SYNERGISTIC APOPTOTIC EFFECTS OF BORTEZOMIB AND METHYLSTAT INHIBITOR ON DIFFERENT MULTIPLE MYELOMA CELL LINES

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by
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ABSTRACT

SYNERGISTIC APOPTOTIC EFFECTS OF BORTEZOMIB AND METHYLSTAT INHIBITOR ON DIFFERENT MULTIPLE MYELOMA CELL LINES

Multiple myeloma is one of the common hematological malignancies that affects plasma cells. Bortezomib, proteasome inhibitor, is an anticancer agent used for the treatment of multiple myeloma while methylstat is a demethylase inhibitor having anticancer potential.

In this study, we investigated antiproliferative and apoptotic effects of methylstat alone or in combination with bortezomib. We also examined the genes involved in methylstat induced apoptosis.

Cytotoxic effects of bortezomib and methylstat on U266 and ARH77 cells were demonstrated by MTT cell proliferation assay. To understand the apoptotic effects of these agents, loss of mitochondrial membrane potential was investigated by JC-1 method while phosphatidylserine localization was investigated by Annexin V assay. Cell cycle analysis in response to Bortezomib and Methylstat alone or in their combination were measured by flow cytometry. Changes in expression profiles of 84 genes underlying apoptosis, cell cycle control, DNA damage repair, and invasion and metastasis in response to Methylstat were determined by PCR Array.

Our results demonstrated that both bortezomib and methylstat have antiproliferative and aoptotic effects in a time and dose dependent manner. Combination of bortezomib and methylstat induced apoptosis significantly as compared to any agent alone. In conclusion, we suggest methylstat as candidate agent for the treatment of MM after *in vivo* analyses.

ÖZET

BORTEZOMİB VE METİLSTAT İNHİBİTÖRÜNÜN FARKLI MULTİPL MYELOMA HÜCRE HATLARI ÜZERİNDEKİ SİNERJİK APOPTOTİK ETKİLERİ

Multiple myeloma, plazma hücrelerini etkileyen hemotolojik malignansilerin yaygın bir türüdür. Multiple myeloma tedavisi için kullanılan bortezomib bir proteazom inhibitörü olarak iş görürken, metilstat ise anti-kanser potansiyele sahip bir demetilaz inhibitörüdür.

Bu çalışmada, hem yalnızca metilstatın, hem de metilstat ve bortezomib kombinasyonunun anti-proliferatif ve apoptotik etkilerini araştırdık. Ayrıca metilstat ile indüklenen apoptozda rol oynayan gen profillerini inceledik.

Bortezomib ve metilstatın U266 ve ARH77 hücreleri üzerindeki sitotoksik etkileri MTT hücre proliferasyon testi ile gösterilmiştir. Bu ajanların apoptotik etkileri ise; mitokondriyal membran potansiyelindeki düşüşler JC-1 kiti, fosfotidil serin lokalizasyonu Annexin V testi ile belirlenmiştir. Hücre siklusu analizleri, flow sitometri kullanılarak gerçekleştirilmiştir.

Metilstata yanıt olarak belli başlı apoptoz, hücre siklusu, DNA hasar tamiri ve invazyon ve metastaz ile ilgili 84 genin ekspresyon profillerindeki değişiklikler PCR Array ile belirlenmiştir.

Sonuç olarak, hem bortezomib hem de metilstatın zamana ve doza bağlı olarak anti-proliferatif ve apoptotik etkilere sahip oldukları tespit edilmiştir. Ajanların tek başlarına ve kombinasyon halinde uygulandığı sonuçlar karşılaştırıldığında, bortezomib ve metilstatın sinerjik olarak apoptozu tetiklediği gösterilmiştir. Sonuç olarak, metilstatın yapılacak *in vivo* ek analizlerden sonra MM'nın daha etkili tedavisi için önemli bir ajan olarak kullanılabileceğini öngörmekteyiz.

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CHAPTER 1

INTRODUCTION

1.1. Multiple Myeloma (MM)

Multiple myeloma (MM) is proliferation of malfunctioning and cancerous plasma cells from a single clone. After the non-Hodgkin lymphoma, MM is the second most common hematological malignancy. Its name comes from "myelo-" (bone marrow) and "-oma" (tumor) (Gupta, 2013).

Von Rustizky introduced the "Multiple Myeloma" term for the first time in 1873. He observed transformation of plasma cells in bone marrow as clumps at many sites, and thus named it multiple myeloma (Gupta, 2013).

In 2001, 14,400 cases and 11,200 deaths; in 2007, 19,900 cases and 10,790 deaths (Gupta, et.al. 2013); while in 2013, 21,700 cases and 10,710 deaths (Kurtin and Bilotti, 2013) were recorded in USA. It is clear that the number of cases is increased and the number of deaths is decreased over time. Increase in the frequency of medical surveillances, more effective treatments and awareness of the disease can explain the decreases in deaths.

Nearby all cases of MM is preceded by 'monoclonal gammopathy of undetermined significance' (MGUS) (Landgren and Kyle, 2009; Weiss and Abadie, 2009) and it especially affects people over the age of 50. Genetic and environmental factors are also effective in the progress of disease from MGUS to MM (Kyle and Kumar, 2009). MM patient's BM microenvironment includes malignant plasma cells producing cytokines and resulting in the bone lesions because of the unbalance between bone resorption and formation (Bataille et al., 1989).

1.1.1. Molecular Biology of MM

The most important hallmarks of the cancer cells are resulting from genetic abnormalities. In MM patients, numerical and structural chromosomal abnormalities can be observed making MM very complex disease (Dewald and Kyle, 1985; Calasanz et al., 1997). Hyperdiploidy and nonhyperdiploidy, translocations, oncogene activation, tumor suppressor gene inactivation and growth factor dysregulation were observed in MM patients (Bottura, 1963).

Most importantly, genetic heterogeneity was observed in multiple myeloma patients (Gupta, 2013; Prideaux et al., 2014). Abnormal class switch recombinations (CSR) translocate the immunoglobulin heavy chain alleles (IGH@), which are at 14q32, with chromosomes 4, 6, 11, 16 and 20 (Prideaux et al., 2014). The most common chromosomal abnormality in myeloma occurs between chromosome 11 and 14 t(11:14)(q13;q32) and involves bcl-1 oncogene. This translocation is observed in 15% of the MM patients and bring oncogenes under the regulation of IGH@ enhancer region and overexpressed (Bergsagel and Kuehl, 2005; Fonseca et al., 2002). This is called as nonhyperdiploidy myeloma (Prideaux et al., 2014).

Hyperdiploidy is observed in nearly 50% of myeloma patients and related with bone disease. It is trisomy of the odd numbered chromosomes, especially for chromosomes 3, 5, 7, 9, 11, 15, 19, and 21. These translocations cause overexpression of cyclin D genes, and thus, deregulating G1/S cell cycle checkpoint. t(4;14)(p16;q32) is the second observed nonhyperdiploidy myeloma. As a result, *fibroblast growth factor receptor 3 (FGFR3)* and a multiple myeloma SET domain containing protein, MMSET (*Wolf-Hirschhorn syndrome candidate 1* (*WHSC1))* genes are overexpressed under the influence of IGH alleles enhancers. *MMSET* gene encodes a protein, which remodels chromatins with the help of its histone methyltransferase activity. Inactivation of *MMSET* genes induce apoptosis and inhibit cellular proliferation. All in all, t(4;14) translocation causes upregulation of cyclin D2 and cyclin D1 (Prideaux et al., 2014).

Translocations, t(6;14) and t(11;14) cause upregulation of CCND3 (encodes cyclin D3) and CCND1 (encodes cyclin D1) genes, respectively. They use the same mechanism with t(4;14), t(14;16) and t(14;20) causing overexpression of MAF family oncogenes (Prideaux et al., 2014). Approximately 6-7% of MM patients have this

translocation. These cells show uncontrolled cell proliferation and escape from apoptosis. The genetic abnormalities start with translocations of oncogenes like cyclin D1 on 11q13, cyclin D3 on 6q21, MMSE on 4p16.3,c-maf on 16q23 and maf-B on 20q11 to the immunoglobulin heavy chain locus on chromosome 14. These changes promote angiogenesis in the BM and proliferation of the cells and very important indicators of MM (Munshi and Avet-Loiseau, 2011).

Also, abnormalities resulting from whole or a part of chromosome 13 are observed in approximately 50% of MM patients and t(4;14) is related with the chromosome 13 monosomy (Nakamura and Merchav, 1989).

Del 17p13 (the p53 locus), Ras mutation, activation of NFκB transcription factor and overexpression of *BCL-2* are also observed in MM patients (Al-farsi, 2013). Proto-oncogenes activation via chromosomal translocations or mutations and tumor suppressor gene inactivation cause the emergence of the MM disease. As a result of translocations, new fusion transcripts may be generated and they can activate protooncogenes. For example, *MYC* expression level is very important for progression of the disease. *c-myc* is a protooncogene and responsible for coding a transcriptional factor stimulating B-cell proliferation and repression of B-cell differentiation (Selvanayagam and Blick, 1988). It is overexpressed in 15% of newly diagnosed tumors and 50% of advanced tumors (Chesi and Bergsagel, 2013).

p53 mutations are detected in 37% of untreated MM tumors with del17p, but not in patients without del17p. These mutations are observed in advanced and agressive stages of disease (Chesi et al., 2014).

Rb1 gene is normally expressed in human hematopoietic cells but there is only one functional copy of *Rb1* gene (other copy inactivated by mutations) in MM patients, hence one copy of gene does not produce active protein product and it stimulates tumorigenesis (Chesi and Bergsagel, 2013). 17% of MM patients has this situation (Cozzolino and Torcia, 1989), while 15% of patients have *N-RAS* and *K-RAS* mutations (Korde et al., 2011).

Table 1.1. Genetic Changes in MM
(Source: Gahrton and Durie, 1996)

Candidate oncogenes	**Localization of genes on chromosomes**	**Activation mechanism**	**Cellular function**
Cyclin D1	11q13	Translocation	Regulator of cell cycle
FGFR3 and *MMSET/WHSC1*	4p16	Translocation	Growth factor receptor and Epigenetic regulator for chromatin remodeling
Cyclin D3	6p21	Translocation	Regulator of cell cycle
c-MAF	16q23	Translocation	Transcription factor
c-MYC	8q24	Translocation	Proliferation, differentiation and apoptosis
BCL-2	18q21		Apoptos inhibition
N-RAS	1p11-13	Point mutation	Growth factor independent proliferation
K-RAS	12p11-12	Point mutation	Growth factor independent proliferation
p53	17p13	Point mutation/ Deletion	Regulator of cell cycle
Rb1	13q14	Deletion	Regulator of cell cycle

DcR3 is a member of TNF receptor superfamily and is responsible for the osteoclast (OC) differentiation (17). In MM patients, DcR3 is overexpressed from malignant plasma cells and T- lymphocytes (Colucci et al., 2009).

IL-3 is a proosteoclastogenic factor and it stimulates OC formation in MM patients. It also plays an important role in the inhibition of osteoblast (OB) differentiation. Consequently, the level of IL3 is higher in BM and MM patient's blood (Merico and Bergui, 1993).

MM cells produce high levels of MIP-1α chemokine and it interacts with CCR1 and CCR5 receptors. It is involved hematopoiesis, OC recruitment, BM and dentritic cell (DC) differentiation. It was shown that inhibition of CRR1 receptor affects the osteoclastogenesis and OC-induced tumor cell proliferation *in vitro* (Vallet, Raje and Ishitsuka, 2007).

MIP-1β is also a chemokine stimulating the formation of bone damage (Roodman, 1997).

TNF-α is produced by MM cells. It has several functions such as OC formation, MM cell proliferation, inhibition of mesenchymal stem cell proliferation and induction of OB apoptosis. First three functions are related with IL-6 secretion from BMSCs (Galson et al., 2012). IL-6 is a cytokine that plays role as a inducer of human osteoclast formation (Kurihara and Bertolini, 1990) and RANKL production in addition to its anti-apoptotic and proliferative effects (Rossi et al., 2005). NF-κB plays a crucial role in activation of IL-6. Firstly, IL-6 to binds its receptor and through RAS/Raf/MEK-ERK signaling pathway activation, it stimulates proliferation of myeloma cells (Hideshima and Richardson, 2001). MAPK and PI3K/Akt/mTOR (mammalian target of rapamycin) signaling pathway also causes proliferation and migration of MM cells (Bruyne and Bos, 2010).

IL-7 was defined as a suppressor of OB differentiation and IL-7 levels are increased in the bone marrow plasma of the MM patients (Giuliani et al., 2012).

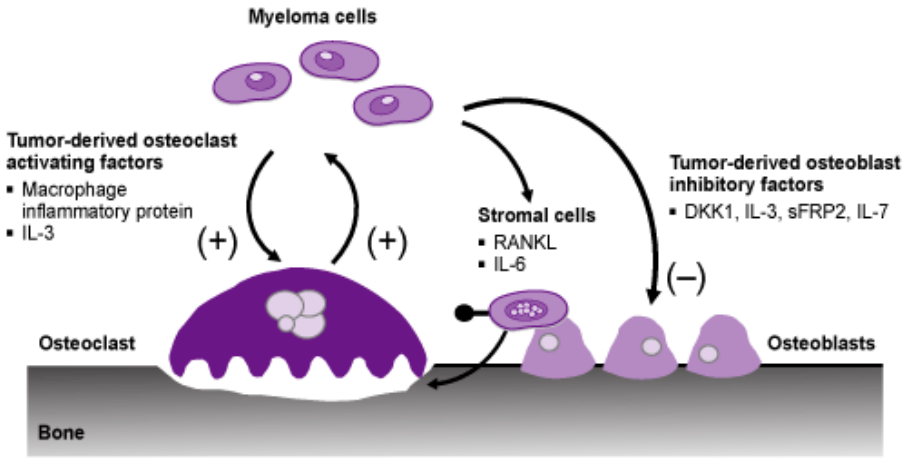

Figure 1.1. Tumor-micro Environmental Interactions in MM
(Source: Roodman, 1997)

1.1.2. Risk Factors for MM

Multiple myeloma is mainly caused by genetic mutations (Prideaux et al., 2014). MM is observed more commonly in developing countries as compared to developed countries. Life expectancy, frequency of medical surveillance and awareness of the disease might cause this difference. The incidence rate of disease increases with the age. The average age of diagnosis is between 62-65 in developed countries and almost 10 years less in developing countries. The frequency of the disease is 1.2 - 1.5 fold higher in males than women (Gupta, 2013). Race is also another risk factor for MM. White people has lower incidence of MM than black people. Black people has almost two times more risk of developing MM (Gahrton et al., 2004).

Overweight and obesity affect the development of MM since obese people can produce more IL-6 protein and it stimulates the proliferation of normal and malignant plasma cells (Larsson and Wolk, 2007). It is also related with exposure to petroleum products and radiation, inflammation, autoimmune diseases and viral infections.

Exposure to the radiation from nuclear warheads in World War II, increased the frequency of having multiple myeloma while the same was true for the exposure to the petroleum products (Gupta, 2013). Also, viral infections such as HIV, Hepatitis, and Herpesvirus 8 might also cause or at least contribute to the disease (Gupta, 2013).

1.1.3. Diagnosis of MM

MM is a neoplastic plasma cell disorder characterized by proliferation of malignant plasma cells in the bone marrow. Mainly, MM patients divided into two groups: Patients those who could show symptoms of disease (active/symptomatic MM) or not (smoldering/ asymptomatic MM) (Durie et al., 2003; Kyle and Rajkumar, 2008).

Symptomatic MM patients have different complications. At diagnosis, nearly 70% of patients have bone pain, 20-60% weakness, 10-20 with hypercalcemia, 15% with loss of weight and 10-20% with infections (Gahrton et al., 2004).

These patients also show additional symptoms such as skeletal lesions, bone marrow failure, renal insufficiency, inhibition of normal blood cells and

immunoglobulin production and increased rate of monoclonal immunoglobulin molecules (M-protein) or immunoglobulin light chains (Bence Jones proteins) in their serum or urine. The amount of these molecules is used as a diagnostic marker for MM patients. So we can say that MM affects the mainly three different organs in the body that are bone, blood and kidneys (International myeloma foundation, 2011).

In order to determine these symptoms, laboratory tests play crucial role. Especially, complete blood count, creatinine and calcium, serum and protein electrophoresis (with immunofixation), M protein quantification, a 24-hour specimen of urine Bence-Jones protein evaluation and free light chain assay are basic methods for diagnostic procedure (Dispenzieri et al., 2008).

Additionally, some imaging methods which are computed tomography (CT or CAT) and magnetic resonance (MRI) are used for diagnosis of MM (Zamagni et al., 2007).

Table 1.2. General Laboratory Tests for Diagnosis of MM
(Source: Multiple Myeloma Disease, 2010)

Diagnostic Test	Purpose	Results
Blood Specimen		
Complete blood count (number of red blood cells, white blood cells, platalets and relative proportion of white blood cells)	Determine the degree to which myeloma is interfering with the normal production of blood cells	Low levels may signal anemia increased risk of infection and poor clotting
Chemistry profile (albumin, calcium lactate dehydogenase (LHD), blood urea nitrogen (BUN), and creatinine)	Assess general health status and the extent of disease	Abnormal levels may indicate kidney damage and increased size/number of tumors
Beta$_2$- microglobulin (B$_2$-M) level	Obtain an indirect measure of the number of cancer cells	Higher levels indicate more extensive disease; aids in staging of disease
C-reactive protein	Obtain an indirect measure of the number of cancer cells	Higher levels indicate more extensive disease
Immunoglobulin levels	Define the levels of antibodies that are overproduced by myeloma cell	Higher levels suggest the presence of myeloma

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Table 1.2 (cont.)

Serum protein electrophoresis	Detect the presence and level of various proteins, including M protein	Higher levels indicate more extensive disease; aids in classification of disease
Immunofixation electrophoresis (IFE; also called immunoelectrophoresis)	Identity the type of abnormal antibody proteins in yhe blood	Aids in classification of disease
Freelite™ serum free light chain assay	Measure immunoglobulin light chains	Abnormal levels and/or ratio suggest the presence of myeloma or a related disease
Urine Specimen		
Urinalysis	Assess kidney function	Abnormal findings may suggest kidney damage
Bence Jones protein level (performed on 24 hour specimen of urine)	Define the presence and level of Bence Jones protein	Presence indicates disease, and higher levels indicate more extensive disease
Urine protein and immunoelectrophoresis	Determine the presence and levels of specific proteins in the urine, including M protein and Bence Jones protein	Presence of M protein or Bence Jones protein indicates disease
Bone/ Bone Marrow Specimen		
Imaging studies (bone) survey, x-ray, magnetic resonance imaging (MRI), computerized tomograph (CT), positron emission tomography (PET)	Assess changes in the bone structure and determine the number and size of tumors in the bone	-
Biopsy (on either fluid aspirated from the bone marrow or on bone tissue)	Determine the number and pecentage of normal and malignant plasma cells in the bone marrow	Presence of myeloma cells confirms the diagnosis, and higher percentage of myeloma cells indicate more extensive disease
Cytogenetic analysis (e.g., fluorescence in situ hybridization (FISH))	Assess the number and normalcy of chromosomes and identity the presence of translocations (mis-matching of choromosomes parts)	Loos of certain chromosomes (deletions) or translocations may be associated eith poor outcome

1.1.4. Stages of MM

In MM, there are two different staging systems known as Durie-Salmon Staging System and International Staging System.

Four factors play an important role in Durie-Salmon Staging System (Jacobson and Hussein, 2003): (I) The amount of abnormal monoclonal immunoglobulin in the blood or urine, (II) The amount of hemoglobin in the blood, (III) The amount of calcium in the blood, (IV) The severity of bone damaged based on x-rays.

Like Durie-Salmon Staging System, The International Staging System separated MM into three stages. System is mainly based on levels of serum beta-2 microglobulin and serum albumin which are powerful indicator for MM patient's prognosis (Greipp and Miguel, 2005).

1.1.5. Treatment Strategy for MM

Because of the high heterogeneity of MM, it still remains an incurable disease and it gives different clinical response against the therapeutic agents and drugs. Thus, detection of conventional treatment strategies play crucial role for patients with MM (Ria et al., 2014).

Treatment strategies in MM depend on classification and stage of disease. Additionally, patient's age, general health condition, lifestyle and previous myeloma treatments are also important (Woon, 2012). Treatment strategies of MM are summarized in Table 1.5.

Table 1.3. Treatment Strategies of MM
(Source: Multiple Myeloma Disease, 2012)

Theraphy	Description
Conventional (standart dose) chemotheraphy	The use of drug(s), administered alone or in combination, to kill cancer cells; some examples are melphalan (Alkeran,® GlaxoSmithKline) and cyclophosphamide
High dose chemotheraphy and stem cell transplantation	The use of higher doses of chemotherapy drugs followed by transplantation of **hematopoietic stem cells** to replace healthy cells damaged by the chemotherapy.
Radiation theraphy	The use of high-energy rays to damage cancer cells and prevent them from growing
Supportive theraphy	Therapies that decrese symptoms and manage complications of the disease and its treatment, such as bisphosphonates for bone disease, low-dose radiation therapy and analgesics for pain relief, **growth factors**, antibiotics, intravenous immunoglobulin, orthopedic interventions, drugs (primarily **anticoagulants**) to prevent and reduce the severity of blood clots.

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Table 1.3 (cont.)

New and emerging treatments	**Velcade (bortezomib)** for injection: Proteosome inhibitor
	Revlimid (lenalidomide): It is an immunomodulatory agent which provide combinational theraphy with dexamethasone
	Kyprolis (carfilzomib): New type of proteasome inhibitor which is used for in patients who have at least 2 prior therapies including Velcade and immunomodulatory agent
	Thalidomid: Provide combinational theraphy with dexamethasone as front-line theraphy
	Doxil (doxorubicine): Chemotherapic agent which is used in combination with Velcade for individuals who previously received theraphy other than Velcade.
	Steroids (corticosteroids): Dexamethasone and prednisone are example and they can be used alone or in combination with other therapies.

1.1.5.1. Bortezomib

In 2003, Food and Drug Administration (FDA) approved bortezomib for the treatment of relapsed MM and mantle cell lymphoma (Adams and Kauffman, 2004) and it became the first proteasome inhibitor used for the treatment of MM (Takimoto and Calvo, 2008).

Bortezomib ($C_{19}H_{25}BN_4O_4$) is a dipeptidil boranic acid targeting the catalytic site (chymotrypsin-like activities) of 26S nuclear or cytosolic proteosome complex by boron atom and inhibit its function. In addition to inhibition of proteasome, it also triggers apoptosis and inhibits tumor cell proliferation in MM cells (Mitsiades et al., 2010; Hideshima and Richardson, 2001). The molecular structure of bortezomib was shown in Figure 1.2.

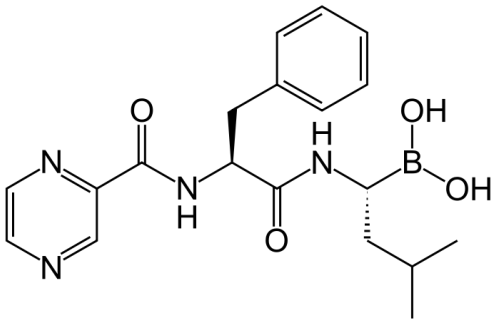

Figure 1.2. Chemical Structure of bortezomib / Peptide boronic acid / Velcade
(Source: Dai et al., 2011)

1.1.5.1.1. Biology of the Proteasome

All eukaryotes and archea and also some bacteria have proteasomes (Peters et al., 1994). Responsible for intracellular protein degredation through cutting the peptide bonds in the polypeptides. Unfolded, misfolded or nonfunctional proteins are degraded by proteosomes. These proteins are recognized by polyubiquitinated protein chain that contains 76 amino acid length polypeptides. It requires ATP for performing its function (Sánchez-Serrano, 2006). 26S proteasomes have two different region: 20S catalytic core and 19S regulatory subunit. 26S proteasomes firstly recognizes ubiquitin- marked proteins and transfer them from 19S regulatory subunit to 20S catalytic core (Wang and Maldonado, 2006). 20S catalytic core includes four stacked subunits, 2-α and 2-β. Two outher α-rings interact with the 19S regulatory subunit and two inner β-rings gather seven β subunits. β subunits show catalytic activity and broad substrate specificity. If taken cross section of β subunits, post-glutamyl (β1), tryptic (β2) and chymotryptic site (β5) are seen and bortezomib shows its inhibition effect on the chymotryptic site (Adams, 2003).

1.1.5.1.2. Proteasome Inhibition by Bortezomib

Proteasome inhibition inhibits degradation of pro-apoptotic factors and promotes the activation of programmed cell death in neoplastic cells. Additionally, this inhibition

may prevent the features of angiogenesis and metastasis of cancer cells *in vivo* (Almond and Cohen, 2002).

Bortezomib application results in proapoptotic protein's upregulation, anti-apoptotic protein supression such as Bcl-2, Bcl-XL, STAT-3, down-regulation of expression level of several proteins, inhibition of NFκB and its anti-apoptotic target genes. Inhibition of proteasome via bortezomib stimulates ER stress resulting in calcium release, cytochrome-c release from mitochondria and stimulates the downstream activation of caspase-9 (Landowski et al., 2005). Bortezomib triggers proapoptotic cascade genes and down-regulates survival genes in multiple myeloma cells (Mitsiades and Mitsiades, 2002).

Inhibition of proteasome by bortezomib results in the supression of nuclear factor–κB (NFκB) pathway (Adams, 2004). NFκB is a transcription factor that found inactive form in the cytoplasm when it is bound to an inhibitory partner protein IκB. As IκB is ubiquitinated or phosphorylated by cytokines or other cellular process, it degrades by proteasome resulting in activation of NFκB. Then NFκB moves from cytoplasm to nucleus where it stimulates the transcription of several genes whose protein products block the apoptotic pathways and promote cell proliferation and regulate the adhesion molecules (Karin et al., 2004).

Application of bortezomib, inhibits the degradation of IκB and resulting in continues inactivation of NFκB.

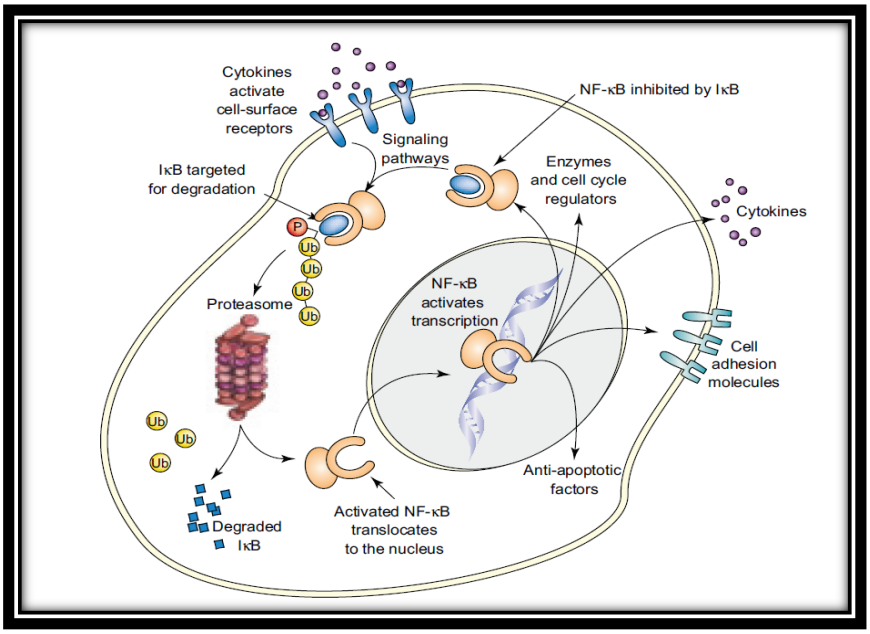

Figure 1.3. NF-κB activation pathway
(Source: Adams, 2003)

1.1.5.2. Epigenetics in MM

Epigenetic regulation is one of the most important gene expression regulatory mechanisms in eukaryotes. It is not related with the sequences of genomic DNA, but it regulates gene expression through the changes on genomic DNA. These changes are histone methylation, acetylation, ubiquitinylation, phosphorylation and sumoylation.

Epigenetic processes affect the gene silencing or activation (Kovalchuk and Baulch, 2008). There different enzymes involved in epigenetic regulation of gene expressions. Histone methylation is orginized by methyle transferases and regulates transcription, DNA damage response, formation of heterochromatin and X-chromosome inactivation. It affects the lysine or arginine residues of histones via histone methyltransferases (HMTs) and histone demethylases (HDMs). Histone demethylases indicate their cellular effects into two ways: Flavin adenine dinucleotide (FAD)-dependent HDMs and Jumonji C domain-containing HDMs (JHDMs) (Luo et al., 2011).

1.1.5.2.1. Jumonji C Domain

JmjC domains have been identified in the jumonji family of transcription factors and it is protected from bacteria to human during evolution. JmjC domain-containing proteins are a kind of histone demehylase. They are involved in many important biological processes like tumor suppressor, gene expression and chromatin remodelling. It was shown by Wang et al. that methylstat selectively inhibits the Jumonji C domain containing histone demethylases in cells and it is important inhibitory molecules of different cancer pathways (Balciunas and Ronne, 2000).

Methylstat ($C_{28}H_{31}N_3O_6$) is a chemical is responsible for inhibition of Jumonji C domain containing histone demethylases in cells and stimulates the methylation of histone lysine residues; H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20. Figure 1.5. shows the molecular structure of methylstat (Anon, 2012; Luo et al., 2012).

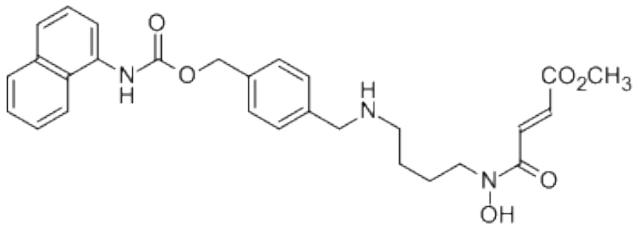

Figure 1.4. Chemical Structure of methylstat

Methylstat plays important roles in the inhibition of cell proliferation, cell cycle arrest and angiogenesis. Methylstat treatment induced transcriptional activation of p53 resulting in the inhibition of angiogenesis, cell cycle arrest and induction of apoptosis (Blackburn and Jerry, 2002). p53 expression level is regulated by the histone lysine residue hypermethylation in p53 promoter (Cho et al., 2014). p53 expression level is regulated by the histone lysine residue hypermethylation in p53 promoter. On the other hand, dose dependent Methylstat treatment inhibits the VEGF-stimulated blood vessel formation (Cho et al., 2014).

1.2. Aim of the Study

It is clear that the number of MM cases is increased day by day and the number of deaths is decreased over time. Increase in the frequency of medical surveillances and awareness of the disease are possible explanations for the increasing cases.

Several treatment strategies are currently being used including chemotheraphy and stem cell transplantation. However, success rates of these treatments are not satisfactory new treatment strategies are needed to get the complete cure of MM.

In this study, we firstly aimed to determine the cytotoxic and apoptotic effects of bortezomib and methylstat on U266 and ARH77 MM cells. Then, we aimed to determine possible synergistic antiproliferative and apoptotic effects of combination of methylstat and bortezomib. Finally, we examined the changes in expression pattern of apoptotic genes in response to methylstat.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

Bortezomib was obtained from LCLabs (USA). The stock solution of bortezomib was prepared in dimethyl sulfoxide (DMSO) at a concentration of 10mmol/ml, stored at -20°C and diluted in cell culture medium.

Methylstat was obtained from Sigma Aldrich (USA). The stock solution of M methylstat was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mM, stored at -20°C and diluted in cell culture medium.

Penicillin-streptomycin, RPMI1640, fetal bovine serum (FBS) and phosphate buffered saline (PBS) were obtained from Invitrogen (Paisley, UK). Bradford dye and Comassie blue, bovine serum albumin (BSA), trypan blue solution, dimethyl sulfoxide (DMSO) and agarose were obtained from Sigma (USA). MTT Cell Proliferation Kit was obtained from Biological Industries. Caspase-3 Colorimetric Assay Kit was obtained from BioVision (USA). APO LOGIX JC-1 Assay Kit was obtained from Cell Technology (USA). AnnexinV-FITC/PI Assay Kit was obtained from BD Pharmingen. Total RNA isolation kit was obtained from Macherey-Nagel (Germany). Reverse transcriptase (Moroney Murine Leukemia Virus Reverse Transcriptase), Taq DNA polymerase and primers were obtained from Fermentas (USA).

2.2. Cell Lines, Culture Conditions and Maintenance

U266 and ARH77 human MM cells were kindly obtained from German Collection of Microorganisms and Cell Cultures (Germany). The cells were grown and maintained in RPMI1640 growth medium containing 1% L-glutamine, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO_2. Medium was replaced in every 4 days.

2.3. Measurement of Cell Growth by MTT Assay

Anti-proliferative effects of bortezomib and methylstat on U266 and ARH77 cells were determined by MTT cell proliferation assay. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide) is a yellow auxiliary agent that reduced formazan crystals in living cells.

$2x10^4$ MM cells were inoculated in each well of 96- well plate. The cells were incubated in the absence or presence of bortezomib (from 1to 500 nM) or methylstat (from 1 to 40 μM) for 48 and 72 h. After incubation periods, 20 μl of MTT solution was added to each well and the cells were incubated at 37°C, 5% CO_2 containing incubator for 3 hours. Then, plates were centrifuged at 1800 rpm for 10 minutes. Supernatant was removed and 150 μl DMSO was added into each well to dissolve the formazan crystals. Finally, the absorbance values were measured at 570 nm by a spectrophotometer (Thermo Electron Corporation Multiskan Spectrum, Finland).

In order to detect the possible synergistic effects of bortezomib in combination with methylstat, $2x10^4$ MM cells were seeded into each well of 96- well plate and exposed to different bortezomib concentrations (from 1 to 160 nM) together with constant methylstat (4.2 μM) concentration. After the incubation, the same procedure explained above is applied for this set of experiments.

2.4. Measurement of Apoptosis

In order to detect apoptotic effects of bortezomib and methylstat on MM cells, the cells were tested by changes in caspase-3 enzyme activity by using caspase-3 colorometric assay kit, loss of mitochondrial membrane potential by using JC-1 mitochondrial membrane potential detection kit and location of phosphotidylserine by using Annexin V-FITC kit.

2.4.1. Detection of the Changes in Caspase-3 Activity

Changes in the activity of caspase-3 enzyme, which is an important mediator of apoptosis, were detected by caspase-3 colorometric assay kit. as indicated by the manifacturer instructions (BioVision Research products, USA). This assay is based on spectrophotometric emmision of chromophore p-nitroanilide (pNA) after cleaving from DEVD-pNA, that is a labeled substrate for caspase-3 enzyme.

Initially, increased concentration of bortezomib (10, 20 and 40 nM) and methylstat (2,2 μM) were applied seperately, and different concentration of bortezomib (from 10 to 40 nM) also applied with constant methylstat (2,2 μM) dose onto $1x10^6$ U266 cells/2 ml in each well of 6-well plates . Same procedure were conducted with 5, 10 and 20 nM doses of bortezomib and 4,2 μM methylstat for ARH77 cells.

Bradford method, by using bovine serum albumin (BSA) as a standart, was used to determine total protein concentration.

2.4.2. Detection of the Loss of Mitochondrial Membrane Potential

In order to confirm previous tests, we detected the loss of mitochondrial membrane potential, which is another crucial sign of apoptosis by JC-1 mitochondrial membrane potential detection kit. JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) is a cytoflurimetric, lipophilic and cationic dye which aggregates in mitochondria and gives red fluorescence in living cells, whereas in apoptotic or dead cells, dye cannot pass through mitochondria and stays in cytoplasm as monomer form and gives green fluorescence in apoptotic cells. Samples were measured at 485 and 535 nm for green fluorescence and at 560 and 595 nm for red fluorescence by using spectrophotometer (Thermo, Varioskan Flash). Ratios of green/ red fluorescence were calculate to analysis the loss of mitochondrial membrane potential rate.

$1x10^6$ cells/2 ml cells were plated into each well of 6 well plates and indicated concentrations of bortezomib and methylstat in caspase-3 assay were applied to MM cells. After 72 hours incubation at 37°C in 5% CO_2, cells were collected and analysed

for loss of MMp. The same set of experiments were run for the cells exposed to combinations of bortezomib and methylstat.

2.4.3. Detection of the Apoptotic Cell Population by Annexin-V Assay

Apoptosis can also be determined by detecting the location of phosphatidylserine (PS) in the cell membrane. Phosphatidylserine (PS) stays at the inner part of the membrane in healthy cells while faces with the extracellular matrix of apoptotic cells. Binding of Annexin to phosphatidylserine provides to apoptotic cell population in different concentration of agents exposed MM cells.

Bortezomib and methylstat were applied onto the 500,000/2 ml of MM cells and incubated for 72 h. The cells were collected and washed for two times with cold PBS. Pellets were re-suspended with 1X binding buffer and transferred into glass FACS tubes. 5 µl propidium iodide (PI) dye and 5 µl FITC were added onto each sample. Finally, 400 µl of 1X binding buffer were added and results were analyzed by flow cytometry (FACSCanto, BD). After the tubes were incubated for 15 min in the dark.

2.5. Detection of Cell Cycle Progression

Cell cycle analysis is based on the determination of the amount of dsDNA by using propidium iodide (PI), a DNA-binding dye. Then, population of the cells at different stages of cell cycle are analyzad via the amount of fragmented DNA, by flow cytometry. Basically, increasing concentrations of bortezomib and methylstat were added onto 1x106 MM cells/2 ml in each well of 6-well plates and incubated at 37°C, in 5% CO2 containing incubator. Collected cells were centrifuged at 1200 rpm for 5 minutes. Supernatants were discarded and the pellet was resuspended with 1 ml cold PBS. In order to fix the cells, 4 ml cold absolute ethanol (at -20°C) were added onto the cells while they are gently vortexed. Then, the cells were incubated at -20°C for overnight for further analysis. In the following day, cells were collected, washed with cold PBS and pellets were re- suspended 200 µl PBS including 0. 1 %triton X 100. 20 µl (200 µg/ml in distilled water) of RNase-A enzyme was added to each sample and incubated at 37°C for 0.5 h. At the end of the incubation, cells were stained with 20 µl

(1 μg/ml in distilled water) PI dye and incubated for 10 min at room temperature. Finally, they were analysed by flow cytometer.

2.6. Total RNA Isolation and cDNA Synthesis

Total RNAs from bortezomib and methylstat applied MM cells were isolated by NucleoSpin RNA II Purification Kit as described by the manufacturer. Initially, $1x10^6$ MM cells/2 ml were added into each well of 6 well plate and incubated for 72 hours at 37°C, 5% CO_2 incubator. Then, the cells were collected in a microcentrifuge tube and total RNA isolation was achieved. Amounts of RNAs were measured by nanodrop (260/280 and 260/230 ratios). 1000 ng RNA was used to synthesis cDNA. PCR mixture was prepared with random hexamer primer, buffer, dNTP mix, RNase inhibitor and reverse transcriptase. Mixtures were incubated at 42°C for 1 hour and to stop the reaction, they were incubated at 72°C for 10 minutes.

2.7. PCR Array

Human Cancer Pathway Finder RT^2 Profiler PCR Array System (SABiosciences Corporation, USA) was used to determine the changes in expression levels of apoptosis related genes in response to methylstat. Expression profiles of 84 genes playing important roles in DNA damage repair, cell cycle control, cell senesence, apoptosis, signal transduction and transcription, angiogenesis, adhesion and also metastasis and invasion were analyzed by RT^2 Profiler PCR Array.

Table 2.1. Ingredients of PCR Array Experimental Coctail

Prob Master Mix	10 μl
Diluted First Strand cDNA Synthesis Reaction	5 μl
H_2O	4 μl

After preparing the coctail (Table 2.1), 25 µl of experimental coctail was added to each well of the PCR Array, which is a PCR plate preloaded with validated real-time PCR primers for a panel of pathway-focused genes.

Afterwards, PCR Array plates were sealed with the adhesive film. Then, one plate was placed in real-time thermal cycler (Roche LightCycler 480, Germany), and reaction was executed.

Table 2.2. Two-step cycling program executed in Roche LightCycler 480

Cycles	**Duration**	**Temperature**
1	10 min	95 °C
45	10 sec	95 °C
	30 sec	60 °C
	1 sec	72 °C
1	30 sec	40 °C

CHAPTER 3

RESULTS

3.1. MTT Cell Proliferation Assay on U266 and ARH77 Cells

3.1. Bortezomib and Methylstat Showed Antiproliferative Effect on Multiple Myeloma Cells

U266 and ARH77-cells were treated with increasing concentrations of bortezomib and methylstat for 48 and 72 hours and MTT cell proliferation assay was carried out in order to determine the anti-proliferative effects of agents on these cells.

The cells were exposed to bortezomib from 1 to 500 nM and to methylstat from 1 to 40 µM. The results showed that there were dose- and time- dependent decreases in the cell proliferation as compared to untreated controls ($p<0.05$).

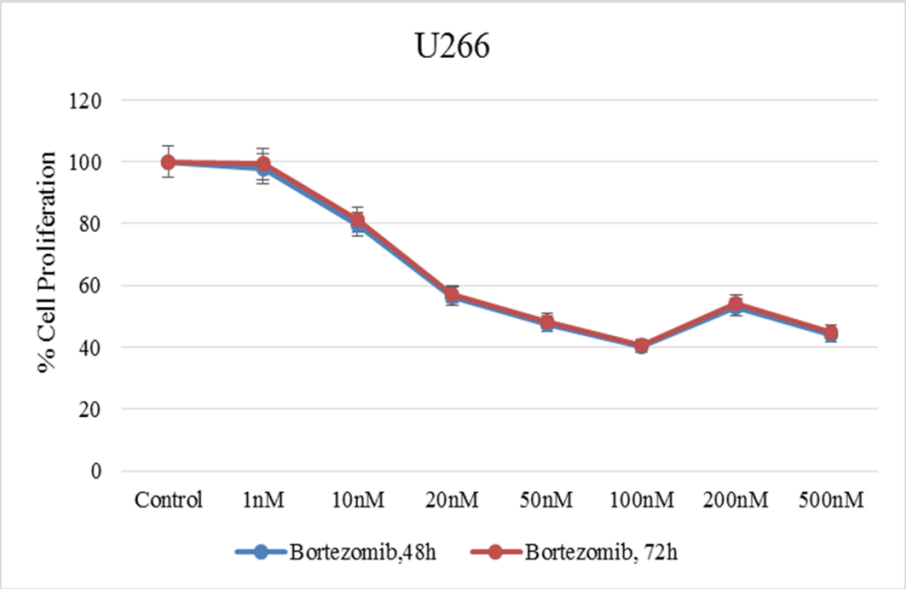

Figure 3.1. Cytotoxic effects of bortezomib on U266 cells ($p<0.05$) (Error bars represents standart deviations (SD), $p<0.05$ was considered as significant)

There were 3, 11, 44, 53, 60, 48 and 57% decreases in the cell proliferation when the cells were exposed to 1-, 10-, 20-, 50-, 100-, 200- and 500 nM bortezomib, respectively. As a result, IC_{50} concentration of bortezomib on U266 cells was calculated from cell proliferation plots and found to be 41 nM (Figure 3.1).

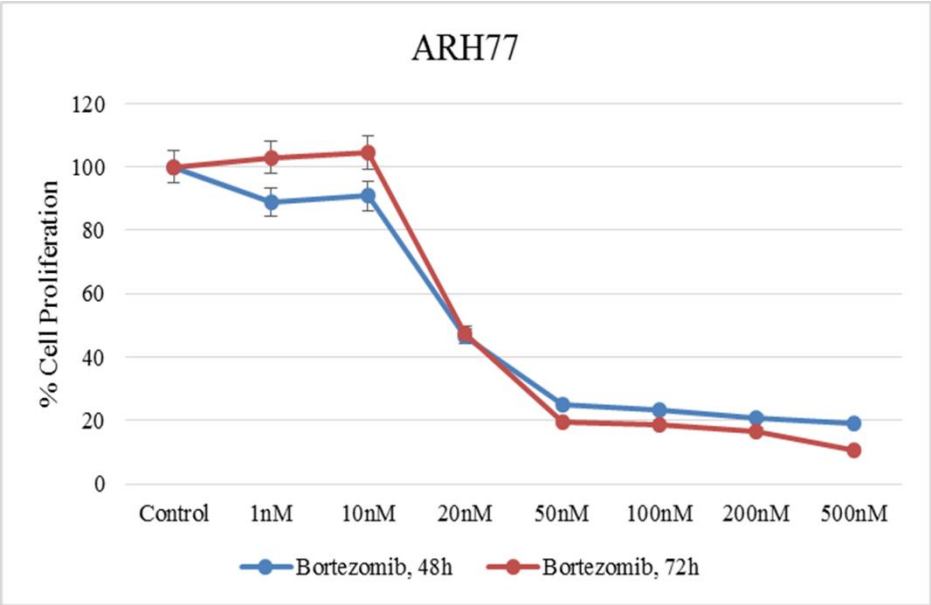

Figure 3.2. Cytotoxic effects of bortezomib on ARH77 cells ($p<0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

On the other hand, there were 12-, 10-, 54-, 76-, 77-, 80- and 82% decreases in the cell proliferation when the cells were exposed to 1-, 10-, 20-, 50-, 100-, 200- and 500 nM bortezomib, respectively. Finally, IC_{50} value of bortezomib on ARH77 cells was calculated from cell proliferation plots and found to be 19 nM (Figure 3.2).

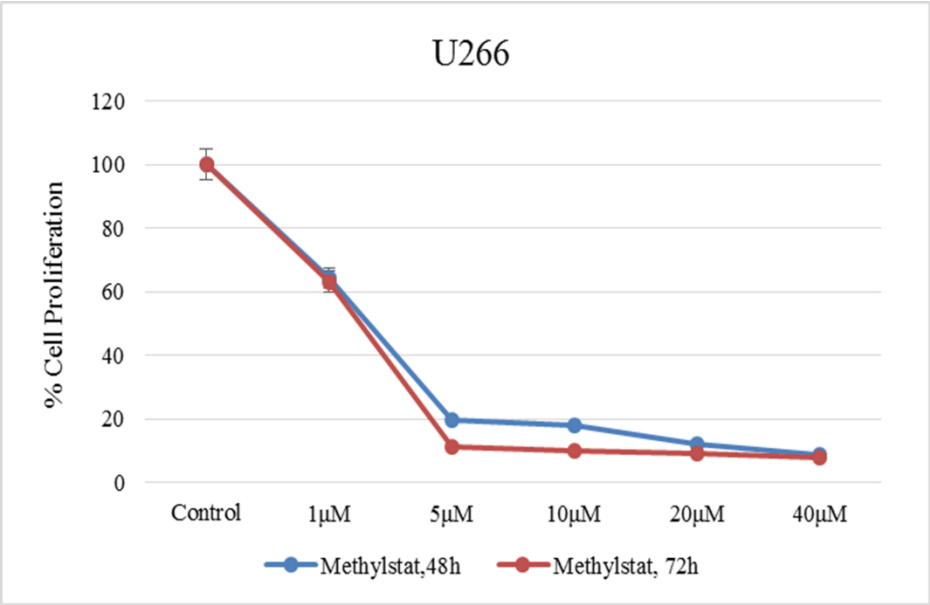

Figure 3.3. Cytotoxic effects of methylstat on U266 cells ($p<0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

There were 36-, 81-, 83-, 88- and 92% decreases in the cell proliferation when U266 cells were exposed to 1-, 5-, 10-, 20- and 40 µM methylstat, respectively. IC_{50} value of methylstat on U266 cells was calculated from cell proliferation plots and found to be 2.2 µM (Figure 3.3).

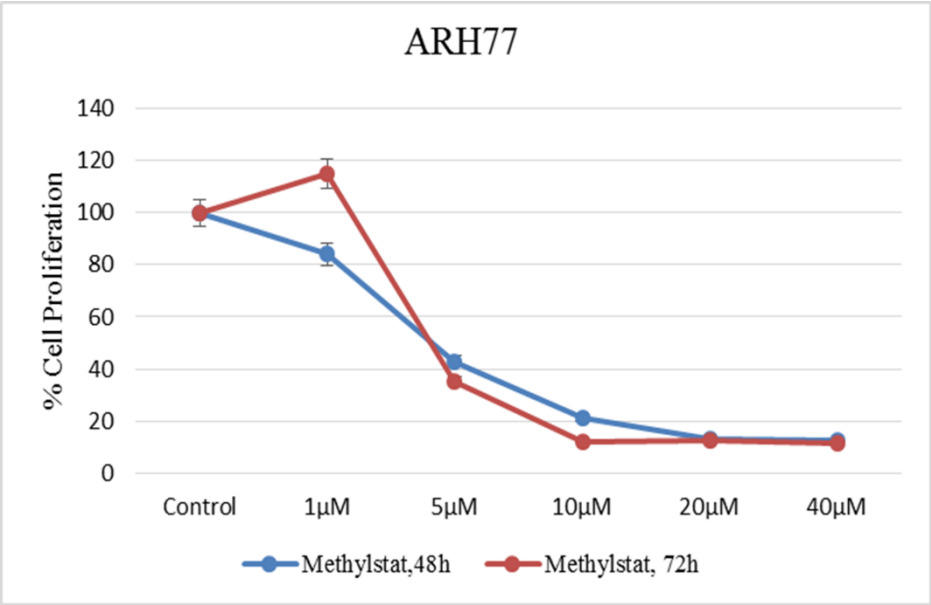

Figure 3.4. Cytotoxic effects of methylstat on ARH77 cells ($p<0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

On the other hand, 16-, 58-, 80-, 87- and 88 % decreases in the proliferation of ARH77 cells were detected in response 1-, 5-, 10-, 20- and 40 µM methylstat, respectively. The IC_{50} value of methylstat on ARH77 cells was calculated from cell proliferation plots and found to be 4.2 µM (Figure 3.4).

3.1.2. Bortezomib in Combination with Methylstat Decreased Proliferation of Multiple Myeloma Cells Significantly

In order to examine the possible synergistic cytotoxicity, the cells were exposed to increasing concentrations of bortezomib from 1- to 160 nM together with 2.2 µM methylstat for U266 and 4.2 µM methylstat for ARH77 cells.

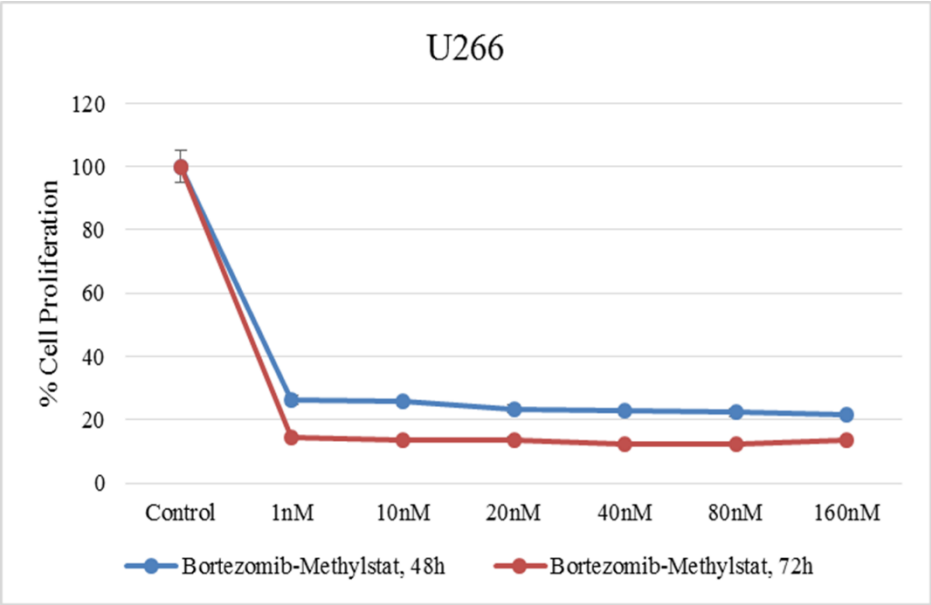

Figure 3.5. Cytotoxic effects of bortezomib in combination with methylstat on U266 cells ($p<0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

There were 70 to 74% decreases in proliferation of U266 cells exposed to increasing combinations of bortezomib and methylstat (1- to 160 nM) with 2.2 µM methylstat (IC_{50} value of U266 cells for 48 h and 72 h) as compared to untreated control cells ($p<0.05$). The IC_{50} value of drug combination on U266 cells was calculated from cell proliferation plots and found to be 0.6 nM for 48 h and 0.5 nM for 72 h (Figure 3.5).

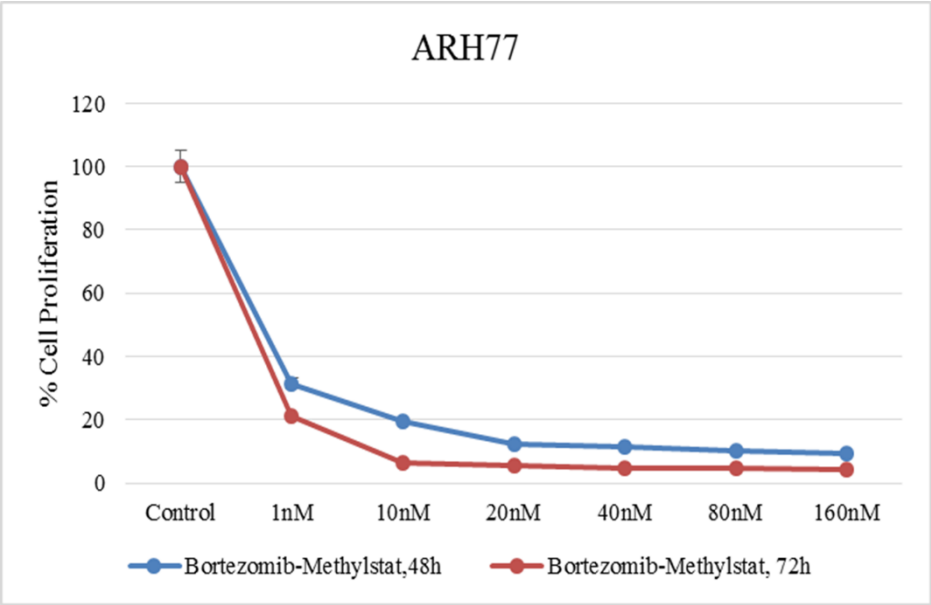

Figure 3.6. Cytotoxic effects of bortezomib in combination with methylstat on ARH77 cells ($p<0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

There were 79 to 96% decreases in the cell proliferation when the cells were exposed to combination of bortezomib and methylstat (1 to 160 nM) and 4.2 µM methylstat (IC_{50} value for ARH77 cells after 48 and 72 h). The IC_{50} value of drug combination on ARH77 cells was calculated from cell proliferation plots and found to be 0.6 nM for 48 h and 0.5 nM for 72 h. (Figure 3.6).

3.2. Evaluation of Apoptosis in U266 and ARH77 Cells

3.2.1. Bortezomib and Methylstat Increased Caspase-3 Enzyme Activity in Multiple Myeloma Cells

In order to evaluate the apoptotic effects of bortezomib and methylstat on U266 and ARH77 cells, alterations in caspase-3 enzyme activity and mitochondrial membrane potential (JC-1) were examined.

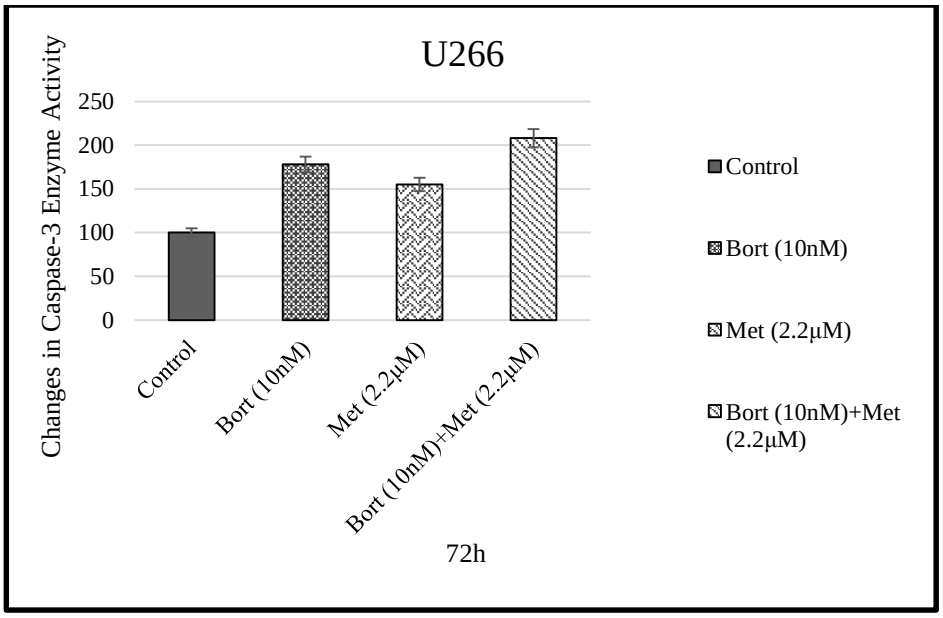

Figure 3.7. Changes in caspase-3 enzyme activity in response to bortezomib and methylstat alone, and their combinations on U266 cell lines, $p<0.05$ (Error bars represents standart deviations, $p<0.05$ was considered as significant)

Treatment of U266 cells for 72 h with 10 nM bortezomib, 2.2 µM methylstat and combination of them resulted in 1.7-, 1.5- and 2- fold increases in caspase-3 activity, respectively, as compared to untreated controls ($p<0.05$) (Figure 3.7).

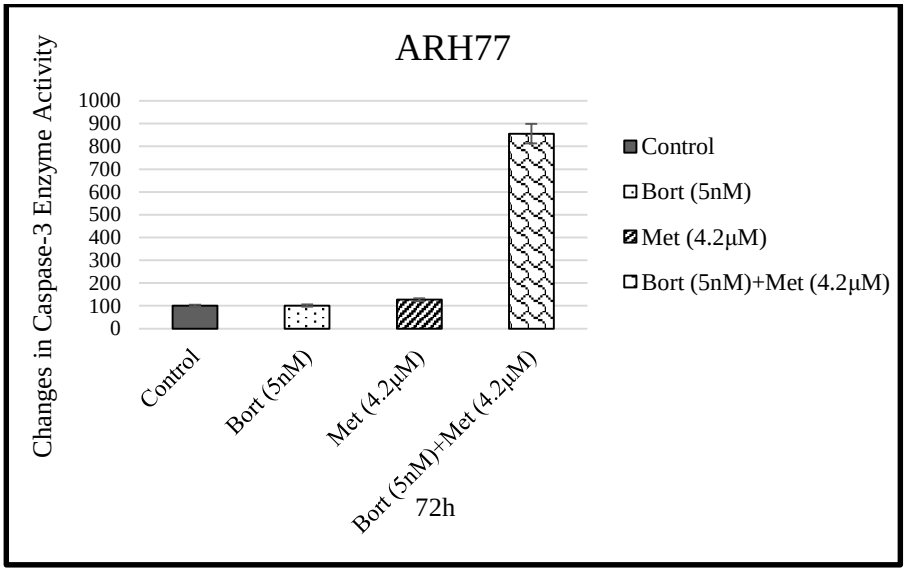

Figure 3.8. Changes in caspase-3 enzyme activity in response to bortezomib and methylstat alone, and their combinations on ARH77 cell lines

On the other hand, treatment of ARH77 cells for 72 h with 5 nM bortezomib, 4.2 μM methylstat and combination of them resulted in 1-, 1.2- and 8.5- fold increases in caspase-3 activity, respectively, as compared to untreated controls ($p<0.05$) (Figure 3.8).

3.2.2. Bortezomib and Methylstat Induced Loss of Mithocondrial Membrane Potential in Multiple Myeloma Cells

Combination of bortezomib with methylstat resulted in loss of MMP significantly as compared to any agent alone and untreated control group. There were 29- and 43.2- fold increases in response to 10 and 20 nM bortezomib while 2.2 μM methylstat application resulted in 18.6- fold increase in loss of MMP in U266 cells. Combination of the same doses of bortezomib with 2.2 μM methylstat induced 111.6- and 180.3- fold increases in loss of MMP of U266 cells ($p<0.05$) (Figure 3.9).

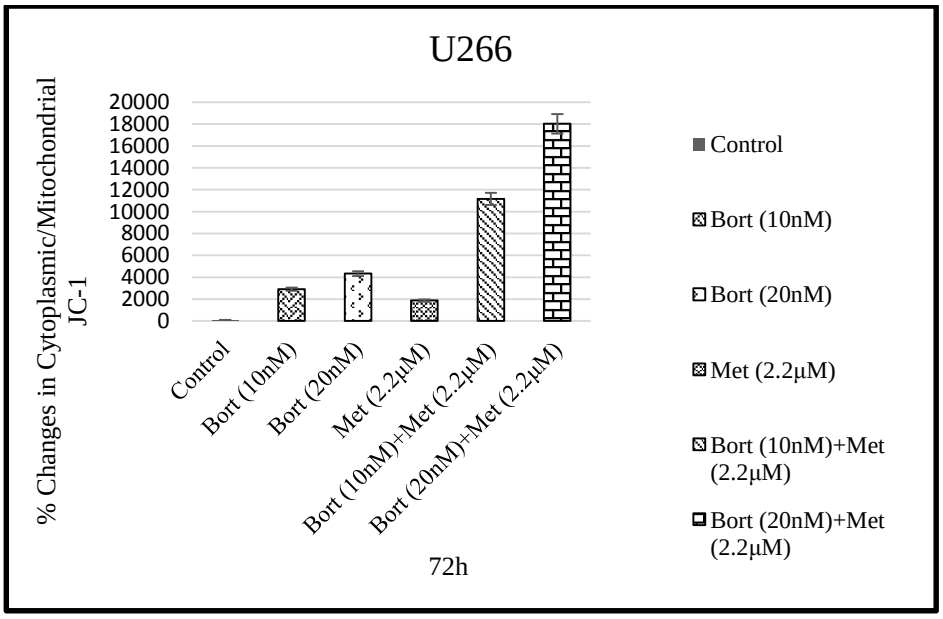

Figure 3.9. Changes in mitochondrial membrane potantial in response to bortezomib and methylstat alone, and their combinations in U266 cell lines ($p<0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

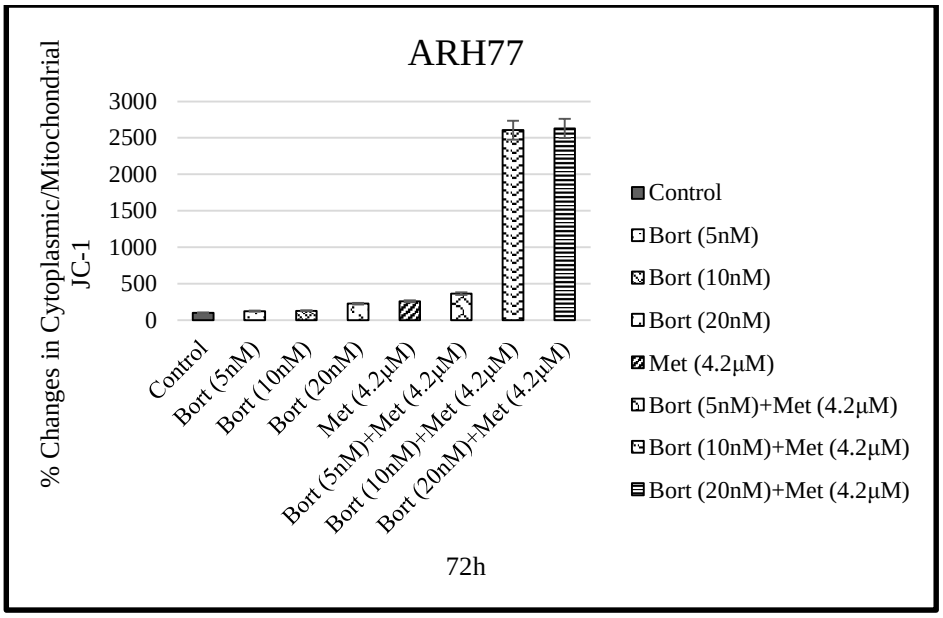

Figure 3.10. Changes in mitochondrial membrane potantial in response to bortezomib and methylstat alone, and their combinations on ARH77 cell lines ($p<0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

Treatment of ARH77 cells with 5-, 10- and 20 nM bortezomib resulted in 1.2-, 1.2-, and 2.2-fold increases in loss of MMP while 4.2 μM methylstat caused a 2.5-fold loss of MMP. The same concentrations of bortezomib in combination with 4.2 μM methylstat caused 3.6-, 26- and 26.3- fold increases in loss of MMP as compared to untreated control cells ($p<0.05$) (Figure 3.10).

3.2.3. Bortezomib and Methylstat Induced Apoptosis of U266 and ARH77 Cells

The degree of apoptosis, as measured by flow cytometry, is quantitatively expressed as the percentage of cells that were Annexin V- positive in the presence of bortezomib and methylstat.

There are 4-, or 10- and 12.5- fold increases in the percentage of apoptotic cell populations in U266 cells, exposed to 10 nM bortezomib or 2.2 μM methylstat alone and their combination, respectively, as compared to untreated control cells ($p<0.05$) (Figure 3.11).

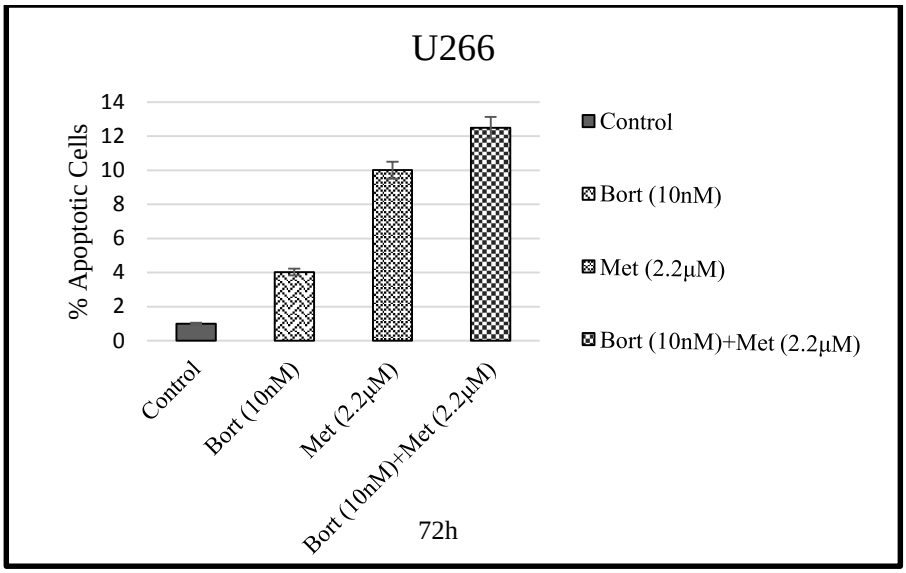

Figure 3.11. Changes in % apoptotic cell population in response to bortezomib and methylstat alone, and their combinations in U266 cell lines ($p<0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

The same results were obtained for ARH77 cells. Combination of bortezomib and methylstat induced apoptosis significantly as compared to any agent alone ($p<0.05$) (Figure 3.12).

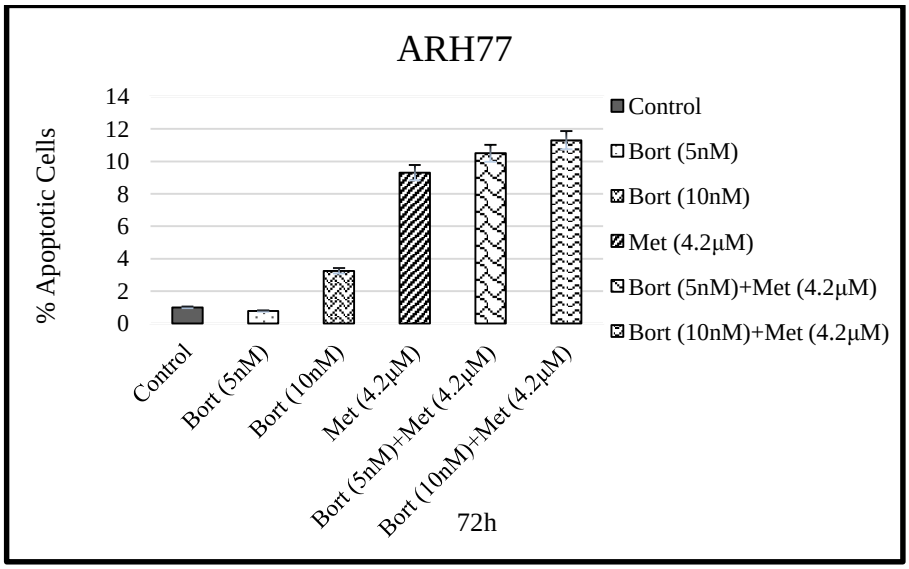

Figure 3.12. Changes in % apoptotic cell population in response to bortezomib and methylstat alone, and their combinations in ARH77cell lines ($p<0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

3.3. Effects of Bortezomib and Methylstat on Cell Cycle Progression on Human U266 and ARH77 Multiple Myeloma Cells

Cytostatic effects of bortezomib and methylstat on cell cycle progression of U266 and ARH77 cells were evaluated by PI staining in flow cytometry.

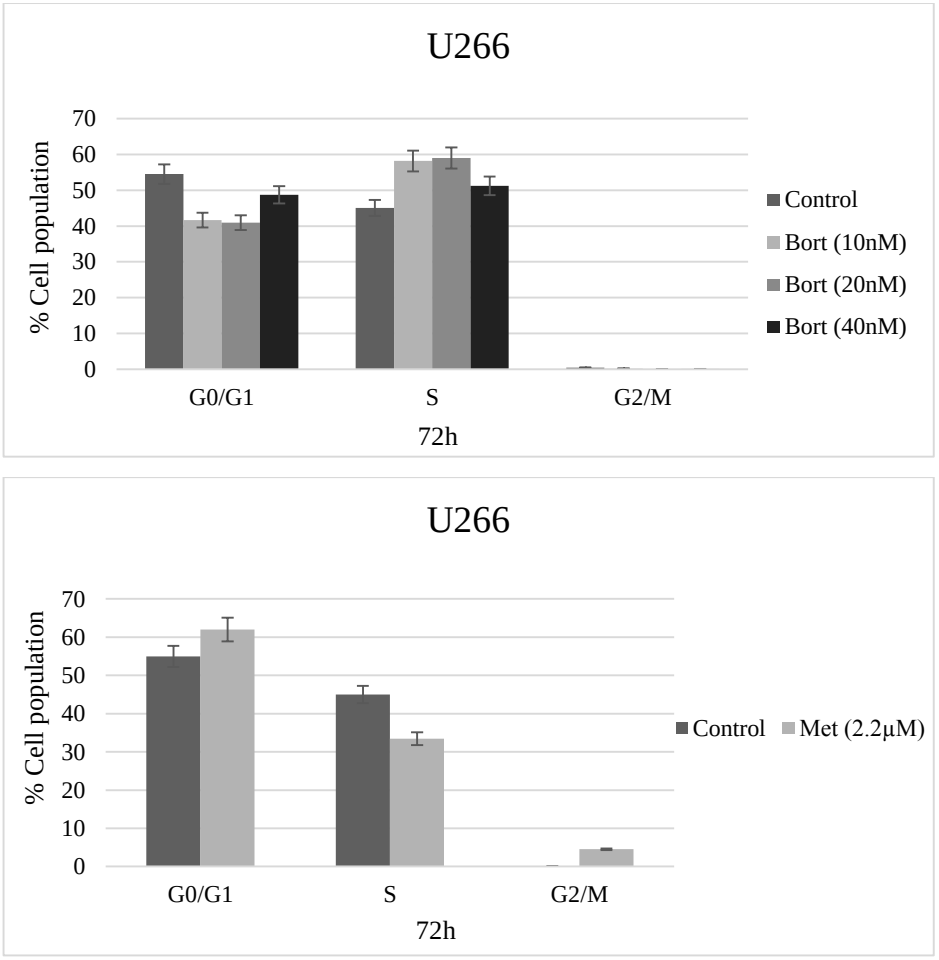

Figure 3.13. Effects of bortezomib and methylstat on cell cycle progression of U266 cells ($p<0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

The data showed that, the proportion of S phase cells significantly increased in response to 10- and 20 nM bortezomib, which is accompanied with slight decreases in the percentage of G1 phase cells (Figure 3.13).

After incubation with methylstat the proportion of G0/G1 phase cells significantly increased and cells in S phase slightly decreased (Figure 3.13). Meanwhile, the fraction of cells in G2/M phase increased accordingly ($p<0.05$).

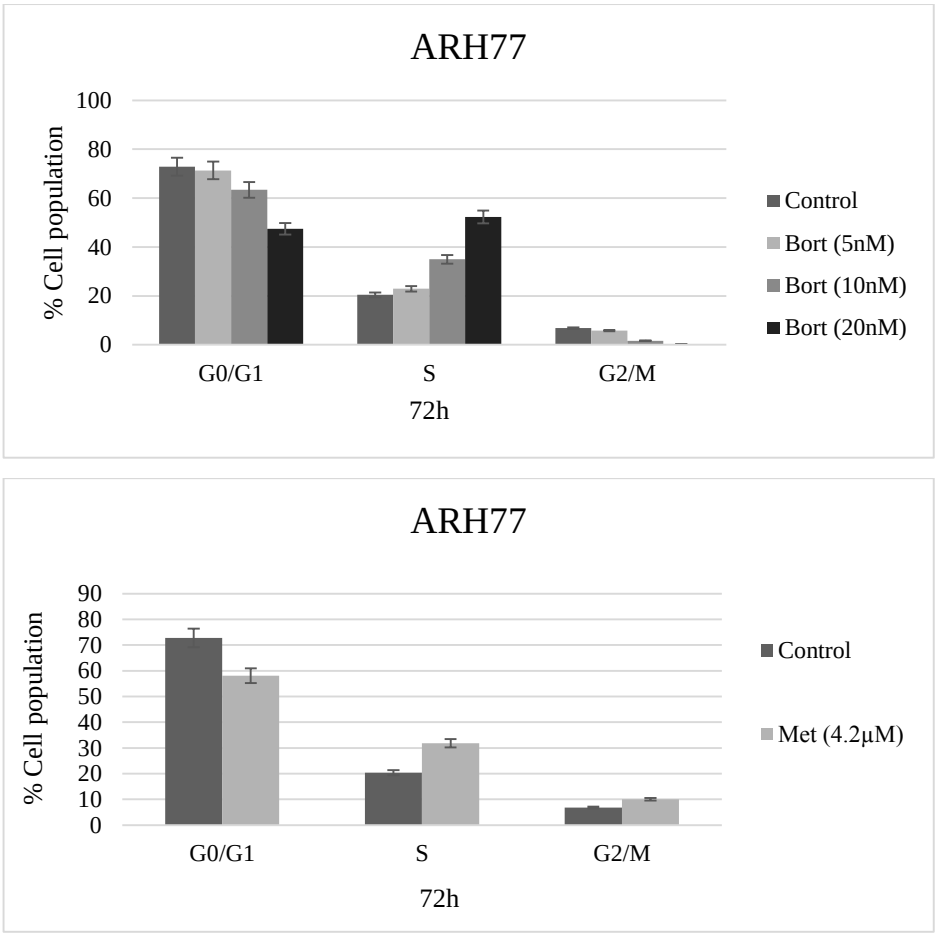

Figure 3.14. Effects of bortezomib and methylstat on cell cycle distribution of ARH77 ($p<0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

After the treatment of ARH77 cells with 5-, 10- and 20 nM bortezomib for 72 h, the cells were analyzed for alterations in their cell-cycle distribution (Figure 3.14). As observed in Figure 3.14.; the proportion of S phase cells significantly increased in a dose-dependent manner while slight decreases in the G2 phase cells were determined. Meanwhile, the fraction of cells in G0/G1 phase decreased in a dose-dependent manner.

After incubation with 4.2 μM methylstat for 72 h, ARH77 cells were analyzed for alterations in their cell-cycle distribution (Figure 3.14). The proportion of S phase cells significantly increased following the treatment the G2 phase cells also increased

slightly. Meanwhile, the fraction of cells in G0/G1 phase increased as compared to untreated control cells ($p<0.05$).

3.4. Effects of Bortezomib in Combination with Methylstat on Cell Cycle Progression

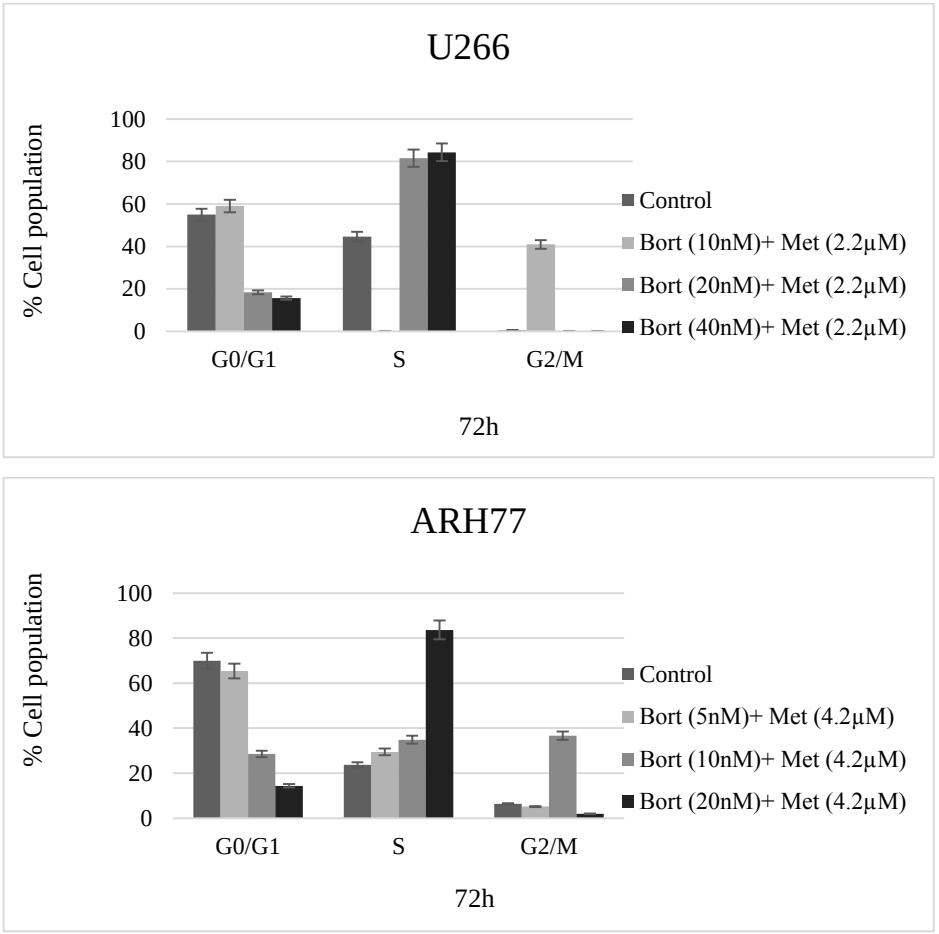

Figure 3.15. Effects of bortezomib and methylstat on cell cycle distribution of U266 and ARH77 cells ($p>0.05$) (Error bars represents SD, $p<0.05$ was considered as significant)

The proportion of S phase cells significantly increased while G0/G1 phase cell population increased following the treatment with 20 and 40 nM bortezomib in combination with 2.2 µM methylstat.

Treatment of ARH77 cells with increasing concentrations of bortezomib and 4.2 μM methylstat resulted in increases in population of cells in S phase and decreases of the cells in G0/G1 phase.

3.3.3. Assessment of Human Cancer Pathway Finder PCR Array Results

U266 and ARH77 cells were treated with increasing concentrations of methylstat (1.1 and 2.2μM and 2.1 and 4.2 μM, respectively) and expression levels of 84 genes related with cancer were determined by Human Cancer Pathway Finder PCR Array. As shown in Figure 3.16 and 3.18, there were 5 and 4 genes upregulated more than 2-times in response to methylstat in U266 and ARH77 cells, respectively. Moreover there were 5 and 1 genes downregulated for more than 2-fold in response to methylstat in U266 and ARH77 cells, respectively (Figure 3.17 and 3.19). These genes changed significantly were found to be related with tumor suppression, apoptosis, metastasis, and/or angiogenesis. As compared with untreated control group, 7 housekeeping genes, 3 positive PCR control groups, and 2 negative PCR control groups, expression levels of anti-apoptotic AKT1, AVEN, BAG1, BCL2L2 and RELA; and apoptosis-triggering FASLG, NGFR, TNF, TNFRS10B and TNFRS1B genes were found to be the most changed ones in U266 cell and expression levels of anti-apoptotic NFKB1, and apoptosis-triggering BCL2L11, CASP7, TNFRSF21 and TNFSF8 genes were found to be the most changed ones in ARH77 cell.

Table 2.3. Plate layout of PCR Array

	1	2	3	4	5	6	7	8	9	10	11	12
A	AKT1	APAF1	AVEN	BAD	BAG1	BAK1	BAX	BBC3	BCL2	BCL2L1	BCL2L10	BCL2L11
B	BCL2L13	BCL2L2	BID	BIK	BIRC2	BIRC3	BIRC5	BOK	CAD	CASP1	CASP10	CASP12
C	CASP14	CASP2	CASP3	CASP4	CASP5	CASP6	CASP7	CASP8	CASP8AP2	CASP9	CFLAR	CRADD
D	DFFA	DIABLO	endoG	FADD	FAM96A	FAM96B	FAS	FASLG	HMGB1	HRK	HSP90B1	HTRA2
E	LRDD	MCL1	NFKB1	NFK2	NGFR	PMAIP1	PTEN	REL	RELA	RELB	SOCS2	SOCS3
F	STAT1	STAT5A	STAT5B	TNF	TNFRSF10A	TNFRSF10B	TNFRSF10C	TNFRSF10D	TNFRSF11B	TNFRSF1A	TNFRSF1B	TNFRSF21
G	TNFRSF25	TNFSF8	TNFSF10	TNFSF11	TP53	TP53I3	TRAF1	TRAF2	TRAF3	TRAF5	TRAF6	TRAF7
H	ACTB	B2M	GAPDH	HPRT1	RPL13A	18S	YWHAZ	RT+	RT+	RT+	RT-	RT-

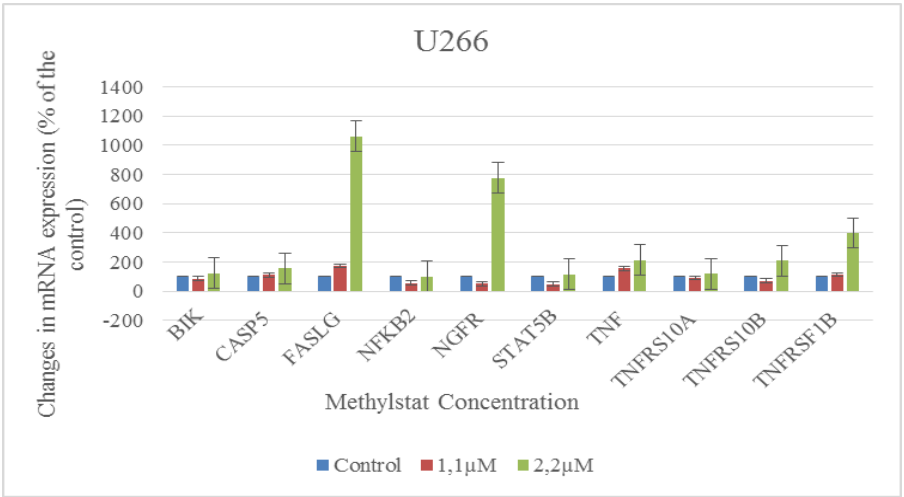

Figure 3.16. Changes in expression levels of apoptotic genes in response to methylstat in U266 cells (Error bars represents SD)

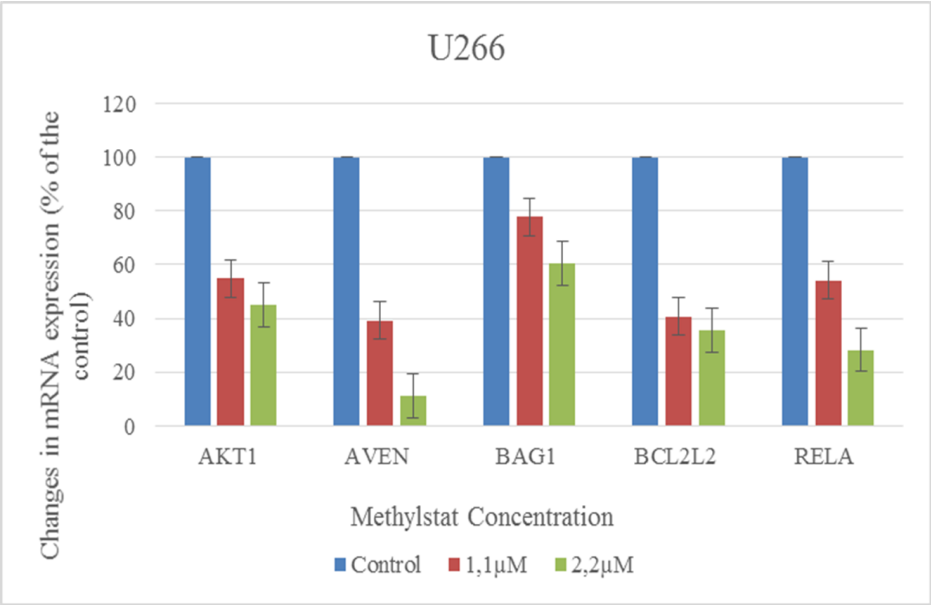

Figure 3.17. Changes in expression levels of anti-apoptotic genes in response to methylstat in U266 cells (Error bars represents SD)

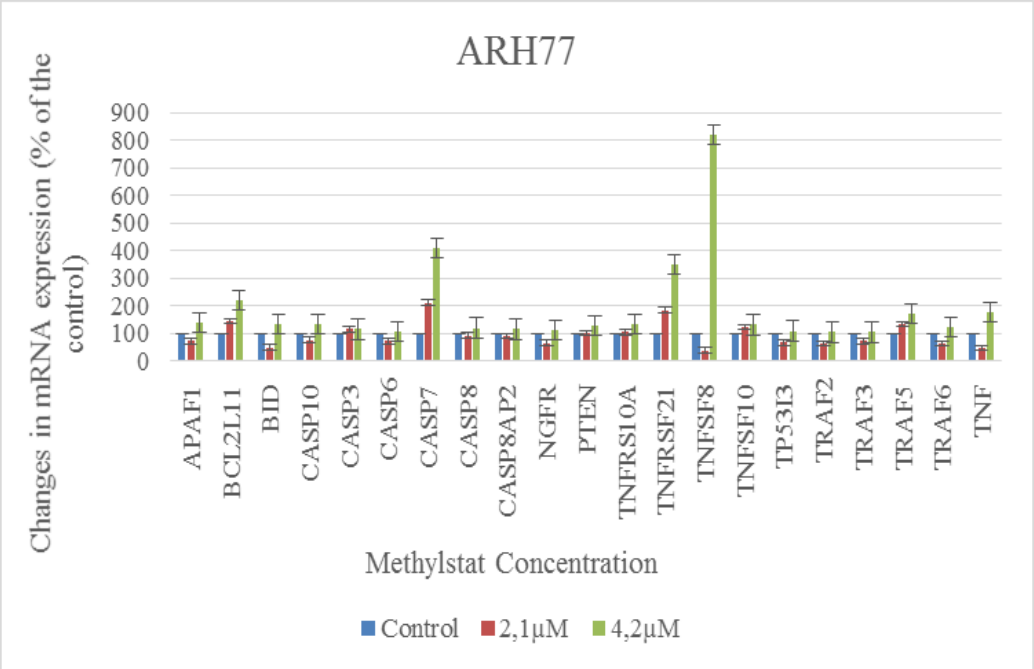

Figure 3.18. Changes in expression levels of 4 apoptotic genes in response to methylstat in ARH77 cells (Error bars represents SD)

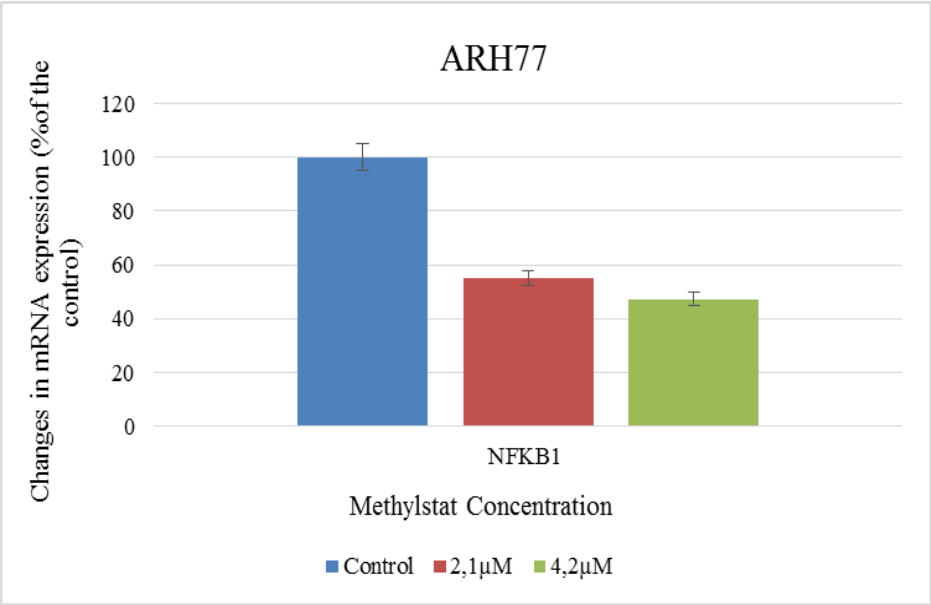

Figure 3.19. Changes in expression levels of NFKB1 gene in response to methylstat (Error bars represents SD)

The anti-apoptotic AVEN gene expression level decreased 8.8-fold in U266 cells whereas the expression levels of Fas ligand (FASLG), nerve growth factor receptor (NGFR), tumor necrosis factor (TNF), tumor necrosis factor receptor superfamily 10B (TNFRSF10B) and tumor necrosis factor receptor superfamily 1B (TNFRSF1B) increased 10.6-, 7.7-, 2.1-, 2- and 3.9 -fold, respectively in U266 cells as compared to untreated controls.

The anti-apoptotic NFKB1 gene expression level decreased 2-fold in ARH77 cells whereas the expression levels of BCL2-like 11 (BCL2L11), caspase 7 (CASP7), tumor necrosis factor receptor superfamily 21 (TNFRSF21) and tumor necrosis factor superfamily 8 (TNFSF8) increased 2.1-, 4-, 3.4- and 8.1 -fold, respectively in ARH77 cells, as compared to untreated controls.

CHAPTER 4

CONCLUSION

Multiple myeloma is a malignancy of B cells, which is characterized by abnormal proliferation of plasma cells (Mateos and Miguel, 2007). Unfortunately, its incidence is increasing day by day, and lots of new treatment strategies are being investigated in order to cure this disease. It was known that various chemicals and natural products are used for the treatment of MM. One of these agents is a proteasome inhibitor. Bortezomib effects are shown lots of studies. Untill now, it was shown that proteasome inhibitor, bortezomib, induces apoptosis in vitro on different cell lines. Agent induces p53 activity and inhibits the overexpression of Bcl-2 (Almond and Cohen, 2002). Methylstat is also used as an inhibitor of the Jumonji C domain-containing histone trimethyl demethylases. Until now, the effects of bortezomib on Multiple Myeloma cells have been showed by molecular approaches at protein level. However, methylstat is a new drug and there are only a few studies on different cancer types. Cell cycle and angiogenesis effects of methylstat are shown by Cho et al. on the human umbilical vascular endothelial cells (HUVECs). Agent showed anti-proliferative effects on HUVECs and indicated cell cycle arrest at G0/G1 phase of HUVECs in a dose- and time- dependent (Cho et al., 2014).

In this study, we examined the cytotoxic and apoptotic effects of bortezomib and methylstat with specific emphasis on methylstat on U266 and ARH77 Multiple Myeloma cells. For this purpose, U266 and ARH77 cells were firstly treated with increasing concentrations of bortezomib and methylstat alone and then their combinations were applied to the cells. Their cytotoxic effects were determined by MTT cell proliferation assay. Our results showed there were dose- and time- dependent decreases in cell proliferation in response to bortezomib and methylstat. IC_{50} values were calculated from cell proliferation plots and found to be 41 nM for U266 and 19nM for ARH77 cells in respons to bortezomib, 2.2 μM for U266 and 4.2 μM for ARH77 cells in response to methylstat the combination results also showed that IC_{50} values decreased as compared to bortezomib and methylstat alone and were calculated as 0.5

nM for U266 and 0.6 nM for ARH77 cells. Then, apoptosis was evaluated by measuring the changes in caspase-3 enzyme activity and loss of mitochondrial membrane potential (MMP). Treatment of U266 cells for 72 h with 10 nM bortezomib and 2.2 μM methylstat resulted in 1.7- and 1.5- fold increases, respectively, in caspase-3 activity as compared to untreated controls. Moreover, the treatment of ARH77 cells for 72 h with 5 nM bortezomib, 4.2 μM methylstat resulted in 1- and 1.2- fold increases, respectively, in caspase-3 activity as compared to untreated controls. In order to determine possible synergistic effects of bortezomib and methylstat, we assessed combinational treatments of bortezomib with methylstat. When we applied the IC_{50} values of methylstat (2.2 μM for U266 cells and 4.2 μM for ARH77 cells) and bortezomib (10 nM for U266 cells and and 5 nM forARH77 cells) synergistic antiproliferative effects on U266 and ARH77 cells were observed as compared to any agent alone and untreated controls. Treatment of U266 and ARH77cells for 72 h with combination of drugs resulted in 2- and 8.5- fold increases, respectively.

In order to confirm caspase-3 enzyme activity results and examine the roles of mitochondria in bortezomib and methylstat-induced apoptosis, we also determined the loss of mitochondrial membrane potential. The results were in agreement with our previous data and have shown that all these chemicals induce apoptosis through inducing loss of MMP. Combination of bortezomib with methylstat (demethylase inhibitor) caused loss of MMP synergistically as compared to any agent alone and untreated control group.

To detect the degree of apoptosis, Annexin V assay was used in the presence of bortezomib and methylstat. There are 4- , 10- and 12.5- fold increases in apoptotic cell populations for U266 cells, exposed to 10 nM bortezomib, 2.2 μM methylstat and their combination, respectively when compared to control cells. There are 1- , 3.2-, 9.3- 10- and 11.3- fold increases in apoptotic cell populations for ARH77 cells, exposed to 5 and 10 nM bortezomib, 4.2 μM methylstat and combination of them, respectively when compared to control cells.

Cell cycle analysis was performed to determine the DNA contents of cells with the help of PI dye. Combination of bortezomib and methylstat arrested cells at the S phase. The fraction of cells in S phase increased accordingly. These results indicated

that combination of bortezomib and methylstat-induced apoptosis could be cell cycle-dependent.

RT-PCR results of this study have shown for the first time that methylstat treatment may be resulted in the upregulation of FASLG, NGFR, TNF, TNFRS10B and TNFRS1B apoptosis- triggering genes in U266 cells and BCL2L11, CSP7, TNFRSF21 and TNFSF8 apoptosis- triggering genes in ARH77 cells in a dose-dependent manner. Furthermore, there were significant decreases in the expression levels of AKT1, AVEN, BAG1 BCL2L2 and RELA anti-apoptotic genes in U266 cells and NFKB1 anti-apoptotic gene in ARH77 cells in response to increasing concentrations of methylstat. These results suggested that methylstat affects lots of genes and pathway in the cells.

While we treated U266 and ARH77 cells with methylstat for 72 hours, expression levels of both some apoptotic genes and some anti-apoptotic genes were increased. However, the highest increase was determined in the expression level of FASLG and TNFSF8 genes, which are apoptotic.

In conclusion, taken together all these results revealed that there were significant increases in antiproliferative effects of bortezomib in combination with methylstat on U266 and ARH77 multiple myeloma cells.

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