00	50.00	100.00	200.00	400.00	600.00	800.00	1000.00	1200.00	1600.00
% Cell Proliferation	100.00	99.79	98.46	90.73	83.79		65.79			
% Cell Proliferation	100.00		98.88	92.81	82.61	76.70	70.47	66.04	58.80	60.63
% Cell Proliferation	100.00		90.18	90.68	82.26	73.18	69.67	60.05	54.39	51.83
Avg % Cell Proliferation	100.00	99.79	95.84	91.41	82.89	74.94	68.65	63.04	56.59	56.23
Effect	0.00	0.00	0.04	0.09	0.17	0.25	0.31	0.37	0.43	0.44

AZA (nM)	0.00	50.00	100.00	200.00	400.00	600.00	800.00	1000.00	1200.00	1600.00
% Cell Proliferation	100.00	89.78	77.98	65.28	58.83		52.38			
% Cell Proliferation	100.00		78.78	62.29	54.34	53.70	52.92	49.61	47.36	45.38
% Cell Proliferation	100.00		75.12	63.25	56.60	54.98	56.70	53.16	50.19	45.89
Avg % Cell Proliferation	100.00	89.78	77.29	63.61	56.59	54.34	54.00	51.38	48.78	45.63
Effect	0.00	0.10	0.23	0.36	0.43	0.46	0.46	0.49	0.51	0.54

Table 6. Results of combined MTT-assays performed on Kasumi-1 cells with serial concentrations of XB05 and 5'azacytidine. Percent proliferation is reported with serial concentration of XB05 and 5'azacytidine.

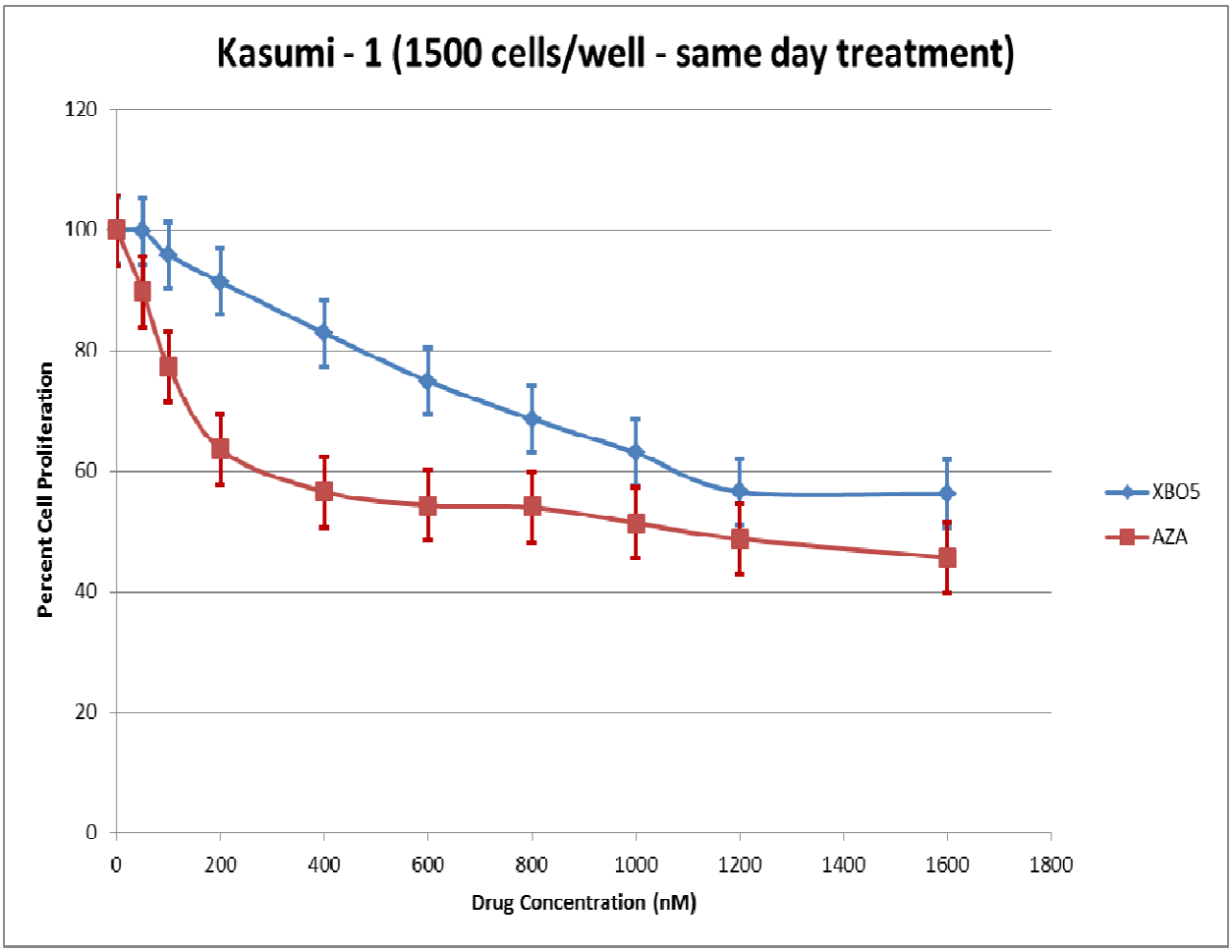


Figure 8. Percent cell proliferation in Kasumi-1 cell line is represented on graph showing decreased proliferation with increasing concentration. Graphical representation of the combined results of three MTT-assays.

These results demonstrate that the cytotoxicity of both drugs increases with increasing concentration; however, the rate of decline in percent viability of cells with azacytidine is greater than seen with XB05.

These results were then exported to CompuSyn® software to calculate the IC_{50} for both drugs in Kasumi-1 cell line.

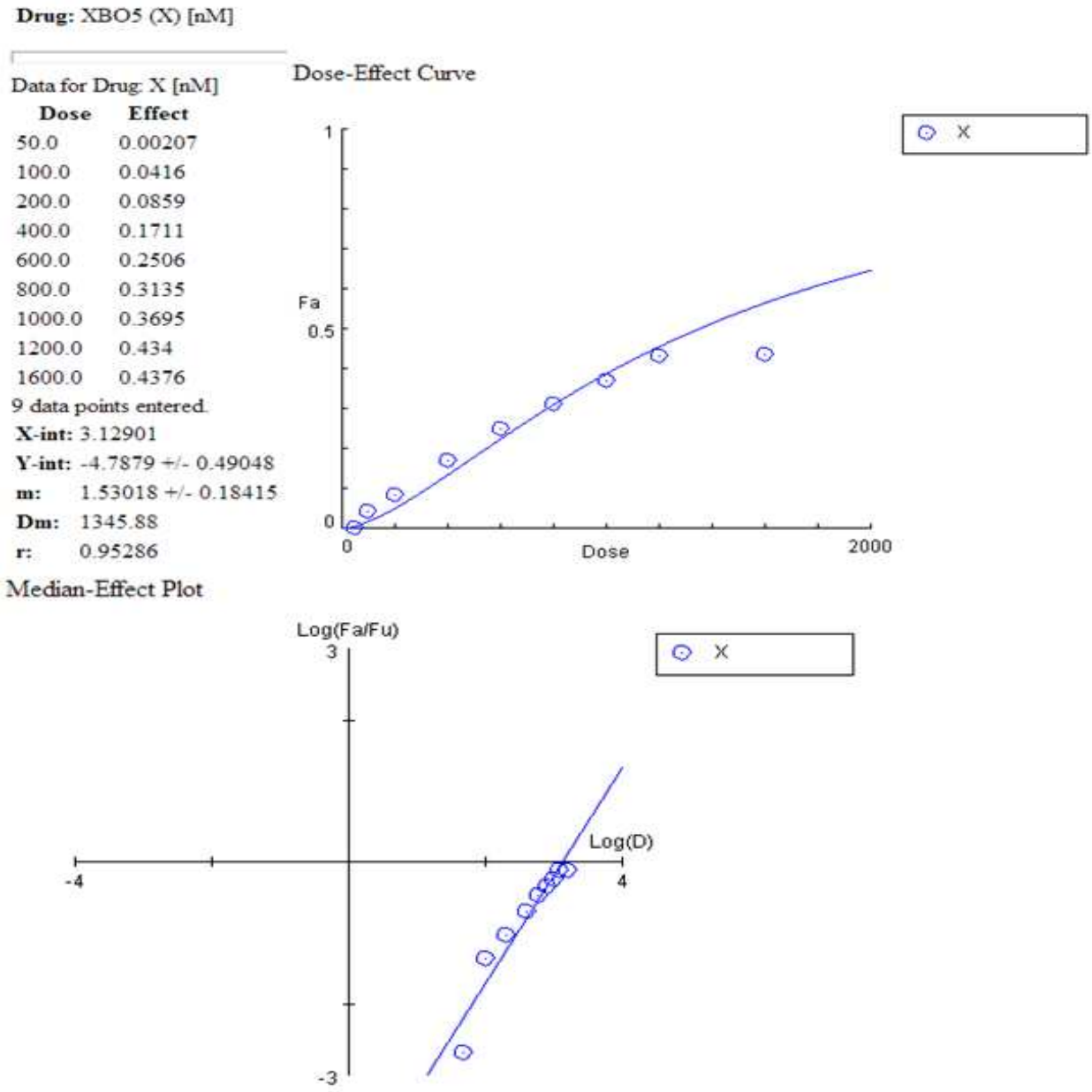


Figure 9. Using the ComuSyn software, IC_{50} for XB05 in Kasumi-1 cell line is calculated which was 1345.88nM. Dose-effect curve is shown which based on the existing data points shows predicted decrease in cell proliferation with increasing dose. Also, based on increasing dose, log decrease in cell proliferation is also shown.

Drug: AZA (A) [nM]

Data for Drug: A [nM]

Dose	Effect
50.0	0.1022
100.0	0.227
200.0	0.3639
400.0	0.434
600.0	0.4566
800.0	0.46
1000.0	0.4861
1200.0	0.5122
1600.0	0.5436

9 data points entered.

X-int: 2.96951

Y-int: -1.7917 +/- 0.19715

m: 0.60336 +/- 0.07402

Dm: 932.198

r: 0.95115

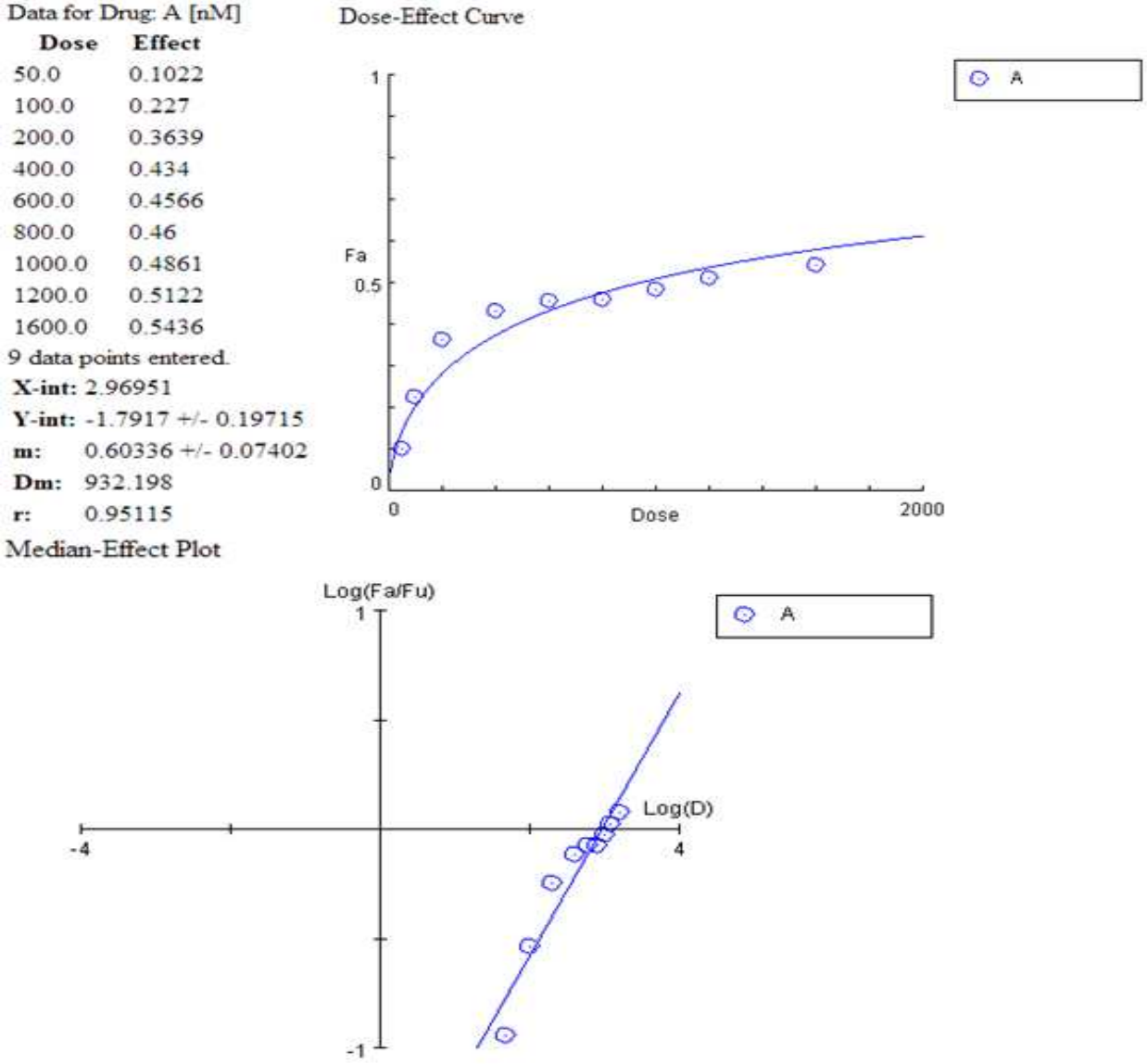


Figure 10. Using the ComuSyn software, IC_{50} for 5'AZA in Kasumi-1 cell line is calculated which was 932.198nM. Dose-effect curve is shown which based on the existing data points shows predicted decrease in cell proliferation with increasing dose. Also, based on increasing dose, log decrease in cell proliferation is also shown.

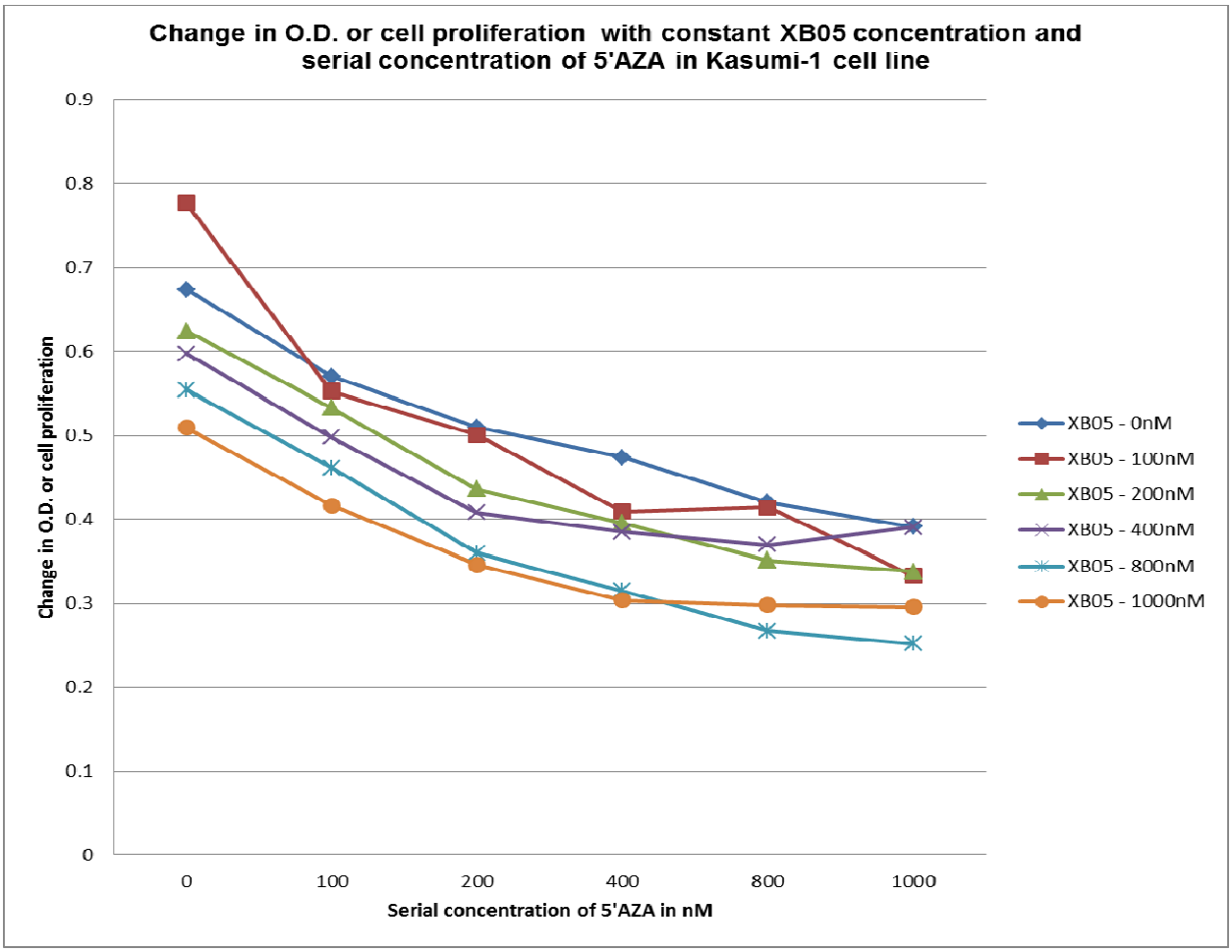


Figure 11. Results of the first MTT-assay performed in Kasumi-1 cell line following combination treatment with XB05 and 5'azacytidine. XB05 concentration was kept constant and 5'azacytidine was added in serial concentrations. Refer to supplementary data.

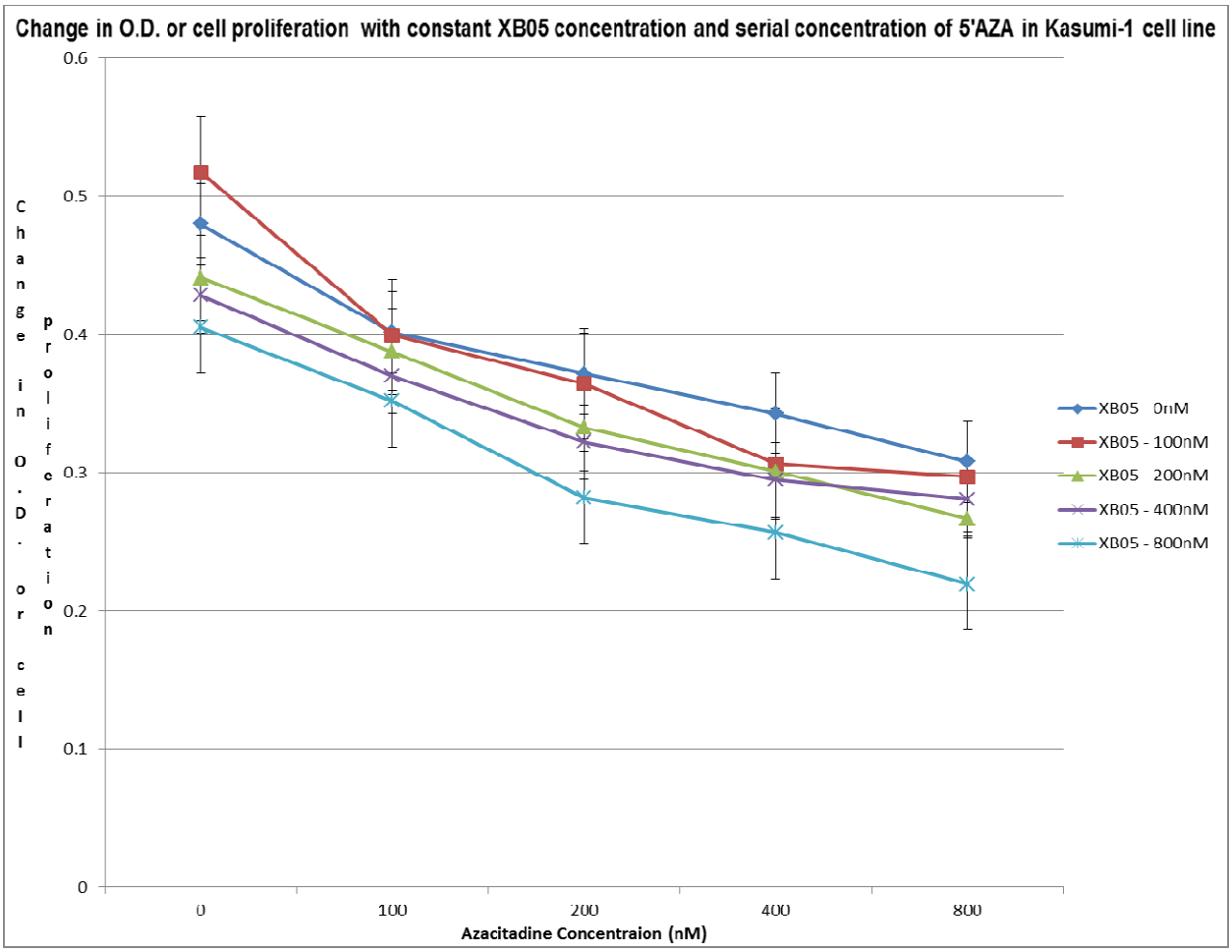


Figure 12. Results of the second MTT-assay performed in Kasumi-1 cell line following combination treatment with XB05 and 5'azacytidine. XB05 concentration was kept constant and 5'azacytidine was added in serial concentrations. Refer to supplementary data.

nM	0.00	50.00	100.00	200.00	400.00	800.00	1000.00
XBO5	1.01	0.91	0.81	0.74	0.64	0.38	0.24
XBO5	0.90	0.91	0.75	0.84	0.68	0.24	0.23
XBO5	0.89	0.86	0.83	0.78	0.61	0.19	0.16
XBO5	0.90	0.84	0.77	0.72	0.55	0.35	0.45
AVG	0.92	0.88	0.79	0.77	0.62	0.29	0.27
% Cell Proliferation	100.00	95.53	85.71	83.46	67.36	31.39	29.30
nM	0.00	50.00	100.00	200.00	400.00	800.00	1000.00
AZA	0.91	0.79	0.78	0.63	0.50	0.35	0.37
AZA	0.89	0.82	0.69	0.61	0.52	0.40	0.41
AZA	0.88	0.80	0.77	0.74	0.61	0.44	0.40
AZA	0.91	0.87	0.78	0.70	0.64	0.44	0.41
AVG	0.90	0.82	0.75	0.67	0.57	0.41	0.40
% Cell Proliferation	100.00	90.98	84.04	74.49	63.19	45.50	44.19

Table 7. First MTT-assay performed on U937 cells with serial concentrations of XB05 and 5'azacytidine. Percent proliferation is reported with serial concentration of XB05 and 5'azacytidine.

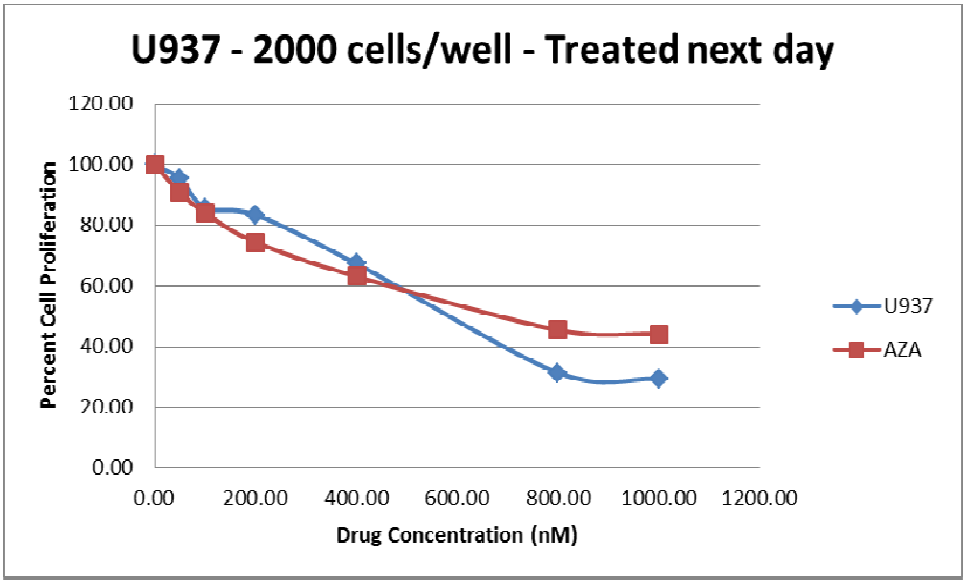


Figure 13. Percent cell proliferation in U937 cell line is represented on graph showing decreased proliferation with increasing concentration.

nM	0.00	50.00	100.00	200.00	400.00	600.00	800.00	1000.00
XBO5	0.65	0.71	0.64	0.58	0.39	0.17	0.29	0.22
XBO5	0.77	0.93	0.81	0.64	0.29	0.15	0.15	0.08
XBO5	0.61	0.75	0.70	0.58	0.33	0.22	0.08	0.07
XBO5	0.75	0.79	0.77	0.61	0.38	0.39	0.01	0.15
Avg	0.70	0.80	0.73	0.60	0.35	0.23	0.13	0.13
% Cell Proliferation	100.00	114.36	104.92	86.22	49.71	33.56	19.20	18.38
nM	0.00	50.00	100.00	200.00	400.00	600.00	800.00	1000.00
AZA	0.77	0.66	0.64	0.58	0.47	0.36	0.30	0.22
AZA	0.73	0.64	0.58	0.55	0.45	0.34	0.29	0.26
AZA	0.75	0.64	0.58	0.51	0.38	0.36	0.32	0.29
AZA	0.75	0.59	0.55	0.53	0.44	0.35	0.35	0.29
Avg	0.75	0.63	0.59	0.54	0.43	0.35	0.31	0.27
% Cell Proliferation	100.00	83.84	78.19	72.17	57.68	46.84	41.72	35.24

Table 8. Second MTT-assay performed on U937 cells with serial concentrations of XB05 and 5'azacytidine. Percent proliferation is reported with serial concentration of XB05 and 5'azacytidine.

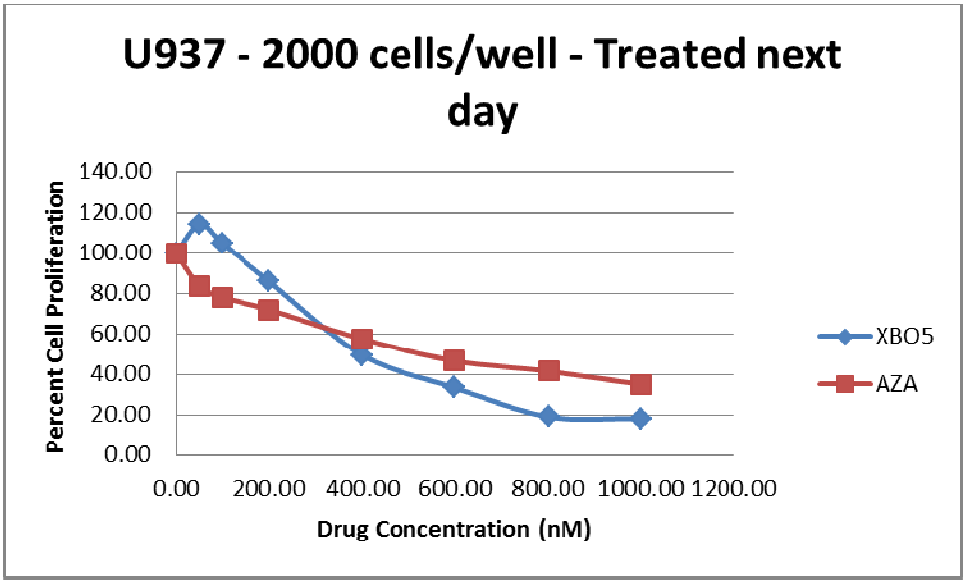


Figure 14. Percent cell proliferation in U937 cell line is represented on graph showing decreased proliferation with increasing concentration.

nM	0.00	50.00	100.00	200.00	400.00	600.00	800.00	1000.00
XBO5	0.72	0.75	0.66	0.53	0.34	0.13	0.03	0.01
XBO5	0.75	0.85	0.81	0.59	0.31	0.33	0.06	0.02
XBO5	0.63	0.69	0.75	0.67	0.33	0.13	0.04	0.12
XBO5	0.74	0.66	0.64	0.61	0.40	0.05	0.41	0.35
Avg	0.71	0.74	0.72	0.60	0.34	0.16	0.14	0.12
% Cell Proliferation	100.00	103.95	101.16	84.40	48.45	22.41	19.05	17.50
nM	0.00	50.00	100.00	200.00	400.00	600.00	800.00	1000.00
AZA	0.72	0.64	0.63	0.55	0.42	0.35	0.33	0.25
AZA	0.69	0.66	0.59	0.53	0.44	0.34	0.31	0.25
AZA	0.68	0.63	0.65	0.54	0.41	0.40	0.36	0.28
AZA	0.71	0.69	0.68	0.50	0.45	0.40	0.35	0.29
Avg	0.70	0.66	0.64	0.53	0.43	0.37	0.34	0.27
% Cell Proliferation	100.00	94.15	91.42	75.64	61.57	53.53	48.15	38.46

Table 9. Third MTT-assay performed on U937 cells with serial concentrations of XB05 and 5'azacytidine. Percent proliferation is reported with serial concentration of XB05 and 5'azacytidine.

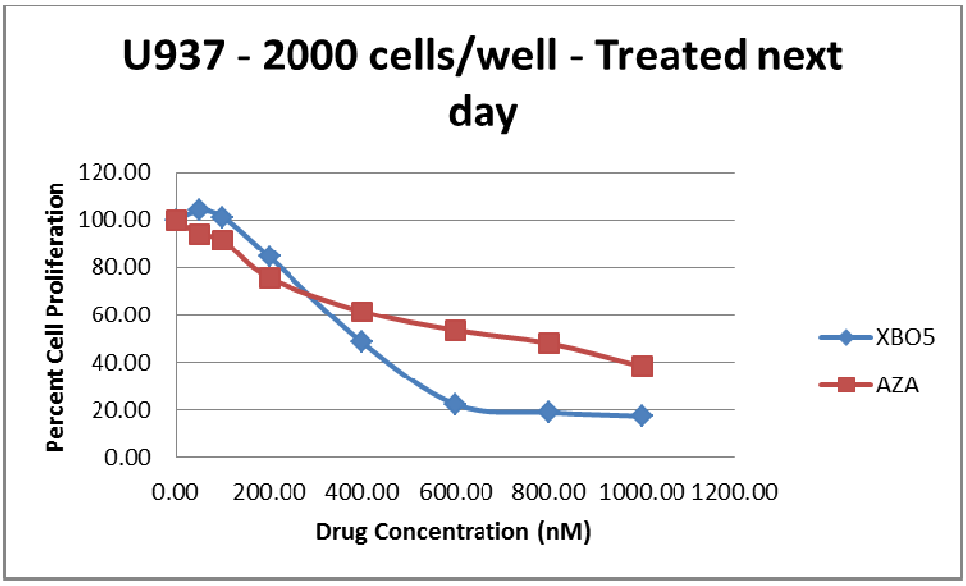


Figure 15. Percent cell proliferation in U937 cell line is represented on graph showing decreased proliferation with increasing concentration.

XBO5 (nM)	0.00	50.00	100.00	200.00	400.00	600.00	800.00	1000.00
% Cell Proliferation	100.00	95.53	85.71	83.46	67.36		31.39	29.30
% Cell Proliferation	100.00	114.36	104.92	86.22	49.71	33.56	19.20	18.38
% Cell Proliferation	100.00	103.95	101.16	84.40	48.45	22.41	19.05	17.50
Avg % Cell Proliferation	100.00	104.61	97.27	84.69	55.17	27.98	23.22	21.73
Effect	0.00	-0.05	0.03	0.15	0.45	0.72	0.77	0.78

AZA (nM)	0.00	50.00	100.00	200.00	400.00	600.00	800.00	1000.00
% Cell Proliferation	100.00	90.98	84.04	74.49	63.19		45.50	44.19
% Cell Proliferation	100.00	83.84	78.19	72.17	57.68	46.84	41.72	35.24
% Cell Proliferation	100.00	94.15	91.42	75.64	61.57	53.53	48.15	38.46
Avg % Cell Proliferation	100.00	89.66	84.55	74.10	60.81	50.19	45.13	39.30
Effect	0.00	0.10	0.15	0.26	0.39	0.50	0.55	0.61

Table 10. Results of combined MTT-assays performed on U937 cells with serial concentrations of XB05 and 5'azacytidine. Percent proliferation is reported with serial concentration of XB05 and 5'azacytidine.

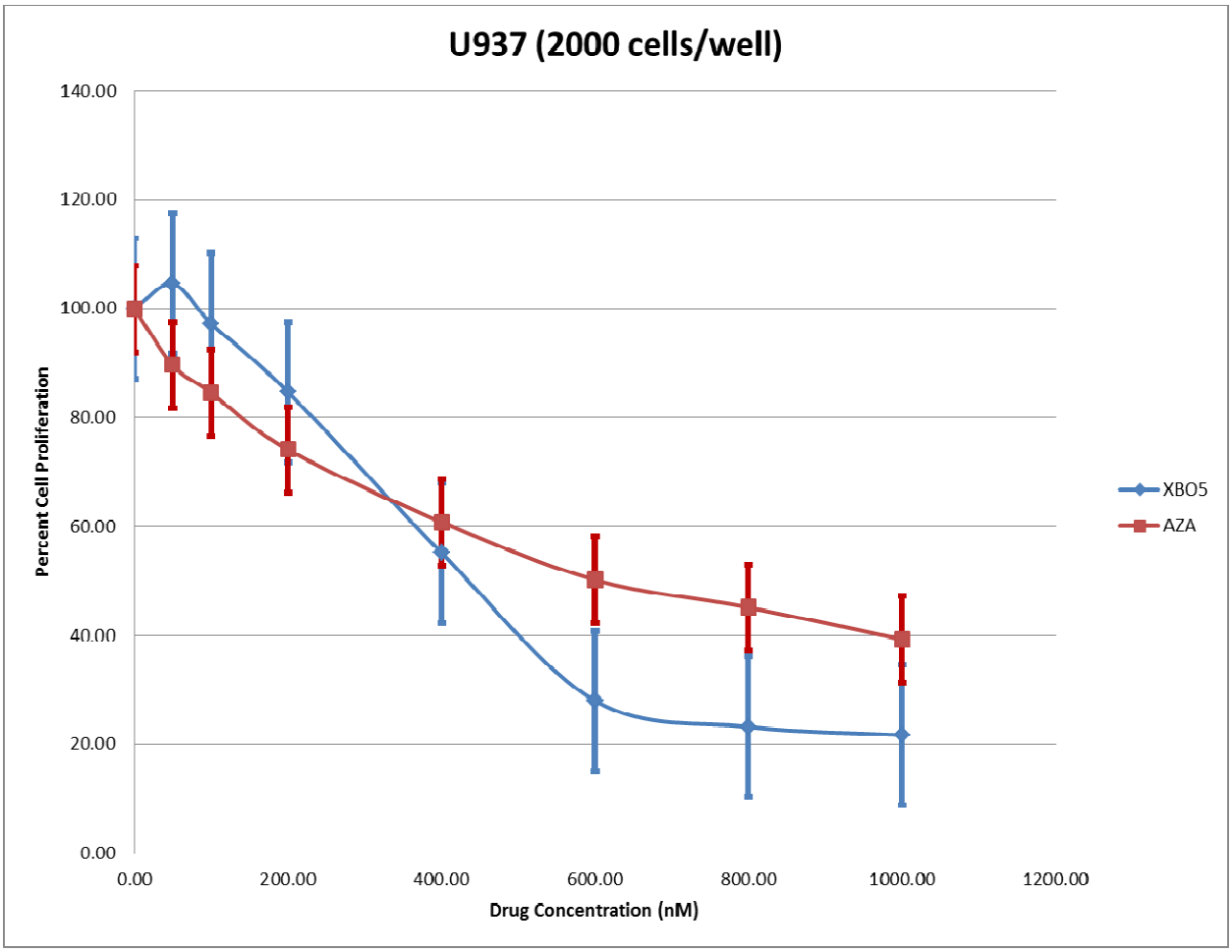


Figure 16. Percent cell proliferation in U937 cell line is represented on graph showing decreased proliferation with increasing concentration. Graphical representation of the combined results of three MTT-assays.

Results of the assay show that with the increasing concentrations of both XB05 and 5'azacytidine, percent cell proliferation decreases; however, the rate of decline in percent cell proliferation of cells with XB05 is greater than seen with 5'azacytidine.

These results were then exported to CompuSyn® software to calculate the IC_{50} for both drugs in U937 cell line. The results obtained from the CompuSyn® software are reported below. IC_{50} for XB05 is 463.297nM and for 5'azacytidine is 633.538nM.

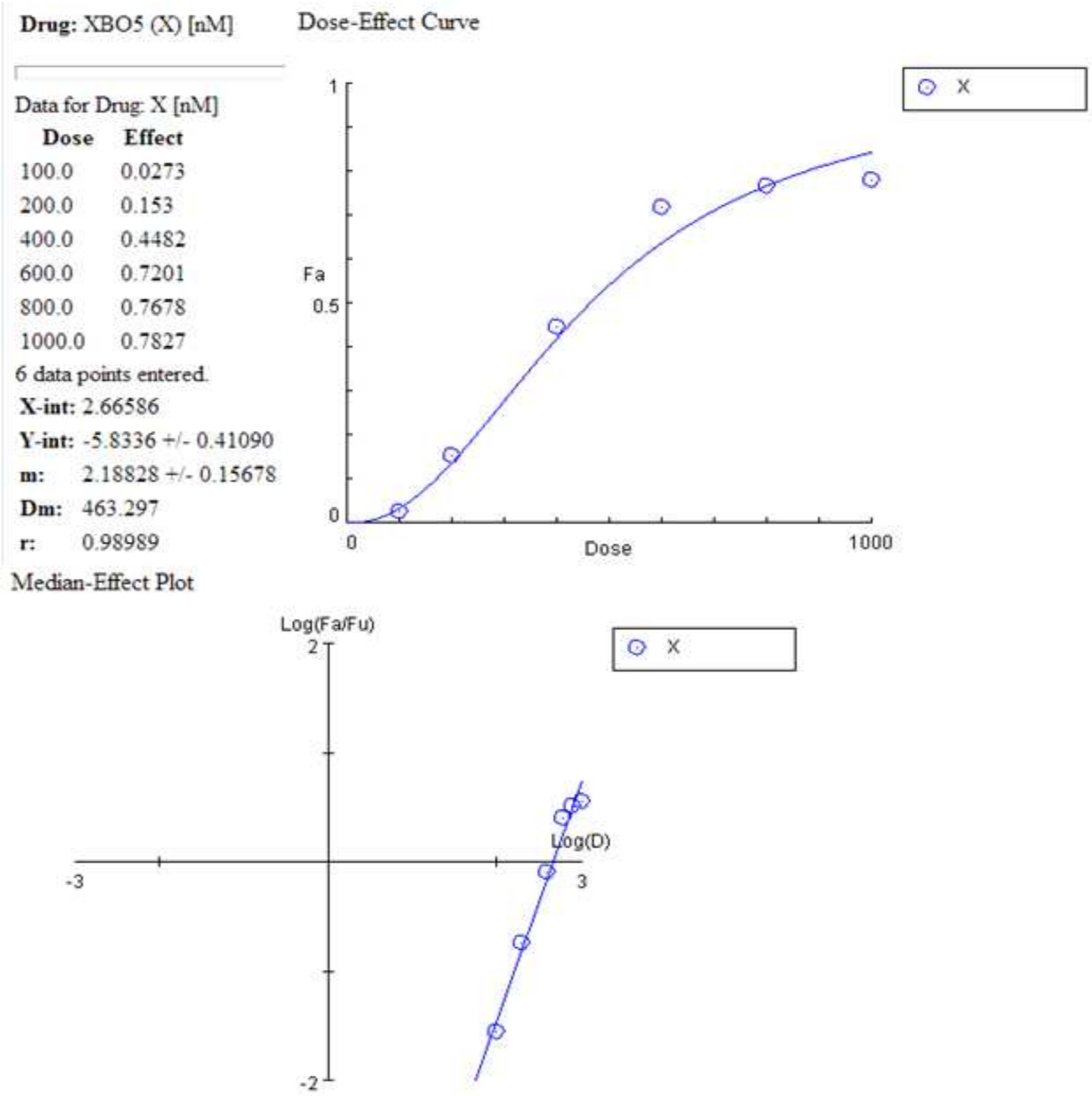


Figure 17. Using the ComuSyn software, IC_{50} for XB05 in U937 cell line is calculated which was 463.297nM. Dose-effect curve is shown which based on the existing data points shows predicted decrease in cell proliferation with increasing dose. Also, based on increasing dose, log decrease in cell proliferation is also shown.

Drug: AZA (A) [nM]

Data for Drug: A [nM]

Dose	Effect
50.0	0.103
100.0	0.1544
200.0	0.2589
400.0	0.3918
600.0	0.4981
800.0	0.5487
1000.0	0.607

7 data points entered.

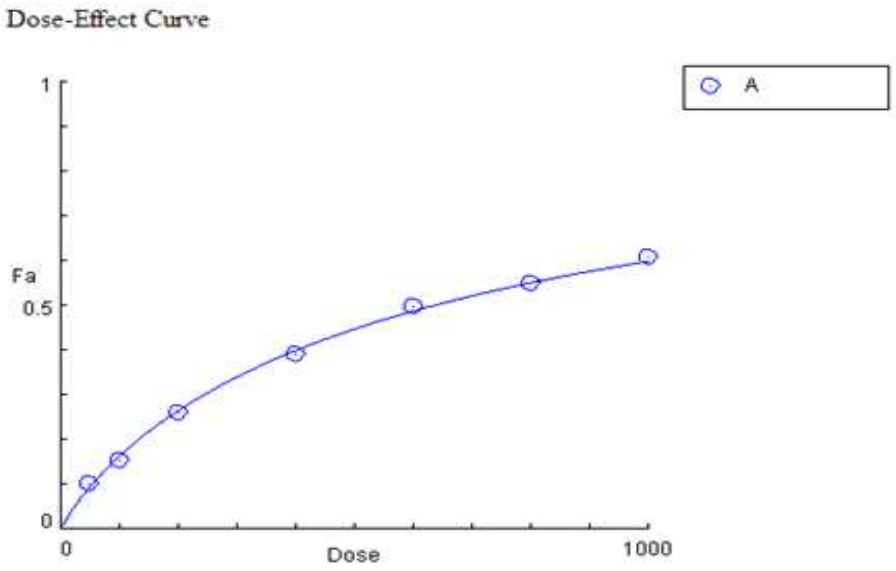
X-int: 2.80177

Y-int: -2.4730 +/- 0.05170

m: 0.88267 +/- 0.02060

Dm: 633.538

r: 0.99864



Median-Effect Plot

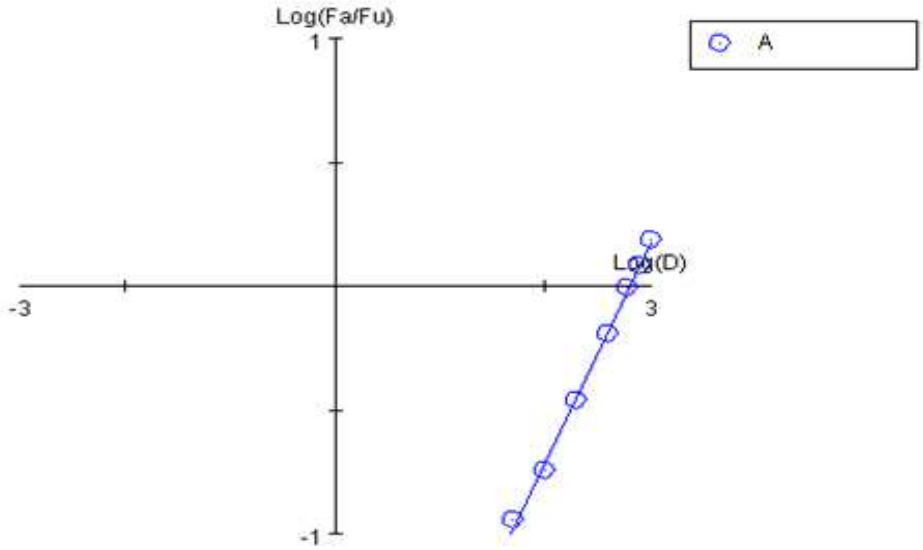


Figure 18. Using the ComuSyn software, IC_{50} for 5'AZA in U937 cell line is calculated which was 633.538nM. Dose-effect curve is shown which based on the existing data points shows predicted decrease in cell proliferation with increasing dose. Also, based on increasing dose, log decrease in cell proliferation is also shown.

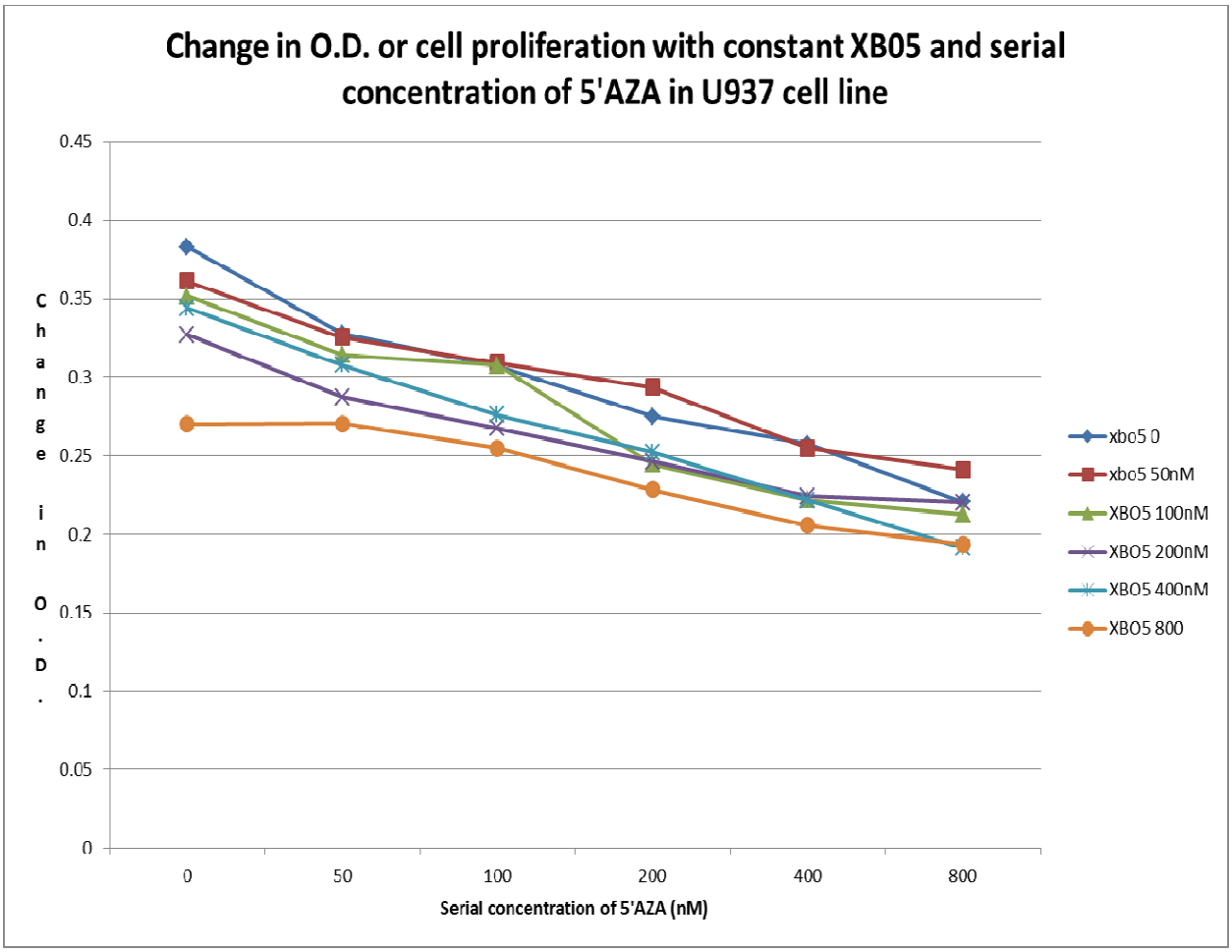


Figure 19. Results of the first MTT-assay performed in U937 cell line following combination treatment with XB05 and 5'azacytidine. XB05 concentration was kept constant and 5'azacytidine was added in serial concentrations. Refer to supplementary data.

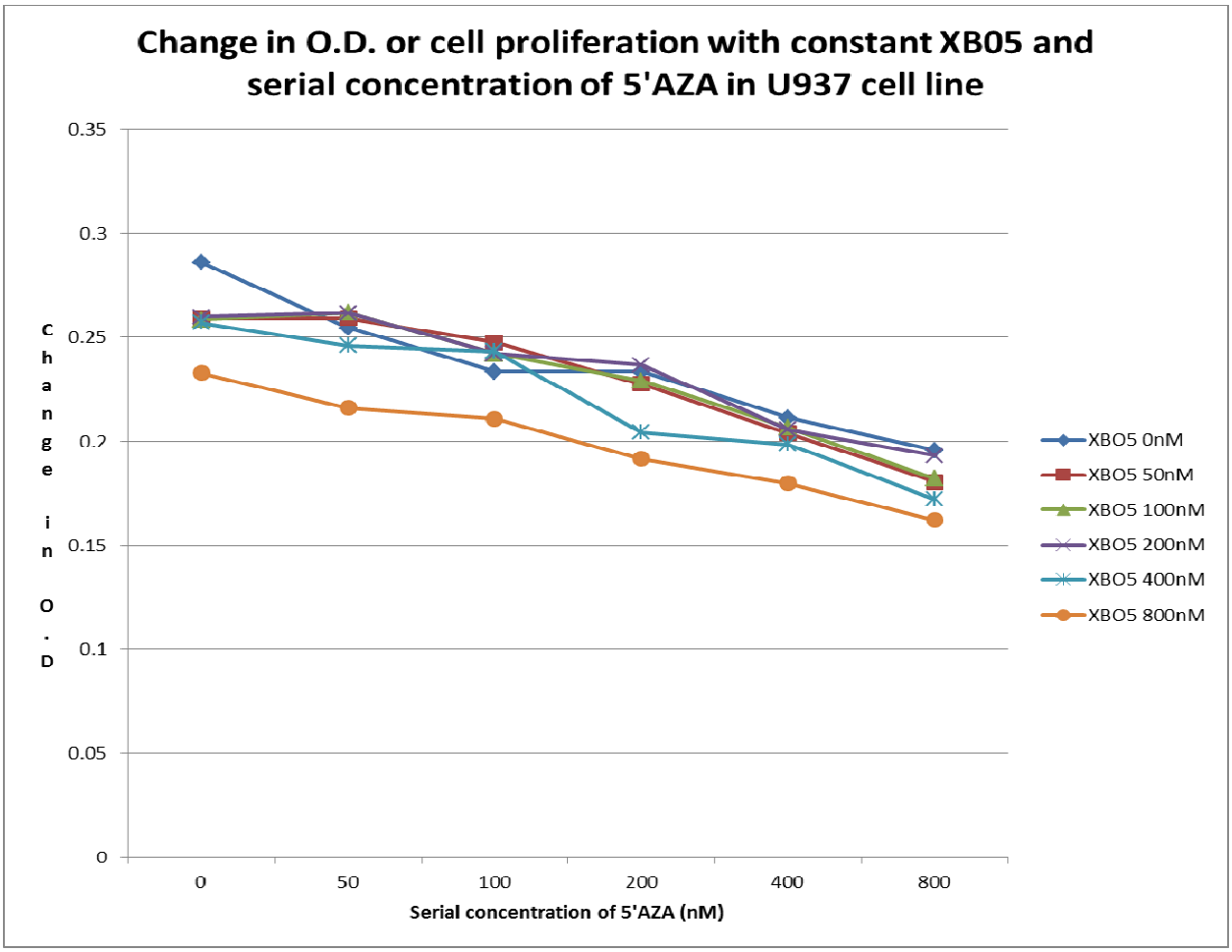


Figure 20. Results of the second MTT-assay performed in U937 cell line following combination treatment with XB05 and 5'azacytidine. XB05 concentration was kept constant and 5'azacytidine was added in serial concentrations. Refer to supplementary data.

POLYMERASE CHAIN REACTION (PCR):

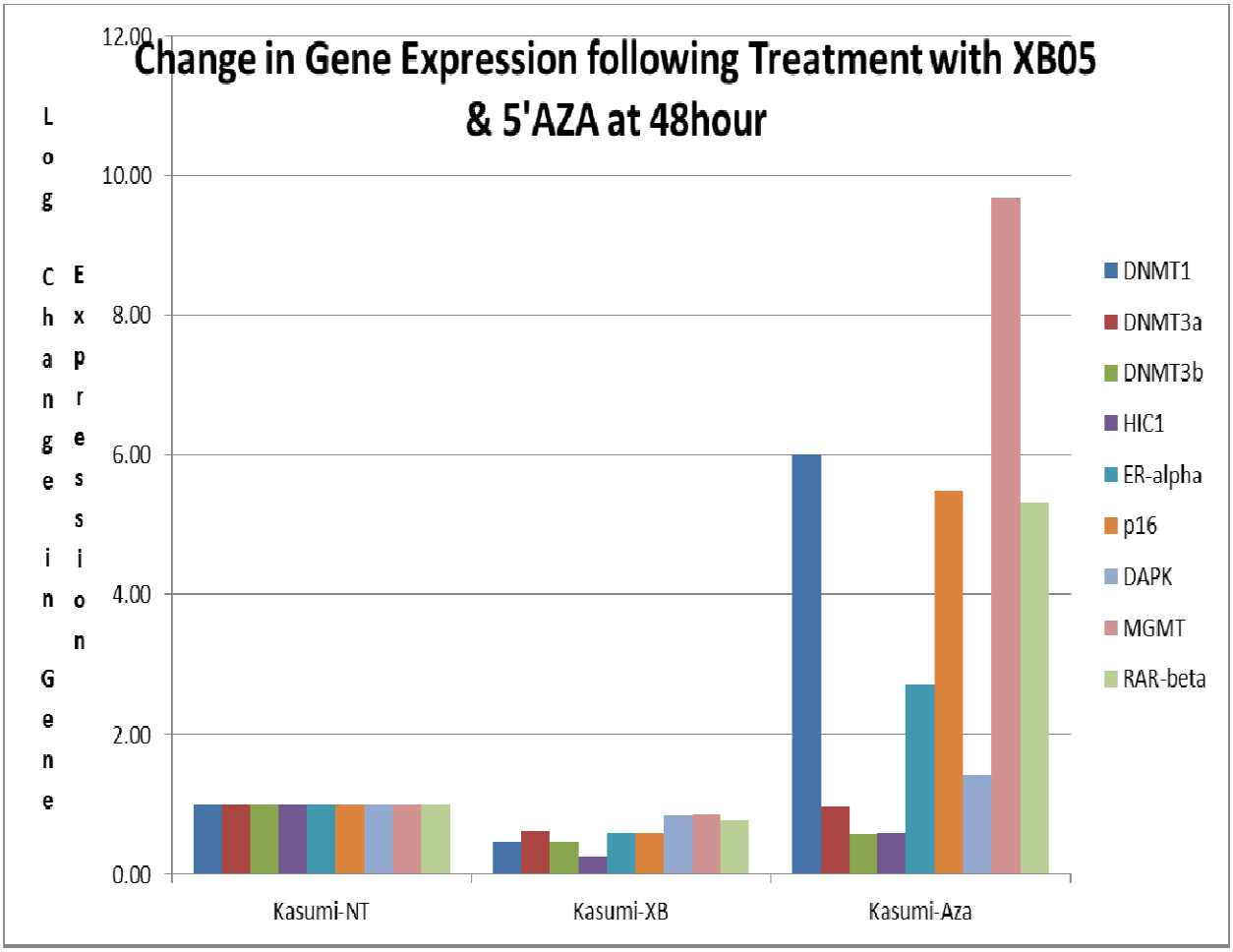


Figure 21. Results of PCR performed on Kasumi-1 cell line without treatment and following treatment with XB05 and 5'azacytdine at 48-hour interval are shown. At 48 hours, following treatment with XB05, expression of three DNMTs (DNMT1, 3a and 3b) decreased in Kasumi-1 cell line as compared to non-treated cells. Increased expression of methylated genes was not seen following XB05 treatment at 48 hours. Following treatment with 5'azacytidine at 48 hours, expression of DNMT1 increased; there was no change in the expression of DNMT3a and expression of DNMT3b increased. However, expression of genes thought to methylated in leukemia increased following treatment with 5'azacytidine at 48 hours. Refer to supplementary data.

.

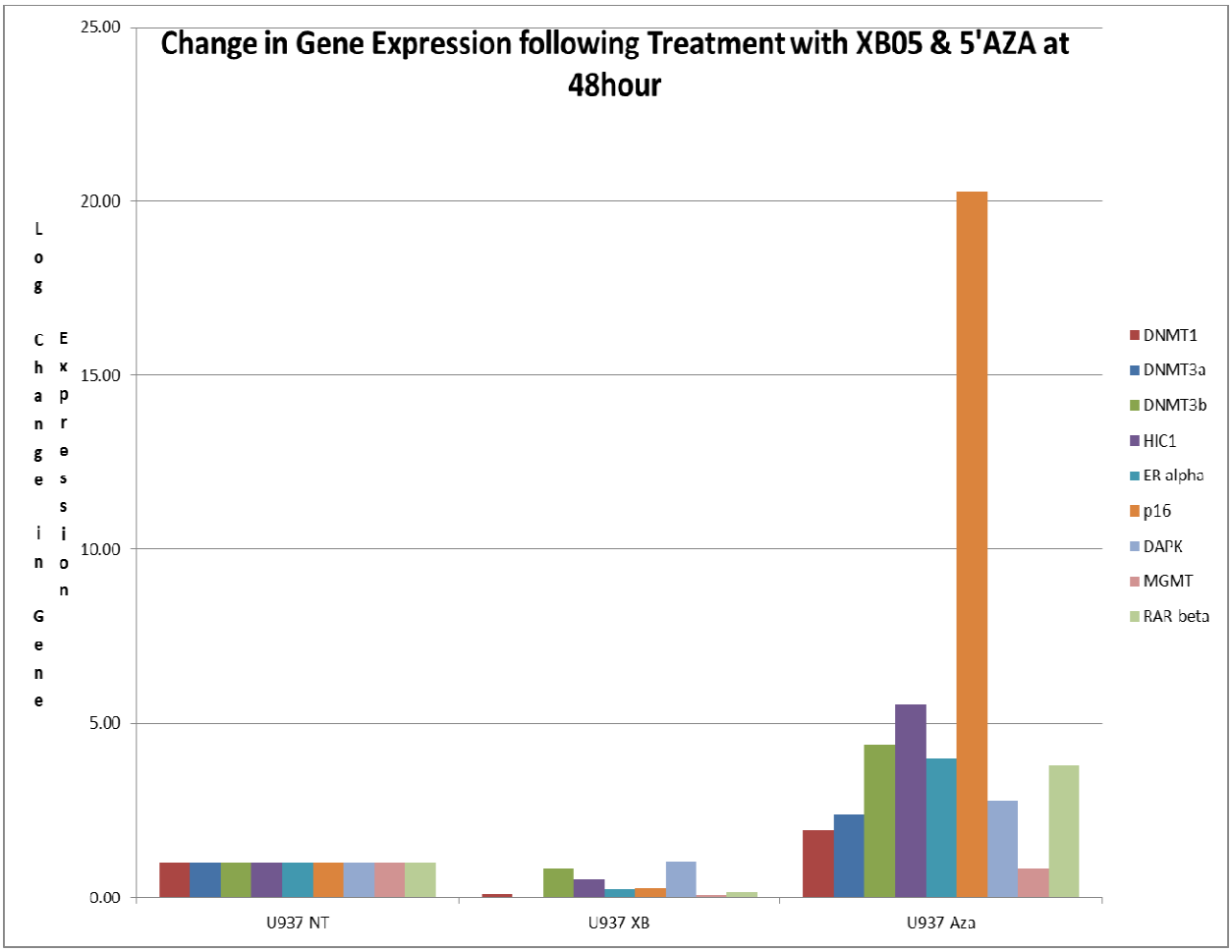


Figure 22. Results of PCR performed on U937 cell line without treatment and following treatment with XB05 and 5'azacytidine at 48-hour interval are shown. At 48 hours, following treatment with XB05, expression of all three DNMTs (DNMT1, 3a and 3b) decreased. Again, there was no increased expression of methylated genes noted at 48 hours following treatment with XB05. Following treatment with 5'azacytidine, expression all three DNMTs increased. Also, expression of genes except MGMT increased at 48 hours after 5'azacytidine treatment. Refer to supplementary data.

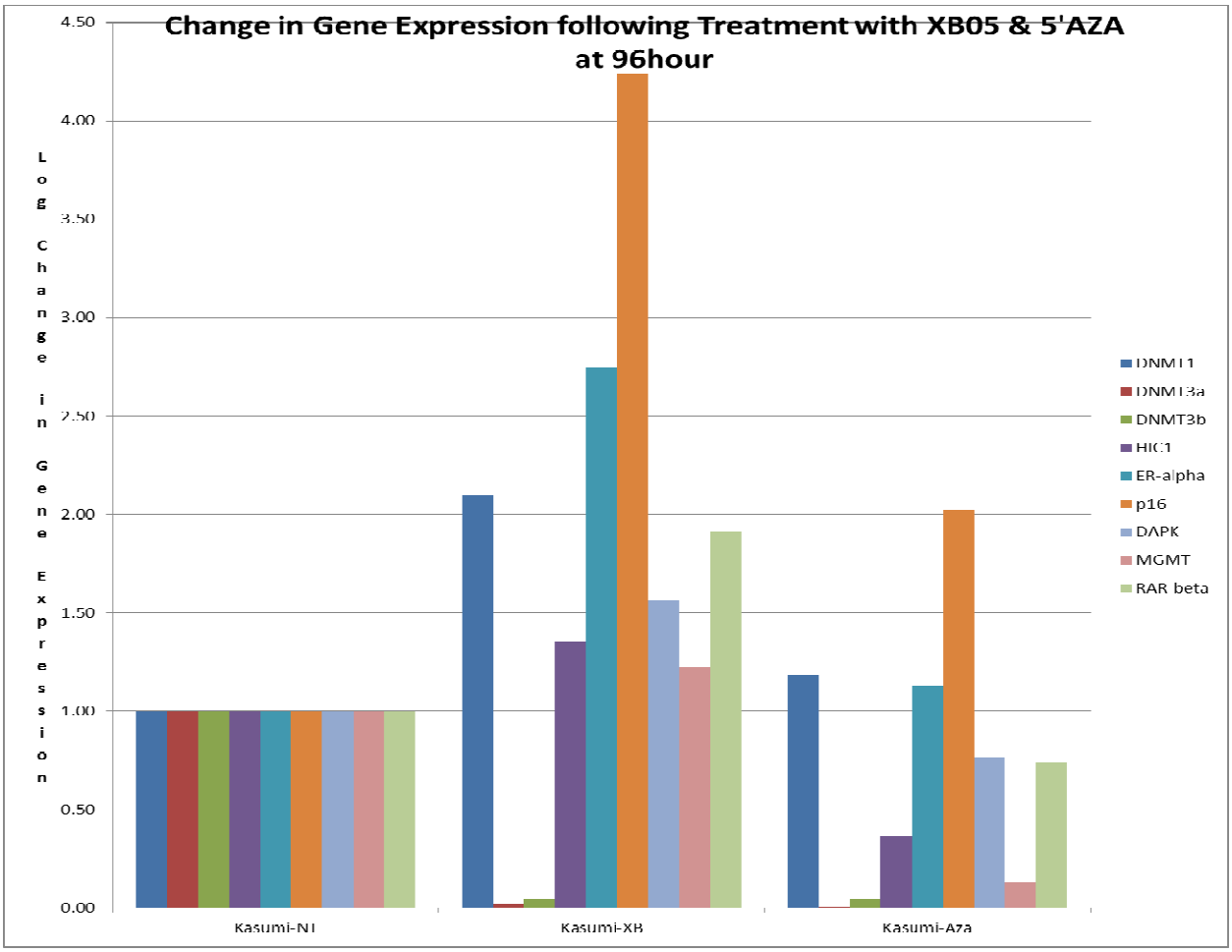


Figure 23. Results of PCR performed on Kasumi-1 cell line without treatment and following treatment with XB05 and 5'azacytidine at 96-hour interval are shown. At 96 hours following treatment with XB05, expression of DNMT1 increased. However, expression of DNMT3a and 3b decreased. Also, expression of genes methylated in leukemia increased following treatment with XB05 at 96 hours. Following treatment with 5'azacytidine at 96 hours, expression of DNMT1 increased but the expression of DNMT3a and 3b decreased. Expression of only ER-alpha and p16 increased, but expression of other genes decreased following treatment with 5'azacytidine. Refer to supplementary data.

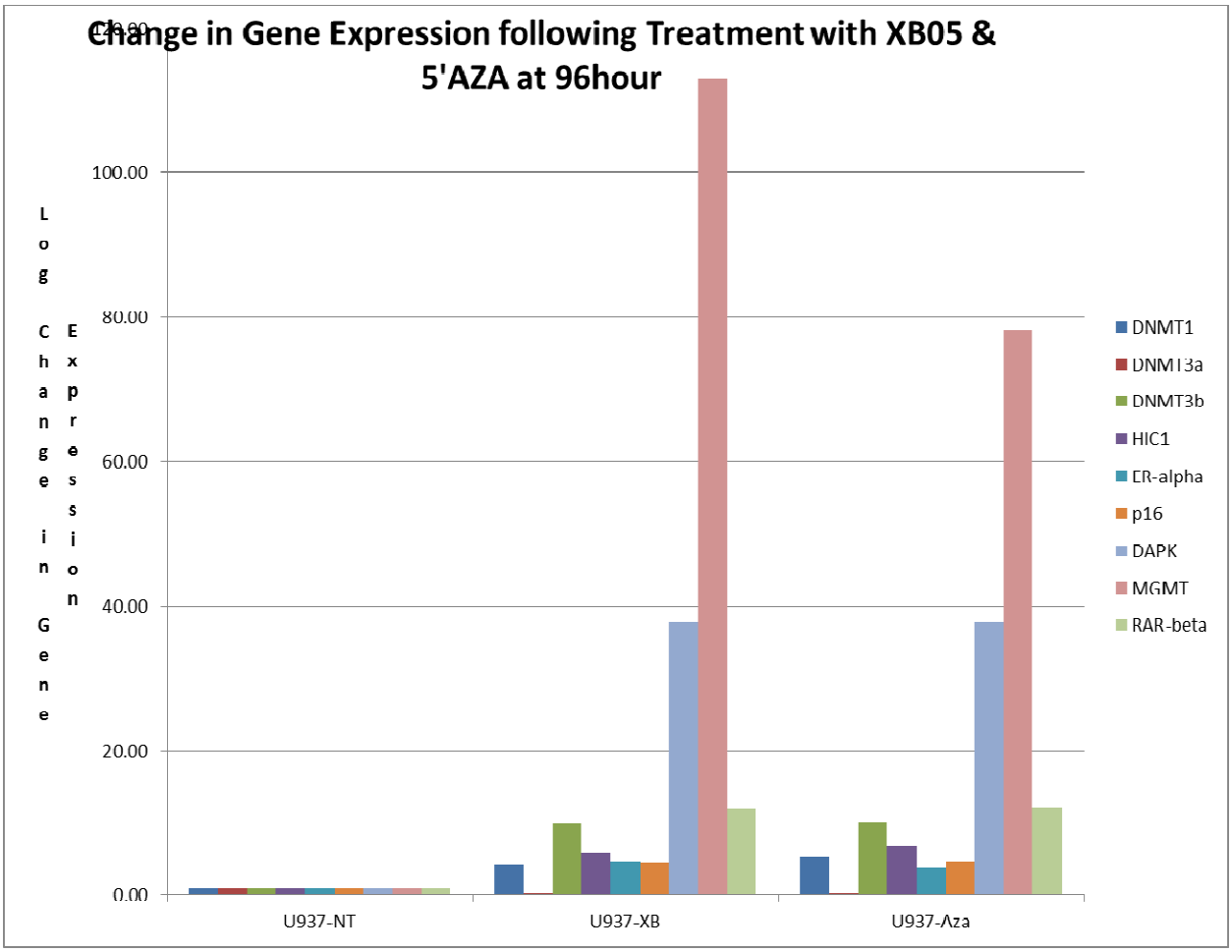


Figure 24. Results of PCR performed on U937 cell line without treatment and following treatment with XB05 and 5'azacytidine at 96-hour interval are shown. At 96 hours, following treatment with XB05 expression of DNMT3a decreased while expression of DNMT1 and 3b increased. Expression of tumor suppressor genes methylated in leukemia increased following XB05 treatment at 96 hours. Following treatment with 5'azacytidine, expression of DNMT3a decreased again, while expression of DNMT1 and 3b increased. Expression of genes methylated in leukemia also increased following treatment with 5'azacytidine. Refer to supplementary data.

EpiTect Methyl II PCR Array (Qiagen®)

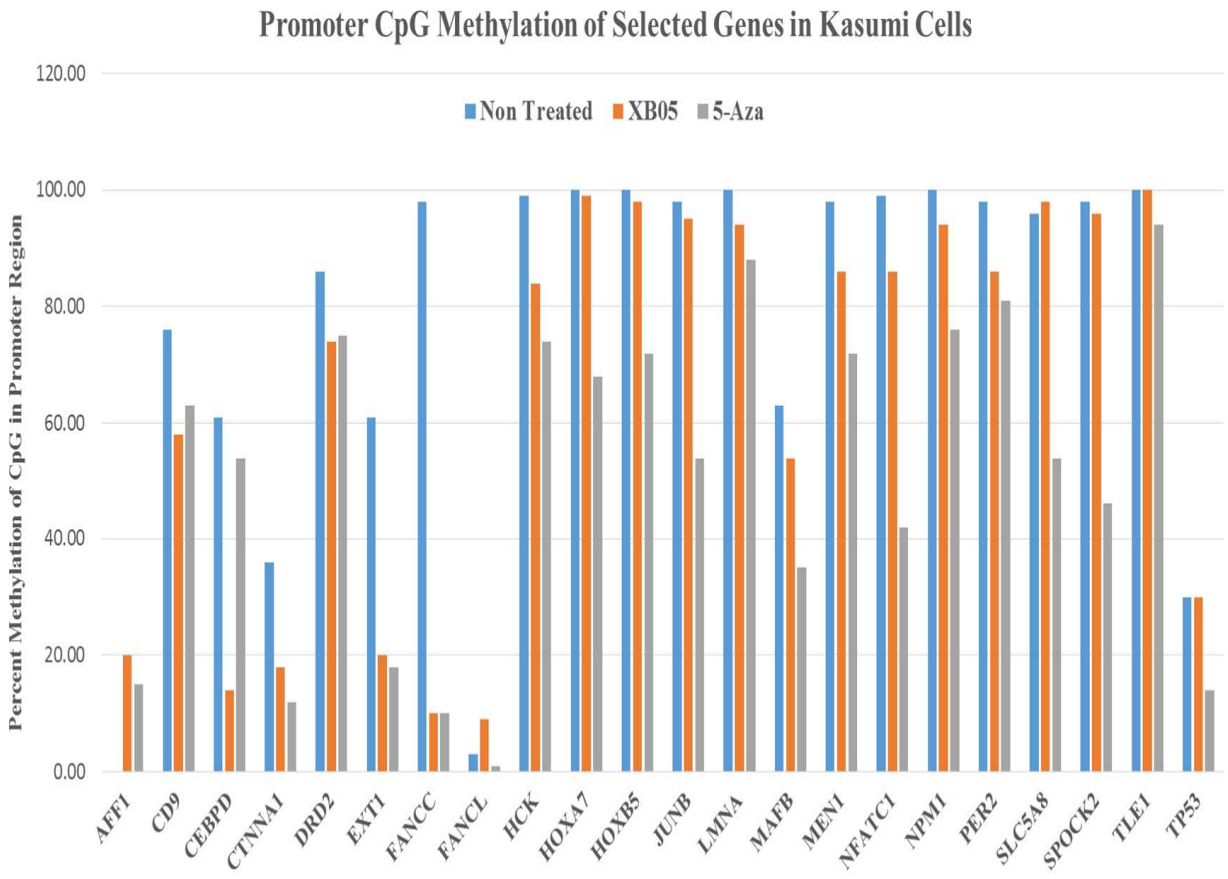


Figure 25. EpiTect Methyl II PCR array results of percent methylation of CpG islands in the promoter regions of selected genes in Kasumi cell line. In Kasumi-1 cell line, following treatment with XB05, percent methylation of promoter regions of oncogenes such as AFF1, FANCL and SLC5A8 increased, percent methylation of TLE1 did not change and percent methylation of FANCC and HCK decreased. However, there was no significant change in percent methylation of oncogenes such as HOXA7, HOXB5, JUNB, MAFB, NFATC1 and NPM1. Percent methylation of promoter regions of tumor suppressor genes such as CD9, CEBPD, CTNNA1, DRD2, EXT1, LMNA, MEN1, PER2 and SPOCK2 decreased following treatment with XB05 in Kasumi-1 cell line while it remained unchanged for TP53. Following treatment of Kasumi-1 cell line with 5'AZA, there was increased percent methylation of promoter regions of oncogenes such as AFF1 and FANCL while percent methylation decreased in FANCC, HCK, HOXA7, HOXB5, JUNB, MAFB, NFATC1, NPM1, SLC4A8 and TLE1. 5'AZA treatment also resulted in decreased promoter methylation in tumor suppressor genes such as CD9, CEBPD, CTNNA1, DRD2, EXT1, LMNA, MEN1, PER2, SPOCK2 and TP53.

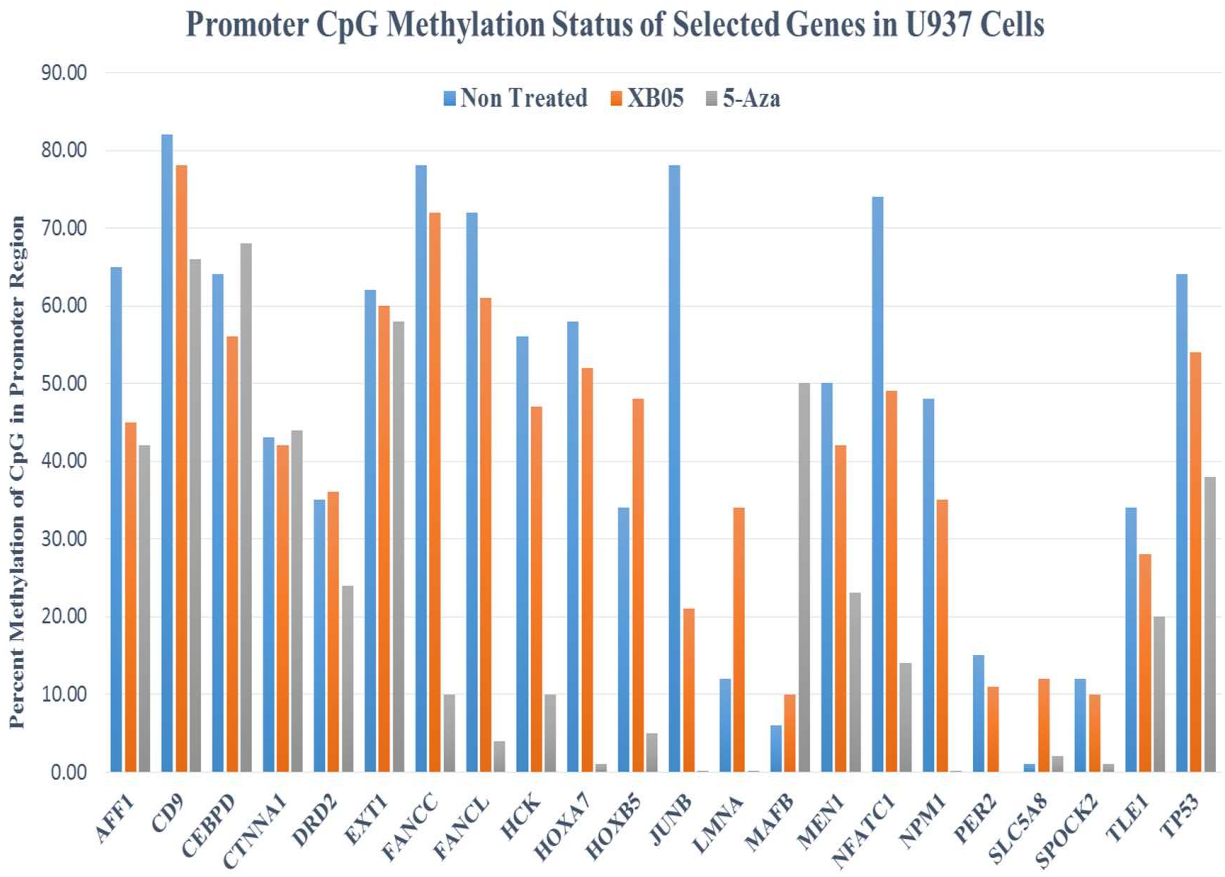


Figure 26. EpiTect Methyl II PCR array results of percent methylation of CpG islands in the promoter regions of selected genes in U937 cell line. In U937 cell line, following treatment with XB05, percent methylation of promoter regions of oncogenes such as HOXB5, MAFB and SLC5A8 increased and percent methylation of AFF1, FANCC, FANCL, HCK, HOXA7, JUNB, NFATC1, NPM1 and TLE1 decreased. FANCC and HOXA7 promoter methylation decreased at a relatively small level. Percent methylation of promoter regions of tumor suppressor genes such as CD9, CEBPD, MEN1, PER2, SPOCK2 and TP53 decreased following treatment with XB05 in Kasumi-1 cell line while it remained relatively unchanged for CTNNA1 and EXT1 and increased for DRD2 and LMNA. Following treatment of Kasumi-1 cell line with 5'AZA, there was increased percent methylation of promoter regions of oncogenes such as MAFB while percent methylation decreased in AFF1, FANCC, FANCL, HCK, HOXA7, HOXB5, JUNB, NFATC1, NPM1, SLC5A8 and TLE1. 5'AZA treatment also resulted in decreased promoter methylation in tumor suppressor genes such as CD9, DRD2, EXT1, LMNA, MEN1, PER2, SPOCK2 and TP53 while percent promoter methylation increased in CEBPD and CTNNA1.

Western Blots:

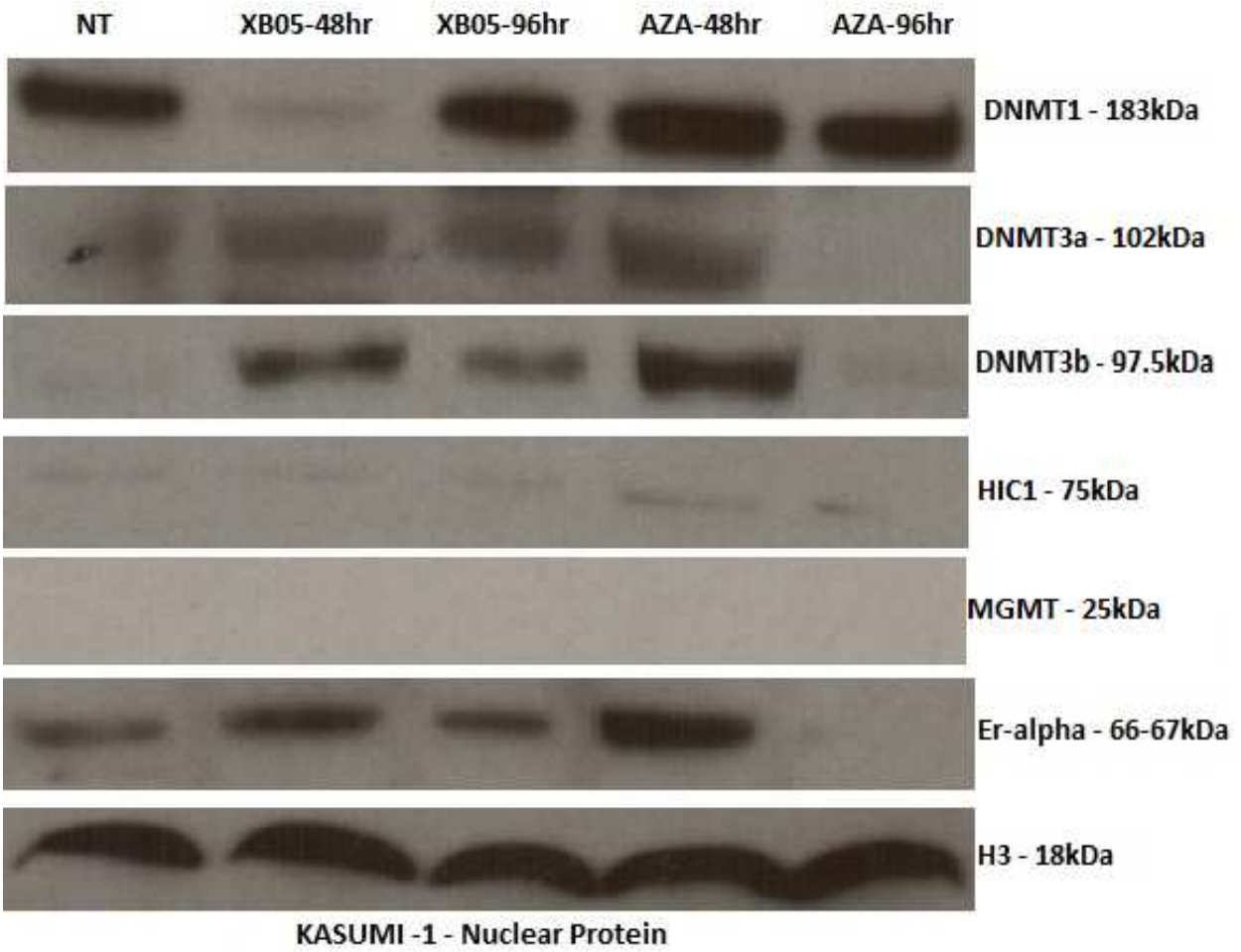


Figure 27. Western blot performed on nuclear proteins from Kasumi-1 cell line. After treatment with XB05, the expression of DNMT3a and 3b and ER-alpha increased at 48 and 96hours; however, there was no change noted in the expression of MGMT or HIC1. The expression of DNMT1 decreased at 48 hours. Treatment with 5'azacytidine did not result in decrease in DNMT1, 3a or 3b. ER-alpha expression was slightly increased at 48hours. Histone protein H3 was used as the internal control for this experiment.

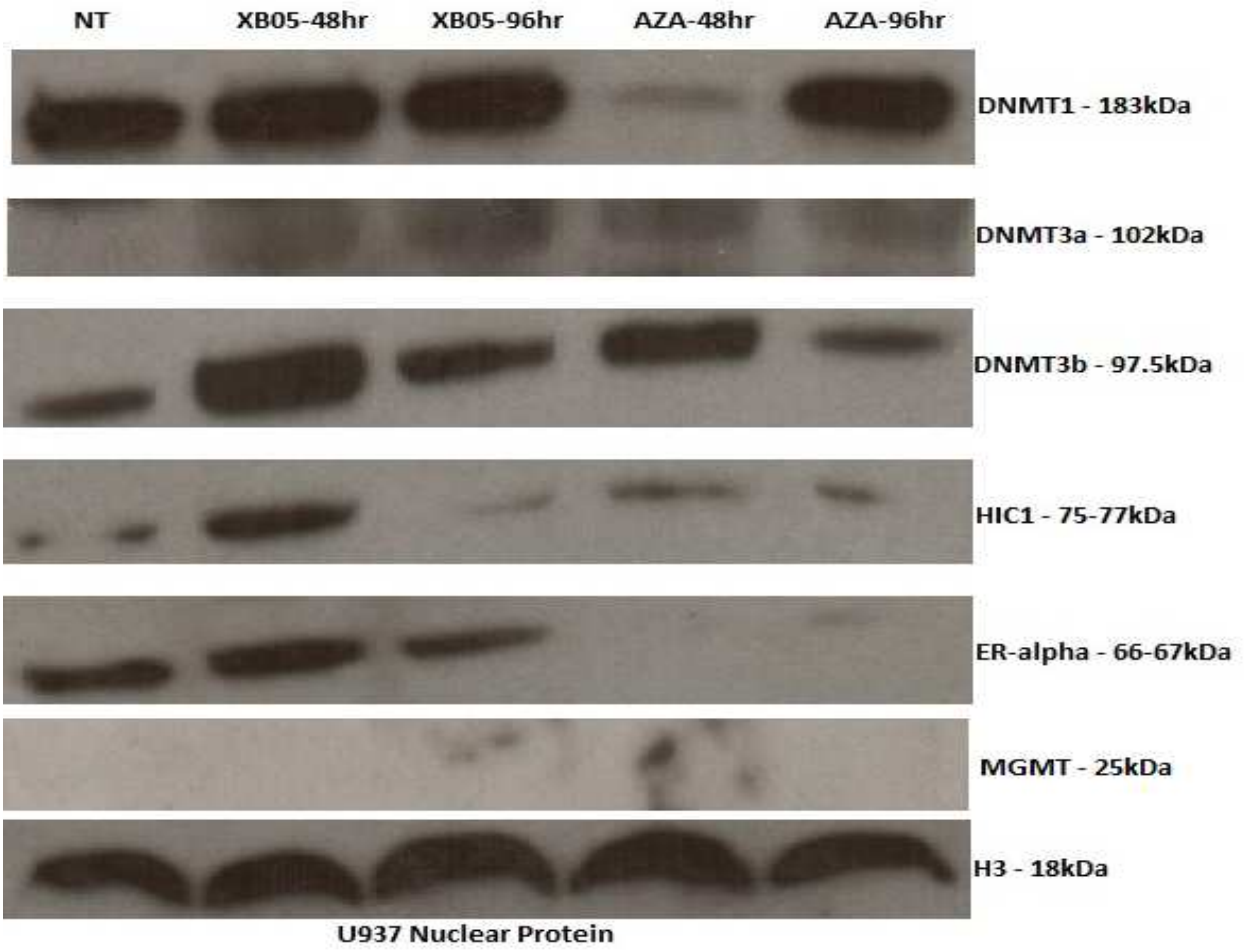


Figure 28. Western blot performed on nuclear proteins from U937 cell line. After treatment with XB05, there was increased expression of DNMT3a and 3b at 48hours. However, the expression of DNMT3b decreased at 96hours. There was no change noticed in the expression of DNMT1 after treatment with XB05. HIC1 and ER-alpha expression slightly increased at 48hours. There was no change in the expression of MGMT after both XB05 and 5'azacytidine treatments. H3 histone expression was used as an internal control. Treatment with 5'azacytidine did decrease the expression of DNMT1 at 48hours and DNMT3b at 96hours. There was no increase in the expression of HIC1 or ER-alpha noticed. Histone protein H3 was used as the internal control for this experiment.

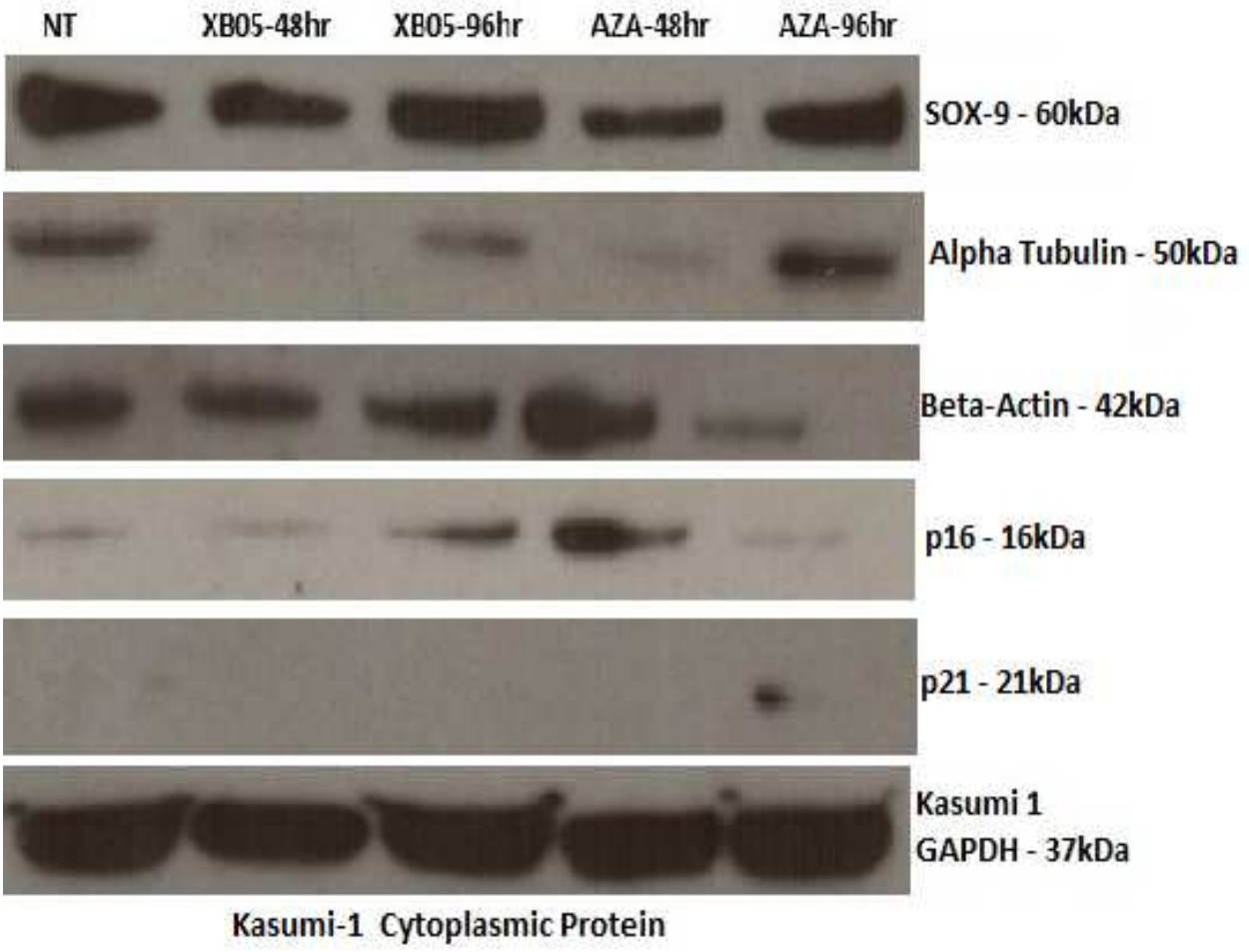


Figure 29. Western blot performed on cytoplasmic proteins from Kasumi-1 cell line. Based on the western blot results, no correlation was found between the expression of SOX-9 and susceptibility to XB05. However, expression of SOX-9 decreased at 48 hours both after XB05 and 5'azacytidine treatments. We did notice that the expression of alpha tubulin decreased following treatment with XB05 at 48 and 96hours, and with 5'azacytidine at 48hours. Changes in the expression of beta-actin were also noted after treatment with both XB05 and 5'AZA. Expression of p16 slightly increased at 96 hour after treatment with XB05 and with 5'azacytidine at 48hours. There was no change noticed in the expression of p21 both with XB05 and 5'azacytidine treatments. GAPDH was used as internal control for cytoplasmic protein expression.

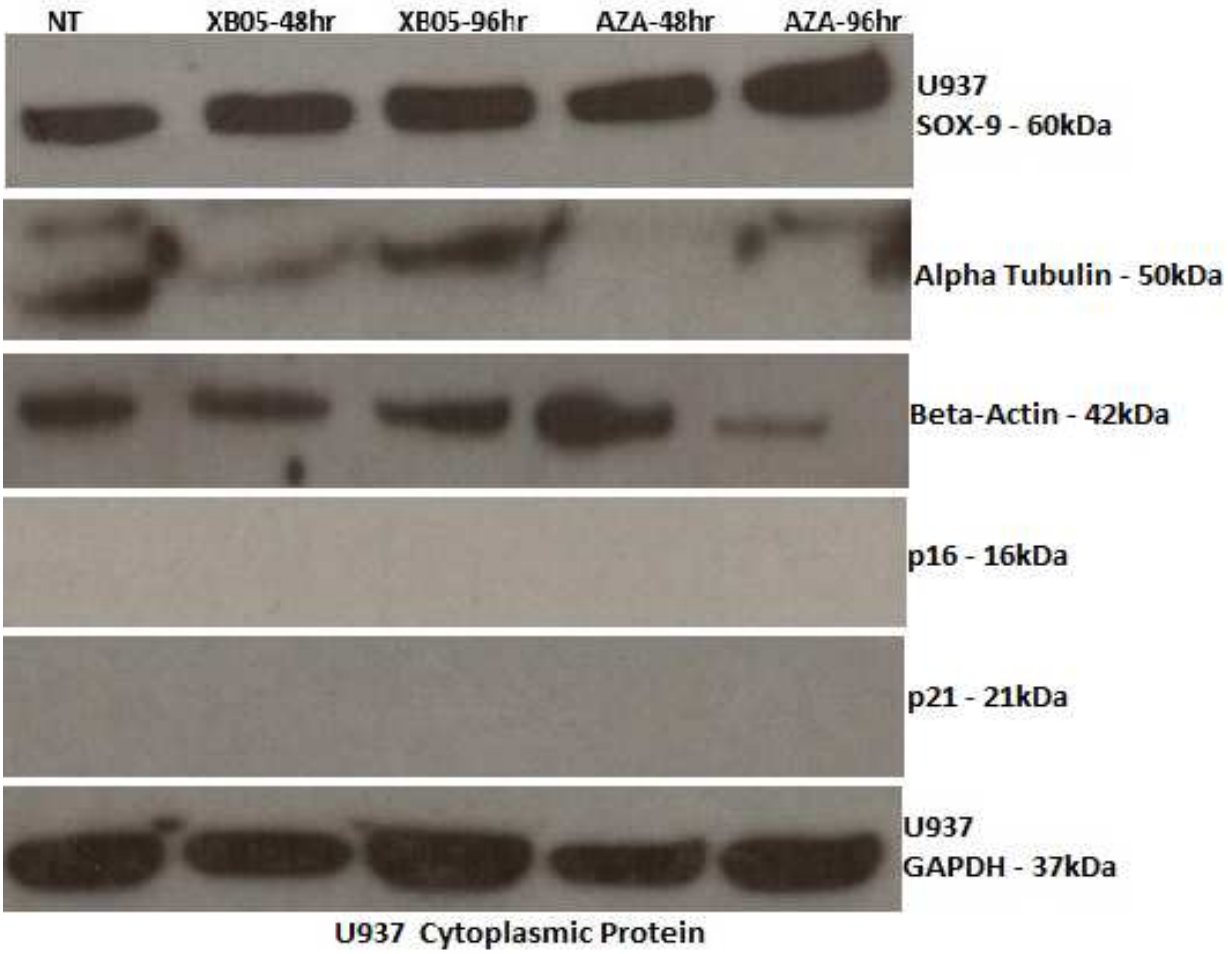


Figure 30. Western blot performed on cytoplasmic protein fractions of U937 cell line showed that baseline level of SOX-9 was lower which may explain its susceptibility to XB05. SOX9 expression increased at 96 hour following treatment with 5'azacytidine. There was no expression of p16 or p21 noticed in non-treated and treated cells. Expression of alpha tubulin decreased at 48hours following treatment with XB05 and both at 48 and 96 hours following treatment with 5'azacytidine. Expression of beta-actin did not change following treatment with XB05; however, it first increased at 48 hours and then decreased after 96 hour treatment with 5'azacytidine. GAPDH was used as internal control for cytoplasmic protein expression.

***Previous studies performed in our laboratory have demonstrated that when DMSO (vehicle for XB05) is used at high concentration alone, it does not affect the expression of various cellular proteins.**[29]

Immunohistochemistry (IHC):

*Despite extreme care, the slides accumulated bubbles. This is likely related the mounting media we used for these slides.

<u>Kasumi cell line:</u>

Figure 31. Power 40x. DNMT1 staining for Kasumi – cell line; NT (A), AZA 48hrs (B) and AZA 96hrs (C). IHC staining of non-treated and 5'AZA treated cells at 48hrs and 96hrs did not show change in nuclear expression of DNMT1.

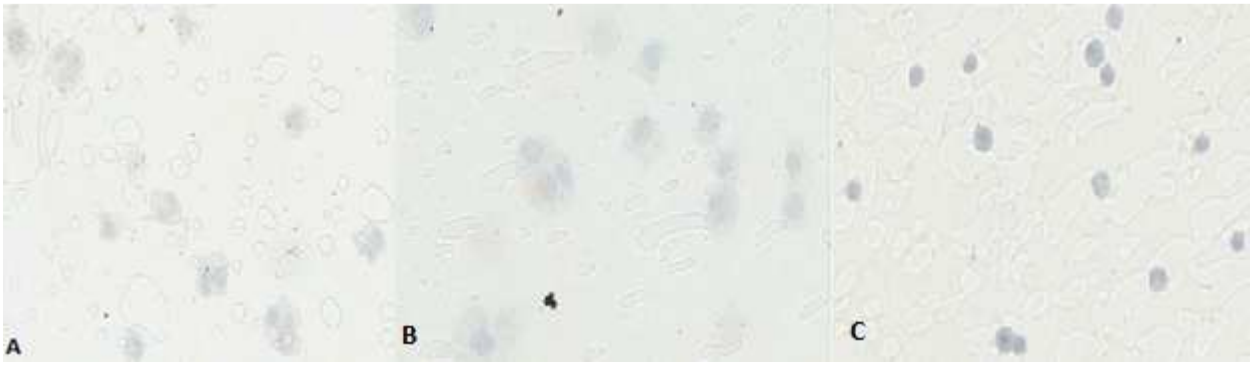


Figure 32. Power 40x. DNMT1 staining for Kasumi – cell line; NT (A), XB05 48hrs (B) and XB05 96hrs (C). IHC staining of non-treated and 5'AZA treated cells at 48hrs and 96hrs did not show change in nuclear expression of DNMT1.

Figure 33. Power 40x. HIC1 staining for Kasumi – cell line; NT (A), AZA 48hrs (B) and AZA 96hrs (C). Non-treated Kasumi-1 cells did not show any positive staining for HIC1. Treatment with 5'azacytidine at 48 hours did not result in re-expression of HIC1; however, at 96 hours, slightly increased expression is noted mostly in the cytoplasm of the cells.

Figure 34. Power 40x. HIC1 staining for Kasumi – cell line; NT (A), XB05 48hrs (B) and XB05 96hrs (C). Non-treated Kasumi-1 cells did not show any positive staining for HIC1. Treatment with XB05 at 48- and 96-hours did not result in re-expression of HIC1.

U937 cell line:

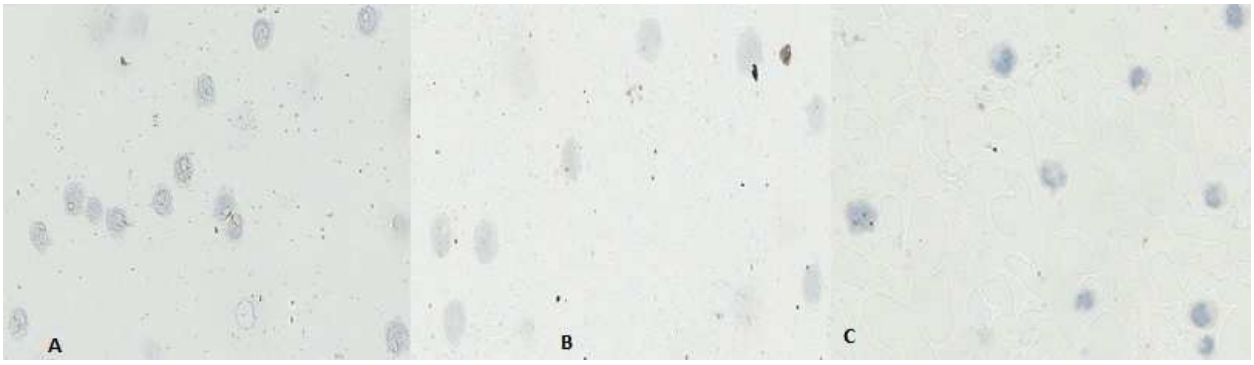


Figure 35. Power 40x. DNMT1 staining for U937 – cell line; NT (A), AZA 48hrs (B) and AZA 96hrs (C). Non-treated U937 cells showed nuclear expression of DNMT1 on IHC staining. The expression of DNMT1 was lost following treatment with 5'azacytidine both at 48 and 96 hours.

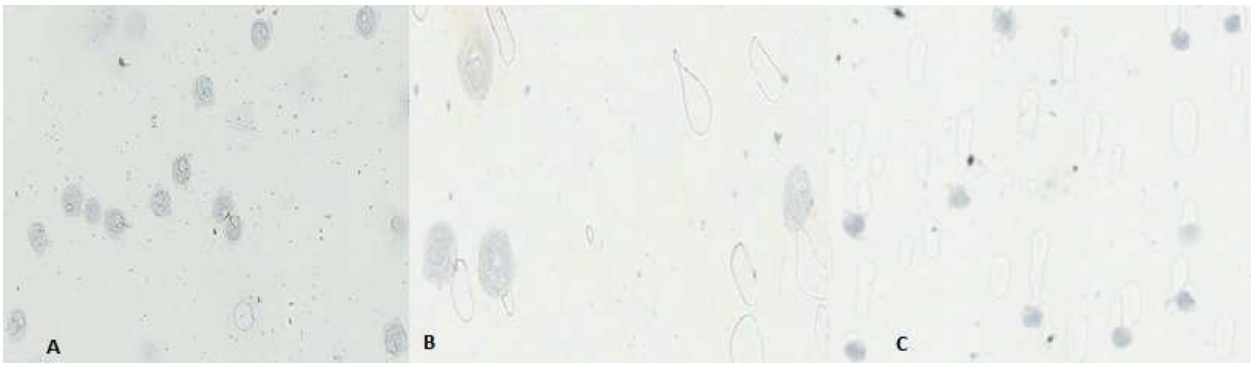


Figure 36. Power 40x. DNMT1 staining for U937 – cell line; NT (A), XB05 48hrs (B) and XB05 96hrs (C). Non-treated U937 cells showed nuclear expression of DNMT1 on IHC staining. The expression of DNMT1 was lost following treatment with XB05 both at 48 and 96 hours.

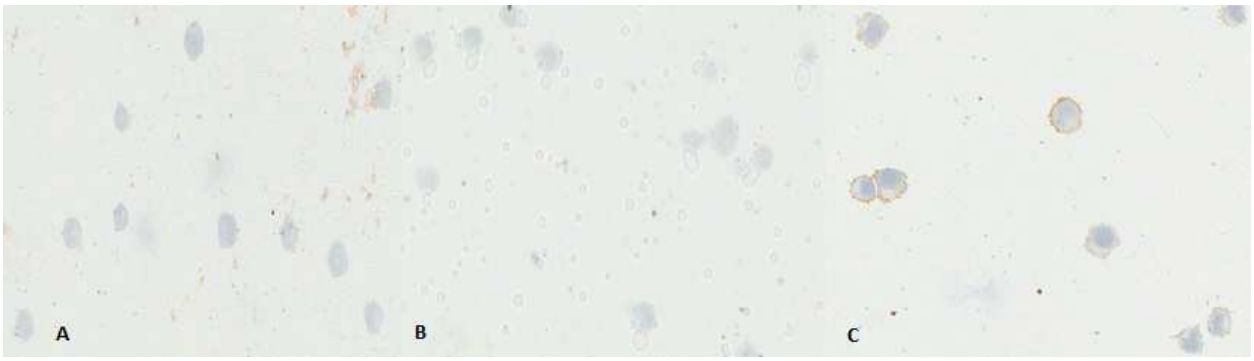


Figure 37. Power 40x. HIC1 staining for U937 – cell line; NT (A), AZA 48hrs (B) and AZA 96hrs (C). Non-treated U937 cells showed no nuclear expression of HIC1 on IHC staining. At 48 hours, treatment with 5'azacytidine did not result in re-expression of HIC1; however, at 96 hours, strong expression of HIC1 is seen.

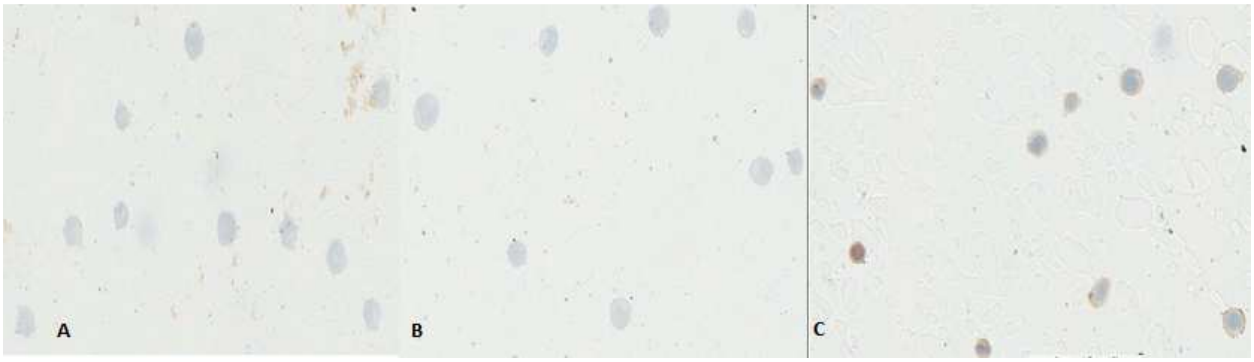


Figure 38. Power 40x. HIC1 staining for U937 – cell line; NT (A), XB05 48hrs (B) and XB05 96hrs (C). Non-treated U937 cells showed no nuclear expression of HIC1 on IHC staining. At 48 hours, treatment with XB05 did not result in re-expression of HIC1; however, at 96 hours, strong expression of HIC1 is seen.

Discussion

AML is a clonal stem cell malignancy resulting from proliferation and accumulation of immature hematopoietic cells in the bone marrow, peripheral blood and other tissues. Current therapeutic options are highly toxic and very poorly tolerated in elderly patients. Recently, FDA approved decytabine (a DNA hypomethylating agent) for AML treatment in elderly patients. It was shown to provide complete response in 47 percent patients with excellent tolerability in this patient population.

We recently discovered a novel small molecule XB05 which on NCI-60 screen was shown to be cytotoxic in certain cell lines while not in others. We have performed preliminary studies in out laboratory demonstrating the in-vitro efficacy of XB05 in certain cell lines. Expanding further on our current knowledge of the mechanisms of action of XB05, we performed in-vitro studies to demonstrate its efficacy in AML cell lines.

MTT-assays were performed on two AML cell lines (Kasumi-1 and U937) as described in the methods and material section. Preliminary data form MTT-assays in comparison with a known DNA-hypomethylating agent, 5'azacytidine showed promising results. Results from three MTT-assays performed on Kasumi-1 cell lines following treatment with XB05 and 5'azacytidine in serial concentrations were combined. XB05 demonstrated decrease in percent cell proliferation with increasing concentration reaching IC_{50} of 1345.8nM based on calculations done with CompuSyn software. 5'Azacytidine which is a known nucleoside analogue and DNMT-hypomethylating agent similarly showed declining percent cell proliferation with increasing concentration

reaching IC_{50} of 932.2nM. This clearly demonstrates the cytotoxicity of XB05 relative to 5'azacytidine and shows that IC_{50} for XB05 is very comparable to 5'azacytidine. Further MTT-assays were performed where the concentration of XB05 was kept constant and serial 5'azacytidine was added to the wells. Results of two MTT-assays performed keeping the Xb05 drug concentration constant were combined. The results from these MTT-assays demonstrated that XB05 further enhances the cytotoxicity of 5'azacytidine, thereby lowering the IC_{50} for the drug. This finding may have significant implications for the further development of the drug as it may be combined with 5'azacytidine in the treatment of AML and help reduce treatment associated with side-effects.

Similarly designed MTT-assays were performed on U937 cell line. The results of the comparative MTT-assays (assays where XB05 and 5'azacytidine cytotoxicity is compared) showed that XB05 was more cytotoxic than 5'azacytidine. The IC_{50} for XB05 was 463.3nM and for 5'azacytidine was 633.5nM. MTT-assays performed while keeping the XB05 concentration constant and using serial concentrations of 5'azacytidine (refer to methods and materials for assay design) showed that XB05 sensitized the cells to the cytotoxic effects of 5'azacytidine and thereby further enhancing its cytotoxicity. As mentioned above for Kasumi-1 cell line, these results again demonstrate that further development of XB05 is needed especially for combinatorial regimens where adverse effects related to established cytotoxic agents can be decreased without sacrificing the efficacy.

Analysis of gene expression following treatment with XB05 and 5'azacytidine at 48 and 96 hours was done with PCR. In the Kasumi-1 cell line, following treatment with

XB05 at 48 hours, expression of DNMT1, 3a and 3b decreased relative to non-treated samples. We had expected XB05 not to affect the transcription of DNMTs as it is thought to be inhibitory to the DNMT enzymes, thus reducing the methylation of the promoter sequences of commonly suppressed genes in AML. Decreased expression of DNMTs following treatment with XB05 may be the result of off-target effect of XB05 where it is likely inhibiting the transcription of different genes through a yet unknown mechanism. Expression of genes methylated in AML also slightly decreased in comparison to the untreated samples. Again this may be related to across the board inhibition of gene transcription machinery. However, the results from PCR following 96 hour treatment with XB05 were completely opposite. Expression of DNMT3a and 3b decreased, but the expression of all the other genes increased significantly. Again, we don't expect XB05 to inhibit or increase the transcription of DNMTs; however, increased expression of commonly suppressed genes in AML does demonstrate that XB05 is likely inhibiting the DNMT enzymes at the protein level and thus causing the re-expression of genes.

Following treatment with 5'azacytidine in the Kasumi-1 cell line at 48 hours, we saw increased expression of DNMT1 and decreased expression of DNMT3a and 3b relative to the non-treated samples. The expression of HIC1 also decreased; however, the expression of rest of the genes increased following the treatment. 5'Azacytidine definitely can cause decreased expression of certain genes directly as it also a nucleoside analogue. Re-expression of commonly methylated genes in AML demonstrates that 5'azacytidine can also inhibit the function of DNMTs. At 96 hours, expression of DNMT3a and 3b is decreased significantly relative to the non-treated

samples. Expression of HIC1, DAPK1, MGMT and RAR-beta also decreased. Expression of DNMT1, ER-alpha and p16 increased. This likely demonstrates that at 48 hours, 5'azacytidine is mostly a DNA-hypomethylating agent; however, at 96 hours, it suppresses the transcription of various genes secondary to its anti-nucleoside properties.

PCR analysis done on U937 cell line following treatment with XB05 for 48 hours showed that the expression of all the genes decreased relative to non-treated samples. This effect as in the Kasumi-1 cell line may the result of off-target effect of XB05 where it is inhibiting the expression of genes. However, at the 96 hours, it was seen that the expression of DNMT3a continued to be suppressed, but expression of all the other genes increased relative to the non-treated samples. This likely reflects the DNA-hypomethylating effect of XB05 where there is increased expression of the previously suppressed genes. Why the expression of DNMT3a continued to be suppressed cannot be explained based on the experiments done so far but can be hypothesized to be again the result of off-target effect of XB05.

Expression of all the genes except p16 increased in the U937 cell line following treatment with 5'azacytidine at 48 hours. This could be the result of differential hypomethylation of DNA following treatment with 5'azacytidine. However, at 96 hours, expression of DNMT3a was suppressed but the expression of the other genes increased relative to the non-treated samples. This effect can be explained partially related to the hypomethylation and partially to anti-nucleoside effects of 5'azacytidine.

Results from EpiTect Methyl II PCR assay showed that XB05 affects the promoter region methylation of both oncogenes and tumor suppressor genes. However, demethylation of promoter regions in oncogenes was not as robust as noticed in tumor suppressor genes. Increasing the methylation of promoters of oncogenes will result in decreased expression of oncogenes and decreased methylation of tumor suppressor genes will result in their increased expression and in essence inhibiting tumor growth. Following treatment with 5'AZA, promoter region methylation decreased in most oncogenes and tumor suppressors at the same level. This shows that 5'AZA is not selective in targeting promoter region methylation in tumor suppressor genes and can result in increased expression of oncogenes through demethylation of their promoter regions. XB05 showed better targeting of promoter region methylation in tumor suppressor genes as opposed to similar regions in oncogenes. This observation is quite striking and provides additional evidence to further support the development of XB05 for clinical use.

Western blot experiments were performed to further understand the mechanism of action of XB05 at protein level. Nuclear and cytoplasmic fractions were separated as per protocol described in the appendix. 30ug of protein was loaded for both the nuclear and cytoplasmic fractions. Both fractions were pure with R^2 of over 95% in reference to BSA standards.

Western blot was performed for nuclear fractions extracted from Kasumi-1 cell line following treatment with XB05 and 5'azacytidine at 48- and 96-hours. Following treatment with XB05, both at 48- and 96-hours, the expression of DNMT1 decreased

although more pronounced at 48-hours. There was no change in DNMT1 expression noted following treatment with 5'azacytidine. Expression of DNMT3a and 3b was low to absent in the non-treated cell line. No change in the level of HIC1 or MGMT was noted following treatment with both drugs at both time intervals. Level of ER-alpha expression increased at 48 hours following treatment with both XB05 and 5'azacytidine. Western blot performed on the cytoplasmic fraction showed that levels of beta-actin and p16 increased slightly following treatment with XB05. Increase in their level was more following treatment with 5'azacytidine. No increased expression of other protein was noted following treatment with XB05. These results to some extent demonstrate that treatment with XB05 can suppress the activity of DNMTs and cause re-expression of genes methylated in AML. The results of western blot were comparable for both the drugs which substantiates that XB05 can be developed as an effective adjunct to currently existing treatment regimens and thus decrease the treatment associated side-effects.

In the U937 cell line, contrary to our expectation, we did not see the decrease in the level of DNMTs following treatment with XB05. Instead, levels increased following treatment. At the same time, increase in the level of HIC1 and ER-alpha was noted. Treatment with 5'azacytidine did not result in increased level of proteins such as HIC. ER-alpha or MGMT. DNMT1 level had decreased at 48 hours. Treatment with XB05 did not result in significant change in the levels of cytoplasmic proteins evaluated.

Level of SOX9 protein was also interrogated with western blot in both cell lines. U937 cell line had slightly lower level of SOX9 expression in the non-treated samples

which may be associated with its increased susceptibility to XB05 cytotoxicity. We did not see a similar correlation with baseline SOX9 expression in Kasumi-1 cell line and its susceptibility to XB05.

On IHC, slight re-expression of HIC1 was noted in the Kasumi-1 cell line following treatment with 5'azacytidine at 48 hours but not at 96 hours. No baseline increased expression of DNMT1 was noted and no change in expression was noted following treatment with XB05 or 5'azacytidine. U937 cells did show increased nuclear expression of DNMT1 at baseline which was lost following treatment with both XB05 and 5'azacytidine. Also, as we had expected the HIC1 was re-expressed following treatment with XB05 and 5'azacytidine at 96 hours.

Validation in vitro experiments performed evaluating the efficacy a novel small molecule XB05 were described in this thesis paper. Cytotoxic effects of XB05 were clearly demonstrated in both cell lines and the cytotoxicity was comparable to the known DNA-hypomethylating agent 5'azacytidine. Also, when used in combination, XB05 increased the cytotoxicity of 5'azacytidine. The ability to lower the dose of a cytotoxic agent without losing the efficacy is particularly important from clinical perspective. This observation substantiates our belief that further development of XB05 would help in combinatorial therapy approaches thus reducing treatment related side-effects. Also, in terms of inducing the expression of previously suppressed genes in AML, XB05 fared well and its effects at mRNA and protein level were again comparable to 5'azacytidine.

Some of the results of the experiments performed did not turn out to be as expected. This may entirely be secondary to technical issues or related to poor quality

of some of the antibodies used especially for western blots and IHC. Further repetition of these experiments may be needed to optimize the conditions and hopefully, results will be more consistent and reflective of hypothesized mechanism of action of XB05. Also, other gene targets and proteins may need to be evaluated. Regardless, these experiments demonstrate that XB05 has cytotoxic effects in AML and further development of this agent may be needed especially from the perspective of combinatorial treatment approaches and reduction of adverse effects.

Conclusions

Management of AML in elderly is challenging with limited treatment options which are poorly tolerated. DNA-hypomethylating agents have been successfully employed in the treatment of this patient subset. XB05 is a novel synthetic molecule which is selectively toxic to malignant cells. In-vitro studies have demonstrated that it is active in leukemia & suppresses the expression of DNMTs resulting in re-expression of tumor suppressor genes. Further studies to elucidate the mechanisms of action of this novel agent may be needed for eventual development for clinical use.

APPENDIX

MTT-Assay

XBO Drug Dilutions:

Weigh 1.5ml Eppendorf tube: x gm

Weight of the tube with 2uL of XBO5: y gm

Weight of the drug: y – x = z gm

Then add 250uL of DMSO (Dimethyl sulfoxide) to each mg of XBO5, which will give ***15.7mM of XBO5***.

Further make a 10,000ul stock of 1mM of XBO5 from 15.7mM in 100% DMSO

M1 * V1 = M2 * V2

1mM * 10,000µl = 15.7mM * V2

Vol = 636.9ul

=> 636.9ul of 15.7mM XBO5 + 9363.1ul of 100% DMSO = 10,000ul of 1mM of XBO5

In order to make XBO5 more soluble in RPMI, make further dilutions each time prior to using the drug for MTT assay or treatment in the flask.

Make 100uM XBO5:

M1 * V1 = M2 * V2

100uM * 2000uL = 1000uM * V2

V2 = 200uL

=> 200uL of 1mM of XBO5 + 1800uL of RPMI = 2000uL of 100uM of XBO5

Now make another dilution of 10uM

M1 * V1 = M2 * V2

10uM * 5000uL = 100uM * V2

V2 = 500uL

=> 500uL of 100uM of XBO5 + 4500uL of RPMI = 5000uL of 10uM of XBO5

Now make further dilutions for treatment in wells. Make three times concentrated drug dilutions for desired final concentration of the drug in each well. Add 50uL of drug to 100uL of media in wells to get desired concentration:

For Kasumi 1 cell line ->
Make 150nM for 50nM in well
300nM for 100nM in well
600nM for 200nM in well
1200nM for 400nM in well
1800nM for 600nM in well
2400nM for 800nM in well
3000nM for 1000nM in well
3600nM for 1200nM in well
4800nM for 1600nM in well

Table 11. Serial dilutions of XB05 for treatment of Kasumi-1 cell line.

Drug Concentration (nM)	Stock	Stock Volume (uL)	RPMI volume (uL)	Total Volume (uL)
4800	10uM (10,000nM)	720	780	1500
3600	10uM (10,000nM)	540	960	1500
3000	10uM (10,000nM)	450	1050	1500
2400	4800nM	750	750	1500
1800	3600nM	750	750	1500
1200	2400nM	750	750	1500
600	1200nM	750	750	1500
300	600nM	750	750	1500
150	300nM	750	750	1500

Table 12. Calculations for serial XB05 concentrations to be used for treatment of Kasumi-1 cell line.

For U937 cell line ->
Make 150nM for 50nM in well
300nM for 100nM in well
600nM for 200nM in well
1200nM for 400nM in well
1800nM for 600nM in well
2400nM for 800nM in well
3000nM for 1000nM in well

Table 13. Serial dilutions of XB05 for treatment of U937 cell line.

Drug Concentration (nM)	Stock	Stock Volume (uL)	RPMI volume (uL)	Total Volume (uL)
3000	10uM	450	1050	1500
2400	10uM (10,000nM)	360	1140	1500
1800	10uM (10,000nM)	270	1230	1500
1200	2400nM	750	750	1500
600	1200nM	750	750	1500
300	600nM	750	750	1500
150	300nM	750	750	1500

Table 14. Calculations for serial XB05 concentrations to be used for treatment of U937 cell line.

DMSO will be used as the control. Concentration of DMSO in the control wells will correspond to the highest drug concentration used in the experiment to ensure that the cells are not being directly killed by the vehicle (DMSO).

First, 10% DMSO was made from 100% DMSO following the dilutions used for XBO5.

= 200uL 100% DMSO + 1800uL RPMI

Then, 1% DMSO was made from 10% DMSO again following the XBO5 dilutions.

= 500uL 10% DMSO + 4500uL of RPMI

Then for Kasumi-1 MTT assay;

Dilute 720uL of 1% DMSO in 780uL of RPMI

For U937 MTT-assay;

Dilute 450uL of 1% DMSO in 1050uL of RPMI

5’Azacytadine Drug Dilutions:

Stock concentration 5’AZA = 500uM in RPMI

Make working concentration of 10uM from 500uM stock to use for further drug dilutions to be used in MTT assays.

M1 * V1 = M2 * V2

10uM * 5000uL = 500uM * V2

V2 = 100uL

100uL of 500uM of 5’AZA + 4900uL RPMI = 10uM of 5’AZA

Make further dilutions as described above for XBO5.

Control for 5’azacytadine will be RPMI.

For combination drug treatments, XB05 concentration was kept constant and serial concentrations of azacytadine were used. Six time concentrated drug was added to each well in the volume of 25uL for a total volume of 150uL after addition of both drugs.

XB05 and azacytadine drug concentrations for Kasumi 1 and U937 cell lines using working drug concentration of 10uM->

Make 300nM for 50nM in well
600nM for 100nM in well
1200nM for 200nM in well
2400nM for 400nM in well
4800nM for 800nM in well
6000nM for 1000nM in well

Table 15. Serial dilutions of azacytadine for treatment of Kasumi-1 and U937.

Drug Concentration (nM)	Stock	Stock Volume (uL)	RPMI volume (uL)	Total Volume (uL)
6000	10uM (10,000nM)	6000	4000	10,000
4800	10uM (10,000nM)	7200	7800	15,000
2400	4800nM	7500	7500	15,000
1200	2400nM	7500	7500	15,000
600	1200nM	7500	7500	15,000
300	600nM	7500	7500	15,000

Table 16. Calculations for serial XB05 concentrations to be used for treatment of U937 cell line.

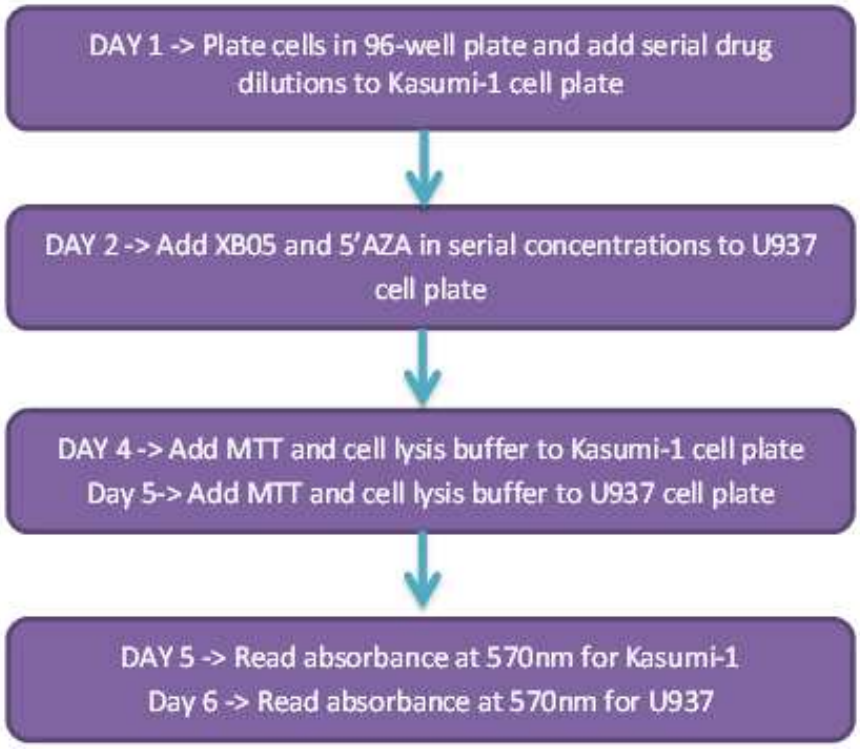


Figure 39. Outline of the plan for MTT-assays.

	1 (0nM)	2 (50nM)	3 (100nM)	4 (200nM)	5 (400nM)	6 (600nM)	7 (800nM)	8 (1000nM)	9 (1200nM)	10 (1600nM)	11	12
A (XBO5)												
B (XBO5)												
C (XBO5)												
D (XBO5)												
E (5'AZA)												
F (5'AZA)												
G (5'AZA)												
H (5'AZA)												Blank

Figure 40. Plate Design for Kasumi-1 MTT experiment.

	1 (0nM)	2 (50nM)	3 (100nM)	4 (200nM)	5 (400nM)	6 (600nM)	7 (800nM)	8 (1000nM)	9	10	11	12
A (XBO5)												
B (XBO5)												
C (XBO5)												
D (XBO5)												
E (5'AZA)												
F (5'AZA)												
G (5'AZA)												
H (5'AZA)												Blank

Figure 41. Plate Design for U937 MTT experiment.

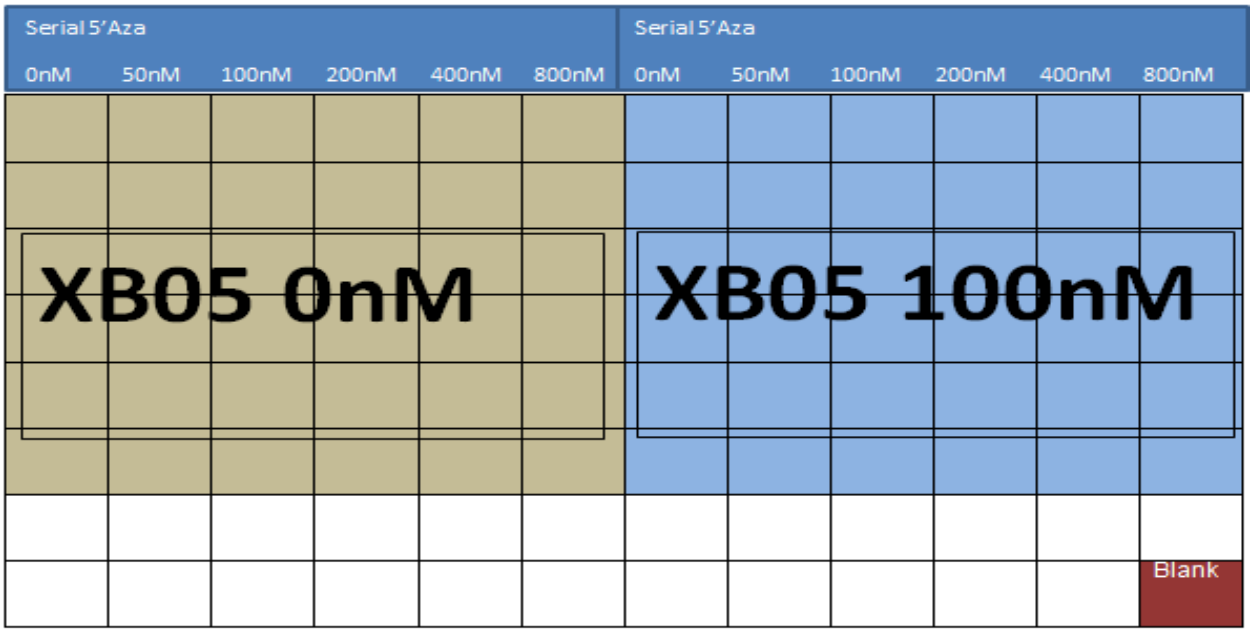


Figure 42. Plate design for drug combination experiments.

XB05 concentration was increased accordingly in the other plates to maximum concentration of 800nM.

RNA Extraction Protocol – TRIzol® Reagent:

TRIzol® Reagent is a ready-to-use reagent, designed to isolate high quality total RNA (as well as DNA and proteins) from cell and tissue samples within one hour. It is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components which facilitate the isolation of a variety of RNA species of large or small molecular size. It maintains the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization.

• Chloroform
• Isopropyl alcohol
• 75% ethanol (in DEPC-treated water)
• RNase-free water or 0.5% SDS
• Centrifuge and rotor capable of reaching up to 12,000 × g
• Polypropylene microcentrifuge tubes
• Water bath or heat block (55–60℃)

Table 17. Materials needed for RNA isolation.

Sample preparation – suspension cells:

1. Harvest cells by centrifugation and remove media.

2. Add 0.75 mL of TRIzol® Reagent per 0.25 mL of sample. Do not wash cells before addition of TRIzol® Reagent to avoid increased chance of mRNA degradation.

3. Lyse cells in sample by pipetting up and down several times.

Phase separation

1. Incubate the homogenized sample for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex.

2. Add 0.2 mL of chloroform per 1 mL of TRIzol® Reagent used for homogenization. Cap the tube securely.

3. Shake tube vigorously by hand for 15 seconds.

4. Incubate for 2–3 minutes at room temperature.

5. Centrifuge the sample at 12,000 × g for 15 minutes at 4℃.

Note: The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is ~50% of the total volume.

6. Remove the aqueous phase of the sample by angling the tube at 45° and pipetting the solution out. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase.

7. Place the aqueous phase into a new tube and proceed to the RNA Isolation Procedure.

RNA Isolation Procedure

RNA precipitation

1. Add 0.5 mL of 100% isopropanol to the aqueous phase, per 1 mL of TRIzol® Reagent used for homogenization.

2. Incubate at room temperature for 10 minutes.

3. Centrifuge at 12,000 × g for 10 minutes at 4℃.

4. Proceed to RNA wash.

RNA wash

1. Remove the supernatant from the tube, leaving only the RNA pellet.

2. Wash the pellet, with 1 mL of 75% ethanol per 1 mL of TRIzol® Reagent used in the initial homogenization.

3. Vortex the sample briefly, then centrifuge the tube at 7500 × g for 5 minutes at 4℃. Discard the wash.

4. Vacuum or air dry the RNA pellet for 5–10 minutes.

5. Proceed to RNA resuspension.

RNA resuspension

1. Resuspend the RNA pellet in RNase-free water by passing the solution up and down several times through a pipette tip.

2. Incubate in a water bath

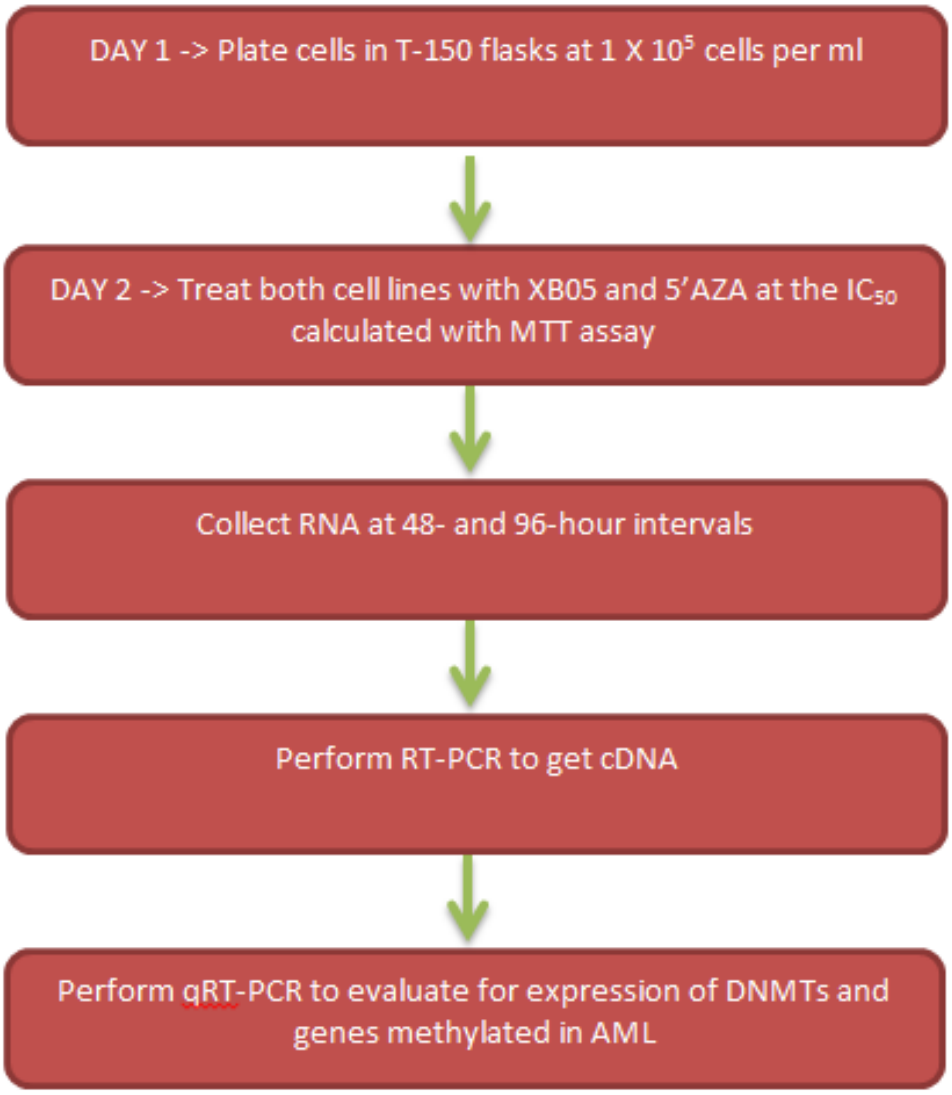


Figure 43. Outline of experiments for RNA extraction, cDNA and RT-PCR.

High Capacity RNA-to-cDNA Kit

PCR INSTRUMENT: Applied Biosystems 9800 Fast Thermal Cycler with 96-Well Aluminum Sample Block Module

Kit components

Component	Quantity in 50 reaction kit
2X RT Buffer Mix	1 bottle, 500 µL
20X RT Enzyme Mix	1 tube, 50 µL

Table 18. Kit components for high capacity RNA-to-cDNA PCR.

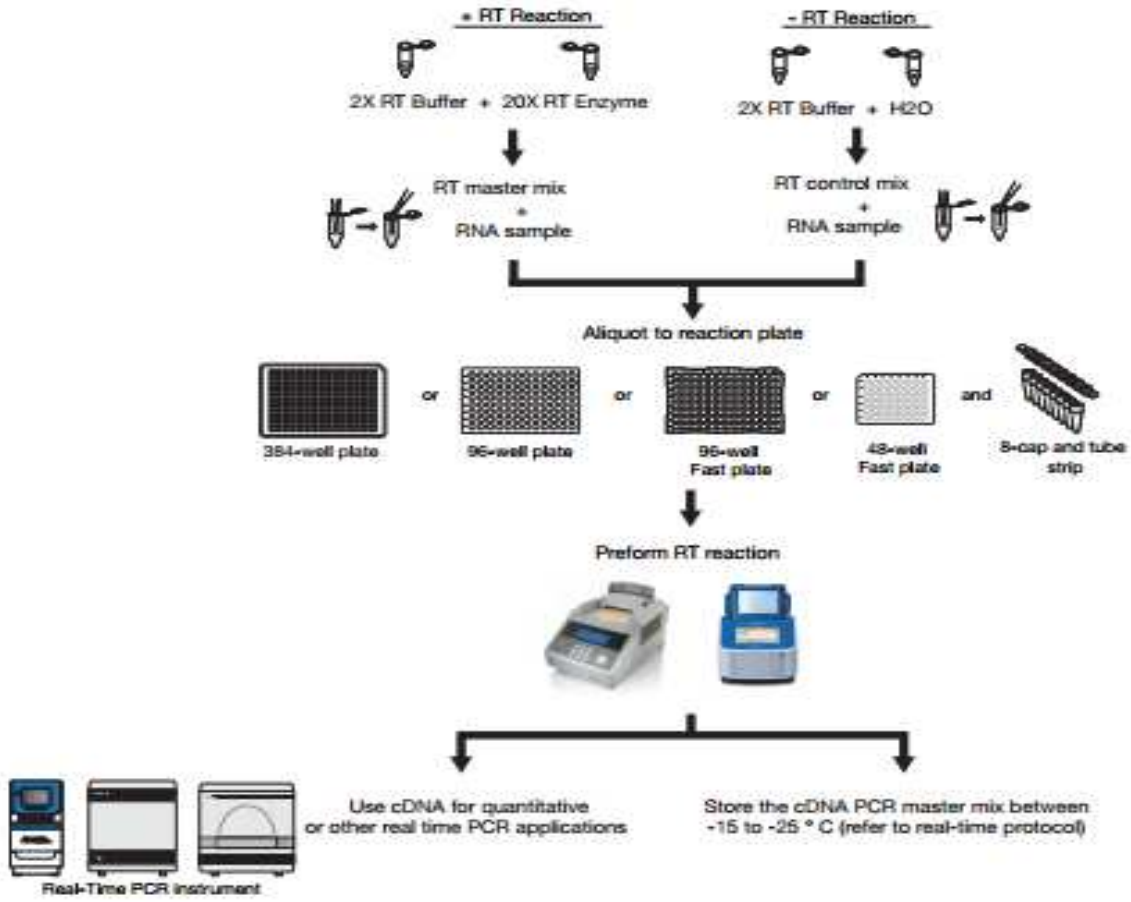


Figure 44. General overview of RT-PCR experiment.

Component	Volume/Reaction (µL)	
	+RT	–RT
2X RT Buffer	10.0	10.0
20X RT Enzyme Mix	1.0	–
Nuclease-free H_2O	Q.S.‡ to 20 µL	Q.S. to 20 µL
Sample	up to 9 µL	up to 9 µL
Total per Reaction	20.0	20.0

Table 19. Volumes for each components of RT-PCR reaction.

	Step 1	Step 2	Step 3
Temperature (°C)	37	95	4
Time	60 min	5 min	∞

Table 20. Time settings for three steps of RT-PCR reaction.

Fast SYBR® Green Master Mix Protocol

Fast SYBR® Green Master Mix, supplied in a 2X concentration, is a convenient premix to perform real-time PCR using SYBR® Green I dye.

• SYBR® Green I Dye
• AmpliTaq® Fast DNA Polymerase, UP (Ultra Pure)
• Uracil-DNA Glycosylase (UDG)
• ROX™ dye Passive Reference
• dNTPs
• Optimized buffer components

Table 21. Components of SYBR Green master mix.

Primers, template, and water are the only components needed by the investigator.

INSTRUMENT NEEDED

• Applied Biosystems 7500 Fast Real-Time PCR System

Principle:

The SYBR Green I dye chemistry uses the SYBR Green I dye to detect PCR products by binding to double-stranded DNA formed during PCR.
The process works as follows:
1. When Fast SYBR® Green Master Mix is added to a sample, SYBR Green I dye immediately binds to all double-stranded DNA.
2. During the PCR, AmpliTaq® Fast DNA Polymerase, UP amplifies the target sequence, which creates the PCR product, or “amplicon.”
3. The SYBR Green I dye then binds to each new copy of double stranded DNA.
4. As the PCR progresses, more amplicon is created. Because the SYBR Green I dye binds to all double-stranded DNA, the result is an increase in fluorescence intensity proportional to the amount of double-stranded PCR product produced.

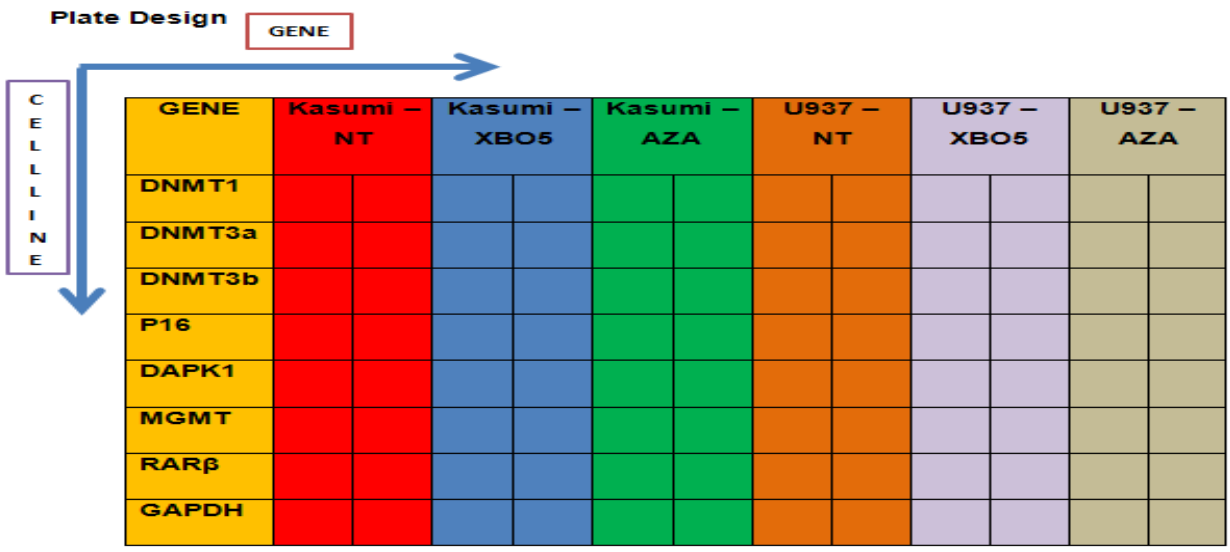


Figure 45. Plate design for qRT-PCR reaction for the study.

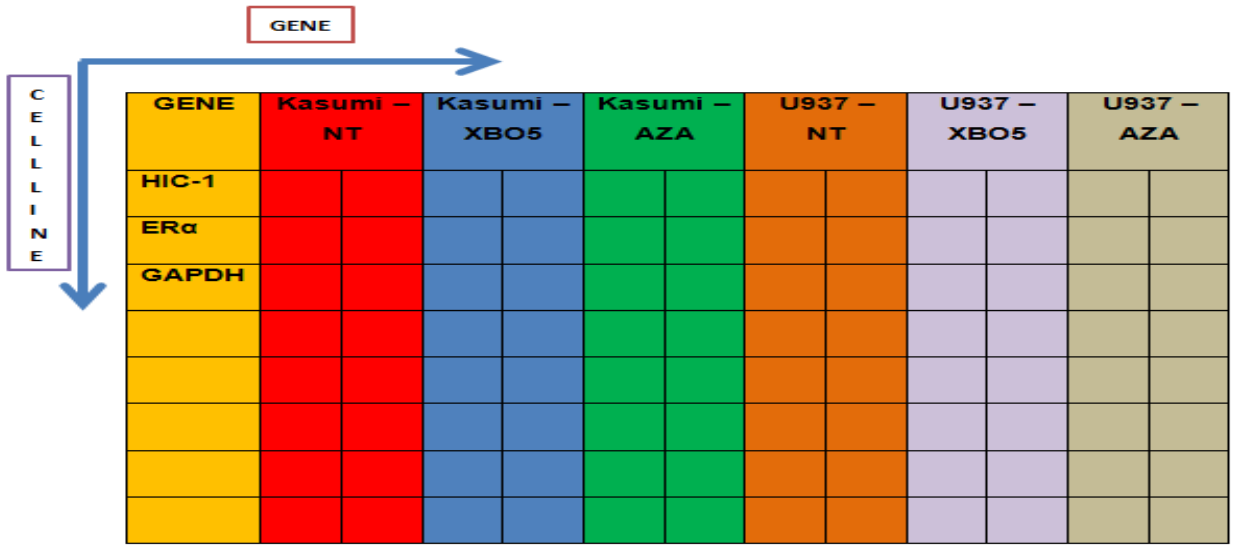


Figure 46. Plate design for qRT-PCR reaction for the study.

Component	Volume for One 20-µL Reaction (µL)
Fast SYBR® Green Master Mix (2✕)	10.0
Forward and Reverse Primers	Variable
cDNA template + RNase-free water	Variable
Total Volume	20.0

Table 22. Volumes for components of SYBR Green qRT-PCR reaction.

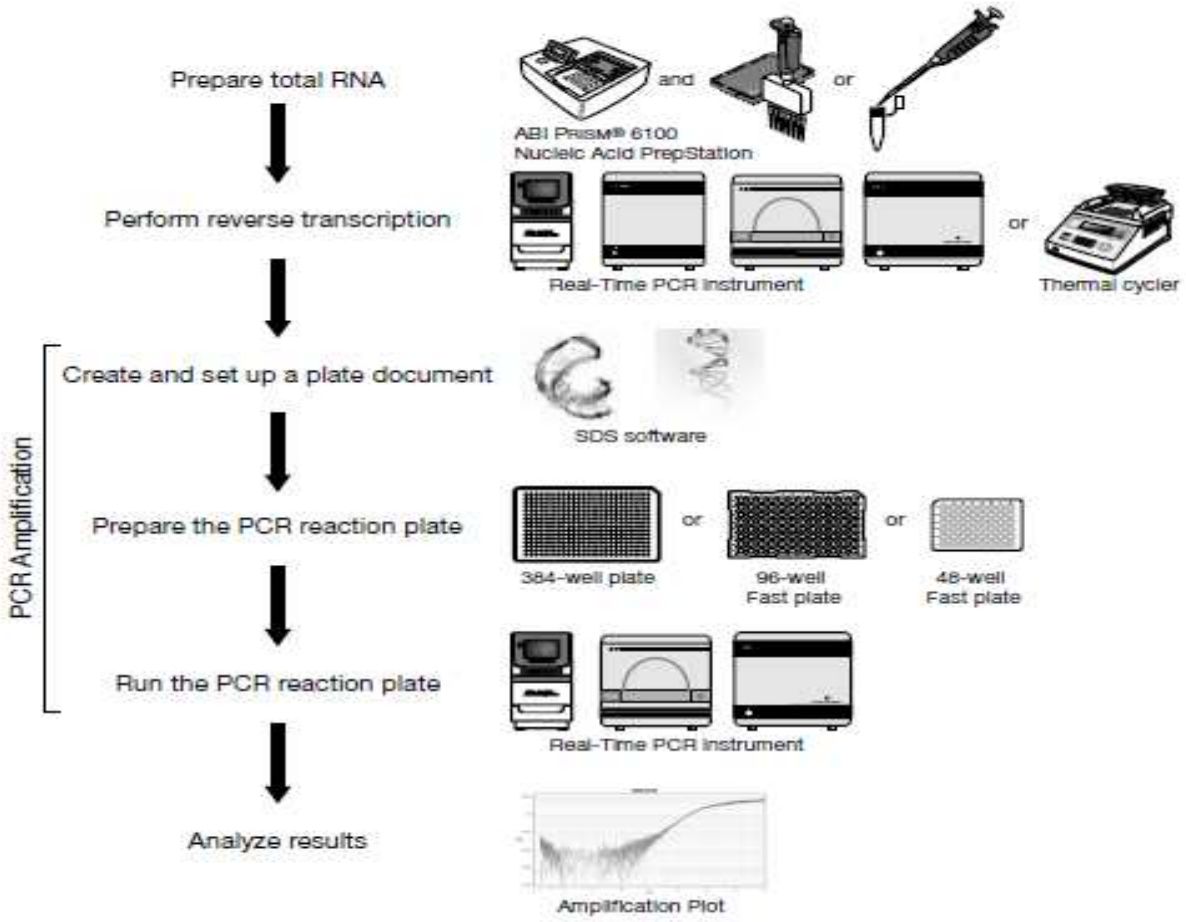


Figure 47. Overview of the qRT-PCR reaction for the study.

Select Fast Mode				
Instrument	**Step**	**Temperature (°C)**	**Duration**	**Cycles**
• Step One • StepOne Plus • 7500 Fast	AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 sec	HOLD
	Denature	95	3 sec	40
	Anneal/Extend	60	30 sec	

Table 23. Time settings for the three steps of qRT-PCR reaction.

EpiTect Methyl II PCR *(adapted from Qiagen EpiTect Methyl II PCR Handbook)*

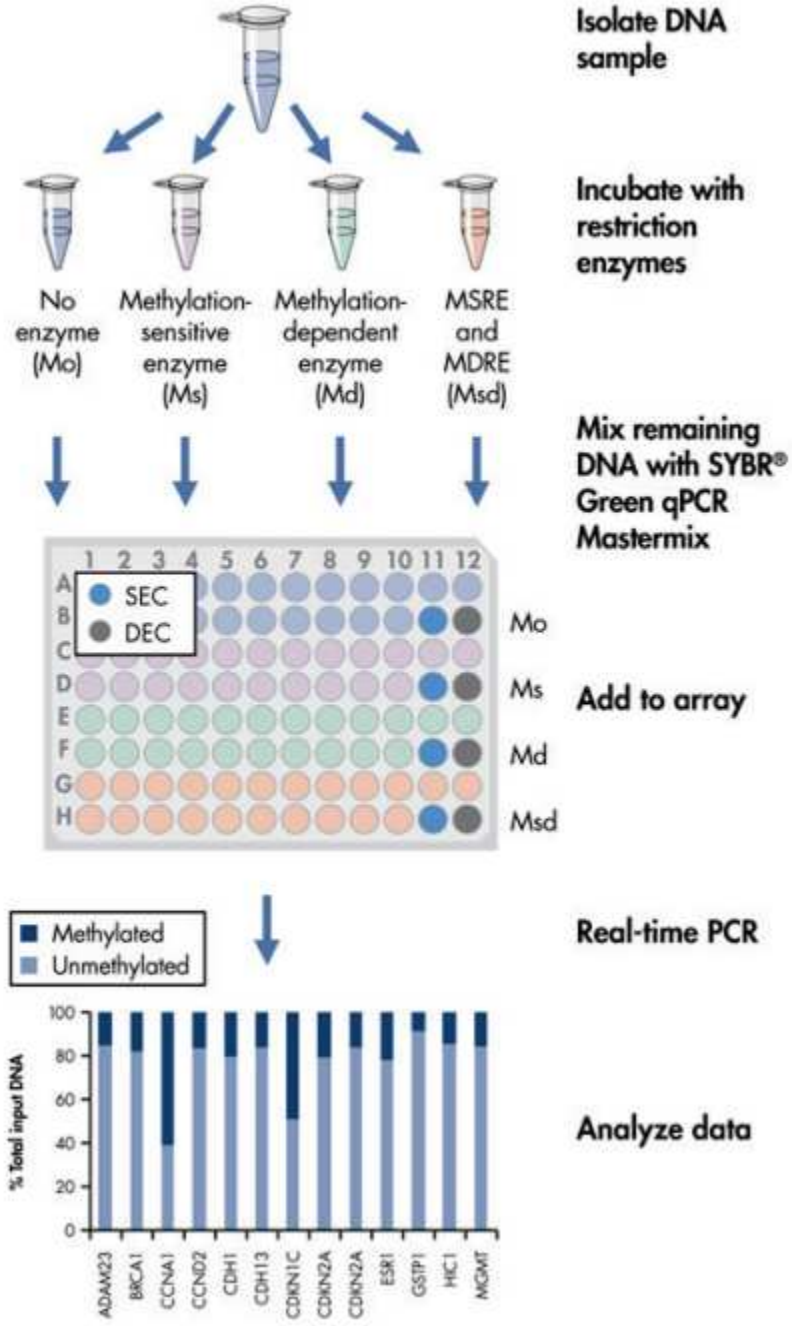


Figure 48. EpiTect Methyll II PCR Procedure overview.

Procedure overview:

The input genomic DNA is aliquoted into four equal portions and subjected to mock (no enzyme), methylation-sensitive (MSRE), methylation-dependent (MDRE), and double (MSRE and MDRE) restriction endonuclease digestion. After digestion, the enzyme reactions are mixed directly with qPCR master mix and are dispensed into a PCR Array plate containing pre-aliquoted primer mixes.

RT-PCR is carried out using cycling conditions provided by the manufacturer. The data obtained (ΔCT values) are exported into the data analysis spreadsheet provided by the manufacturer which automatically calculates the relative amount of methylated and unmethylated DNA fractions.

The product of the mock (no enzyme) digestion represents the total amount of input DNA. In the methylation-sensitive digestion (Ms) reaction, the MSRE will digest unmethylated and partially methylated DNA. The remaining hypermethylated DNA — DNA in which all CpG sites are methylated — will be detected by real-time PCR. In the methylation-dependent digestion (Md) reaction, the MDRE will preferentially digest methylated DNA. The remaining unmethylated DNA will be detected by real-time PCR. In the double digestion (Msd) reaction, both enzymes are present, and all DNA molecules (both methylated and unmethylated) will be digested. This reaction measures the background and the fraction of input DNA refractory to enzyme digestion.

The assay provides gene methylation status as percentage unmethylated (UM) and percentage methylated (M) fraction of input DNA. Unmethylated represents the fraction of input genomic DNA containing no methylated CpG sites in the amplified region of a gene. Methylated represents fraction of input genomic DNA containing two or more methylated CpG sites in the targeted region of a gene.

Each well of EpiTect Methyl II PCR Array plate contains a different primer assay mixed with an inert dye. This dye does not affect assay performance or fluorescence detection.

EXPERIMENT:

Restriction digestion

- Restriction enzyme digestions are performed using the EpiTect Methyl II DNA Restriction Kit (cat. no. 335452).
- Reaction mix without enzymes as below:

Component	Volume
Genomic DNA (1 µg)	Variable
5x Restriction Digestion Buffer	26 µl
RNase-/DNase-free water	Variable
Final volume	**120 µl**

Table 24. Volume for components of EpiTect Methyl II PCR reaction.

- RNase-/DNase-free water is added to make the final volume 120 µl. Vortex and centrifuge the components.

- Four enzyme digestion reactions are set up as below and should contain equal amounts of genomic DNA – MMo, Ms, Md, and Msd.

Component	M_o	M_s	M_d	M_{sd}
Reaction mix from step 3	28 µl	28 µl	28 µl	28 µl
Methylation-sensitive enzyme A	–	1 µl	–	1 µl
Methylation-dependent enzyme B	–	–	1 µl	1 µl
RNase-/DNase-free water	2 µl	1 µl	1 µl	–
Final volume	**30 µl**	**30 µl**	**30 µl**	**30 µl**

Table 25. Volumes for four reactions in EpiTect Methyl II PCR assay.

- Mix the reaction components thoroughly by pipetting up and down to gently but do not vortex.

- All four tubes are incubated at 37℃ for 6 h in a heating block or thermal cycler.

- Stop the reaction after incubation by heat-inactivating the enzymes at 65℃ for 20 mins.

PCR

- PCR reaction mix for for each of the 4 digestions (Mo, Ms, Md, and Msd) is prepared in a 1.5 ml tube as below:

Component	M_o	M_s	M_d	M_{sd}
PCR master mix	330 µl	330 µl	330 µl	330 µl
M_o digest	30 µl	–	–	–
M_s digest	–	30 µl	–	–
M_d digest	–	–	30 µl	–
M_{sd} digest	–	–	–	30 µl
RNase-/DNase-free water	300 µl	300 µl	300 µl	300 µl
Final volume	**660 µl**	**660 µl**	**660 µl**	**660 µl**

Table 26. Final reaction volume for four reactions in EpiTect Methyl II PCR assay.

- Vortex and centrifuge the contents to the bottom of the tube.

- Add 25 µl of the Mo reaction to each well in rows A and B of the 96-well EpiTect Methyl II Signature PCR Array. Add 25 µl of the Ms reaction to each well in rows C and D. Add 25 µl of the Md reaction to each well in rows E and F. Finally, add 25 µl of the Msd reaction to each well in rows G and H.

Digest	Well	1	2	3	4	5	6	7	8	9	10	11	12
M_o	A	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	B	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC
M_s	C	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	D	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC
M_d	E	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	F	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC
M_{sd}	G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	H	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC

Figure 49. Plate design for EpiTect Methyl II PCR assay.

- Seal the wells of the plate and centrifuge the plate for 1 min at 2000 rpm to remove any air bubbles.

- Program the thermal cycler as per manufacturer's instructions described below:

Temperature	Time	Number of cycles
95°C	10 min*	1 cycle
99°C 72°C	30 s 1 min	3 cycles
97°C 72°C	15 s 1 min†	40 cycles
According to instrument recommendations	Melting curve segment	

Table 27. Time settings for EpiTect Methyl II PCR assay.

Coomassie (Bradford) Protein Assay Kit

Bradford protein assay is a quick and ready-to-use modification of the Bradford coomassie-binding, colorimetric method for total protein quantitation. When coomassie dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465nm to 595nm with a concomitant color change from brown to blue.

Standard Microplate Protocol

1. Pipette 5µL of each standard or unknown sample into 96-well microplate.

2. Add 250µL of the Coomassie Reagent to each well and mix with plate shaker for 30 seconds.

3. Remove plate from shaker and incubate plate for 10 minutes at room temperature (RT).

4. Measure the absorbance at 595nm with a plate reader.

5. Prepare a standard curve by plotting the average Blank-corrected 595nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000µg/mL)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Table 28. Plate design for Coomassie assay and BSA serial dilutions.

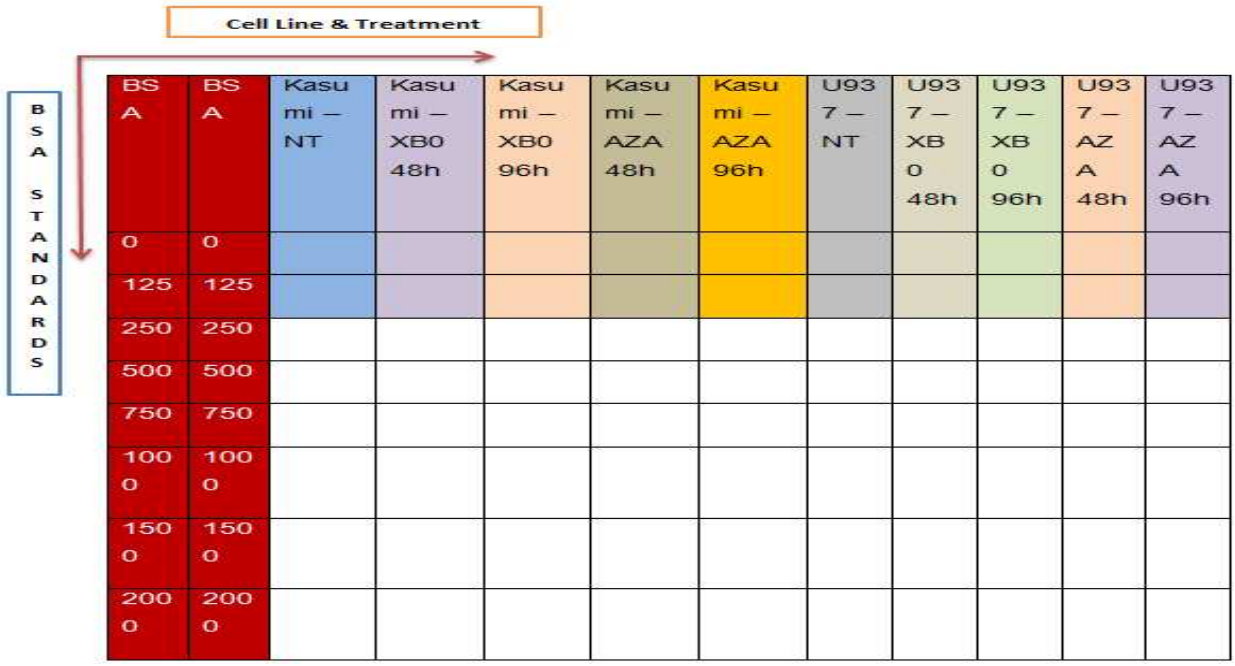


Figure 50. Plate design for Coomassie assay.

Western Blot

Protocol for treatment of cells in flasks, protein extraction and western blot:

Step 1: Plate ~100,000 cells per ml or 1,500,000 cells per flask in a T-150 flask (total volume of media = 15ml) on day one.

Step 2: On day two, add XBO5 and 5'AZA at IC_{50} concentrations to the individual flasks to get desired molar concentration.

Step 3: Incubate for three days and extract the cell pellet by centrifuging at 1000 rpm for five minutes. Repeat the same steps for untreated cells.

Step 4: Extract nuclear and cellular proteins using protocol separately.

Step 5: Add different concentrations of the extracted proteins to commercially available 10-well Bio-Rad (4-20%) SDS-PAGE gel and run at 100volts for 1 hour.

Step 6: Transfer proteins from gel to the PVDF membrane using XCell II™ Blot Module (Invitrogen®) and transfer media at 100mA for 2 hours. Activate the membrane with methanol for 30 secs.

Step 7: After transfer, block the membrane with 5% milk at 4ºC overnight.

Step 8: Wash the membrane with PBS-T for five minutes times three.

Step 9: Incubate the membrane with commercially available primary antibody at 1:500 at 4*C overnight.

Step 10: Wash with PBS-T for five minutes times three.

Step 11: Incubate the membrane with commercially available secondary antibody at 1:1000 for 1 hour at room temperature.

Step 12: Wash with PBS-T every five minutes times three.

Step 13: Using ECL Western Blot detection kit and darkroom development techniques, develop film to detect and quantify the expression of these proteins in untreated and treated cells.

Protein Extraction Protocol:

- Wash pellet twice in cold 5ml of 1x PBS
- Add 100-500µl of ice-cold extraction buffer B to the pellet.
- Transfer contents to Eppendorf tubes.
- Perform three cycles of freeze-thaw. Freeze the tubes at -80ºC for five minutes and thaw at room temperature (37ºC).
- Recover cytoplasmic extracts (supernatant) by centrifuging at 12,000rpm for one minute.
- Resuspend pellets in 40-60µl of buffer C.

- Incubate at 40ºC for 30 minutes.
- Recover nuclear extracts (supernatant) by centrifuging at 12,000rpm for five minutes.

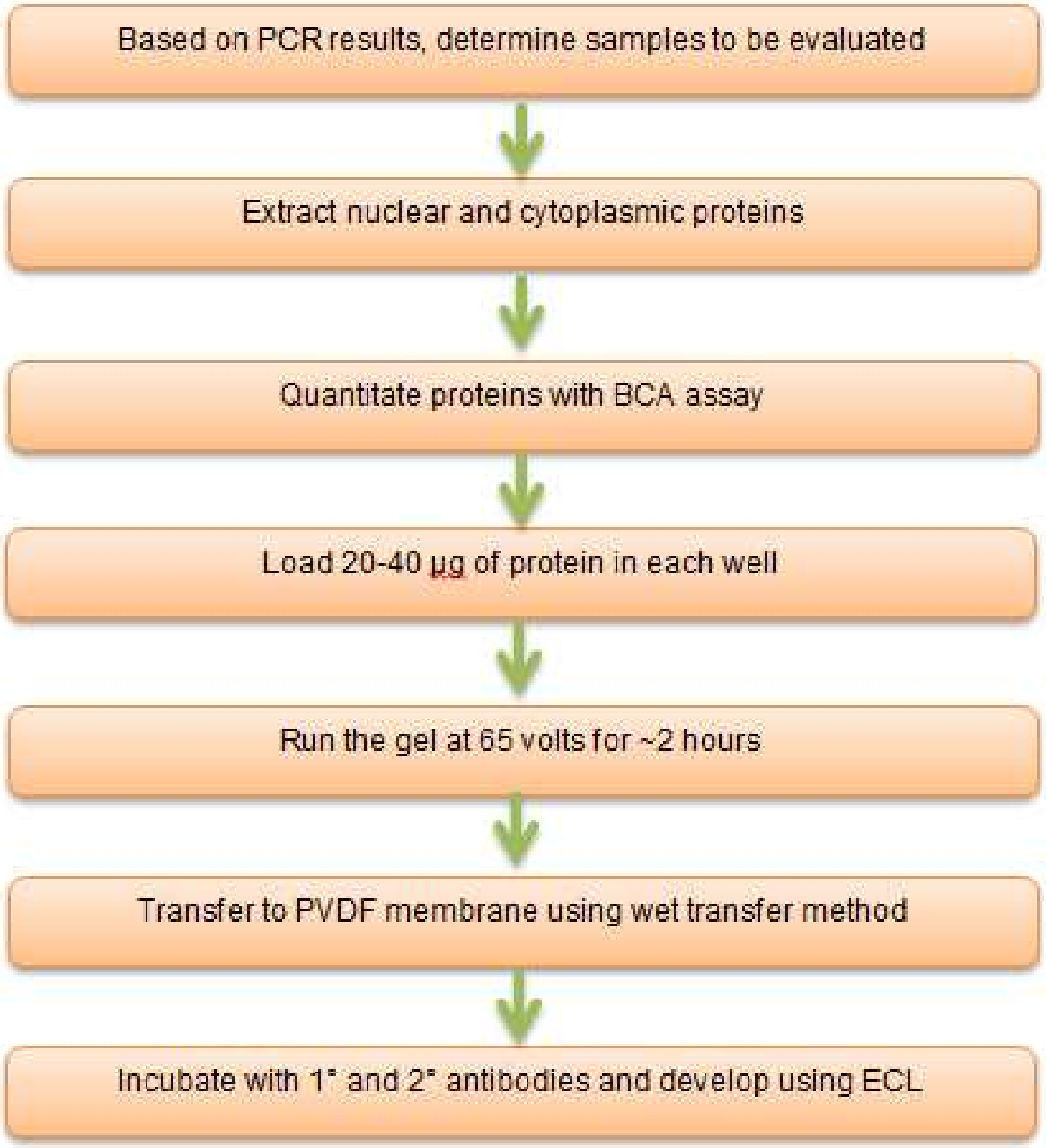


Figure 51. Overview of western blot experiment for the study.

Content	Volume	Molar concentration
1M Hepes (pH 7.9)	100µl	10nM
1M $MgCl_2$	15µl	1.5nM
2M KCl	50µl	10mM
Leupeptin (1mg/ml)	10µl	1µg/ml
Aprotinin (5mg/ml)	2µl	1µg/ml
100mM PMSF (phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride)	100µl	1mM
H_2O	9723µl	
Total	10,000µL	

Table 29. Components of Buffer B.

Content	Volume	
1M Hepes (pH 7.9)	200µl	20nM
1M $MgCl_2$	15µl	1.5nM
2M KCl	2100µl	420mM
Leupeptin (1mg/ml)	10µl	1µg/ml
Aprotinin (5mg/ml)	2µl	1µg/ml
EDTA	4µl	0.2mM
Glyceral	2500µl	25%
100mM PMSF (phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride)	100µl	1mM
H_2O	5069µl	
Total	10,000µl	

Table 30. Components of Buffer C.

Primary Antibody	Product Number	Idiotype	Dilution	Company	Experiment
Histone H3	(FL-136): sc-10809	Rabbit polyclonal IgG	1:500	Santa Cruz Biotech	WB
GAPDH	(G-9): sc-365062	Mouse monoclonal IgG_1	1:500	Santa Cruz Biotech	WB
MGMT	(C-20): sc-8825	Goat polyclonal IgG	1:500	Santa Cruz Biotech	WB
DNMT1	(C-17): sc-10222	Goat polyclonal IgG	1:500 for WB & 1:25 for IHC	Santa Cruz Biotech	WB & IHC
DNMT3a	(H-295): sc-20703	Rabbit polyclonal IgG	1:500	Santa Cruz Biotech	WB
DNMT3b	(T-16): sc-10236	Goat polyclonal IgG	1:500	Santa Cruz Biotech	WB
HIC1	(H-6): sc-271499	Mouse monoclonal IgG2b	1:500 for WB & 1:25 for IHC	Santa Cruz Biotech	WB & IHC
SOX9	(H-90): sc-20095	Rabbit polyclonal IgG	1:500	Santa Cruz Biotech	WB
Beta-actin	A3853	Moue monoclonal IgG	1:500	Sigma Aldrich	WB
ER-Alpha	ab2746	Mouse monoclonal IgG	1:100	Abcam	WB
P16-INK4A	P16D25	Rabbit polyclonal	1:500	Cell Signaling	WB

Table 31. List of primary antibodies and dilutions used for Western Blot and Immunohistochemistry.

Table 31. (cont'd)

P21	(0.N.488): sc-71811	Mouse monoclonal IgG2a	1:500	Santa Cruz Biotech	WB
α Tubulin	(H-300): sc-5546	Rabbit polyclonal IgG	1:500	Santa Cruz Biotech	WB

Primary Antibody	Product Number	Idiotype	Dilution	Company	Experiment
Goat anti-rabbit IgG-HRP	sc-2030	IgG	1:1000	Santa Cruz Biotech	WB
Goat anti-mouse IgG-HRP	sc-2031	IgG	1:1000 for WB & 1:50 for IHC	Santa Cruz Biotech	WB & IHC
Donkey anti-goat IgG-HRP	sc-2020	IgG	1:1000 for WB & 1:50 for IHC	Santa Cruz Biotech	WB & IHC

Table 32. List of secondary antibodies used for Western Blot and Immunohistochemistry.

Immunohistochemistry (IHC)

Materials Required

• Wash Buffer (Tris-buffered saline with 0.05% Tween-20 Detergent): Dissolve contents of one BupH TBS Pack in 500mL of ultrapure water. Add 2.5mL of 10% Tween-20 Detergent and mix well. Store reconstituted buffer at 4℃.

• Primary Antibody: Dilute antibody with Universal Blocker Blocking Buffer at 1:25.

• HRP-conjugated secondary antibody: Dilute antibody with Universal Blocker Blocking Buffer at 1:50.

• Coverslips

Protocol:

1. Permeabilize cells on slide with 0.05% TritonX.

2. Wash slide two times for 3 minutes with Wash Buffer.

3. Add blocking buffer to the slide and incubate for 30 minutes.

4. Blot excess blocker from the tissue sections. Apply the Primary Antibody and incubate tissue at 4*C overnight.

5. Wash slide two times for 3 minutes with Wash Buffer.

6. Apply the HRP Secondary Antibody and incubate tissue for 30 minutes.

7. Wash slides three times for 3 minutes each with Wash Buffer.

8. Remove the DAB/Metal Concentrate (10X) from -20°C storage and mix well by inverting the bottle. Remove quantity required for use and immediately return bottle to -20℃.

9. Prepare a 1X working solution of the DAB/Metal Concentrate (10X) by adding the Stable Peroxide Buffer and mixing well.

10. Add the Metal Enhanced DAB Substrate Working Solution to the slide and incubate for 15mins.

11. Wash slide two times for 3 minutes each with Wash Buffer and counterstain with hematoxylin.

12. Mount slide with Mounting Media and apply coverslip after washing with distilled water.

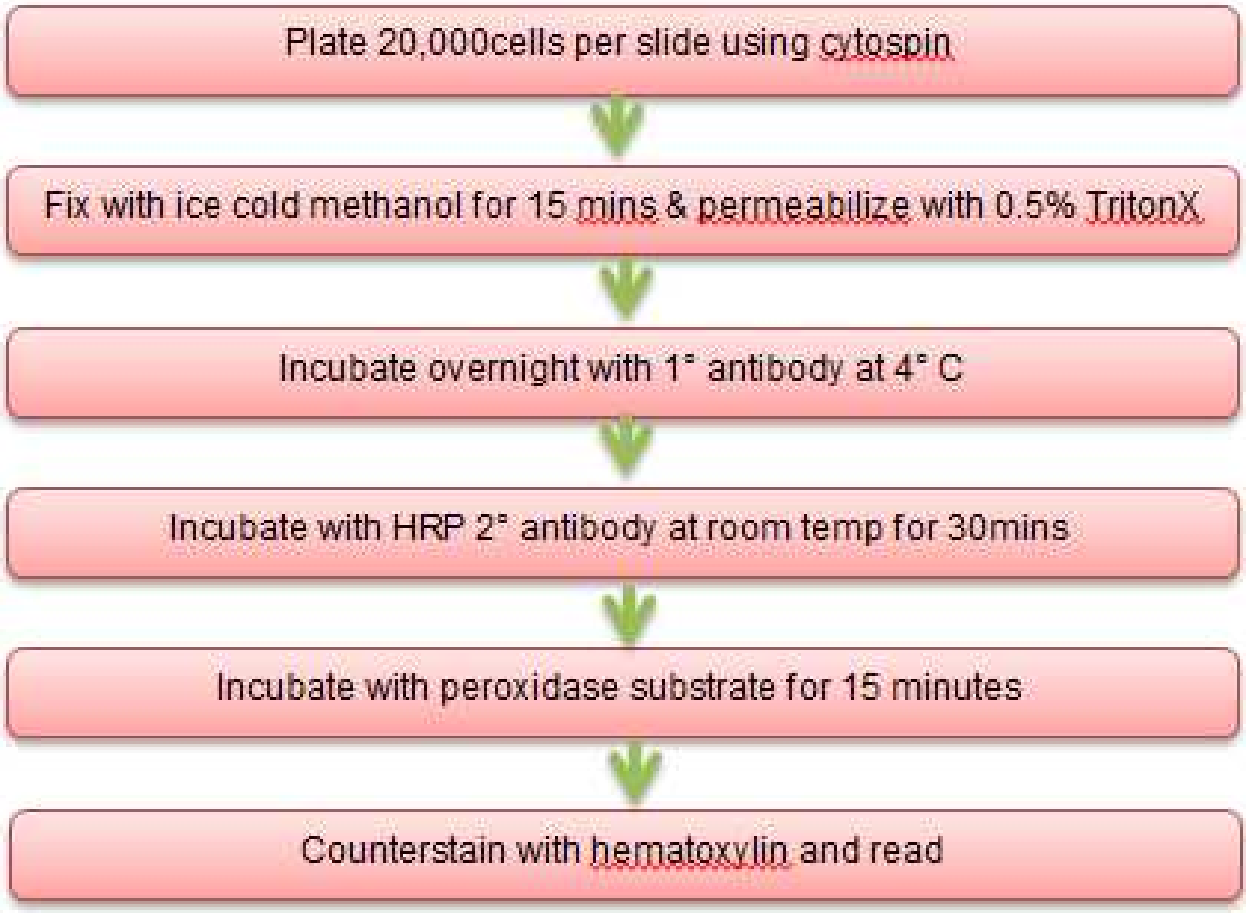


Figure 52. Outline of IHC experiment for the study.

SUPPLEMENTARY DATA

Results of the first MTT-assays performed in Kasumi-1 cell line following combination treatment with XB05 and azacytadine are outlined below. XB05 concentration was kept constant and azacytadine was added in serial concentrations.

XB05 in nM	XB05 0	XB05 0	XB05 0	XB05 0	XB05 0	XB05 0	XB05 100	XB05 100	XB05 100	XB05 100	XB05 100	XB05 100
AZA 9nM)->	0.00	100.00	200.00	400.00	800.00	1000.00	0.00	100.00	200.00	400.00	800.00	1000.00
	0.51	0.55	0.35	0.43	0.36	0.36	0.68	0.51	0.45	0.36	0.35	0.27
	0.72	0.66	0.56	0.50	0.40	0.41	0.78	0.57	0.50	0.33	0.39	0.36
	0.72	0.58	0.54	0.48	0.41	0.41	0.78	0.57	0.52	0.42	0.42	
	0.73	0.58	0.55	0.51	0.46	0.40	0.85	0.59	0.54	0.48	0.48	
	0.68	0.53	0.56	0.51	0.47	0.41	0.84	0.57	0.55	0.46	0.43	0.38
	0.68	0.52	0.50	0.42	0.43	0.36	0.73	0.51	0.45	0.41	0.42	0.33
	0.67	0.57	0.51	0.47	0.42	0.39	0.78	0.55	0.50	0.41	0.41	0.33

Table 33. Results of the first MTT-assays performed in Kasumi-1 cell line following combination treatment with XB05 (0 and 100nM) and serial azacytadine.

XB05 in nM	XB05200	XB05200	XB05200	XB05200	XB05200	XB05200	XB05 400	XB05 400	XB05 400	XB05 400	XB05 400	XB05 400
AZA 9nM)->	0.00	100.00	200.00	400.00	800.00	1000.00	0.00	100.00	200.00	400.00	800.00	1000.00
A	0.54	0.50	0.38	0.41	0.36	0.32	0.61	0.51	0.40	0.37	0.36	0.31
B	0.65	0.55	0.44	0.39	0.34	0.34	0.62	0.49	0.39	0.36	0.36	0.39
C	0.63	0.55	0.44	0.41	0.34	0.35	0.58	0.50	0.40	0.40	0.35	
D	0.68	0.53	0.46	0.39	0.36	0.34	0.61	0.49	0.40	0.40	0.39	0.44
E	0.62	0.53	0.45	0.39	0.32	0.33	0.58	0.52	0.40	0.39	0.36	0.43
F	0.63	0.55	0.45	0.37	0.40	0.35	0.60	0.48	0.46	0.39	0.40	
G	0.62	0.53	0.44	0.40	0.35	0.34	0.60	0.50	0.41	0.38	0.37	0.39

Table 34. Results of the first MTT-assays performed in Kasumi-1 cell line following combination treatment with XB05 (200 and 400nM) and serial azacytadine.

XB05 in nM	XB05 800	XB05 800	XB05 800	XB05 800	XB05 800	XB05 800	XB05 1000	XB05 1000	XB05 1000	XB05 1000	XB05 1000	XB05 1000
AZA 9nM)->	0.00	100.00	200.00	400.00	800.00	1000.00	0.00	100.00	200.00	400.00	800.00	1000.00
A	0.50	0.48	0.36	0.33	0.29	0.28	0.56	0.44	0.35	0.33		0.28
B	0.57	0.48	0.37	0.29	0.26	0.26	0.53	0.40	0.33	0.28		0.30
C	0.52	0.44	0.36	0.32	0.26	0.26	0.50	0.39	0.33	0.28	0.27	
D	0.60	0.44	0.36	0.31	0.25	0.26	0.46	0.41	0.33	0.29	0.30	0.31
E	0.57	0.47	0.37	0.32	0.27	0.25	0.49	0.43	0.35	0.32	0.31	
F	0.57	0.46	0.34	0.32	0.27	0.21	0.51	0.43	0.38	0.34	0.31	
G	0.55	0.46	0.36	0.32	0.27	0.25	0.51	0.42	0.35	0.30	0.30	0.30

Table 35. Results of the first MTT-assays performed in Kasumi-1 cell line following combination treatment with XB05 (800 and 1000nM) and serial azacytadine.

Combined results of two MTT-assays performed in Kasumi-1 cell line following combination treatment with XB05 and azacytadine are outlined below. XB05 concentration was kept constant and azacytadine was added in serial concentrations.

XB05 in nM	XB05 0	XB05 0	XB05 0	XB05 0	XB05 0	XB05 100	XB05 100	XB05 100	XB05 100	XB05 100
AZA in nM->	0.00	100.00	200.00	400.00	800.00	0.00	100.00	200.00	400.00	800.00
	0.67	0.57	0.51	0.47	0.42	0.78	0.55	0.50	0.41	0.41
	0.29	0.23	0.23	0.21	0.20	0.26	0.25	0.23	0.20	0.18
AVG	0.48	0.40	0.37	0.34	0.31	0.52	0.40	0.36	0.31	0.30

Table 36. Combined results of two MTT-assays performed in Kasumi-1 cell line following combination treatment with XB05 (0 and 100nM) and serial azacytadine.

XB05 in nM	XB05 200	XB05 200	XB05 200	XB05 200	XB05 200	XB05 400	XB05 400	XB05 400	XB05 400	XB05 400
AZA in nM->	0.00	100.00	200.00	400.00	800.00	0.00	100.00	200.00	400.00	800.00
	0.62	0.53	0.44	0.40	0.35	0.60	0.50	0.41	0.38	0.37
	0.26	0.24	0.23	0.21	0.18	0.26	0.24	0.24	0.21	0.19
AVG	0.44	0.39	0.33	0.30	0.27	0.43	0.37	0.32	0.30	0.28

Table 37. Combined results of two MTT-assays performed in Kasumi-1 cell line following combination treatment with XB05 (200 and 400nM) and serial azacytadine.

XB05 in nM	XB05 800	XB05 800	XB05 800	XB05 800	XB05 800
AZA in nM->	0.00	100.00	200.00	400.00	800.00
	0.55	0.46	0.36	0.32	0.27
	0.26	0.24	0.20	0.20	0.17
AVG	0.41	0.35	0.28	0.26	0.22

Table 38. Combined results of two MTT-assays performed in Kasumi-1 cell line following combination treatment with XB05 (800nM) and serial azacytadine.

Results of the first MTT-assays performed in U937 cell line following combination treatment with XB05 and azacytadine are outlined below. XB05 concentration was kept constant and azacytadine was added in serial concentrations.

XB05 in nM	XBO5 0	XBO5 0	XBO5 0	XBO5 0	XBO5 0	XBO5 0	XBO5 50	XBO5 50	XBO5 50	XBO5 50	XBO5 50	XBO5 50
AZA nM ->	0.00	50.00	100.00	200.00	400.00	800.00	0.00	50.00	100.00	200.00	400.00	800.00
	0.41	0.33	0.29	0.29	0.24	0.23	0.35	0.31	0.29	0.26	0.26	0.26
	0.39	0.31	0.33	0.28	0.27	0.20	0.37	0.32	0.30	0.27	0.25	0.22
	0.38	0.36	0.31	0.28	0.26	0.23	0.39	0.36	0.31	0.32	0.24	0.23
	0.37	0.32	0.32	0.25	0.27	0.23	0.39	0.35	0.35	0.32	0.26	0.26
	0.38	0.34	0.32	0.30	0.26	0.22	0.35	0.32	0.32	0.30	0.28	0.26
	0.37	0.31	0.28	0.25	0.25	0.21	0.33	0.29	0.29	0.28	0.24	0.23
AVG	0.38	0.33	0.31	0.28	0.26	0.22	0.36	0.33	0.31	0.29	0.25	0.24

Table 39. Results of the first MTT-assays performed in U937 cell line following combination treatment with XB05 (0 and 50nM) and serial azacytadine.

XB05 in nM	XBO5 100	XBO5 100	XBO5 100	XBO5 100	XBO5 100	XBO5 100	XBO5 200	XBO5 200	XBO5 200	XBO5 200	XBO5 200	XBO5 200
AZA nM ->	0.00	50.00	100.00	200.00	400.00	800.00	0.00	50.00	100.00	200.00	400.00	800.00
	0.37	0.32	0.31	0.24	0.21	0.21	0.34	0.30	0.27	0.24	0.20	0.23
	0.36	0.30	0.35	0.25	0.24	0.21	0.33	0.32	0.27	0.25	0.23	0.23
	0.36	0.33	0.32	0.26	0.23	0.21	0.33	0.27	0.28	0.24	0.23	0.22
	0.36	0.32	0.30	0.24	0.23	0.23	0.31	0.27	0.27	0.25	0.24	0.23
	0.35	0.31	0.29	0.25	0.21	0.21	0.33	0.29	0.26	0.24	0.23	0.22
	0.32	0.30	0.29	0.23	0.20	0.22	0.32	0.28	0.26	0.24	0.21	0.19
	0.35	0.31	0.31	0.24	0.22	0.21	0.33	0.29	0.27	0.25	0.22	0.22

Table 40. Results of the first MTT-assays performed in U937 cell line following combination treatment with XB05 (100 and 200nM) and serial azacytadine.

XB05 in nM	XBO5 400	XBO5 400	XBO5 400	XBO5 400	XBO5 400	XBO5 400	XBO5 800	XBO5 800	XBO5 800	XBO5 800	XBO5 800	XBO5 800
AZA nM ->	0.00	50.00	100.00	200.00	400.00	800.00	0.00	50.00	100.00	200.00	400.00	800.00
	0.35	0.30	0.29	0.25	0.23	0.15	0.20	0.28	0.23	0.22	0.21	0.19
	0.34	0.30	0.27	0.27	0.22	0.21	0.28	0.28	0.27	0.23	0.21	0.19
	0.35	0.32	0.27	0.26	0.23	0.21	0.29	0.26	0.25	0.23	0.21	0.19
	0.33	0.32	0.27	0.25	0.23	0.19	0.29	0.27	0.26	0.24	0.20	0.22
	0.35	0.32	0.28	0.25	0.22	0.20	0.30	0.27	0.27	0.22	0.20	0.20
	0.35	0.28	0.27	0.24	0.21	0.19		0.26	0.24	0.24	0.21	0.18
AVG	0.34	0.31	0.28	0.25	0.22	0.19	0.27	0.27	0.25	0.23	0.21	0.19

Table 41. Results of the first MTT-assays performed in U937 cell line following combination treatment with XB05 (400 and 800nM) and serial azacytadine.

Combined results of two MTT-assays performed in U937 cell line following combination treatment with XB05 and azacytadine are outlined below. XB05 concentration was kept constant and azacytadine was added in serial concentrations.

XB05 in nM	XB05 0	XB05 0	XB05 0	XB05 0	XB05 0	XB05 100	XB05 100	XB05 100	XB05 100	XB05 100
AZA nM ->	0.00	100.00	200.00	400.00	800.00	0.00	100.00	200.00	400.00	800.00
	0.68	0.63	0.55	0.51	0.42	0.66	0.52	0.48	0.38	0.39
	0.38	0.31	0.28	0.26	0.22	0.35	0.31	0.24	0.22	0.21
	0.53	0.47	0.41	0.38	0.32	0.51	0.41	0.36	0.30	0.30

Table 42. Combined results of two MTT-assays performed in U937 cell line following combination treatment with XB05 (0 and 100nM) and serial azacytadine.

XB05 in nM	XB05 200	XB05 200	XB05 200	XB05 200	XB05 200	XB05 400	XB05 400	XB05 400	XB05 400	XB05 400
AZA nM ->	0.00	100.00	200.00	400.00	800.00	0.00	100.00	200.00	400.00	800.00
	0.69	0.66	0.52	0.46	0.40	0.75	0.60	0.54	0.48	0.38
	0.33	0.27	0.25	0.22	0.22	0.34	0.28	0.25	0.22	0.19
	0.51	0.47	0.38	0.34	0.31	0.55	0.44	0.39	0.35	0.29

Table 43. Combined results of two MTT-assays performed in U937 cell line following combination treatment with XB05 (200 and 400nM) and serial azacytadine.

XB05 in nM	XB05 800	XB05 800	XB05 800	XB05 800	XB05 800
AZA nM ->	0.00	100.00	200.00	400.00	800.00
	0.65	0.56	0.46	0.39	0.35
	0.27	0.25	0.23	0.21	0.19
	0.46	0.41	0.35	0.30	0.27

Table 44. Combined results of two MTT-assays performed in U937 cell line following combination treatment with XB05 (200 and 400nM) and serial azacytadine.

Previously, we had performed MTT-assays on HCT116 (colon cancer cell line) following treatment with XB05 and DMSO to evaluate if the concentration of DMSO that was used for reconstitution of XB05 was toxic to cells. The results below demonstrate that DMSO even at high concentrations was not cytotoxic to cells.

Concentration ->	0.00	100.00	200.00	400.00	800.00	1600.00
HCT116 vehicle control (DMSO)	1.00	0.93	0.92	0.92	0.87	0.88
	1.00	0.90	0.91	0.87	1.07	0.83
avg	1.00	0.92	0.92	0.89	0.97	0.86
stdev	0.00	0.02	0.01	0.03	0.15	0.04
Hct116 treated with XBO5	1.00	0.89	0.80	0.53	0.24	0.10
	1.00	1.03	0.92	0.57	0.26	0.08
avg	1.00	0.96	0.86	0.55	0.25	0.09
stdev	0.00	0.10	0.08	0.03	0.01	0.02

Table 45. MTT-assays on HCT116 (colon cancer cell line) following treatment with XB05 and DMSO.

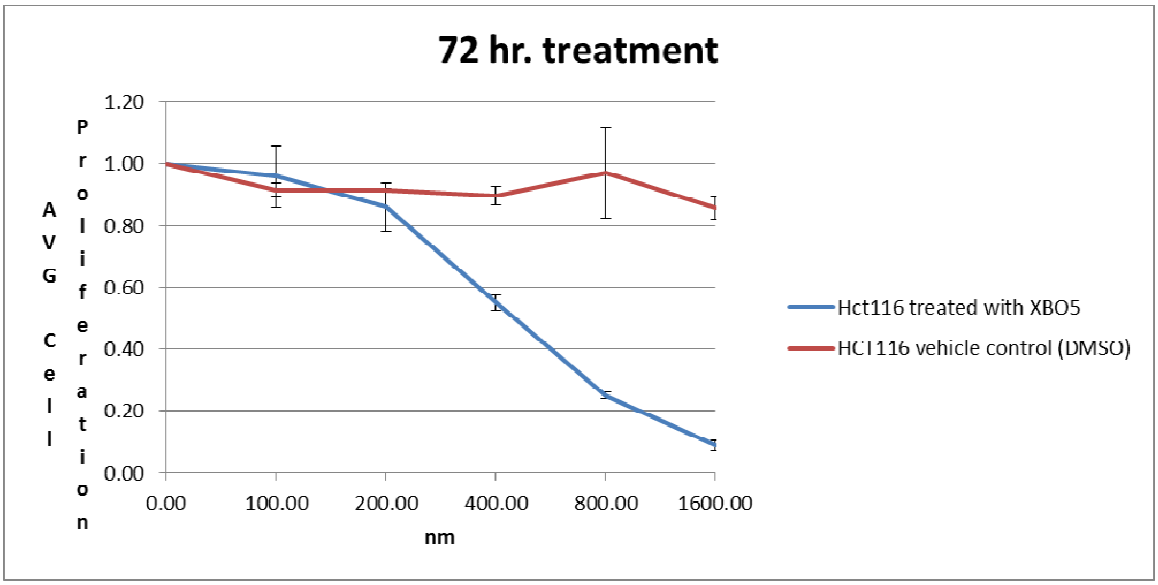


Figure 53. Graphical representation of the MTT-assays on HCT116 (colon cancer cell line) following treatment with XB05 and DMSO.

Target Name	CT	CT Mean	CT SD	ΔCT	ΔCT Mean	ΔCT SE	ΔΔCT	Gene Expression
GAPDH	24.62	24.46	0.22					
GAPDH	24.31	24.46	0.22					
GAPDH	24.00	23.99	0.02					
GAPDH	23.97	23.99	0.02					
GAPDH	27.56	27.44	0.17					
GAPDH	27.33	27.44	0.17					
DNMT1	26.56	26.63	0.10		2.17		0.00	1.00
DNMT1	26.70	26.63	0.10					
DNMT1	27.35	27.28	0.09		3.30		-1.13	0.46
DNMT1	27.22	27.28	0.09					
DNMT1	26.81	27.02	0.30		-0.42		2.59	6.02
DNMT1	27.23	27.02	0.30					
DNMT3a	24.59	24.65	0.09		0.19		0.00	1.00
DNMT3a	24.71	24.65	0.09					
DNMT3a	24.80	24.86	0.08		0.87		-0.68	0.62
DNMT3a	24.91	24.86	0.08					
DNMT3a	27.69	27.70	0.02		0.25		-0.07	0.96
DNMT3a	27.71	27.70	0.02					
DNMT3b	21.71	21.92	0.31		-2.54		0.00	1.00
DNMT3b	22.14	21.92	0.31					
DNMT3b	22.35	22.59	0.34		-1.39		-1.15	0.45
DNMT3b	22.83	22.59	0.34					
DNMT3b	25.70	25.70	0.01		-1.75		-0.79	0.58
DNMT3b	25.69	25.70	0.01					
HIC1	25.13	25.04	0.13		-1.08		0.00	1.00
HIC1	24.94	25.04	0.13					
HIC1	26.62	26.57	0.07		0.37		-2.01	0.25
HIC1	26.52	26.57	0.07					
HIC1	28.80	28.76	0.06		-0.25		-0.74	0.60
HIC1	28.72	28.76	0.06					
ER alpha	25.72	25.69	0.05		-0.43		0.00	1.00
ER alpha	25.66	25.69	0.05					
ER alpha	25.70	25.99	0.41		-0.20		-0.78	0.58
ER alpha	26.28	25.99	0.41					
ER alpha	27.41	27.23	0.26		-1.79		1.45	2.72
ER alpha	27.04	27.23	0.26					
p16	27.06	27.24	0.25		2.77		0.00	1.00
p16	27.42	27.24	0.25					
p16	27.41	27.54	0.18		3.56		-0.78	0.58
p16	27.67	27.54	0.18					
p16	27.65	27.76	0.16		0.32		2.46	5.48
p16	27.88	27.76	0.16					
DAPK	25.15	25.26	0.15		0.79		0.00	1.00
DAPK	25.37	25.26	0.15					
DAPK	24.96	25.05	0.12		1.06		-0.27	0.83
DAPK	25.13	25.05	0.12					
DAPK	27.81	27.73	0.12		0.28		0.51	1.42
DAPK	27.65	27.73	0.12					
MGMT	33.11	33.11	0.00		8.64		0.00	1.00
MGMT	33.11	33.11	0.00					
MGMT	33.09	32.85	0.35		8.86		-0.22	0.86
MGMT	32.60	32.85	0.35					
MGMT	33.08	32.81	0.37		5.37		3.27	9.68
MGMT	32.55	32.81	0.37					
RARb	26.80	26.81	0.02		2.34		0.00	1.00
RARb	26.82	26.81	0.02					
RARb	26.68	26.72	0.06		2.74		-0.40	0.76
RARb	26.76	26.72	0.06					
RARb	27.42	27.38	0.06		-0.07		2.41	5.31
RARb	27.34	27.38	0.06					

Table 46. PCR data for Kasumi-1 cell line – 48 hour treatment with XB05 and 5'AZA.

Sample Name	Target Name	Cт	Cт Mean	Cт SD	ΔCт	ΔCт Mean	ΔCт SE	ΔΔCт	Gene Expression
U937-NT	GAPDH	26.56	27.29	1.04					
U937-NT	GAPDH	28.03	27.29	1.04					
U937-XB	GAPDH	24.74	24.67	0.11					
U937-XB	GAPDH	24.59	24.67	0.11					
U937-Aza	GAPDH	28.94	29.05	0.16					
U937-Aza	GAPDH	29.17	29.05	0.16					
U937-NT	DNMT1	27.13	27.07	0.08		-0.23		0.00	1.00
U937-NT	DNMT1	27.01	27.07	0.08					
U937-XB	DNMT1	27.43	27.44	0.01		2.77		-3.00	0.12
U937-XB	DNMT1	27.45	27.44	0.01					
U937-Aza	DNMT1	27.80	27.87	0.10		-1.19		0.96	1.94
U937-Aza	DNMT1	27.94	27.87	0.10					
U937-NT	DNMT3a	27.50	27.58	0.12		0.29		0.00	1.00
U937-NT	DNMT3a	27.67	27.58	0.12					
U937-XB	DNMT3a	25.02	25.15	0.18		0.49		-24.38	0.00
U937-XB	DNMT3a	25.28	25.15	0.18					
U937-Aza	DNMT3a	28.18	28.09	0.12		-0.96		1.25	2.38
U937-Aza	DNMT3a	28.01	28.09	0.12					
U937-NT	DNMT3b	28.23	28.28	0.07		0.98		0.00	1.00
U937-NT	DNMT3b	28.33	28.28	0.07					
U937-XB	DNMT3b	25.85	25.91	0.09		1.24		-0.26	0.84
U937-XB	DNMT3b	25.97	25.91	0.09					
U937-Aza	DNMT3b	27.66	27.90	0.34		-1.15		2.14	4.39
U937-Aza	DNMT3b	28.15	27.90	0.34					
U937-NT	HIC1	29.47	29.41	0.09		-0.33		0.00	1.00
U937-NT	HIC1	29.34	29.41	0.09					
U937-XB	HIC1	27.82	27.72	0.15		0.98		-0.94	0.52
U937-XB	HIC1	27.61	27.72	0.15					
U937-Aza	HIC1	28.56	28.69	0.19		-2.60		2.47	5.55
U937-Aza	HIC1	28.83	28.69	0.19					
U937-NT	ER alpha	27.57	27.38	0.28		-2.35		0.00	1.00
U937-NT	ER alpha	27.18	27.38	0.28					
U937-XB	ER alpha	26.70	26.65	0.08		-0.09		-1.90	0.27
U937-XB	ER alpha	26.59	26.65	0.08					
U937-Aza	ER alpha	27.25	27.13	0.17		-4.16		2.01	4.02
U937-Aza	ER alpha	27.01	27.13	0.17					
U937-NT	p16	27.70	27.64	0.10		0.34		0.00	1.00
U937-NT	p16	27.57	27.64	0.10					
U937-XB	p16	26.74	26.78	0.07		2.12		-1.78	0.29
U937-XB	p16	26.83	26.78	0.07					
U937-Aza	p16	24.71	25.05	0.48		-4.00		4.34	20.27
U937-Aza	p16	25.40	25.05	0.48					
U937-NT	DAPK	28.51	28.72	0.30		1.43		0.00	1.00
U937-NT	DAPK	28.93	28.72	0.30					
U937-XB	DAPK	26.06	26.04	0.04		1.37		0.06	1.04
U937-XB	DAPK	26.01	26.04	0.04					
U937-Aza	DAPK	29.08	29.01	0.10		-0.05		1.48	2.78
U937-Aza	DAPK	28.94	29.01	0.10					
U937-NT	MGMT	Undetermined	35.53			8.24		0.00	1.00
U937-NT	MGMT	35.53	35.53						
U937-XB	MGMT	38.33	36.42	2.70		11.75		-3.51	0.09
U937-XB	MGMT	34.51	36.42	2.70					
U937-Aza	MGMT	37.34	37.53	0.27		8.47		-0.24	0.85
U937-Aza	MGMT	37.72	37.53	0.27					
U937-NT	RARb	27.74	27.68	0.08		0.39		0.00	1.00
U937-NT	RARb	27.63	27.68	0.08					
U937-XB	RARb	27.44	27.57	0.19		2.90		-2.52	0.17
U937-XB	RARb	27.70	27.57	0.19					
U937-Aza	RARb	27.50	27.52	0.03		-1.53		1.92	3.79
U937-Aza	RARb	27.55	27.52	0.03					

Table 47. PCR data for U937 cell line – 48 hour treatment with XB05 and 5'AZA.

Sample Name	Target Nan	Ct	Ct Mean	Ct SD	ΔΔCt	Gene Expression	
Kasumi-NT	GAPDH	26.06	26.32	0.36			
Kasumi-NT	GAPDH	26.57	26.32	0.36			
Kasumi-XBO	GAPDH	27.98	27.97	0.03			
Kasumi-XBO	GAPDH	27.95	27.97	0.03			
Kasumi-AZA	GAPDH	26.95	26.75	0.29			
Kasumi-AZA	GAPDH	26.54	26.75	0.29			
Kasumi-NT	DNMT1	26.56	26.63	0.10	0.00	1.00	
Kasumi-NT	DNMT1	26.70	26.63	0.10			
Kasumi-XB	DNMT1	27.45	27.21	0.34	1.07	2.10	
Kasumi-XB	DNMT1	26.97	27.21	0.34			
Kasumi-Aza	DNMT1	26.76	26.82	0.09	0.25	1.19	
Kasumi-Aza	DNMT1	26.88	26.82	0.09			
Kasumi-NT	DNMT3a	24.59	24.65	0.09	0.00	1.00	
Kasumi-NT	DNMT3a	24.71	24.65	0.09			
Kasumi-XB	DNMT3a	32.45	32.13	0.44	-5.83	0.02	
Kasumi-XB	DNMT3a	31.82	32.13	0.44			
Kasumi-Aza	DNMT3a	31.88	32.23	0.49	-7.15	0.01	
Kasumi-Aza	DNMT3a	32.58	32.23	0.49			
Kasumi-NT	DNMT3b	21.71	21.92	0.31	0.00	1.00	
Kasumi-NT	DNMT3b	22.14	21.92	0.31			
Kasumi-XB	DNMT3b	28.02	27.94	0.11	-4.37	0.05	
Kasumi-XB	DNMT3b	27.87	27.94	0.11			
Kasumi-Aza	DNMT3b	26.82	26.88	0.10	-4.53	0.04	
Kasumi-Aza	DNMT3b	26.95	26.88	0.10			
Kasumi-NT	HIC-1	25.76	25.69	0.09	0.00	1.00	
Kasumi-NT	HIC-1	25.63	25.69	0.09			
Kasumi-XBO	HIC-1	26.86	26.91	0.06	0.44	1.35	
Kasumi-XBO	HIC-1	26.95	26.91	0.06			
Kasumi-AZA	HIC-1	27.41	27.57	0.22	-1.45	0.37	
Kasumi-AZA	HIC-1	27.73	27.57	0.22			
Kasumi-NT	ER-Alpha	25.71	25.63	0.11	0.00	1.00	
Kasumi-NT	ER-Alpha	25.55	25.63	0.11			
Kasumi-XBO	ER-Alpha	25.81	25.82	0.01	1.46	2.74	
Kasumi-XBO	ER-Alpha	25.83	25.82	0.01			
Kasumi-AZA	ER-Alpha	25.82	25.88	0.09	0.18	1.13	
Kasumi-AZA	ER-Alpha	25.95	25.88	0.09			
Kasumi-NT	p16	27.06	27.24	0.25	0.00	1.00	
Kasumi-NT	p16	27.42	27.24	0.25			
Kasumi-XB	p16	26.72	26.80	0.11	2.08	4.24	
Kasumi-XB	p16	26.88	26.80	0.11			
Kasumi-Aza	p16	26.55	26.66	0.15	1.01	2.02	
Kasumi-Aza	p16	26.76	26.66	0.15			
Kasumi-NT	DAPK	25.15	25.26	0.15	0.00	1.00	
Kasumi-NT	DAPK	25.37	25.26	0.15			
Kasumi-XB	DAPK1	26.15	26.27	0.17	0.64	1.56	
Kasumi-XB	DAPK1	26.39	26.27	0.17			
Kasumi-Aza	DAPK1	26.17	26.07	0.14	-0.38	0.77	
Kasumi-Aza	DAPK1	25.97	26.07	0.14			
Kasumi-NT	MGMT	33.11	33.11	0.00	0.00	1.00	
Kasumi-NT	MGMT	33.11	33.11	0.00			
Kasumi-XB	MGMT	34.44	34.47	0.04	0.29	1.22	
Kasumi-XB	MGMT	34.49	34.47	0.04			
Kasumi-Aza	MGMT	34.40	36.47	2.93	-2.93	0.13	
Kasumi-Aza	MGMT	38.54	36.47	2.93			
Kasumi-NT	RARb	26.80	26.81	0.02	0.00	1.00	
Kasumi-NT	RARb	26.82	26.81	0.02			
Kasumi-XB	RARb	27.45	27.52	0.10	0.93	1.91	
Kasumi-XB	RARb	27.59	27.52	0.10			
Kasumi-Aza	RARb	27.61	27.67	0.09	-0.43	0.74	
Kasumi-Aza	RARb	27.73	27.67	0.09			

Table 48. PCR data for Kasumi-1 cell line – 96 hour treatment with XB05 and 5'AZA.

Target Nar	Ct	Ct Mean	Ct SD	ΔΔCt	Gene Expression	
GAPDH	25.87	25.74	0.18			
GAPDH	25.61	25.74	0.18			
GAPDH	27.95	28.08	0.19			
GAPDH	28.21	28.08	0.19			
GAPDH	28.45	28.13	0.46			
GAPDH	27.81	28.13	0.46			
DNMT1	27.13	27.07	0.08	0.00	1.00	
DNMT1	27.01	27.07	0.08			
DNMT1	27.33	27.32	0.01	2.08	4.24	
DNMT1	27.32	27.32	0.01			
DNMT1	27.16	27.07	0.12	2.39	5.24	
DNMT1	26.99	27.07	0.12			
DNMT3a	27.50	27.58	0.12	0.00	1.00	
DNMT3a	27.67	27.58	0.12			
DNMT3a	32.40	32.38	0.04	-2.46	0.18	
DNMT3a	32.36	32.38	0.04			
DNMT3a	32.32	32.29	0.04	-2.32	0.20	
DNMT3a	32.27	32.29	0.04			
DNMT3b	28.23	28.28	0.07	0.00	1.00	
DNMT3b	28.33	28.28	0.07			
DNMT3b	27.19	27.29	0.15	3.33	10.04	
DNMT3b	27.40	27.29	0.15			
DNMT3b	27.48	27.34	0.20	3.34	10.11	
DNMT3b	27.19	27.34	0.20			
HIC-1	26.97	26.89	0.11	0.00	1.00	
HIC-1	26.82	26.89	0.11			
HIC-1	26.71	26.69	0.04	2.55	5.85	
HIC-1	26.66	26.69	0.04			
HIC-1	26.56	26.51	0.07	2.77	6.84	
HIC-1	26.47	26.51	0.07			
ER-Alpha	25.74	25.76	0.02	0.00	1.00	
ER-Alpha	25.77	25.76	0.02			
ER-Alpha	25.82	25.90	0.11	2.20	4.59	
ER-Alpha	25.98	25.90	0.11			
ER-Alpha	25.91	26.21	0.43	1.94	3.84	
ER-Alpha	26.51	26.21	0.43			
p16	27.70	27.64	0.10	0.00	1.00	
p16	27.57	27.64	0.10			
p16	27.67	27.80	0.19	2.17	4.51	
p16	27.94	27.80	0.19			
p16	27.94	27.80	0.20	2.23	4.70	
p16	27.66	27.80	0.20			
DAPK	28.51	28.72	0.30	0.00	1.00	
DAPK	28.93	28.72	0.30			
DAPK1	25.89	25.83	0.08	5.23	37.64	
DAPK1	25.77	25.83	0.08			
DAPK1	25.89	25.88	0.01	5.24	37.76	
DAPK1	25.87	25.88	0.01			
MGMT	35.53	35.53		0.00	1.00	
MGMT	35.53	35.53				
MGMT	31.22	31.05	0.24	6.82	113.05	
MGMT	30.89	31.05	0.24			
MGMT	31.40	31.64	0.34	6.29	78.31	
MGMT	31.88	31.64	0.34			
RARb	27.74	27.68	0.08	0.00	1.00	
RARb	27.63	27.68	0.08			
RARb	26.44	26.44	0.01	3.58	11.96	
RARb	26.45	26.44	0.01			
RARb	26.45	26.49	0.05	3.59	12.03	
RARb	26.53	26.49	0.05			

Table 49. PCR data for U937 cell line – 96 hour treatment with XB05 and 5'AZA

Standard curve obtained from serial concentrations of BSA (mg/mL) using the Buffer B for dilutions, the same buffer that was used to extract cytoplasmic fractions.

	0.000	0.025	0.125	0.250	0.500	0.750	1.000	1.500	2.000
BSA	0.001	0.015	0.080	0.171	0.323	0.429	0.566	0.743	0.829
BSA	-0.001	0.018	0.084	0.182	0.327	0.417	0.580	0.744	0.827
AVG	0.000	0.017	0.082	0.177	0.325	0.423	0.573	0.744	0.828

Table 50. Average O.D.'s of serial dilutions of BSA for standard curve for cytoplasmic protein.

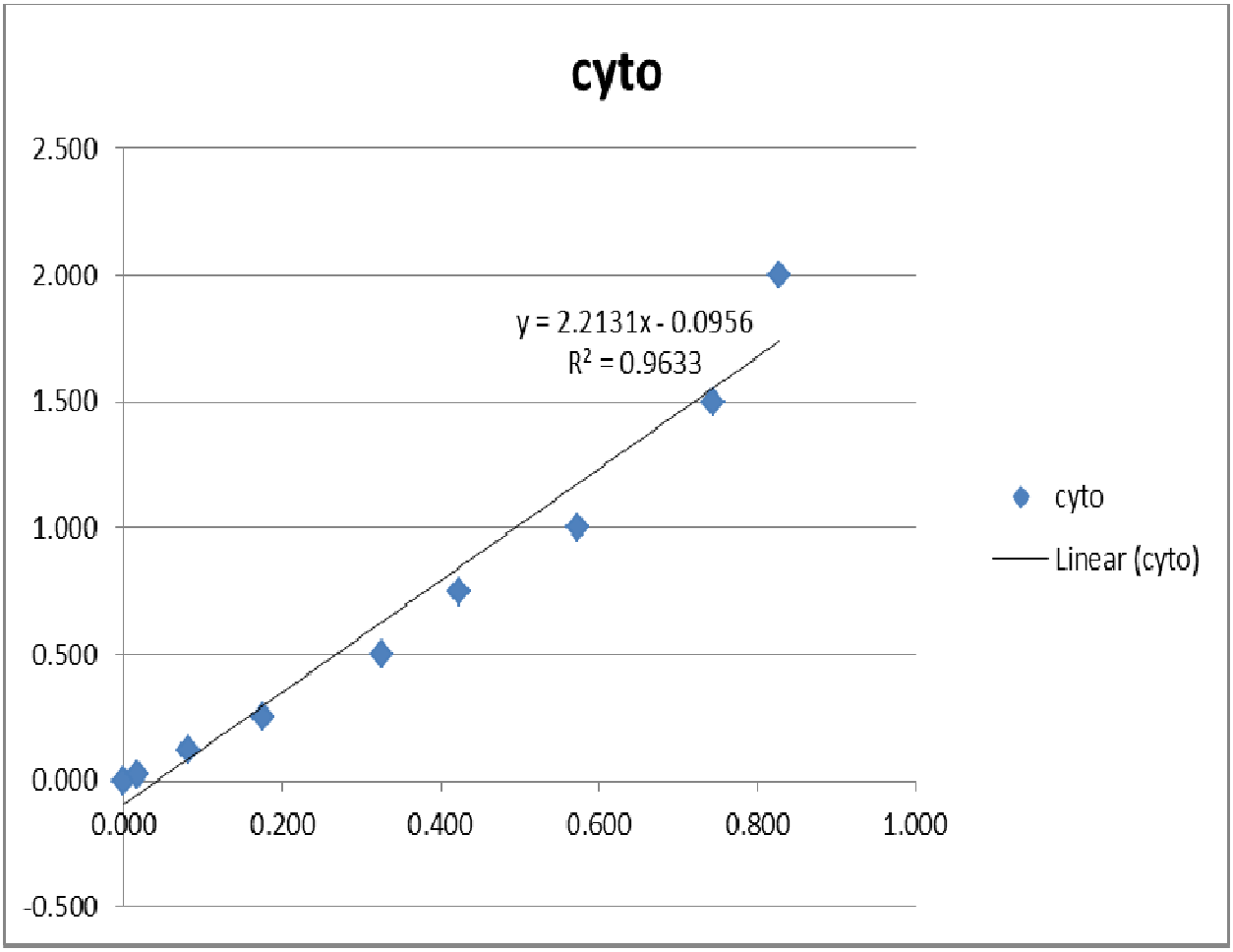


Figure 54. Standard curve for BSA for quantitation of cytoplasmic protein.

Calculation performed to calculate the volume to be loaded in each well for 30ug of protein for cytoplasmic fractions.

	KNT	K XB 48	K XB 96	K A 48	K A 96	UNT	U XB 48	U XB 96	U A 48	U A 96
	0.075	0.055	0.095	0.059	0.123	0.091	0.096	0.122	0.075	0.103
	0.069	0.059	0.096	0.053	0.123	0.079	0.094	0.113	0.067	0.109
AVG	0.072	0.057	0.096	0.056	0.123	0.085	0.095	0.118	0.071	0.106
Protein Quantity (1:20)	0.076	0.069	0.087	0.069	0.099	0.082	0.086	0.097	0.075	0.091
Protein quantity (mg/mL)	1.515	1.379	1.732	1.370	1.976	1.632	1.722	1.930	1.506	1.822
Protein quantity (ug/uL)	1.515	1.379	1.732	1.370	1.976	1.632	1.722	1.930	1.506	1.822
30uq protein	19.8	21.8	17.3	21.9	15.2	18.4	17.4	15.5	19.9	16.5
Laemelli Reagent	19.8	21.8	17.3	21.9	15.2	18.4	17.4	15.5	19.9	16.5
H2O	10.4	6.5	15.3	6.2	19.6	13.2	15.2	18.9	10.1	17.1
Total Volume	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0

Table 51. Cytoplasmic protein calculations for western blot.

Standard curve obtained from serial concentrations of BSA (mg/mL) using the Buffer C for dilutions, the same buffer that was used to extract nuclear fractions.

	0	0.025	0.125	0.25	0.5	0.75	1	1.5	2
BSA	-0.002	0.016	0.077	0.156	0.303	0.422	0.55	0.749	0.831
BSA	0.002	0.012	0.079	0.157	0.314	0.408	0.541	0.739	0.819
AVG	0	0.014	0.078	0.1565	0.3085	0.415	0.5455	0.744	0.825

Table 52. Average O.D.'s of serial dilutions of BSA for standard curve for nuclear protein.

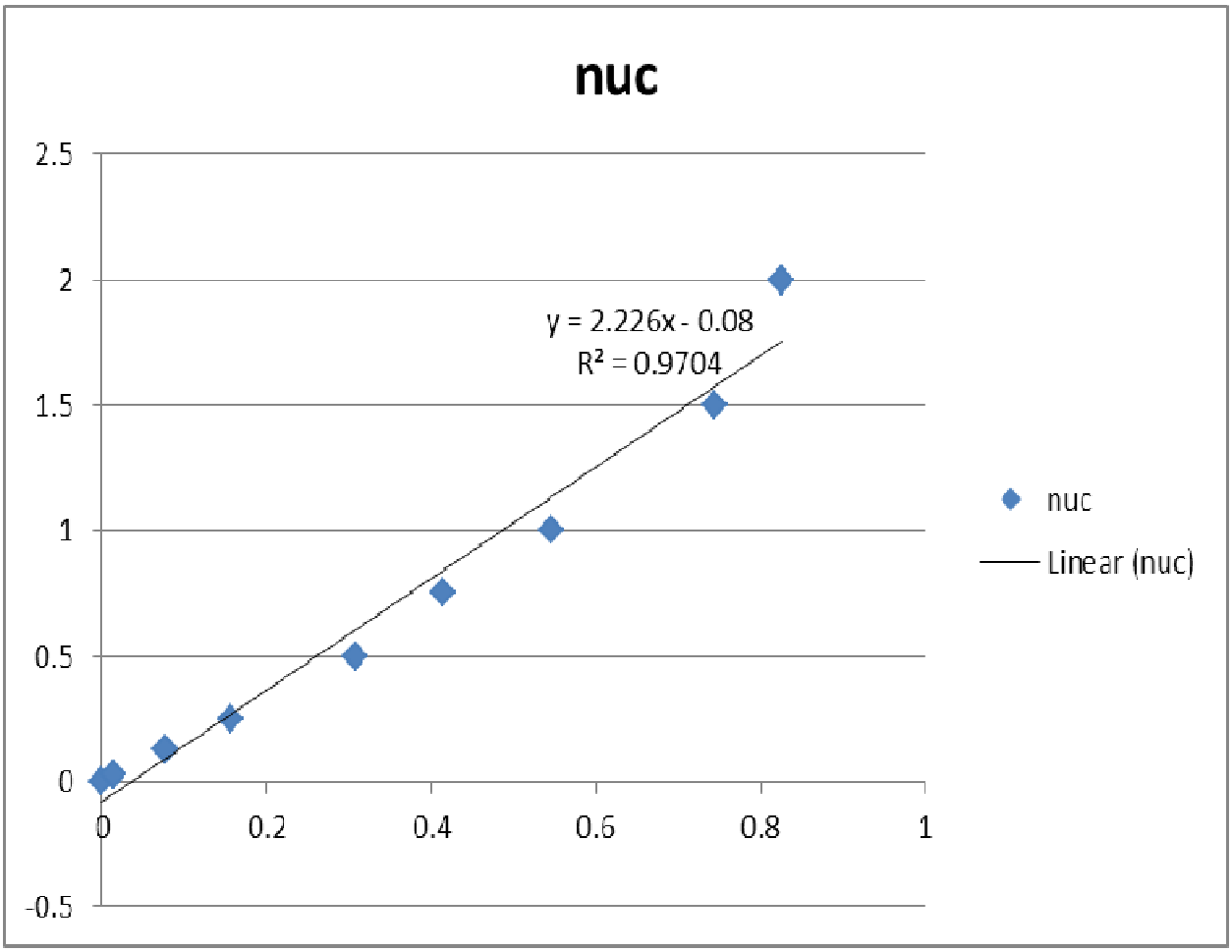


Figure 55. Standard curve for BSA for quantitation of nuclear protein.

Calculation performed to calculate the volume to be loaded in each well for 30ug of protein for nuclear fractions.

	KNT	K XB 48	K XB 96	K A 48	K A 96	UNT	U XB 48	U XB 96	U A 48	U A 96
	0.203	0.131	0.34	0.128	0.159	0.187	0.142	0.181	0.128	0.156
	0.184	0.122	0.347	0.133	0.152	0.179	0.141	0.155	0.136	0.127
AVG	0.194	0.127	0.344	0.131	0.156	0.183	0.142	0.168	0.132	0.142
Protein quantity (1:20)	0.123	0.093	0.19	0.095	0.106	0.118	0.1	0.111	0.095	0.1
Protein quantity (mg/mL)	2.462	1.86	3.81	1.896	2.12	2.363	1.995	2.228	1.905	1.995
Protein quantity (ug/uL)	2.46	1.86	3.81	1.9	2.12	2.36	1.99	2.23	1.9	1.99
uL for 30ug	12.2	16.1	7.9	15.8	14.1	12.7	15	13.5	15.8	15
Laemelli Reagent	12.2	16.1	7.9	15.8	14.1	12.7	15	13.5	15.8	15
H2O	25.6	17.7	34.3	18.4	21.7	24.6	19.9	23.1	18.5	19.9
Total Volume	50	50	50	50	50	50	50	50	50	50

Table 53. Cytoplasmic protein calculations for western blot.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Ferrara, F. and C.A. Schiffer, *Acute myeloid leukaemia in adults.* Lancet, 2013. **381**(9865): p. 484-95.

2. Kern, W. and E.H. Estey, *High-dose cytosine arabinoside in the treatment of acute myeloid leukemia: Review of three randomized trials.* Cancer, 2006. **107**(1): p. 116-24.

3. Shih, A.H., et al., *The role of mutations in epigenetic regulators in myeloid malignancies.* Nat Rev Cancer, 2012. **12**(9): p. 599-612.

4. Kelly, L.M. and D.G. Gilliland, *Genetics of myeloid leukemias.* Annu Rev Genomics Hum Genet, 2002. **3**: p. 179-98.

5. Figueroa, M.E., et al., *DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia.* Cancer Cell, 2010. **17**(1): p. 13-27.

6. Oki, Y. and J.P. Issa, *Epigenetic mechanisms in AML - a target for therapy.* Cancer Treat Res, 2010. **145**: p. 19-40.

7. Gutierrez, S.E. and F.A. Romero-Oliva, *Epigenetic changes: a common theme in acute myelogenous leukemogenesis.* J Hematol Oncol, 2013. **6**: p. 57.

8. Esteller, M., *Epigenetic gene silencing in cancer: the DNA hypermethylome.* Hum Mol Genet, 2007. **16 Spec No 1**: p. R50-9.

9. Ordway, J.M., et al., *Comprehensive DNA methylation profiling in a human cancer genome identifies novel epigenetic targets.* Carcinogenesis, 2006. **27**(12): p. 2409-23.

10. MP, C., *Decitabine: A Review of its Use in Older Patients with Acute Myeloid Leukaemia.* Drugs Aging, 2013. **30**(6): p. 447-58.

11. Estey, E.H., *Acute myeloid leukemia: 2013 update on risk-stratification and management.* Am J Hematol, 2013. **88**(4): p. 318-27.

12. O'Donnell, M.R., et al., *Acute myeloid leukemia.* J Natl Compr Canc Netw, 2012. **10**(8): p. 984-1021.

13. Sweet, K. and J.E. Lancet, *Novel therapeutics in acute myeloid leukemia.* Curr Hematol Malig Rep, 2014. **9**(2): p. 109-17.

14. Yan P, F.D., Murphy M, Tam HH, Rodriguez B, Curfman J, Trimarchi M, Geyer S, Wu YZ, Whitman SP, Metzeler K, Walker A, Klisovic R, Jacob S, Grever MR, Byrd JC, Bloomfield CD, Garzon R, Blum W, Caligiuri MA, Bundschuh R, Marcucci G, *Genome-wide methylation profiling in decitabine-treated patients with acute myeloid leukemia.* Blood, 2012. **120**(12): p. 2466-74.

15. Conway O'Brien, E., S. Prideaux, and T. Chevassut, *The epigenetic landscape of acute myeloid leukemia.* Adv Hematol, 2014. **2014**: p. 103175.

16. Jiang, S., et al., *Reactivating aberrantly hypermethylated p15 gene in leukemic T cells by a phenylhexyl isothiocyanate mediated inter-active mechanism on DNA and chromatin.* J Hematol Oncol, 2010. **3**: p. 48.

17. Lakshmikuttyamma, A., et al., *Reexpression of epigenetically silenced AML tumor suppressor genes by SUV39H1 inhibition.* Oncogene, 2010. **29**(4): p. 576-88.

18. Clark, S.J. and J. Melki, *DNA methylation and gene silencing in cancer: which is the guilty party?* Oncogene, 2002. **21**(35): p. 5380-7.

19. Kulis, M. and M. Esteller, *DNA methylation and cancer.* Adv Genet. **70**: p. 27-56.

20. Estey, E.H., *Epigenetics in clinical practice: the examples of azacitidine and decitabine in myelodysplasia and acute myeloid leukemia.* Leukemia, 2013. **27**(9): p. 1803-12.

21. Geyer, C.R., *Strategies to re-express epigenetically silenced p15(INK4b) and p21(WAF1) genes in acute myeloid leukemia.* Epigenetics, 2010. **5**(8): p. 696-703.

22. Yun-Gyoo Lee, I.K., Sung-Soo Yoon, Seonyang Park, June Won Cheong, Yoo Hong Min, Jeong-Ok Lee, Soo-Mee Bang, Hyeon Gyu Yi, Chul Soo Kim, Yong Park, Byung-Soo Kim, Yeung-Chul Mun, Chu-Myoung Seong, Jinny Park, Jae Hoon Lee, Sung-Yong Kim, Hong Ghi Lee, Yeo-Kyeoung Kim and Hyeoung-Joon Kim, *Comparative analysis between azacitidine and decitabine for the treatment of myelodysplastic syndromes.* British Journal of Haematology, 2013. **161**: p. 339-347.

23. Hagemann, S., et al., *Azacytidine and decitabine induce gene-specific and non-random DNA demethylation in human cancer cell lines.* PLoS One, 2011. **6**(3): p. e17388.

24. Francesca R. Šalipur, E.M.R.-R., Bo Xu, Gerald B. Hammond and Paula J. Bates, *A Novel Small Molecule that Selectively Kills Malignant Cells via an Oxidative Stress-Dependent Mechanism* Free Radical Biology and Medicine, 2013. **68**: p. 110-121.

25. Irwin ME, R.-D.V.N., Chandra J, *Redox control of leukemia: from molecular mechanisms to therapeutic opportunities.* Antioxid Redox Signal, 2013. **18**(11): p. 1349-83.

26. Rushworth, S.A., K.M. Bowles, and D.J. MacEwan, *High basal nuclear levels of Nrf2 in acute myeloid leukemia reduces sensitivity to proteasome inhibitors.* Cancer Res. **71**(5): p. 1999-2009.

27. Rushworth, S.A., et al., *The high Nrf2 expression in human acute myeloid leukemia is driven by NF-kappaB and underlies its chemo-resistance.* Blood. **120**(26): p. 5188-98.

28. Wu, R.P., et al., *Nrf2 responses and the therapeutic selectivity of electrophilic compounds in chronic lymphocytic leukemia.* Proc Natl Acad Sci U S A. **107**(16): p. 7479-84.

29. Salipur, F.R., et al., *A novel small molecule that induces oxidative stress and selectively kills malignant cells.* Free Radic Biol Med, 2014. **68**: p. 110-21.

30. Sun L, M.L., Cabarcas SM, Zhang X, Yang A, Zhang Y, Young MR, Klarmann KD, Keller JR, Farrar WL, *Epigenetic Regulation of SOX9 by the NF-κB Signaling Pathway in Pancreatic Cancer Stem Cells.* Stem Cells, 2013: p. 1-25.

31. Flotho, C., et al., *The DNA methyltransferase inhibitors azacitidine, decitabine and zebularine exert differential effects on cancer gene expression in acute myeloid leukemia cells.* Leukemia, 2009. **23**(6): p. 1019-28.

32. Shen, N., et al., *A nucleolin-DNMT1 regulatory axis in acute myeloid leukemogenesis.* Oncotarget, 2014. **5**(14): p. 5494-509.

33. Garcia-Dominguez, P., et al., *Synthetic approaches to DNMT inhibitor SGI-1027 and effects on the U937 leukemia cell line.* Bioorg Med Chem Lett, 2013. **23**(6): p. 1631-5.

34. Soultanas, P., et al., *Modulation of human DNA methyltransferase activity and mRNA levels in the monoblast cell line U937 induced to differentiate with dibutyryl cyclic AMP and phorbol ester.* J Mol Endocrinol, 1993. **11**(2): p. 191-200.

35. Larizza, L., I. Magnani, and A. Beghini, *The Kasumi-1 cell line: a t(8;21)-kit mutant model for acute myeloid leukemia.* Leuk Lymphoma, 2005. **46**(2): p. 247-55.

36. Asou, H., et al., *Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation.* Blood, 1991. **77**(9): p. 2031-6.

37. Sundstrom, C. and K. Nilsson, *Establishment and characterization of a human histiocytic lymphoma cell line (U-937).* Int J Cancer, 1976. **17**(5): p. 565-77.