CHANGES IN PROTEIN PROFILES IN BORTEZOMIB APPLIED MULTIPLE MYELOMA CELLS

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ABSTRACT

CHANGES IN PROTEIN PROFILES IN BORTEZOMIB APPLIED MULTIPLE MYELOMA CELLS

Multiple Myeloma is a malignant B-cell neoplasm that is characterized by the accumulation of malignant plasma cells in the bone marrow. Over the recent years, several novel agents have been introduced in the treatment of this disease. Bortezomib is the first of a new class of agents known as proteasome inhibitors.

The main objective of the project was basically to both determine the cytotoxic and apoptotic effects of Bortezomib on Multiple Myeloma U-266 cells and compare and explore the differences between Bortezomib applied Multiple Myeloma cells and control group Multiple Myeloma cells, by proteomics studies. In order to achieve our aims in the project, variety of multidisciplinary subjects were come together. Cancer research techniques, biochemical studies at protein level and proteomics were combined in our studies.

In this study, our experimental results demonstrated that Bortezomib has antiproliferative and apoptotic effects on MM U-266 cells. On the other hand, the responsible proteins for the effect mechanism of anti-cancer agent on cells were determined by MALDI-TOF-TOF Mass Spectrometry for the first time. According to the mass spectrometric analysis, 37 protein spots were differentially expressed. Among them, five proteins were newly formed, ten proteins lost, twelve proteins were up-regulated and ten proteins were down-regulated as compared to control group (untreated cells).These differential expressed proteins in response to Bortezomib have different important functions ranging from cell signaling transduction, cell cycle regulation, apoptosis to immunity and defense mechanism.

In conclusion, it was identified which proteins have a central role behind the effect of Bortezomib on MM U-266 cells. The identified proteins may let to be possible to treat other cancer types by same anticancer agent. The data obtained by this study may also be helpful for medical schools and drug designers and may also provide new treatments.

ÖZET

BORTEZOMİB UYGULANAN MULTİPL MİYELOMA HÜCRELERİNİN PROTEİN PROFİLLERİNDEKİ DEĞİŞİKLİKLER

Multilpl Miyelom, bağışıklık sisteminin bir parçası olan B hücrelerinin, hasarlı olarak kemik iliğinde çoğalması ve birikmesiyle oluşan hematolojik bir kanserdir. Son yıllarda, bu hastalığın tedavisi için birçok yeni kimyasal ajan tıp dünyasının hizmetine sunulmuştur. Bortezomib, Multipl Miyelom tedavisinde kullanılan ve tedavi sırasında proteazom inhibitörü olarak görev yaptığı bilinen bir anti-kanser ajandır.

Projenin esas amacı, hem Bortezomibin Multipl Miyeloma hücreleri üzerindeki sitotoksik ve apoptotik etkilerini belirlemek hemde Bortezomib uygulanan bu hücreler ile control grubu hücreleri arasındaki farklılıkları kıyaslamak ve keşfedebilmekti. Bu projede, hedeflerimize ulaşmak için, çeşitli interdisipliner konular biraraya getirilmiştir. Bu hedef doğrultusunda, kanser araştırma teknikleri, protein bazlı biyokimyasal çalışmalar ve proteomik yaklaşımlar kombine edilerek kullanılmıştır.

Bu çalışmadaki deneysel sonuçlarımız, Bortezomibin, MM U-266 hücreleri üzerinde antiproliferatif ve apoptotic etkilere sahip olduğunu gösterdi. Öte yandan, bu anti-kanser ajanın hücreler üzerindeki etki mekanizmasında görev alan proteinler MALDI-TOF-TOF Kütle Spektrometresi ile ilk defa belirlenmiştir. Kütle spektrometresi sonuçlarına gore, otuz yedi protein spotunda farklılık gözlenmiştir. Bunlardan beş spot yeni oluşan ve on spot kaybolan proteinlere karşılık gelmiştir. Ayrıca, on iki spotun seviyesinde artışlar gözlenirken, on ayrı spotun da ekspirasyon seviyelerinde azalma gözlenmiştir. Bu proteinler hücre içerisinde, sinyal iletiminden, hücre bölünmesine, apoptozdan vücudun savunma mekanizmasına kadar değişen önemli fonksiyonlara sahipler.

Sonuç olarak Bortezomibin MM U-266 hücreleri üzerindeki etki mekanizmasında hayati öneme sahip proteinler tanımlanmıştır. Farklılık gösteren bu proteinlerin ve fonksiyonlarının belirlenmesi sayesinde aynı kemoterapatik ajan ile aynı proteinlerin rol aldığı diğer kanser türlerinin de tedavi edilebilirliği mümkün olabilir. Ayrıca buradan elde edilen bulgular, yeni tedavi yöntemleri konusunda tıp fakültelerine ve ilaç dizaynı üzerine çalışan araştırmacılara yeni ufuklar açabilir.

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CHAPTER 1

INTRODUCTION TO MULTIPLE MYELOM

1.1. Cancer

Cancer is one of the most frightning health problems of our age on account of higher death rate. It is known that the number of research groups dealing with the biological basis of cancer formation, progression and new treatment options increase in number with the expansion of the knowledge in the field of cancer research, but, unfortunately, cancer still remains one of the leading causes of death after the cardiovascular diseases in the world (Jemal et al., 2007). According to GLOBOCAN 2008 data, nowadays, approximately 12 million people worldwide caught this disease each year, about 7 million of these patients (~60%) are dying in the same year and it is estimated that 25 million cancer patients are still alive (Bilir, 2008; Jemal et al., 2011). Furthermore, considerable number of scientific studies on cancer statistics show that both the rate of cancer is increasing day by day and diagnosis and treatment options of it require the co-operation of many specialized fields (Parkin et al., 2005; Boyle and Levin, 2008; Jemal et al., 2011 respectively).

The term cancer applies to a set of diseases characterized by unregulated, abnormal cell growth leading to invasion of surrounding tissues from its point of origin and spreads to other parts of the body where they establish secondary areas of growth (King and Robins, 2006; Lieberman and Marks, 2009). A brief definition for cancer would be a group of diseases in which cells no longer respond to normal restraints on growth. In other words, cancer originates from the loss of normal growth control.

The body is made up of hundreds of millions of living cells. Normal body cells grow, divide and die in an orderly fashion. During the early years of a person's life, normal cells divide faster to allow the person to grow. After the person becomes an adult, most of the cells divide only to replace worn-out or dying cells or to repair injuries and the rates of new cell growth and old cell death are kept in balance. On the contrary, cancer cell growth is very different from normal cell growth. Instead of dying, cancer cells continue to grow and form new, anomalous cells.

When normal cells in the body begin to divide for proliferation to replace the death ones, two or more cells begin to come into contact with each other to slow up the rate of division. This behaviour is a consequence of the natural process of arresting cell growth called contact inhibition. It is one of the most significant control mechanism to direct the cells to stop proliferating and it ensures the cells to create a layer only one cell thick, a monolayer (Alberts et al., 2008; Lieberman and Marks, 2009; NCI, 2011).

On the other hand, the behaviour of cancer cells is quite different. These cells do not require growth stimulatory signals and also they are resistant to growth inhibitory signals. When the normal cells regulate the growth, unfortunately, cancer cells continue to divide in an unregulated manner yielding a clump of cells which is called focus. So, the balance between formation and destruction of cells is disrupted. Moreover, cancer cells are also resistant to apoptosis (programmed death process) which is the mechanism by which old or damaged cells normally self-destruct. They have an infinite proliferative capacity and do not become senescent (Şenel and Çırakoğlu, 2003; Alberts et al., 2008; Lieberman and Marks, 2009; NCI, 2011).

All of these data introduce the linked questions : What agents cause cancer? What is the reason of abnormal growth of cancer cells? and How does cancer develop?

Although there are multiple risk factors including genetic (biological) factors, age, obesity, declining immune system, exposure to chemicals and/or radiation, pesticides and virus, environment and lifestyle factors are known to have a major impact to constitute the cancer. Relevant research demonstrates us that of all cancer-related deaths, nearly 5-10% are due to genetic factors, whereas the rest of it, nearly 90-95% are because of lifestyle and environment factors (Anand et al., 2008; Irigaray et al., 2007).

The aberrant growth pattern in cancer stems from many mutations in genes which are responsible for proliferation, differentiation and survival of cells. Cancer cells no longer respond to growth inhibitory signals owing to these genetic changes (Lieberman and Marks, 2009). In Figure 1.1, the difference between normal cells and cancer cells in terms of growth control mechanism can be seen easily. DNA is in every cell and directs all its actions. In a normal cell division, when a cell' s DNA gets damaged, the cell either repairs the damage or goes to apoptosis (programmed cell death) yet, in cancer cell division, the damaged DNA is not repaired, make matters worse, the cell does not die like it should. Instead, this cancer cell goes on making new copies that the body does not need. These new cells will all have the same damaged

DNA as the first cell does. Therefore, if mutation is not repaired properly and before replication occurs, the mutated cells amplify to generate billions of cells that constitute a cancer (King and Robins, 2006; Lieberman and Marks, 2009).

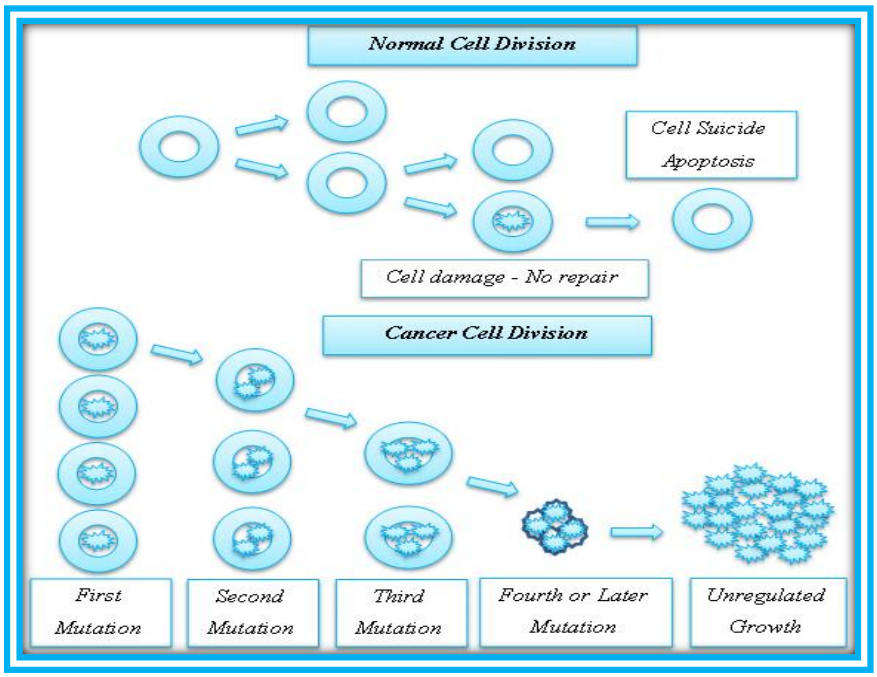


Figure 1.1. Loss of Normal Growth Control

Carcinogenesis, the process by which cancers are developed, is a multistage mechanism resulting from the accumulation of errors in vital regulatory pathways. It is initiated in a single cell, which then multiplies and obtains additional alterations that give it a survival advantage over its neighbours (Figure 1.2) (King and Robins, 2006).

Figure 1.2 represents development of cancer. Each connecting arrow refers several events and many pathways can exist between each stage. First step (Initiation) explains the generation of mutated cells in consequence of mutations. Following step (Promotion) expresses the proliferation of differentiated cells. Step three (Progression) states the formation of cancer. Subsequent to this uncontrolled growth, cancer cells that alter morphological properties, invade other tissues and metastasize.

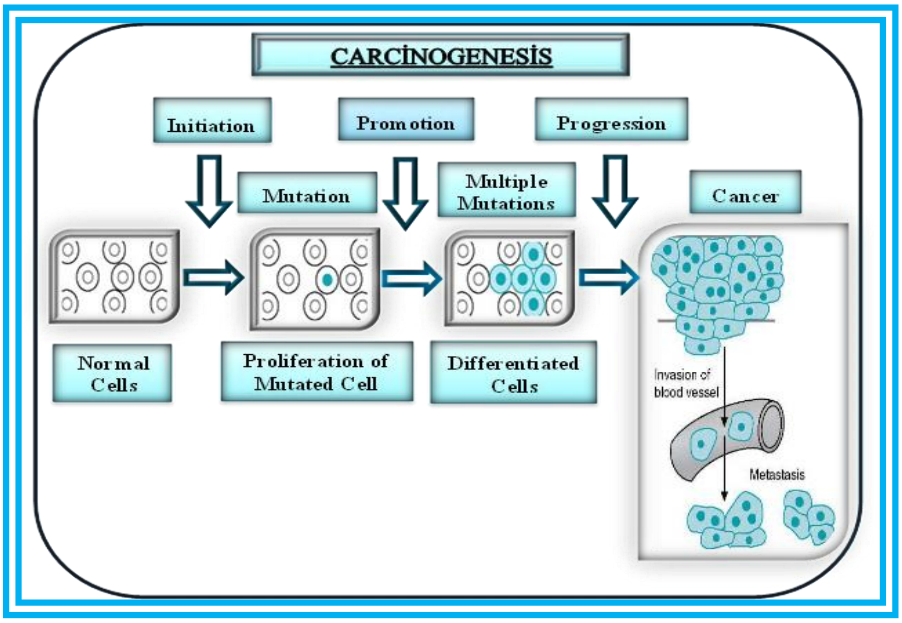


Figure 1.2. Development of Cancer

Six considerable characteristics found in cancer cells are called hallmarks of cancer (Hanahan and Weinberg, 2000). They are as follows :

- ✓ Self-sufficiency in growth signals,
- ✓ Insensitivity to antigrowth signals,
- ✓ Evasion of apoptosis,
- ✓ Limitless replicative potential,
- ✓ Sustained angiogenesis,
- ✓ Tissue invasion and metastasis.

1.2. Multiple Myeloma (MM)

Multiple Myeloma (MM) (also known as myelomatosis, plasma cell myeloma or Kahler's disease) is a malignant B-cell neoplasm that is characterized by the accumulation of malignant plasma cells in the bone marrow. It is known as bone marrow cancer in the society and it is the second most common hematological disorder (Raab et al., 2009). It accounts for 2% of cancer deaths among the most dangerous cancer types (Zaidi and Vesole, 2001). Recently, increasing number of people is being diagnosed as MM. It has a yearly incidence of nearly 14-15 thousand that accounts for approximately 10% of all hematologic cancers in the US (San et al., 1999; Collins,

2005) and the incidence number is almost 3 thousand in Turkey (Lag et al, 2002; Horner et al., 2009). In addition to all of this statistic data, American Cancer Society reports that approximately 20 thousand new cases of MM is diagnosed during 2008 (NCI, 2011).

As mentioned before, MM is a type of cancer formed by malignant plasma cells. Plasma cells mainly take part in the bone marrow which is the soft tissue found inside some hollow bones. These cells are an important part of the immune system.

Even though immune system is composed of several types of cells that endeavour together to fight infections and other diseases, lymphocytes (lymph cells) are the main cell type of it. They can be found in many areas, such as lymph nodes, bone marrow and bloodstream. There are two types of lymphocytes; T cells and B cells.

When B cells respond to an infection, they are activated by helper T cells (CD4 T Cells) in the presence of IL-4, IL-5, IL-6 (cytokines) and CD40 Ligand. These activated B cells mature and change into memory cells and plasma cells (Figure 1.3) (Murphy et al., 2008). Plasma cells are responsible for the making antibodies (immunoglobulins, a kind of protein) that help the body protect from germs and other harmful substances by attacking and killing them (Figure 1.4) (NCI, 2011).

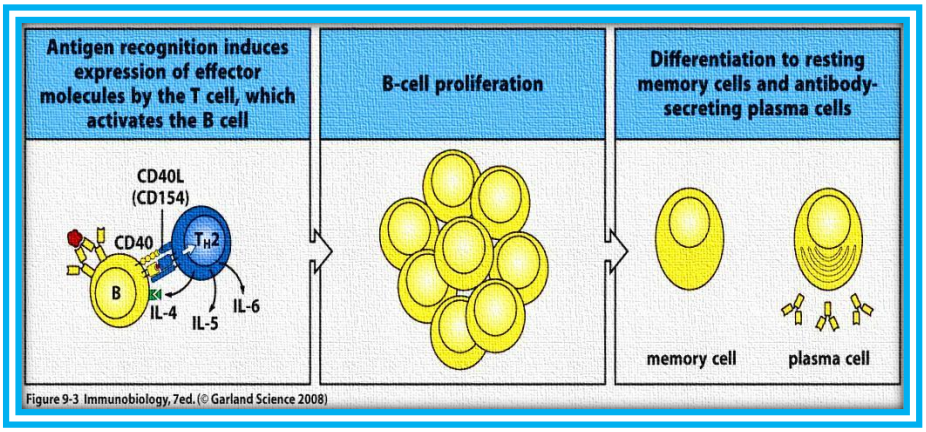


Figure 1.3. The Activation and Differentiation of B Cell
(Source: Murphy et al., 2008)

In MM, the body makes too many plasma cells (myeloma cells) (Bataille and Harousseau, 1997). These cells lose their normal functions and produce much more antibodies called M proteins that the body does not need. Myeloma cells at issue cause the problems in the body in two ways: They crowd out the normal plasma cells so that the antibodies to fight the infections are not made. Additionally, antibodies secreted by

the myeloma cells does not help fight infections. That is why, it should not be expected from this abnormal plasma cells to protect the body.

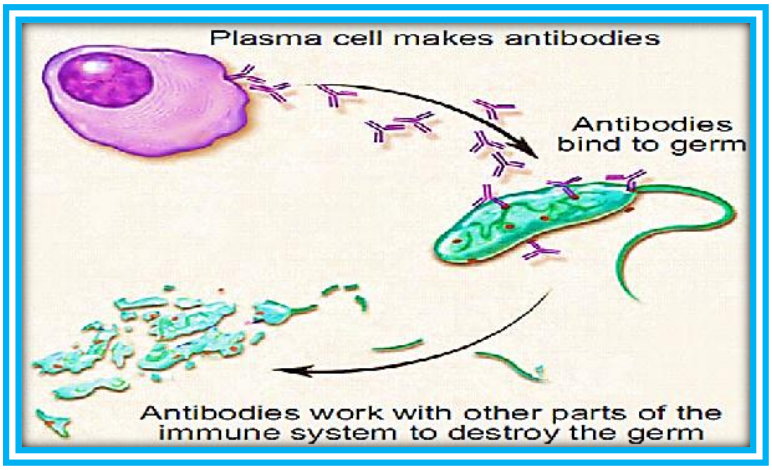


Figure 1.4. Function of the Plasma Cells
(Source: NCI, 2011)

1.2.1. The Classification of MM

Different types of myeloma can be classified into three parts by the type of antibody (immunoglobulin, also called M protein) secreted by malignant plasma cells.

- ✓ Plasma cells can produce a certain types of immunoglobulin (such as IgG),
- ✓ Plasma cells can produce incomplete immunoglobulins, containing only the light chain portion of it,
- ✓ Plasma cells do not produce immunoglobulins or light chains.

Immunoglobulins (Ig) can be classified under the category of glyco-proteins and basically made up of two components; light chains (which has 25 kDa molecular weight and consists of 220 amino acids) and heavy chains (which has 50 kDa molecular weight and consists of 220 amino acids) (Voet et al., 1999). What is more, they are classified by the sort of light (kappa or lambda) or heavy (alpha [IgA], gamma [IgG], mu [IgM], delta [IgD], and epsilon [IgE]) chains, based on differences in the amino acid sequences in the constant region of the light or heavy chains. (Steward, 1984).

The main molecular structure of immunoglobulins is shown in Figure 1.5. Even though antibodies can differ structurally, they are made from the same basic unit (Voet et al., 1999). Light and heavy chain immunoglobulins show a Y shape structure (Steward, 1985).

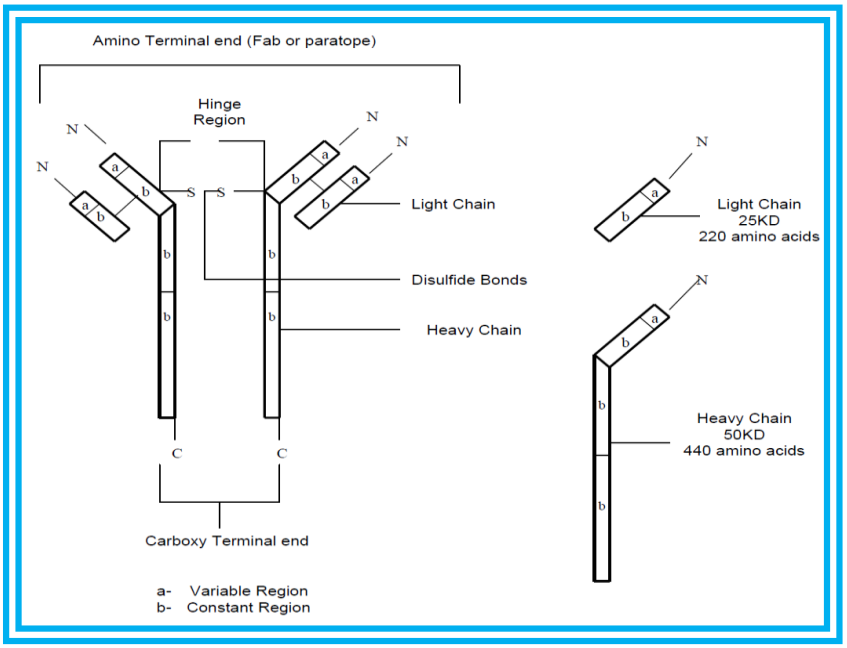


Figure 1.5. Molecular Structure of Immunoglobulin
(Source: Voet et al., 1999)

Although MM cells can produce all classes of immunoglobulins theoretically, IgM type immunoglobulin is the least and IgG kappa is the most common monoclonal protein encountered in MM. Approximately 60% to 70% of all cases of MM is IgG type. Morover, IgA type of MM accounts for about 20% of cases. As compared to IgG type, IgA myeloma cells are less widespread, but still prevalent. IgD and IgE types MM are very rare (Anderson, 2003; Sirohi and Powles, 2004; Multiple Myeloma, 2011).

Some cells produce an incomplete immunoglobulin consisting of only light chains, known as Bence-Jones proteins (Light Chain Myeloma or Bence Jones Myeloma). Most of the time it is seen in the form of kappa, but it can be also lambda type (Hoffbrand et al., 2006; Putnam and Stelos, 2011). In this disease, M protein is found primarily in the urine, rather than in the blood. On the other hand, some rare diseases are associated with plasma cell overproduction of heavy chains only. These are called as heavy chain diseases and they may or may not be similar to myeloma in their characteristics.

A rare form of MM called Nonsecretory Myeloma occurs in about 1% of myeloma patients. In this disease, neoplastic plasma cells do not produce any immunoglobulin chains, heavy or light (Shaw, 2006).

1.2.2. Molecular Pathways of MM

MM is a plasma cell malignancy characterized by very complex cytogenetic and molecular genetic aberrations.

Cancers can be caused by mistakes, defects or certain changes in the DNA called mutations that turn on oncogenes or turn off tumor suppressor genes. DNA is a complex molecule and the fundamental building block for a human's entire genetic makeup. It is a component of virtually every cell in the body. Certain genes that are the parts of the DNA and responsible for promoting cell division are called oncogenes and others that take charge in slowing down cell division or causing cells to die at the appropriate time are known tumor suppressor genes.

Chromosomal translocations and/or point mutations, amplifications of proto-oncogenes and inactivations of tumor supressor genes are the important mechanisms that cause to progression of MM. Oncogenes which are active in this disease and their activation mechanisms are shown in the Table 1.1 (Gahrton and Durie, 1996).

Table 1.1 Genetic Alterations in MM

Oncogenes	**Chrosomal Locations**	**Activation Mechanism**	**Literatures**
c-myc	8q24	Trasnlocation	(Greil et al., 1991), (Cobbold et al., 2010)
bcl-2	18q21	Trasnlocation	(Pettersson et al., 1992)
N-ras	1p11-13	Point Mutation	(Portier et al., 1992)
K-ras	12p11-12	Point Mutation	(Portier et al., 1992)
p53	17p13	Point Mutation Deletion	(Neri et al., 1993), (Portier et al., 1992)
Rb1	13q14	Deletion	(Gahrton and Durie, 1996).
c-MAF	14q16 or 14q20	Trasnlocation	(Sawyer et al., 1998) (Kuehl and Bergsagel, 2002)

In MM, a chromosomal translocation between the immunoglobulin heavy chain gene on chromosome 14, locus q32 (14q32) and an oncogene 11q13 (cyclin D1), 4p16 (FGFR3 & MMSET (Multiple Myeloma SET Domain)), 6p21 (cyclin D3), 16q23 (c-maf), 20q11 (mafB) and 8q24 (mafA), which account for about 40% of all myeloma tumors (Table 1.2), (Bergsagel and Kuehl, 2001; Barillé-Nion et al., 2003; Kyle and Rajkumar, 2004) is frequently observed. These mutations bring about disregulation of the oncogenes that are known to be a significant initiating event in the pathogenesis of MM. The consequence all of these mutations is proliferation of a plasma cell clone and genomic instability that leads to further mutations, deletions and translocations.

Table 1.2. Prevalance of Important Translocations in MM (Adapted from Sawyer et al., 1998; Kuehl and Bergsagel, 2002; Avet-Loiseau et al.,2002).

Translocations	**Prevalance**
11q13 (cyclin D1)	15%
4p16 (FGFR3 & MMSET)	15%
6p21 (cyclin D3)	3%
16q23 (c-maf)	4%
20q11 (mafB)	2%
8q24 (mafA)	1%
TOTAL	40%

Translocations between t(4;14) (p16;q32) or t(14;16) (q32;q23), partial (q14) or complete loss of chromosome 13 and partial loss of 17p13 refer to high risk of MM (Stewart and Fonseca, 2005; Tassone et al., 2006). In addition to these data, translocation t(11;14) (q13;q32) that is activated Bcl-1 oncogene is the most common chromosomal difference (Stewart, 2008). This translocation is also seen in other hematological cancers. Besides, changes in the number of chrosomes are frequently encountered in MM, especially hyperdiploidy is seen at the 3., 5., 7., 9., 11., 15., 19. and 21. chromosomes whereas hypodiploidy is seen at the 8., 13. and X chromosomes (Gahrton and Durie, 1996; Mahindra et al., 2010).

In MM, the chromosome 14 abnormality is observed nearly 50% of all cases of myeloma and aberration of chromosome 13 is also observed in about 50% of cases with 85% monosomy 13, while the remaining 15% constitute deletion of chromosome 13 (Munshi and Avet-Loiseau, 2011; Sawyer, 2011).

1.2.3. Risk Factors for MM

A risk factor can be defined as anything which alterates a person's chance of getting the disease.

The known risk factors for MM are as follows ;

Age is the most crucial risk factor for MM, as nearly 98% of cases are diagnosed in people over the age of 40. It means that only 2% of patients are younger than 40 years old. Furthermore, more than 75% of these patients over the age of 70. Median age for men is about 62 years and for women 61 years at diagnosis (Raab et al., 2009).

It is thought that susceptibility to get MM may increase with the aging process. Because it affects the the people in a negative way in terms of the result of reduction in immune surveillance of evolving cancer or accumulation of toxic substances. So, the risk of the catching MM goes up with age.

MM has two times more common incidence in men than in women. Thus, men are slightly more likely to develop this disease when compared to women (Mitsiades et al., 2004; Sirohi and Powles, 2004).

Race is also risk factor for MM. It is almost twice as common among African Americans than white Americans. The higher incidence of MM in African Americans and the much less frequent occurrence in Asians refer genetic factors (Cohen et al., 1998; Malpas et al., 1998).

Exposure to radiation may increase the risk of multiple myeloma (ACS, 2011).

People who has a sibling or parent with MM is four times more likely to constitute cancer than would be expected. Still, most patients have no affected relatives, so this accounts for only a small number of cases (ACS, 2011).

Some studies have suggested that people in agricultural occupations, workers both in certain petroleum-related industries and leather industries and cosmetologists all seem to have a higher-than-average chance of developing MM. Exposure to herbicides, insecticides, petroleum products, heavy metals, plastics and various dusts including asbestos also appear to be risk factors for the disease. In addition, people exposed to large amounts of radiation, such as survivors of the atomic bomb explosions, have an increased risk for MM, although this accounts for a very small number of cases (ACS, 2011).

Obesity is the most common known risk factor for MM. Being overweight or obese increases a person's risk of developing MM according to a study carried out by the American Cancer Society (ACS, 2011).

Other plasma cell diseases are also important risk factor for people, because these people most prone to get caught to MM. There is a crucial information related to plasma cells and MM. It must be noted that uncontrolled plasma cell growth is not always correspond to MM. Besides, there are two plasma cell related diseases like MM.

One of them is monoclonal gammopathy of undetermined significance (MGUS). In this diseases, similar to MM, abnormal plasma cells produce excess amounts of antibody protein. However, these cells do not form an actual tumor or mass and do not cause any of the other problems seen in MM. The most distinction between MGUS and MM is that MGUS does not give rise to weak bones and anemia.

Scientists who have studied the genes of the plasma cells in patients with MGUS found that the genetic background of these cells resembles myeloma plasma cells more than it resembles normal plasma cells. This finding suggests that these cells are principally malignant, and people with MGUS can go on to develop MM (Weiss and Kuehl, 2010).

Another type of abnormal plasma cell growth disease is solitary or isolated plasmacytoma. It differs from MM with regards to number of tumor. Patients with this disease have only one tumor rather than multiple tumors in different locations as in multiple myeloma. Most often, a solitary plasmacytoma develops in a bone, but it is also rarely found in other tissues. When it begins in other tissues (such as the lungs), it is called an extramedullary plasmacytoma. As many people with a solitary plasmacytoma will develop multiple myeloma (Di Micco and Di Micco, 2005).

1.2.4. Signs and Symptoms of MM

The bone marrow microenvironment has a key role in both bone destructive and tumor growth process in MM (Roodman, 2009). There are two major kinds of bone cells that basically work together to keep bones healthy and strong. One of them is osteoblasts that are responsible for laying down new bones and the other one is osteoclasts which break down worn or dying bones. These cells take part in the bone tissue. The main function of osteoblasts are both bone matrix synthesis and regulation

of the mineralizations by adjusting the calcium-phosphorus balance. The most characteristic morphological features of osteoclasts are their curved cell edges and finger-shaped cell membrane protrusions. These features makes it easy to form bone resorption (the destruction of bones from metabolism by melting physiologically). function. These two types of cells are controlled various growth factors and they are in balance in the bone tissues of healthy individuals. However, this situation is somewhat differ in patients with MM. Because myeloma cells change the balance between osteoblasts (formation of new bones) and osteoclasts (destruction of old bones) in favor of osteoclasts by producing certain growth factors. While dickkopil, interleukin-3 (IL-3) and interleukin-7 (IL-7) which are the growth factors secreted by myeloma cells are inhibiting the functions of osteoblast cells, macrophage inflammatory protein and interleukin-3 (IL-3) activate the role osteoclast cells either directly or indirectly (Terpos and Dimopoulos, 2005; Silvestris et al., 2007; Terpos, 2008) (Figure 1.6). Another important molecule in bone metabolism, RANKL (Receptor Activator for Nuclear Factor KB Ligand), plays also a role to activate the osteoclasts (Edwards et al., 2008). In addition to all of these, myeloma cells encourage the bone marrow stromal cells to the production of interleukin-6 (IL-6), interleukin-1 (IL-1) and Tumor Necrosis Factor (TNF) to increase osteoclast formation and tumor growth (Sirohi and Powles, 2004; Terpos, 2008; Roodman, 2009). These interleukins activates multiple pathways that protect myeloma cells from apoptosis (Zaidi and Vesole, 2001; Bommert et al., 2006).

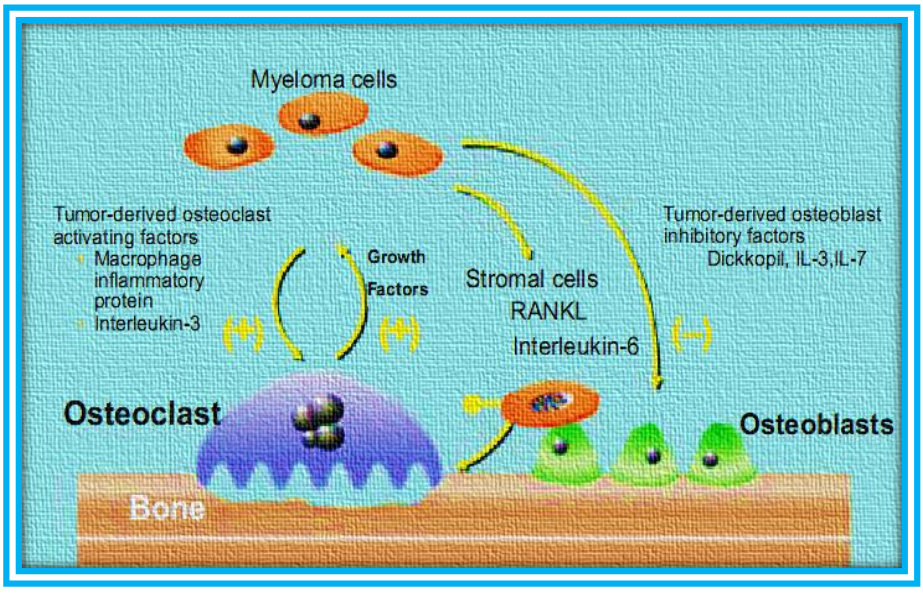


Figure 1.6. Tumor-micro Environmental Interactions in MM
(Source: Roodman, 2009)

As a result of this situation observed in patients with MM, bones are damaged causing pain and sometimes they can even break. Fractured bones and bone pain which is leading manifestation of myeloma in 75% of MM patients (Zaidi and Vesole, 2001)) are major problems.

Another significant symptom of MM is high blood calcium. With the increased bone resorption, calcium in the bones is released. This can result from high blood levels of calcium (hypercalcemia) that can cause dehydration, severe constipation, loss of appetite, drowsy and nausea. Furthermore, renal failure (present 5-30% of MM patients) may commonly develop owing to hypercalcemia both acutely and chronically (Alexanian et al., 1990; Ludwig, 2005; Smith et al., 2006; Wirk, 2011; ACS, 2011).

One of the reason for MM patients to feel themselves very tired and weak is anemia that blocks the proliferation of the new red blood cells by malignant myeloma cells. A reduced number of red blood cells causes weakness, shortness of breath and dizziness. Furthermore, they also hamper the formation of new white blood cells which are significant for immune system. This is why, insufficient white blood cells (leukopenia) lowers resistance to infections (Zaidi and Vesole, 2001) and the lower blood platelet (thrombocytopenia) (Kristinsson, 2010) may cause serious bleeding (Ludwig, 2005; ACS, 2011).

Patients with MM are nearly fifteen times more likely to get infections. This is because, the body is unable to make the antibodies which help fight infection. One of the result of this situation, patients are slow to respond to treatment. Pneumonia is very common and a serious infection determined in myeloma patients (Savage et al., 1982; ACS, 2011).

If MM give rise to the weakening of bones in the spine, they can press on spinal nerves causing sudden severe pain, numbness, and/or muscle weakness. Besides, some abnormal proteins produced by myeloma cells can be toxic to the nerves.

1.2.5. Diagnosis of MM

For the diagnosis of MM, many test and procedures were introduced with the clinic. These crucial studies in terms of the evaluation of MM, their purposes and results are demonstrated in Table 1.3.

To measure the levels of red cells, white cells, and platelets in the blood, the complete blood count (CBC) test can carry out. As myeloma cells occupy too much of the bone marrow, CBC levels in patients body will be low.

So as to assess the blood levels of the different antibodies the test quantitative immunoglobulins can perform. In these test, the levels of immunoglobulins quantified to observe if any of them are anomalously high or low. According to previous studies, we know that the level of one type immunoglobulin may be high while the others are low in MM.

Other laboratory tests to find any abnormal immunoglobulin and measure the total amount of it are Serum Protein Electrophoresis (SPEP) and urine protein electrophoresis (UPEP). Besides, immunofixation or immunoelectrophoresis and urine immunofixation can be carried out in order to detect the which type of antibody is abnormal (Smith et al., 2006).

When the SPEP results are inadaquate, the test that is based on quantifying the lighy chains can be used in certain types of myeloma (Bradwell et al., 2003).

Table 1.3. Laboratory Tests and Procedures to Diagnose the MM
(Source : MMRF, 2010)

BLOOD SPECIMEN		
Diagnostic Test	**Purpose**	**Results**
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	Determine the level of serum protein that reflect both disease activity and renal function	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

(cont. on next page)

Table 1.3. Laboratory Tests and Procedures to Diagnose the MM (cont.)

BLOOD SPECIMEN		
Diagnostic Test	**Purpose**	**Results**
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		
Diagnostic Test	**Purpose**	**Results**
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		
Diagnostic Test	**Purpose**	**Results**
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

Beta-2 microglobulin level is very important to understant the how much disease progress. Actually, this protein is very useful sign for staging of MM. Thus, the level of this protein produced by the malignant plasma cells can be evaluated by a test (Bataille et al, 1992).

The levels of blood urea nitrogen (BUN) and creatinine levels in blood is related with the kidney function. The increased levels of them mean that kidney function is impaired. Similarly, low levels of Albumin and high levels of Calcium in the blood can be a cue of advanced myeloma (Bataille et al., 1992; Jacobson et al., 2003).

Bone marrow biopsy is of great importance in terms of checking the bone marrow. Because, in MM, patients have too many plasma cells in their bone marrow (Smith et al., 2006).

With the progression of technology in imaging area, these types of studies begin to occupy a great place. One of these types of study is Bone x-rays. By the help of method, bone destruction caused by the myeloma cells can be detected (Ozaki, 2007). The computed tomography (CT or CAT) scan can be used as an improved x-ray procedure to take the detailed cross-sectional images of patient's body to determine the damaged bones (Schreiman et al., 1985; Kyle et al., 1985; Winterbottom and Shaw, 2009). One another imaging methos is magnetic resonance (MRI). It can be also useful in looking at bones. It differs from the x-rays and CT in that it utilizes radio waves instead of x-rays. Firstly, this energy is absorbed and then released in a pattern produced by the type of tissue. Next, the pattern of radio waves given off by the tissues are translated into detailed image of the body (Joffe et al., 1988; Lecouvet et al., 2001).

To make a diagnosis of MM, results obtained any single test are not sufficient.

1.2.6. Stages of MM

Staging process is very significant in that it is the sign of cancer progress. Importance of this process stems from determining the appropriate treatment options and prognosis.

MM has been staged with two systems up to now. One of them is Durie-Salmon Staging System and the other one is International Staging System. Although some doctors still use this system, its value is becoming restricted due to newer diagnostic methods. Recently, International Staging System for MM has been developed.

1.2.6.1. The Durie-Salmon Staging System

The Durie-Salmon Staging System was developed in the mid-1970s and most commonly used staging system for patient with MM (Jacobson et al., 2003).

This system is basically depends on four factors ;

- ✓ The amount of abnormal monoclonal immunoglobulin in the blood or urine,
- ✓ The amount of calcium in the blood,
- ✓ The amount of hemoglobin in the blood,
- ✓ The severity of bone damage based on x-rays.

Table 1.4. Durie-Salmon Staging System in MM
(Source : Durie and Salmon, 1975)

Stage	Criteria
I	Hemoglobin level > 10 g / 100 ml, (Hemoglobin level is slightly under the normal level)
	Calcium levels in blood ≤ 12 mg / 100 ml, (Normal calcium levels in blood)
I	Normal bone structure, (Bone x-ray results either appear normal or indicate that only one area of bone damage)
	Relatively low monoclonal immunoglobulin rates in blood or urine ; ✓ IgG value < 5 g / 100 ml ✓ IgA value < 3 g / 100 ml ✓ Urine light chain monoclonal immunoglobulin on electrophoresis < 4 g / 24 hours.
II	A moderate number of malignant plasma cells are present. Features of this stage are between stage I and III.
III	Hemoglobin level > 8.5 g / 100 ml, (Low hemoglobin level)
	Calcium levels in blood > 12 mg / 100 ml, (High calcium levels in blood)
	Advanced lytic bone lesions (Bone x-ray results indicate that three or more areas of bone)
	High monoclonal immunoglobulin rates in blood or urine ; ✓ IgG value > 7 g / 100 ml ✓ IgA value > 5 g / 100 ml ✓ Urine light chain monoclonal immunoglobulin on electrophoresis > 12 g / 24 hours.

By using the system introduced by Durie and Salmon (Durie and Salmon) in 1975, MM has been divided myeloma into three stages. According to this system, the risk of MM increases from Stage I to Stage III. Thus, Stage I indicates the smallest amount of tumor and stage III indicates the largest amount of tumor.

Large amounts of abnormal monoclonal immunoglobulin is an indicator of many malignant plasma cells and advanced MM.

The amount of calcium in the blood is directly related to bone destruction. Because, bones normally include lots of calcium. With the bone damage in MM, calcium in the bones are released into the blood. Multiple areas of bone damage seen on x-rays indicate an advanced stage of multiple myeloma.

Hemoglobin level is another important factor to stage MM. It is mainly responsible for carrying oxygen and the substance of red blood cells. In MM, malignant plasma cells occupy much of the bone marrow, in connection with this, the normal bone marrow cells have not enough space to produce red blood cells adaquately. Therefore, low hemoglobin level is equal to advanced MM.

1.2.6.2. The International Staging System

The International Staging System is similar to Durie-Salmon Staging System in that it also divides MM into three stages. System is based only on the Serum β-2 Microglobulin and Serum Albumin Levels.

Table 1.5. International Staging System in MM
(Source : Greipp et al., 2005)

Stage	Criteria
I	Serum β-2 Microglobulin Level < 3.5 mg / L, Serum Albumin ≥ 3.5 g / 100 ml,
II	Serum β-2 Microglobulin Level < 3.5 mg / L but Serum Albumin < 3.5 g / 100 ml or 3.5 mg / L < Serum β-2 Microglobulin Level < 5.5 mg / L (with any Serum Albumin level)
III	Serum β-2 Microglobulin Level > 5.5 mg / L

1.2.7. Treatment Strategy for MM

1.2.7.1. Bortezomib

Treatment strategy for MM is a very important and vital step for the patients, because MM is currently still an incurable disease (Pandit, 2005; Dmoszyńska, 2008). Bortezomib (Velcade®, Millennium Pharmaceuticals Inc., and Johnson & Johnson Pharmaceutical Research & Development, LLC) (LCLabs, USA) is the first of a new class of anti-cancer agents known as proteasome inhibitor that was entered clinical trials and firstly confirmed by the US Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA) for the treatment of MM (Mateos and Miguel, 2007).

Bortezomib (previously known as PS-341, LDP-341, MLN341) is a dipeptide boronic acid derivative, which specifically and reversibly inhibit proteasome activity and triggers apoptosis in MM by binding the catalytic site of the 26S proteasome with high affinity and specificity with the boron atom in its structure (Elliott and Ross, 2001; Blade et al., 2003; Richardson et al., 2003; Voorhees et al., 2003; Adams and Kauffman,2004; Chauhan et al., 2005). The chemical formula and molecular weight of Bortezomib is $C_{19}H_{25}BN_4O_4$ and 384.24 g / mol respectively. The chemical structure of Bortezomib is as follows (Figure 1.7).

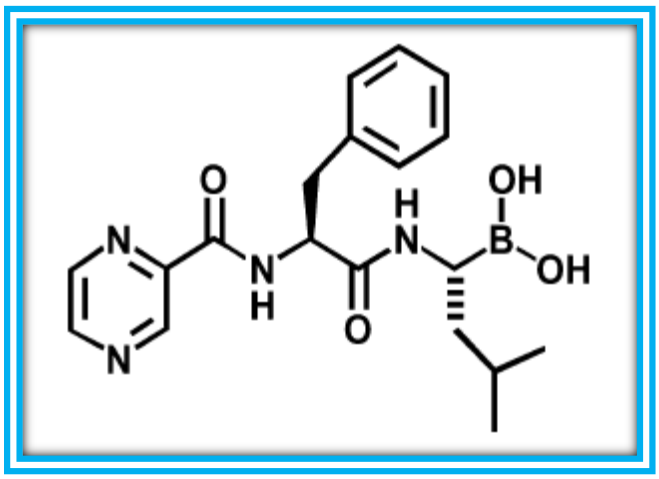


Figure 1.7. Structure of Bortezomib
(Source: Blade et al., 2005)

1.2.7.2. Biology of The Proteasome

The 26S Proteasome plays an essential role in the targeted degradation of intracellular proteins which are responsible for regulating essential cellular functions, such as cell-cycle control, cellular adhesion, proliferation, survival and apoptosis (Matthews et al., 1989; Glickman and Ciechanover, 2002). Lots of studies carried out using proteasome inhibitors have shown that the 26S proteasome takes charge in the elimination of more than 80% of all cellular proteins (Zwickl et al., 1999).

The proteasome is a 2.5-MDa multi-catalytic proteinase complex present in the cytoplasm and nucleus of all eukaryotic cells (Richardson et al., 2003). The primary function of this complex is to degrade proteins which have been tagged with a poly-ubiquitin chain (Adams, 2003; Wolf and Hilt, 2004).

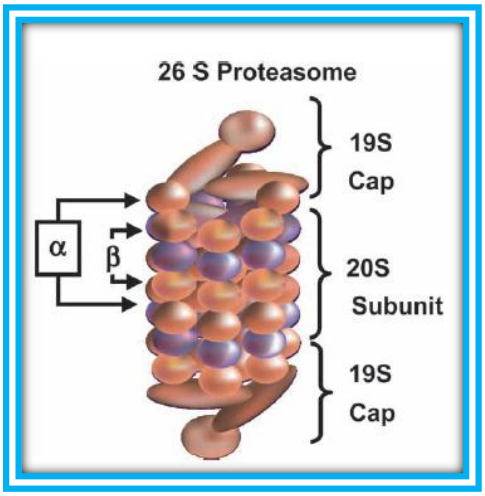


Figure 1.8. Scheme of the 26S Proteasome
(Source: Mateos and Miguel, 2007; Roodman, 2009)

26S proteasome is a cylindrical complex comprised of a 20S catalytic core capped at one or both ends by 19S regulatory subunits (Nussbaum et al., 1998; Richardson et al., 2003; Voorhees et al., 2003; Blade et al., 2005) (Figure 1.8). The 19S regulatory subunits are responsible for binding the poly-ubiquitin chain and cleaving it from the protein substrate. They also contain 6 ATPases that are thought to play a key role to denature the target protein and might deliver the substrate into the proteolytic chamber in the 20S subunit (Zwickl et al., 1999). The 20S catalytic core consists of four stacked rings; two outer α-rings that interact with the 19S subunit to check protein entry into the 20S catalytic core, and two inner β-rings which makes up of seven β subunits.

The β-1, β-2 and β-5 subunits contain the post-glutamyl peptidyl hydrolytic-like, tryptic-like, and chymotryptic-like proteolytic activities of the 26S proteasome, respectively (Groll et al., 1997) (Figure 1.9).

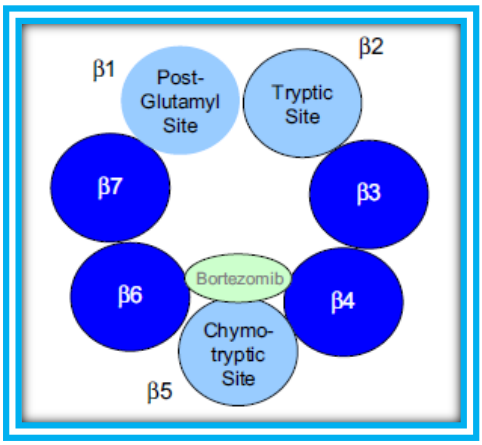


Figure 1.9. Cross Section of β Ring
(Source: Adams and Kauffman, 2004; Landis-Piwowar et al., 2006; Roodman, 2009)

Proteins entering the 20S catalytic core are degraded in a progressive manner, resulting in the release of oligopeptides composing of three to twenty two amino acids in length (Goldberg et al., 1997; Hershko and Ciechanover, 1998; DeMartino and Slaughter, 1999; Delcros et al., 2003).

1.2.7.3. Ubiquitin-Proteasome Pathway (UPP)

The degradations process is chiefly mediated by the ubiquitin–proteasome pathway (UPP). The binding of ubiquitin to the target proteins from its ε-amine of lysine residues involves a series of ATP-dependent enzymatic steps by E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin-ligating) enzymes. This combination forms the poly-ubiquitinated protein which serves as substrate for the destruction of targeted protein by the proteasome (Glickman and Ciechanover, 2002) (Figure 1.10).

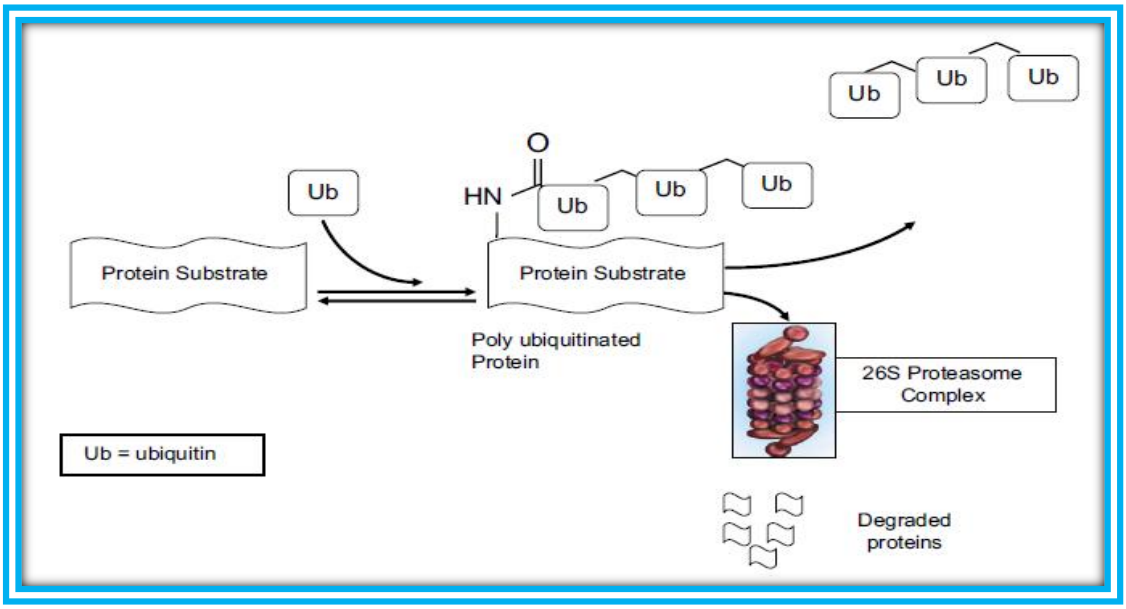


Figure 1.10. Ubiquitin–Proteasome Pathway (UPP)
(Source: Mateos and Miguel, 2007)

1.2.7.4. Proteasome Inhibition by Bortezomib

There is doubtless that inhibition of 26S proteasome affects many vital regulatory pathways. Many significant proteins which are regulated by the proteasome contain cell cycle regulators p21, p27 and cyclin E (the cyclin-dependent kinase inhibitors), pro- and anti-apoptosis factors Bcl-2, Bax, and inhibitory proteins such as inhibitor-kappaB (I-κB). All of these proteins are relevant to cancer initiation and progression. Accumulation of I-κB on proteasome inhibition induces decreased nuclear factor-kappaB (NF-κB) dependent transcription of genes crucial to the promotion of tumorigenesis through the induction of cell proliferation and angiogenesis, suppression of apoptosis, increasement of tumor cell invasiveness and metastasis (Orlowski and Baldwin, 2002; Voorhees et al., 2003; Anderson, 2004).

The UPP takes an essential role in regulation of the NF-κB family of transcription factors. Through the UPP-dependent pathway, NF-κB1 and NF-κB2 are processed into their mature forms. Moreover, the activation of NF-κB is dependent on proteasome activity (Voorhees et al., 2003). NF-κB is normally sequestered in the cytoplasm and rendered inactive by I-κB to hamper its nuclear translocation. However, a variety of stimuli, including cell stressors, prompt the phosphorylation of critical serine residues on I-κB, targeting it for ubiquitination and proteasomal degradation,

which then permits NF-κB to enter the nucleus and mediate transcription. After NF-κB translocates to the nucleus, it initiates the transcription of a wide range of genes that promote cell survival and growth, influences the expression of cell adhesion molecules and cytokine signaling and decreases apoptosis susceptibility. All of these molecules are of great significance in cancer progression (Orlowski and Baldwin, 2002; Voorhees et al., 2003; Richardson et al., 2003) (Figure 1.11).

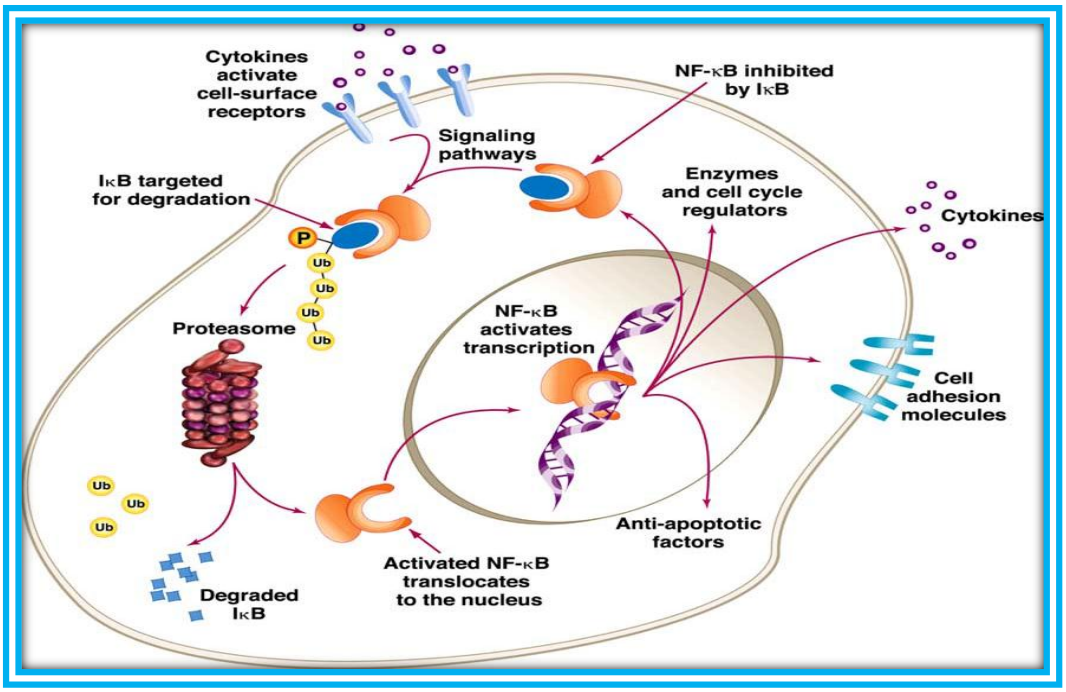


Figure 1.11. The inhibition of NF-κB activation.
(Source: Richardson et al., 2003)

The NF-κB pathway is constitutively active in some cancer cells, especially in MM. If NF-κB is inhibited by I-κB, cancer development is prevented. The role of Bortezomib in NF-κB pathway is to inhibit both NF-κB activation by protecting I-κB from degradation by the 26S proteasome and the adhesion of MM cells to bone marrow stromal cells (BMSCs), bringing about blockade of the adhesion related transcription and then secretion of multiple cytokines (Chauhan et al., 2005) (Figure 1.12).

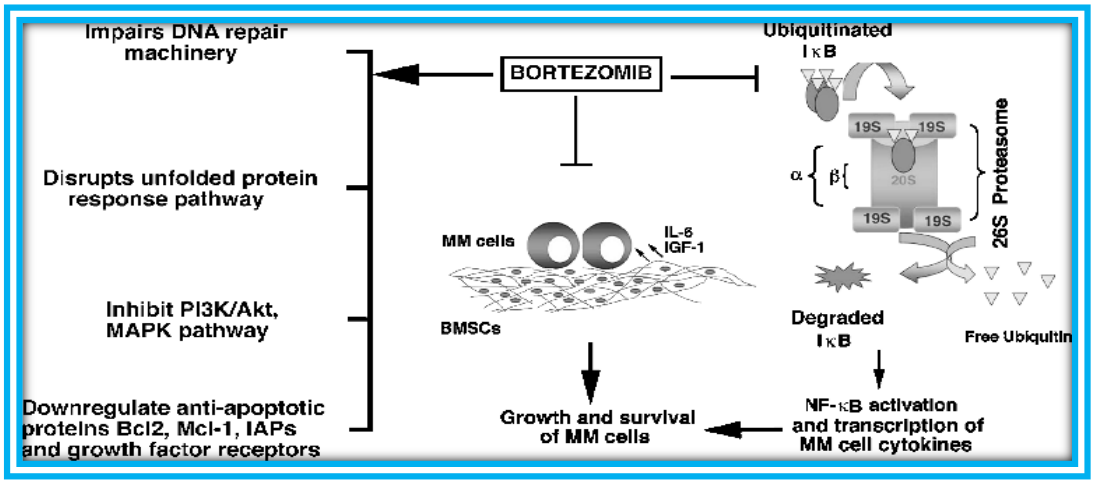


Figure 1.12. Effect of Bortezomib on Various Growth and Survival Pathways in MM (Source: Chauhan et al., 2005)

CHAPTER 2

PROTEOMICS AND MASS SPECTROMETRY

2.1. Proteomics

Proteins are abundant in all organisms and play key roles in most biological events as catalysts, transporters, and messengers. Thus, it is crucial to note that all research related to proteins increase our understanding of their levels, interactions, functions, modifications, regulations, and localization in cells (Graves and Haystead, 2002; Twyman, 2004). Proteomics is a rapidly expanding discipline that aims to gain a comprehensive understanding of proteins. The term proteomics, which is a combination of **prote**in and gen**omics** (Figure 2.1), is used to define the large-scale analysis of a complete set of proteins, the chief components of cells that are responsible for the most significant metabolic pathways in cells or tissues (Wilkins and Pasquali et al., 1996; Wilkins et al., 1996; James, 1997; Pandey and Mann, 2000; Graves and Haystead, 2002; Lane, 2005).

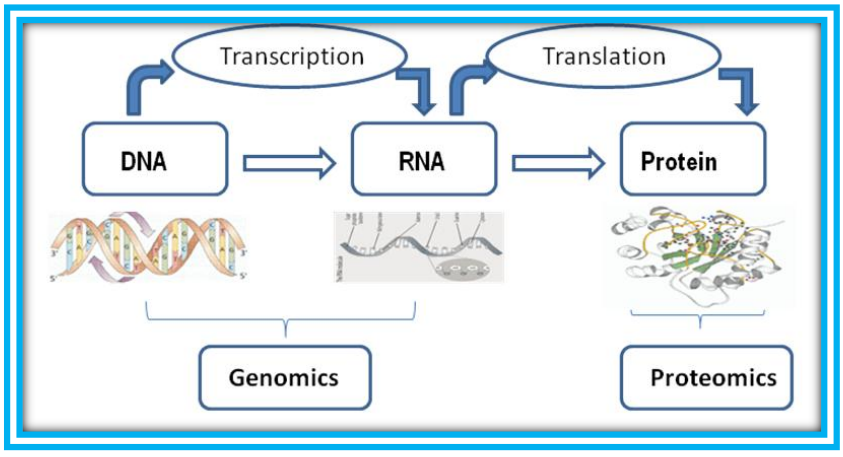


Figure 2.1. Biochemical Interactions Between Genomics and Proteomics (Source: Turan and Mohamed, 2011)

The goal underlying proteomics is not only to identify all proteins in a cell, but also to identify the correlation between the genetic sequence and three-dimensional (3D) protein structure (Graves and Haystead, 2002). In other words, work in proteomics

encompasses the investigation of protein-protein interactions, the connection between the structure of proteins and their function, cellular processes and networks, and to improve protein separation and protein profiling techniques.

The general workflow (Figure 2.2) in a 2D gel-based proteomics experiment and some of the factors affecting the way the experiment is performed are outlined below.

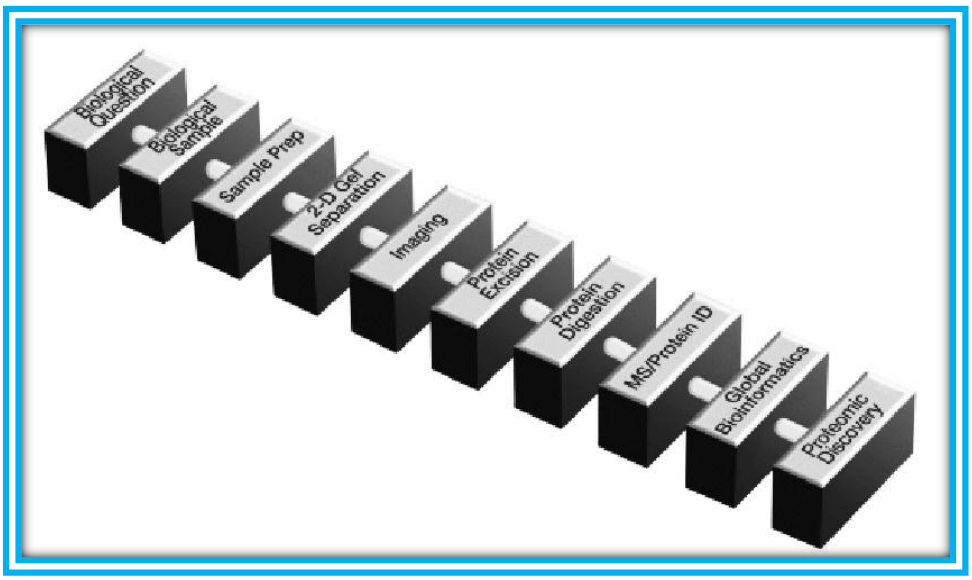


Figure 2.2. Schematic of workflow involved in a proteomics experiment, Bio-rad.

2.1.1. Protein Profiling and its Importance

Protein profiling, an emerging independent subspecialty of proteomics, is poised to provide unprecedented insight into biological events. Quantitative evaluation of protein levels can be accomplished with protein profiling, which shows us unique expression patterns (diseased vs. healthy, treated vs. untreated, experimental vs. control) at the protein level when proteins from one cell type are compared with those of another cell type. The value of protein profiling is increasing daily and there are several reasons why it is of great importance, especially as a potential tool for the early diagnosis of leukemias and other diseases.

One such reason is that it provides a much better understanding of an organism, as it is not always possible or sufficient for scientists to clarify some metabolic pathways, including mechanisms of diseases, exclusively by studying the genome (Graves and Haystead, 2002). Additionally, there are some difficulties associated with

accurately identifying genes solely by dealing with genomic data (Eisenberg, et al., 2000). To overcome this problem, data provided from genomic studies should be supported with data obtained from the study of proteins. Proteomics is often considered as the stage following genomics in the study of biological systems. Compared to genomics, proteomics is much more complicated, as the proteome differs from cell to cell, and under different conditions. This is because distinct genes are expressed in distinct cell types, and to identify even a basic group of proteins produced in a cell, one needs to have a comprehensive understanding of protein-related actions (Graves and Haystead, 2002).

Until recently, such research was carried out using mRNA analysis via different methods, including microarray technology (Schena et al., 1995; Shalon et al., 1996) and serial analysis of gene expression (SAGE) (Velculescu et al., 1995). On the other hand, recent studies demonstrate that mRNA analysis can not be correlated directly with protein levels (Abbott, 1997; Anderson and Seilhamer, 1997; Gygi et al., 1999; Ideker et al., 2001; Dhingraa et al., 2005; Rogers et al., 2008), as mRNA is not always translated into proteins (Lane, 2005). Moreover, the quantity of protein formed for a given quantity of mRNA depends on both the gene that it is transcribed from and the current physiological state of the cell. As such, the level of transcription of a gene provides only a rough estimation of its extent of translation into a protein. Additionally, mRNA produced may go under rapid degradation that causes a reduction in translation, resulting in the production of less protein. In addition, some bodily fluids, such as serum and urine, have no source of mRNA under normal circumstances; therefore, proteomic technologies have emerged as an important addition to genomic studies (Graves and Haystead, 2002).

Proteomics verifies the presence of a protein and provides a direct measure of the quantity present. An additional important reason that protein profiling is crucial is its power to analyze protein modifications. Although a particular cell may have a distinguishable set of proteins at various times or under various conditions, any particular protein may go through a wide range of alterations known as post-translational modifications, which will have critical effects on its function. Phosphorylation is an example of posttranslational modification. Structural proteins can undergo phosphorylation during cell signaling and result in the protein becoming a target for binding to or interacting with a distinct group of proteins that recognize the phosphorylated domain (Hunter, 1995). Ubiquitination is another post translational type

of modification. Ubiquitin is a small protein that can be affixed to certain protein substrates by means of enzymes known as E3 ubiquitin ligases (Glickman and Ciechanovr, 2002). Identifying which protein is poly-ubiquitinated can be helpful in understanding how protein pathways are regulated. In addition to phosphorylation and ubiquitination, proteins can undergo additional modifications via methylation, acetylation, glycosylation, oxidation, sulfation, hydroxylation, nitrosylation, amidation, etc.

These modifications can be assessed only at the protein level and modifications of many proteins expressed by a cell can be determined at the same time using such proteomic methods as phosphoproteomics and glycoproteomics (Graves and Haystead, 2002). In addition to modifications, there is no doubt that protein localization and interactions are of vital importance to their function. Mislocalization of a protein or any problem in signal transduction can turn normal cells into abnormal cells, which is a well-known paradigm in carcinogenesis. Protein profiling using proteomic methods can also be used to characterize these regulatory mechanisms. Another point that emphasizes the importance of protein profiling is that many proteins form complexes with other proteins and/or with nucleic acids, and exert their function in the presence of these molecules.

In summary, protein profiling provides a much better understanding of an organism, in terms of structure and function. Use of protein profiling in the study of multiple proteins, protein forms, and protein families, almost always by comparing two different states (diseased vs. healthy), is expected to expand our understanding of molecular mechanisms.

2.1.2. Biological Sources in Cancer and Proteomics Research

Proteomics technique is basically depends on identifying the protein profile changes by comparing total proteins of two different samples as mentioned earlier. These samples can be obtained from several biological sources to compare the protein contents efficiently. Cancer cell line, human tissue and body fluids are available as a biological source for studying the proteome in cancer (Chen and Yates, 2007).

Cancer cell lines are largely in use in cancer-related proteomics researches, especially in vitro cancer progression studies, which present many advantages. One of

the positive aspects of this sample is that they are both renewable and limitless self-replicating sources which provide large quantities of biological samples for proteomic technique. Another advantage is the predisposition of cancer cell line towards the gene manipulation. An additional benefit of them is their low cost, because they are easily acquired by using the cell culture. Combination of all the benefits resulted in tremendous amount of cell line utilized clinical research. Although there are many advantages of cell lines in the usage of proteomics, of course they are not without drawbacks. The most significant disadvantage is that the possibility of subpopulation formation on account of the phenotypic alterations in cell culture.

Human tissues are another biological source which can be applied to cancer investigations by the method of proteomics. The evaluation of changing protein levels lets broaden our horizon for both disease progression and new therapeutic options. Actually, human tissue have the high physiological/pathological relevance as a positive feature, unfortunately, the study of human tissues have the higher cost as compared to cancer cell line and also they have limited availability.

One of the most studied biological samples in cancer research is body fluids, such as urine, saliva and plasma, since they have good advantages like low cost, easy to obtain from patient, high physiological and pathological relevance. However, characterizing the proteome of that fluid presents significant challenges due to extreme complexity and large dynamic range in protein concentrations compared to cell lines and tissues for the study of proteomics research (Lee et al., 2006).

2.1.3. Exploitation of Protein Profiling in MM

Because malignant plasma cells lose regulation of growth controlling mechanisms, in most cases signaling pathways involving numerous proteins are altered, as mentioned before. When this is taken into account, not surprisingly, expression patterns of growth-inducing and growth-suppressing genes change with malignant transformation (Bártek et al., 1991). Therefore, monitoring these changes is of great importance for understanding carcinogenesis, identifying diagnostic markers, and developing new therapeutics for MM. And also it is very significant for the usage of Bortezomib in other cancer type therapies. The most widely used methods for this involve examination of differential gene expression by assessing the mRNA levels in

the given cells. On the other hand, this methodology comes from a reductionist point of view, as it neglects the dynamic nature of protein translation and further modifications that take place beyond transcription. As such, an approach involving direct proteomic methods might be a better choice for obtaining more accurate insight into what is happening at the cellular level in MM.

It is clear that proteomic techniques and protein profiling are especially valuable for obtaining a deeper understanding of malignant transformation, improved diagnostics, and more accurate prognostic predictions, and for the development of effective therapeutic options. Genomics has provided a considerable body of useful information on the alterations in cancer cells by identifying the genes responsible for tumor suppression and growth, but as long as the complex interactions of proteins and the dynamic nature of protein synthesis are overlooked, genomics will be unable to establish a complete understanding of MM. Because the information stored in genes is reflected in the phenotype by proteins, assessment of proteins in the cell at a given time would provide accurate and detailed insight into the specifics of the myeloma cells.

2.1.4. Techniques for Determining Changes in Protein Profiles

Proteins are important targets for drug discovery and are utilized in cancer research because there are defects in the protein machinery of cells undergoing malignant transformation. Identification of protein profiles is clinically promising in the development of potential new drugs or treatment options to eradicate various malignancies, including, but not limited to, those of hematologic origin. Changes in protein profiles provide a wide variety of critical data. By examining these alterations, proteins that have a profound impact on the progression of diseases can be identified, making the development of individually tailored drugs possible (Twyman, 2004). Because of its importance, recent technologic advances have opened a new era for analyzing changes in protein profiles. To date, many of the high-throughput protein identification and characterization methods developed for proteomics have been applied to protein profiling. Among them, the most widely and efficiently used ones are Two Dimensional Poly Acrylamide Gel Electrophoresis (2D PAGE) and Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI TOF) Mass spectrometry.

2.1.4.1. Gel Electrophoresis

Electrophoresis is a bio-analytical method utilized for the isolation of biomolecules depending on the mobility of charged molecules under the influence of an electric field. When an electric field is applied, any charged molecule in solution will migrate. (Twyman, 2004). Mobility of a molecule is based upon the magnitude of its charge, molecular weight, and structure. Many biopolymers, such as proteins are charged either by coating with the anionic detergent sodium dodecylsulfate (SDS) or by acid-base association-dissociation reactions of amine and carboxylic acid parts of them, so they can also be separated by electrophoretic methods (Mikkelsen and Corton 2004).

Electrophoresis, which is the central component of the proteomic research, is commonly carried out in a gel that is formed by the polymerization of acrylamide, so it is called polyacrylamide gel electrophoresis. The reaction mechanism that results in a gel with a characteristic porosity which depends on the polymerization conditions and monomer concentrations can be seen in Figure 2.3 (Kinter and Sherman 2000).

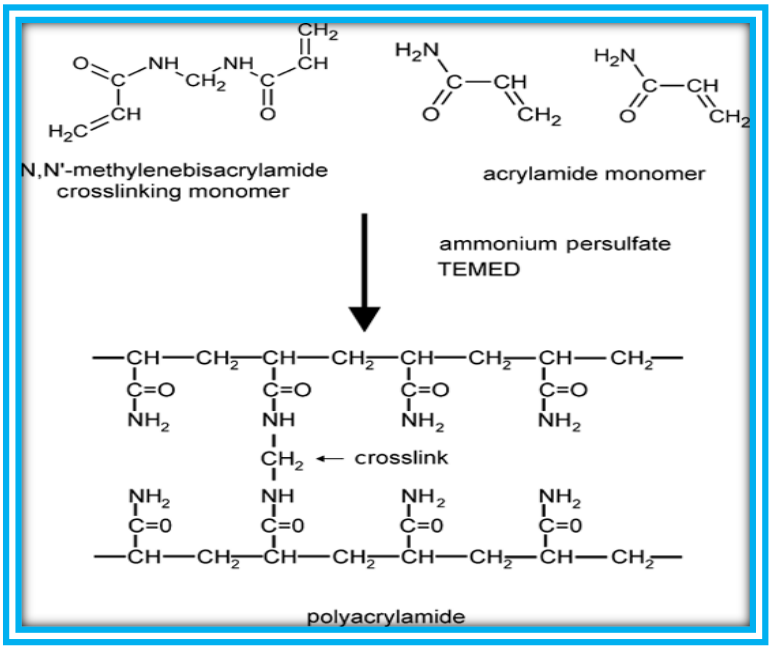


Figure 2.3. Reaction of polyacrylamide gel formation
(Source: Gallagher, 2008)

In the presence of an initiator generally ammonium persulfate and a catalyst commonly tetramethylethylenediamine [TEMED, $(CH_3)_2N(CH_2)_2N(CH_3)_2$], polyacrylamide gels are prepared by the copolymerization of acrylamide monomer with

bis-acrylamide (N,N'-methylene-bisacrylamide). Polymerization is initiated by ammonium persulfate and TEMED.

The degree of cross-linking is very important in the formation of polyacrylamide gel. Because, the lower degrees of cross-linking gel allows to pass larger protein transition.

2.1.4.1.1. One-Dimensional Gel Electrophoresis (1D PAGE)

One-dimensional gel electrophoresis (1D-GE or SDS-PAGE) is the most commonly used protein seperation technique in proteomic studies. This method basically depends on separating proteins according to their molecular weight (size) difference. Most commonly, the strong anionic detergent SDS is used in combination with a reducing agent (mercaptoethanol or DTT) to surround the proteins homogeneously to dissociate them. The denatured polypeptides (simple rod-like shape of the proteins) gain a negative charge in constant proportion to molecular weight by binding SDS. By this way, the newly formed complex starts to migrate through the pores of the polyacrylamide gel towards the positive electrode, when the gel expose to high voltage. As compared to larger ones, smaller proteins migrate further through the gel, depending on their molecular weight (Liebler, 2002).

2.1.4.1.2. Two-Dimensional Gel Electrophoresis (2D PAGE)

SDS-PAGE has been utilized for several decades for the protein separation. The method is delicate to separate only 80-100 different protein components where cell proteomes are intensely complex holding several thousand of proteins. So, it is not sufficient for some comprehensive protein studies. Two-dimensional gel electrophoresis (2D-GE or 2D PAGE) firstly introduced by O'Farrell in 1975 (O'Farrell 1975). 2D PAGE can separate thousands of proteins simultaneously. Additionally, it can be also used to determine the protein alterations given a stress condition. Because of these reasons, it is is still the most accepted protein separation method in proteomics studies. Every single spot formed in 2D PAGE analysis demonstrates an individual protein species and its specific coordinates. The intensity of a spot in the gel implies the amount of that actual protein produced (Bendixen 2005).

2D-GE separates protein mixture in accordance with two distinct properties. In the first dimension, proteins are separated depending on their isoelectric point (pI, is known the pH at which a protein has no net charge) disparity, whereas in the second dimension, the system uses the molecular weight difference. It means that proteins are resolved according to their molecular weight. Isoelectric focusing (IEF) is coupling with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation, generally.

Recently, the importance of 2D-GE system is rapidly increasing, as it not only can be combine with the most biological studies but also has a capacity to separate up to 10000 protein spots on one gel. Furthermore, it can monitorize more than 5000 proteins simultaneously, having nearly 2000 proteins routinely, hinging upon the pore size in acrylamide gels and pH gradient utilized. As compared to 1D-GE, it is more sensitive thanks to the properties of detection and quantification nearly 1 ng amount of protein per spot.

By loading a range of protein markers whose molecular masses are already known in one of the line of the polyacrylamide gel, the molecular weights of the proteins in the sample can be estimated.

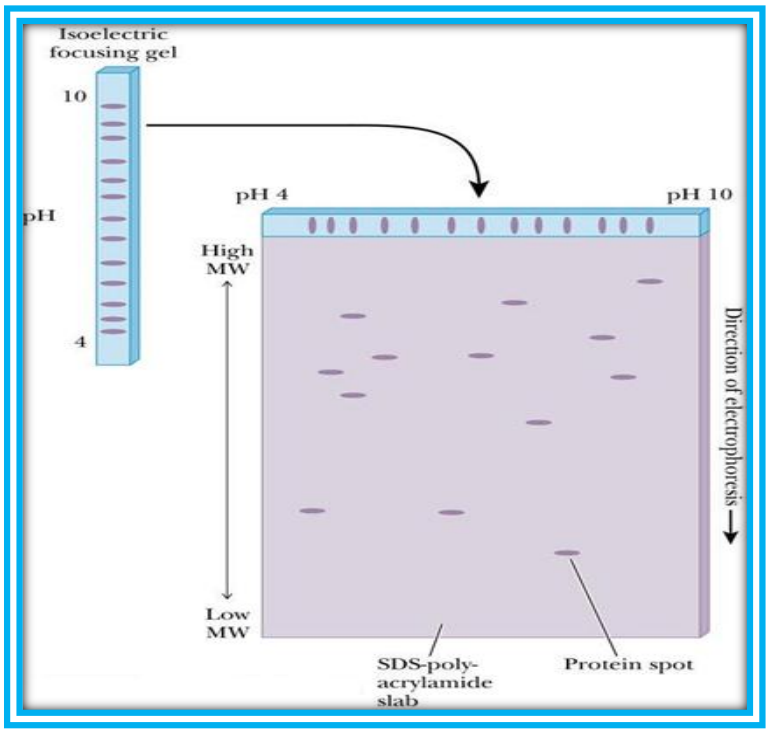


Figure 2.4. Schematic Representation of 2D PAGE
(Source: Nelson and Cox 2008)

2.1.4.2. Detection of Protein Spots and Image Analysis

The last procedure of 2D-PAGE is the visualization of protein spots on gel by staining methods. Detection of protein spots can be commonly achieved by six techniques. These are Coomassie Brillant Blue (CBB) Staining, silver staining, negative staining with metal cations (e.g. zinc imidazole), staining or labeling with organic or fluorescent dyes, detection by radioactive isotopes, and by immunological detection, respectively. Among these methods, CBB staining, silver staining and fluorescence staining are the most preferred detection methods for proteomic researches.

There are some notable characteristics to select the most suitable staining technique for ideal protein detection on 2D PAGE. First of all, it should be sensitive (low detection limit), well-matched with mass spectrometry and reproducible. And also it should possess linear and wide dynamic range. However, there is no method that have all of these properties exactly.

Each type of protein stain has its own characteristics and limitations with regard to the sensitivity of detection and the types of proteins that stain best (Table 2.1).

Table 2.1. Characteristics of protein stains

Gel Stain		Sensitivitiy	Process Time and Steps	Advantages
SYPRO Ruby Protein Gel Stain		1 ng	3 hr / 2 steps	✓ Mass spectrometry compatible, ✓ High detection sensitivity, ✓ High dynamic range, ✓ Reproducility, ✓ Allows protein analysis in fluorescent imagers.
Literature		(Berggren et al., 2000; Nishihara and Champion, 2002; Lilley and Friedman, 2004).		
Coomassie Brillant Blue	**G-250**	10 ng	2.5 hr / 3 steps	✓ Mass spectrometry compatible, ✓ Easily visualized, ✓ Nonhazardous ✓ Low cost
	R-250	40 ng	2.5hr / 2 steps	
	R-350	40 ng	5 hr / 3 steps	
Literature		(Neuhoff et al., 1988; Berggren et al., 2000; Patton, 2002; Mackintosh et al., 2003; Candiano et al., 2004).		
Silver Stain		1 ng	1.5hr / 3 steps	✓ High detection sensitivity, ✓ Low background
Literature		(Heukeshoven and Dernick, 1985; Merril et al., 1986).		

The sensitivity that is achievable in staining is designated by:

- ✓ The amount of stain that binds to the proteins,
- ✓ The intensity of the coloration,
- ✓ The difference in coloration between stained proteins and the residual background in the body of the gel (the signal-to-noise ratio).

Unbound stain molecules can be washed out of the gels without removing much stain from the proteins.

Subsequent to the staining procedure, in order to analyze the spot detection, intensity, size, and shape, background correction, gel matching, normalization, quantification, etc. the gel images necessitate to be turned into digital data by using a charge-coupled device (CCD) camera or a scanner. Following, the image obtained from the CCD camera is analyzed by computer based software, such as DECODON Delta2D, PD Quest or Image J.

Finally, the spots are cut out from gel and digested (in-gel digestion) into peptide fragments with trypsin enzyme to attain the determination of protein spots (newly formed, lost or up- or down-regulated) in polyacrylamide gel. Next, these spots are identified using mass spectrometry and database searches.

2.1.4.3. In-Gel Digestion

In-gel digestion is the most widely used step of sample preparation for the mass spectrometric analysis of proteins. Its significance is coming from the protein databases, because they are generated depending on the peptide masses that cause peptides preferable against intact proteins. In addition to this, mass spectrometry can produce measurement errors with the increasing length of the peptide chain.

The method is introduced by Rosenfeld in 1992. It can be applicable both one and two dimensional polyacrylamide gels (Rosenfeld et al., 1992).

In most of the proteomic studies, trypsin is a universal choice owing to its exceptive properties as a protease for sequencing experiments with tandem mass spectrometry (MS/MS). It cleaves amide bonds in proteins at the C-terminal side of lysine and arginine residues. It forms small peptides which are generally in the mass

range of 600-2500 Da. This is the ideal protein digestion for mass spectrometry. Actually, peptides having 6-20 amino acids are optimum for MS analysis and database searches. There are also different proteases exist and used for in-gel digestion process such as chymotrypsin, pepsin etc. The only difference is their cleavage characteristics.

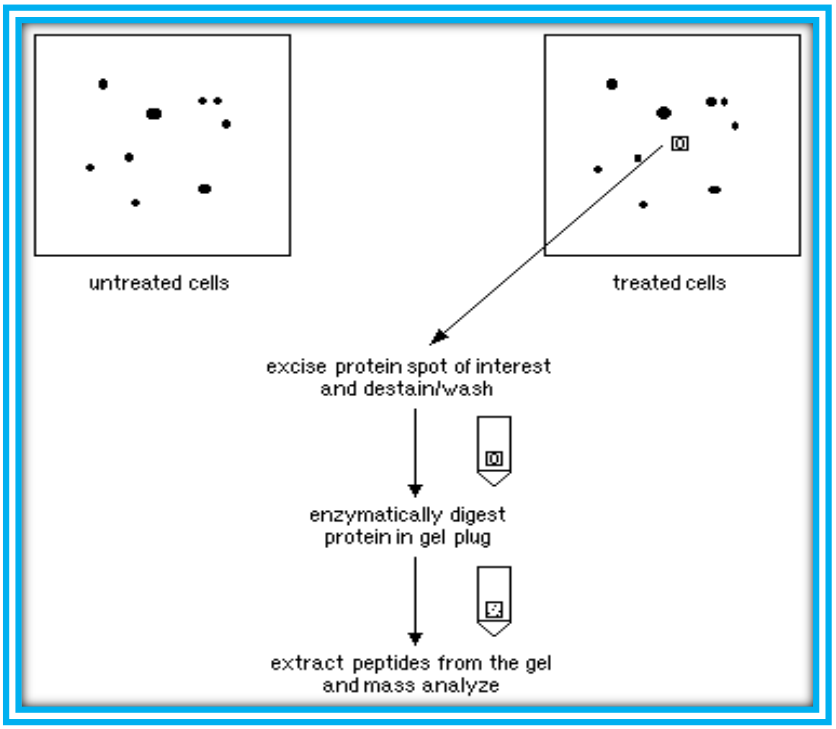


Figure 2.5. Schematic Representation of In-Gel Digestion
(Source: Jiménez et al., 2001)

2.1.4.4. ZipTip (C-18 Colon)

Following the in-gel digestion process, peptides are required to purify to eliminate gel contaminants such as salts, buffers, and detergents. To obtain good spectra, it is essential to keep the salt concentrations in buffers to a minimum. If the gel contaminants do not eliminate from peptides, they can interfere with mass spectrometry either inducing the poor quality mass signal or masking the peptide signals (Yates, 1998). Thus, the obtained peptide mixtures should be purified with ZipTips (Millipore).

2.1.4.5. Mass Spectrometry

Mass Spectrometry (MS) is an analytic technique for the determination of the elemental composition of a molecule. Its principle basically depends on separating molecular ions according to their mass-to-charge ratio (*m/z*). The main purposes of MS studies are molecular mass determination, structure characterization, quantification at trace levels, and mixture analysis.

In 1897, cathode ray tube experiments carried out by Sir J.J. Thomson cause the rising a new field named MS (Thomson, 1897), but it came into prominence with the exploration of soft ionization techniques. Electrospray ionization (ESI) was introduced by Fenn and his co-workers (Fenn et al., 1989) and matrix-assisted laser desorption/ionization (MALDI) was put into the service of science by Karas and Hillenkamp (Karas and Hillenkamp, 1988). MS currently plays a key role in the identification of proteins. High sensitivity (low attomole levels), rapidity, versatility, and the accuracy of the method in the field of biological science enable MS the most attractive properties of MS most comprehensive and used analytical technique.

Mass spectrometer is fundamentally composed of three essential parts, namely the ionization source, the mass analyzer, and the detector (Figure 2.6).

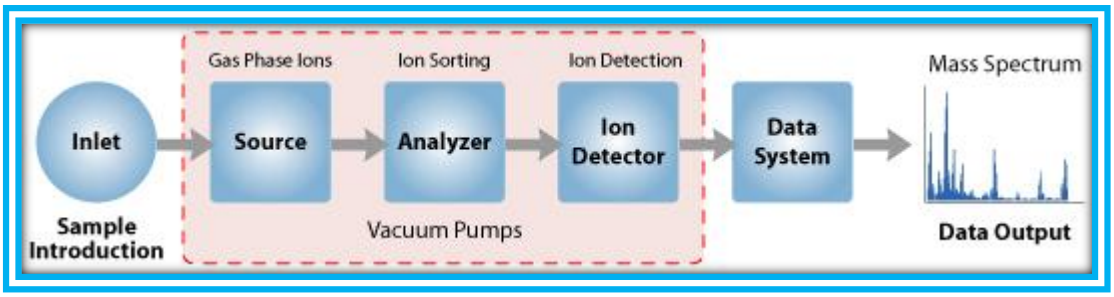


Figure 2.6. Basic Components of a Mass Spectrometer, Premier Biosoft.

The ionization source is the first component of the MS. In MS analysis, the first vital step is to turn the analyte which is neutral into gas phase ionic species to be able to check their motion. If the energies higher than ionization energies, fragmentation happens. Next, ions are accelerated towards a mass analyzer, which is the second component of the MS, through a voltage difference. Then, the charged fragments of the molecular ions are separated according to their m/z ratio. The last component of MS is the detector (photomultiplier, micro-channel plate, electron multiplier) which detects the

ions resolved by the mass analyzer and it transforms the ion beam into a signal. Each of these three parts of mass spectrometer is under vacuum-pump systems. This high vacuum (10^{-4} – 10^{-8} torr) is required to allow the ions move freely in space without colliding or interacting with other species.

2.1.4.5.1. Ionization Methods

Sample ionization is of great significance in mass spectrometric analysis as the compound is required to be charged and ionized. The conversion of neutral molecules into gas phase ionic species is achieved by ionization sources, so that electric and magnetic fields can be used to exert forces on charged particles in a vacuum-pump system for mass analysis. The most common techniques for ionization are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) which are soft ionization techniques.

The result of ionization, ion separation and detection is a mass spectrum that can provide structural information.

2.1.4.5.1.1. Matrix-Assisted Laser Desorption/Ionization (MALDI)

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) was first introduced by Karas and Hillenkamp in 1988 (Karas and Hillenkamp 1988) as mentioned above. MALDI is a method that allows for the ionization and transfer of a sample from a condensed phase to the gas phase. This technique has gained a wide acceptance for analysis of biological macromolecules with masses up to 500 kDa.

In this approach, the sample molecules have to be co-crystallised with a high molar excess of an appropriate matrix, which is a low-molecular weight energy-absorbing organic acid, in a ratio 1:1000, respectively. The amount of matrix must be much more than analyet to both absorb all ot the radiation energy and prevent the analyte from fragmentation.

The functions of the matrix are:

- ✓ Absorb and accumulate the energy of the laser radiation,
- ✓ Protect the analytes from destruction and fragmentation.

A good matrix material should be :

- ✓ Adsorb strongly at the laser wavelength,
- ✓ Chemically inert and stable in vacuum,
- ✓ Be able to embed the analyte,
- ✓ Pure et least 99%,
- ✓ Promote co-desorption of the analyte upon laser irradiation as well as ionisation of the analyte by donating protons.

3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (Beavis and Chait, 1989) and α-cyano-4-hydroxycinnamic acid (CHCA) (Beavis et al., 1992) are the most preferred and popular matrices in proteomic researches.

In MALDI, process begins with the sample preparation. For the preparation, matrix and our sample (analyte) mix in an eppendorf tube, and then this prepared mixture spots to the MALDI Target and allows to dry. After it is dry, the target is transferred in the vacuum chamber of the mass spectrometer. UV-Laser pulses are directed at the sample plate, which carries the co-crystallized peptides and matrix mixture, with a high energy (~10^6 W/cm^2). A large proportion of the laser beam is efficiently absorbed by the matrix crystals inducing evaporation of the matrix, and this energy is transferred to the analyte as heat in a controlled manner (it means that there is no fragmentation). That is the most important stage in MALDI. If there is no problem, analyte molecules are converted into gas-phase ions by gas phase proton-transfer reactions. Finally they are directed into the mass analyzer by appropriate electric fields (Figure 2.7).

MALDI permits the analysis of high molecular weight compounds with high sensitivity. And also it has an ease of use while tolerating small amounts of contaminants like salts and surfactants.

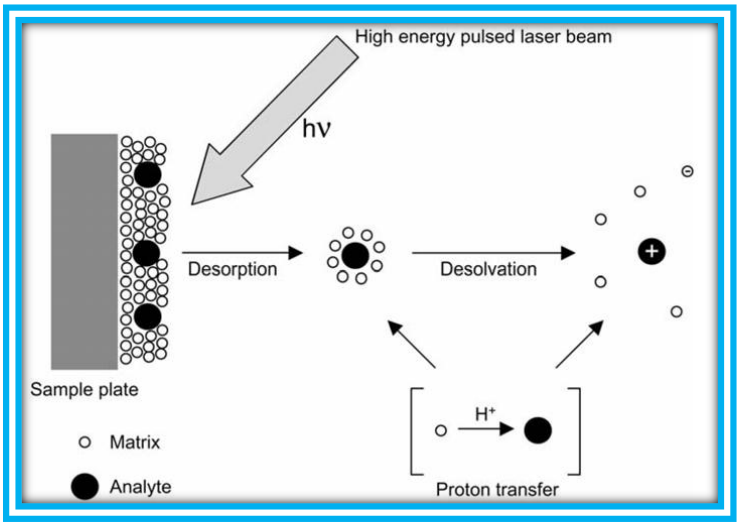


Figure 2.7. Illustration of the principles behind MALDI
(Source: Barker, 1999; Hoffmann and Stroobant, 2003)

2.1.4.5.2. Mass Analyzers

After the ions are generated by ionization sources, a mass analyzer is required to separate them depending on their *m/z* ratios. It is the most remarkable advantage of the mass analyzer to controll and manipulate the ion motion by applying suitable electric and magnetic fields. Hence, the number of ions at each individual *m/z* value can be detected.

The the resolution, the transmission and the upper mass limit are three main crucial properties of an mass analyzer. The resolution identifies the capability of the mass analyzer to separate ions of similar *m/z*. The transmission is correspond to the ratio between the number of ions reaching the detector and the number of ions generated in the sources. The upper mass limit determines the measured highest value of the *m/z* ratio. Besides, mass accuracy, detection sensitivity, and scan speed also affect the performance of the mass analyzer.

Several types of mass analyzers can be used in proteomics study. Time-of-flight (TOF), quadrupole linear, quadrupole ion trap, Fourier Transform Ion Cyclotron Resonance (FT-ICR) analyzers are the most known analyzers useful for biomolecules. All of these analyzers are quite different from each other in terms of both experimental design and performance parameters like resolution, transmission and the upper mass limit.

2.1.4.5.2.1. Time-of-Flight Mass Analyzer (TOF)

A time-of-flight (TOF) mass analyzer was firstly introduced by Stephens in 1946 (Stephens, 1946). It is one of the simplest instrument in terms of its working principle.

Chiefly, TOF analysis is based on accelerating a set of ions to a dedector with the same amount of kinetic energy. All the ions are exposed to high voltage to create an electric field that allows the ions to be accelerated. After acceleration, the ions go to a field-free region where they fly at a velocity that is inversely proportional to their *m/z*. It means that, the smaller ions reach the dedector first because of their greater velocity and the larger ions take longer. Because the ions have the same energy, yet a different mass, the ions reach the dedector at different times. Thus the analyzer is called time-of-flight because the m/z is determine from the ions time of arrival. Finally, required time is measured for the travel of the ion through the length of the field-free region.

The resolution is a very important parameter in proteomics studies. In a TOF mass analyzer, both the length of the flight tube and the accelerating the voltage affect the resolution. So, in order to improve the sensitivity and to increase the resolution, both acceleration voltages can be lowered and reflectron modes which increase the length of the flight tube can be placed at the end of the drift zone (Figure 2.8). In summary, using a long flight tube is the only way to have both high resolution and high sensitivity.

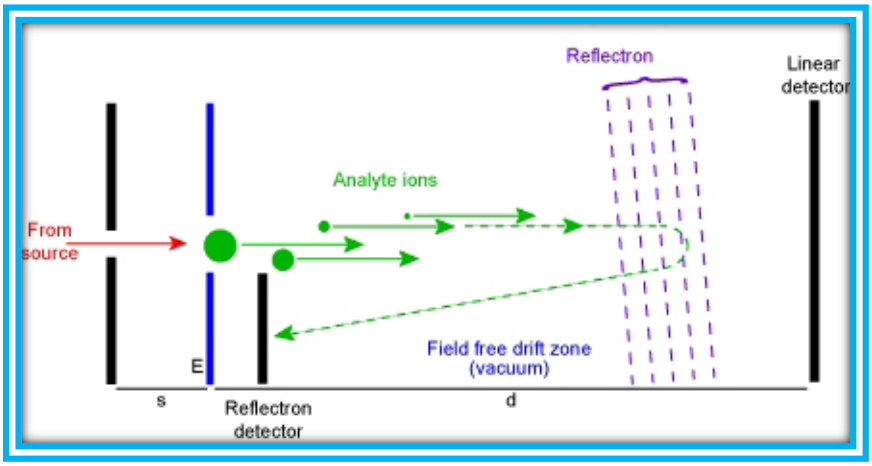


Figure 2.8. Schematic Representation of Reflectron Time-of-Flight Mass Analyzer (Source: The University of Bristol, 2007)

There is no doubt that different mass analyzers can connect with different ionization sources. However, the harmony between mass analyzers and ionization is of great value. Depending on this reality, TOF analyzer is well suited to the MALDI (MALDI-TOF) ionization process, as the laser beam takes individual shots instead of working in continuous procedure. Figure 2.9 shows a MALDI-TOF Mass Spectrometer. Similarly, quadrupole and ion trap can coupled to ESI (Figure 2.10) (Micallef et al., 2010).

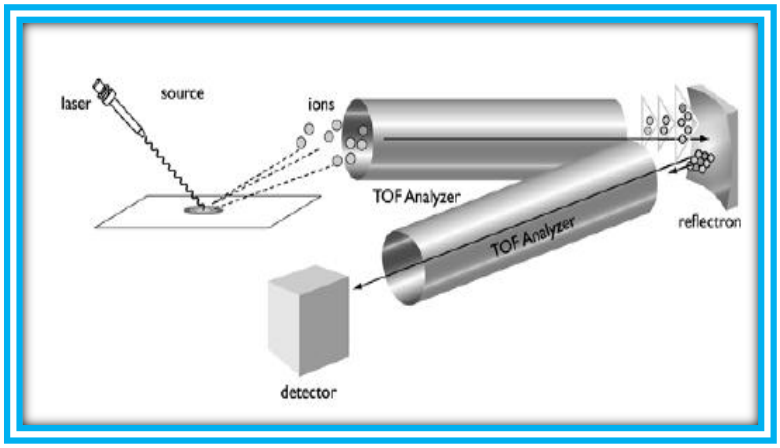


Figure 2.9. A MALDI-TOF Mass Spectrometer
(Source: Liebler 2002)

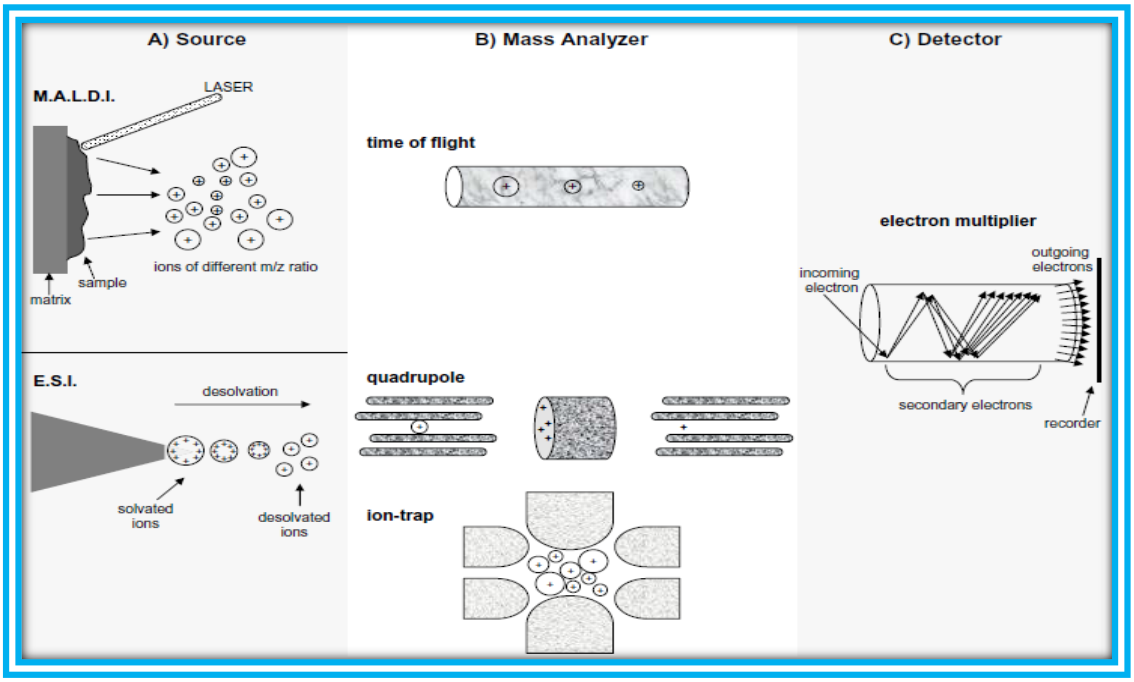


Figure 2.10. A Mass Spectrometer (Representation of the most suitable source-analyzer-dedector combination) (Source: Micallef et al., 2010)

2.1.4.5.3. Detectors

Dedector is the final component of a mass spectrometer. After the ions pass through the analyzer, they arrive and hit the surface of the dedector and then it determines ion abundance for each corresponding ion resolved by the mass analyzer depending on their m/z value and generates a mass spectrum (Micallef et al., 2010).

Detectors can be categorized in two class. First category of detectors are responsible for measuring of the charges that reach the detector directly. Second category of detectors play a central role to increase the intensity of signals. While photographic plate and the Faraday cage are comprised in the first category, photomultiplier, electron multiplier and microchannel detectors are included in the second category.

2.1.4.6. Tandem Mass Spectrometry (MS/MS)

The basic principle of tandem mass spectrometry (MS/MS) is to isolate the precursor ion in the first spectrometer and then analyze the product ions in the second spectrometer. The process begins with selecting an ion. Following, this ion is fragmented through collision (generally with Ar) and the fragments are analyzed by the other spectrometer.

2.1.5. Advantages and Drawbacks of Protein Profiling

Although analysis of important proteins in biological systems is important, there are several drawbacks of protein profiling due to techniques like mass spectrometry in combination with separation tools such as 2D PAGE (Washburn et al., 2001; Wolters et al., 2001; Aebersold and Mann, 2003). First of all, these techniques are labor intensive and time consuming for the analysis of proteins. Improved robotics may increase the frequency with which these techniques are utilized, as well as their efficiency. Secondly, 2D PAGE lacks the sensitivity to detect low quantities of proteins and therefore requires a significant quantity of biological material (Sydor and Nock, 2003; Issaq and Veenstra, 2008). Additionally, most of the time high-quantity proteins can

mask low-quantity proteins that may be important biomarkers for the disease studied. To overcome this type of drawback immunodepletion and multidimensional chromatography may be a reasonable solution (Cho et al., 2005). Through sample purification, low-quantity proteins may be lost due to interactions with other high-quantity proteins; therefore, all steps of purification must be analyzed. Sample collection, processing, and, ultimately, sample measurement are also very crucial for the utilization of protein profiling in many clinical settings. Consistency, use of strict protocols, running replicates for each sample, and increasing the sample count can be a general solution to eliminating such drawbacks. Other obstacles to the use of techniques in protein profiling can be ascribed to poor resolution of spots after 2D PAGE, non-identification of mass spectrometry peaks, and the limitations of databanks for unknown proteins. The advantages and disadvantages of the techniques used for protein profiling are summarized in Table 2.2.

Table 2.2. Advantages and disadvantages of protein profiling techniques (Source: Twyman, 2004; Mohamed et al., 2011)

Techniques	Advantages	Disadvantages
Usage of Cell Lines and Protein Extraction from Cells	✶ Renewable, ✶ Limitless self-replicating, ✶ Predisposition towards gene manipulation, ✶ Low cost.	✶ Subpopulation formation, ✶ Sample contamination, ✶ Protein degradation, ✶ Extraction of membrane proteins.
Two Dimensional Gel Electrophoresis (2D PAGE)	✶ High resolution, ✶ Quantitative, ✶ Qualitative, ✶ Sample variety (cell lines and body fluids), ✶ Many protein analyses in a single run.	✶ Slow, ✶ Lacks automation, ✶ Poor reproducibility, ✶ Labor intensive, ✶ Tedious procedure, ✶ Requires a significant quantity of sample, ✶ Lacks sensitivity for low-quantity proteins.
MALDI-TOF Mass Spectrometry	✶ Fast, ✶ Buffer / salt tolerance, ✶ Off-line, ✶ Mass accuracy, ✶ Mixture of samples okay, ✶ Small and large polypeptide analysis.	✶ Limited databank information, ✶ Limited resolution of peaks, ✶ Low dynamic range of detection.

2.1.6. Aim of the Study

Investigating protein profiles and understanding the dynamic alterations of cellular proteins are of great importance for diagnostic and therapeutic purposes in clinical settings. This is because most diseases, cancers in particular, are reflected as driven by protein alterations in cells. As such, proteomic methods enhance our understanding of the characteristics of various cancers via, making it possible to discriminate healthy and malignant cells more accurately, development of novel therapeutics that target altered protein signaling pathways, and assessment of what changes occur at the protein level in cells in response to treatment with drugs, leading to the evaluation of therapeutic efficacy.

In the light of this valuable knowledge, the objective of this project was mainly to both determine the cytotoxic and apoptotic effects of Bortezomib on Multiple Myeloma U-266 cells and find out the changes in protein profiles of MM U-266 cells when they exposed to Bortezomib, by proteomics studies.

The cytotoxic effects were determined by measuring the IC-50 value using XTT cell proliferation assay and apoptotic effects were assessed measuring the changes in caspase-3 enzyme activity and loss of mitochondrial membrane potential (MMP). After assigning the MM U-266 cells as a control while, Bortezomib applied MM U-266 cells as a Bortezomib, protein isolation was carried out from these two compartments separately. Following to the extraction of total protein content, a comparison of protein profiles in these two different states was carried out using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Differentially expressed protein spots (increased and/or decreased or appear and/or disappear) were in-gel digested and identified by MALDI-TOF-TOF Mass Spectrometry, and database searches were performed (Mascot search engine and NCBInr protein database) in order to discover the differences in details between Bortezomib applied and control group MM U-266 cells at the level of proteins. The functions of Bortezomib related proteins were also discussed.

Proteomic study provides an excellent opportunity to find the differentiated proteins depending on Bortezomib effect. For future perspectives, these differentiated proteins can let to be possible to treat other cancer types by same anti-cancer agent. Additionally, the data obtained by this study can be helpful for medical schools and drug designers and may also provide new treatments.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Medias

Medias were listed in Appendix A.

3.1.2. Chemicals, Reagents and Solutions

Reagents and solutions were presented in Appendix B.

3.2. Methods

3.2.1. Cell Lines and Culture Conditions

U-266 human MM cells were kindly obtained from the German Collection of Microorganisms and Cell Cultures (Germany). The cells were grown and maintained in RPMI-1640 growth medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen) at 37°C in 5% CO_2. Medium was refreshed every 3 days. In order to passage the cells, whole cell suspension was taken from tissue culture flask (75cm^2) into a sterile falcon tube (50ml) and then centrifuged at 1000 rpm (~800g) for 10 minutes (min) at room temperature. After centrifugation, the supernatant was removed and the pellet was washed with 3-4 mL Phosphate Buffered Saline (PBS) and centrifugation process was repeated. In the following step, supernatant was removed from the tube and the pellet was resuspended with 15 mL of RPMI-1640 (10% FBS and 1% Penicillin-Streptomycin). Then, it was transferred into a sterile 75cm^2 filtered tissue culture flask and incubated in CO_2 incubator.

3.2.2. Thawing the Frozen Cells

Cells (1ml) were removed from frozen storage at -196°C (Liquid Nitrogen) and quickly thawed in a water bath at 37°C so as to acquire the highest percentage of viable cells. When the ice crystals melted, the content was immediately transferred into a sterile filtered tissue culture flask ($25cm^2$) containing 5-6 ml of RPMI-1640 growth medium and incubated overnight at 37°C in 5% CO_2. After incubation, cells were passaged as mentioned before.

3.2.3. Freeze the Cells

Cells taken from tissue culture flask were centrifuged at 1000 rpm for 10 minutes at room temperature. After centrifugation, the supernatant was carefully removed and the pellet was resuspended by the addition of 4.5ml FBS and 0.5ml dimethyl sulfoxide (DMSO). Then, gentle pipetting was applied and the cell suspension was transferred to the cryogenic vials (1 ml) by labeling. At the following step, these cryogenic vials were lifted to freezing compartment -80°C, for overnight by wrapping in cotton wool in a polystyrene box to prevent the cells from shock. Finally, they were transferred to liquid nitrogen dewar for long-term storage.

3.2.4. Cell Viability Assay

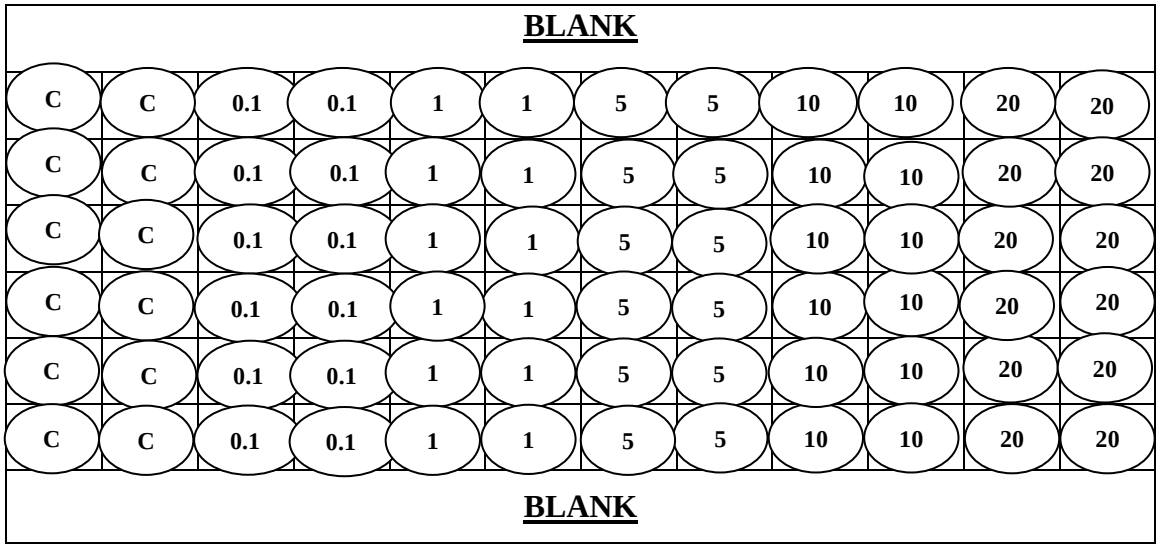
The assay primarily based on distinguishing dead cells from alive cells with the addition of trypan blue dye. ~30 µl of trypan blue dye was mixed ~30 µl of cells (1:1 ratio, volume/volume) in order to measure the viability of cells. When cells were treated with trypan blue dye, viable cells would be normally impermeable to it, whereas dead cells would permeate by virtue of breakdown in membrane integrity. Thus, cells can be observed as unstained cells that are alive or blue stained cells that are dead under a microscope. By applying this assay, cells were counted using a hemocytometer in the presence of trypan blue solution, under microscope. Then the percentage of viable cells was calculated. Cell viability assay was conducted before each experiment.

3.2.5. Cell Proliferation Assay

Anti-proliferative effects of Bortezomib on U-266 MM cells were determined by XTT cell proliferation assay. The principle of this assay is based on the ability of metabolic active cells to reduce the tetrazolium salt XTT to orange colored compounds of formazan by their mitochondrial enzyme. These formazan molecules formed is water soluble and can be measurable at the wavelength of 492 nm with a spectrophotometer.

Briefly, the test procedure includes cultivation of cells in a 96-well plate, adding the XTT reagent (The procedure for preparation of XTT Reaction Solution that was used in XTT Assay was given in Appendix B) and incubation for 72 hours. During incubation an orange color is formed. The greater the number of the active cells in the well, the greater the activity of mitochondria enzymes and the higher the concentration of the formazan compounds (dye) formed, which can then be measured and quantitated with a spectrophotometer.

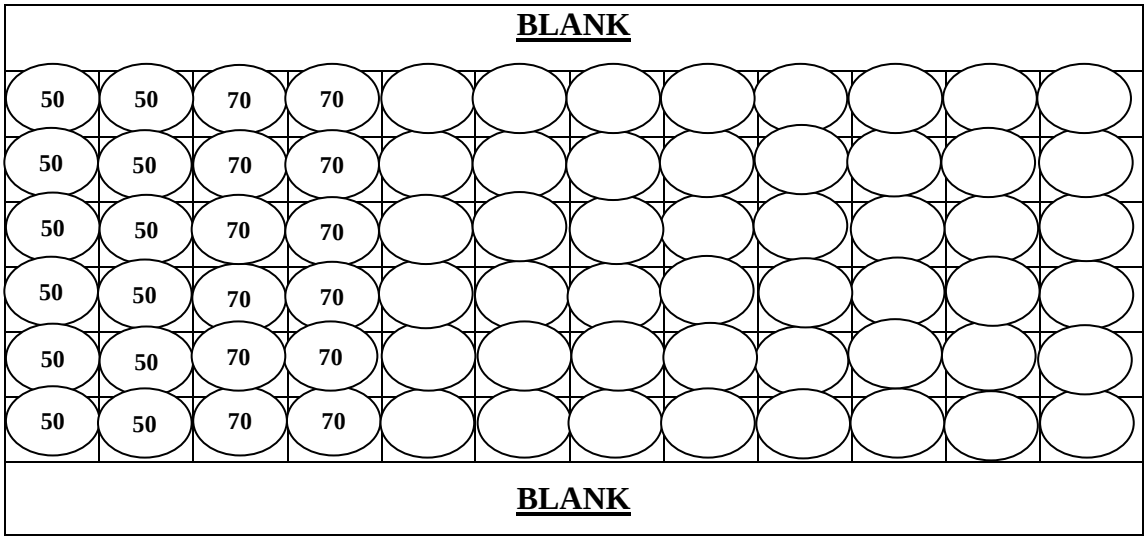
$2x10^4$ U-266 cells (100 µl) were seeded into 96-well plates containing 200 µl growth medium in the absence or presence of increasing concentrations of Bortezomib (Figure 3.1)



96 Well Plate A

Figure 3.1. The Dosage of Bortezomib (nM) Applied on MM U-266 Cells

(cont. on next page)



96 Well Plate B

Figure 3.1. The Dosage of Bortezomib (nM) Applied on MM U-266 Cells (cont.)

In Figure 3.1 ;

- ✓ C is correspond to control group (100 µl ($2x10^4$) MM U-266 Cells + 100 µl RPMI-1640 Growth Medium),
- ✓ 0.1 is correspond to 0.1 nM Bortezomib concentration (100 µl ($2x10^4$) MM U-266 Cells + 100 µl 0.1 nM Bortezomib),
- ✓ 1 is correspond to 1 nM Bortezomib concentration (100 µl ($2x10^4$) MM U-266 Cells + 100 µl 1 nM Bortezomib),
- ✓ 5 is correspond to 5 nM Bortezomib concentration (100 µl ($2x10^4$) MM U-266 Cells + 100 µl 5 nM Bortezomib),
- ✓ 10 is correspond to 10 nM Bortezomib concentration (100 µl ($2x10^4$) MM U-266 Cells + 100 µl 10 nM Bortezomib),
- ✓ 20 is correspond to 20 nM Bortezomib concentration (100 µl ($2x10^4$) MM U-266 Cells + 100 µl 20 nM Bortezomib),
- ✓ 50 is correspond to 50 nM Bortezomib concentration (100 µl ($2x10^4$) MM U-266 Cells + 100 µl 50 nM Bortezomib),
- ✓ 70 is correspond to 70 nM Bortezomib concentration (100 µl ($2x10^4$) MM U-266 Cells + 100 µl 70 nM Bortezomib),

After 72 hour incubation period at 37°C in 5% CO_2, they were treated with 50 µl XTT reagent for 4 hours at CO_2 incubator. After that, the absorbances of the samples were read under 492 nm wavelength of light by Elisa reader (Thermo Electron Corporation Multiskan Spectrum, Finland) and graphed cell proliferation plots. Finally, IC50 value (the concentration of drug that inhibits 50% of cell proliferation as compared to untreated control) of Bortezomib was calculated from cell proliferation plots.

3.2.6. Evaluation of Apoptosis

3.2.6.1. Measurements of Caspase-3 Enzyme Activity

Also called programmed cell death or cell suicide, apoptosis is an integral and necessary part of life cycle of organisms. In the human body, about a hundred thousand cells are produced every second by mitosis and a similar number of them die by apoptosis (Vaux and Korsmeyer, 1999). The apoptotic mode of cell death can be described as a process which plays an important role in the improvement and homeostasis of multicellular organism and in the regulation and maintenance of the cell populations in tissues upon pathological and physiological conditions (Jacobson et al., 1997; Meier et al., 2000; Hengartner, 2000; Leist and Jaattela, 2001). Apoptosis can be triggered by various stimuli from outside or inside the cell. For example, ligation of cell surface receptors, DNA damage as a cause of defects in DNA repair mechanisms, treatment with cytotoxic drugs or irradiation, a lack of survival signals, contradictory cell cycle signaling or developmental death signals (Gewies, 2003). It is doubtless that apoptotic processes are of widespread biological and biochemical significance. A major biochemical feature of apoptosis is the sequential activation of the caspases (**c**ysteine-dependent **asp**artate- specific prote**ases**), a family of proteases whose substrate include large protein precursors of enzymes capable of destroying DNA (endonucleases); lamin and actin (proteases); and proteins involved in DNA repairing, RNA splicing, signal transduction and transcription factors (Thornberry and Lazebnik, 1998; Hengartner, 2000; Green 2000; King and Robins, 2006).

One of the vital members of these caspases family is Caspase-3 enzyme which is the key component in that it takes part in the center of apoptotic pathways (Bratton et

al., 2000). This enzyme is also responsible for the cleavage of important cellular substrates resulting in the classical biochemical and morphological changes associated with the apoptotic phenotype. The afore-cited enzyme is activated either in response to the ligation of cell surface death receptors (extrinsic apoptosis pathways) or in response to signals originating from inside the cell (intrinsic apoptosis pathways) (Figure 3.2) (MacFarlane and Williams, 2004).

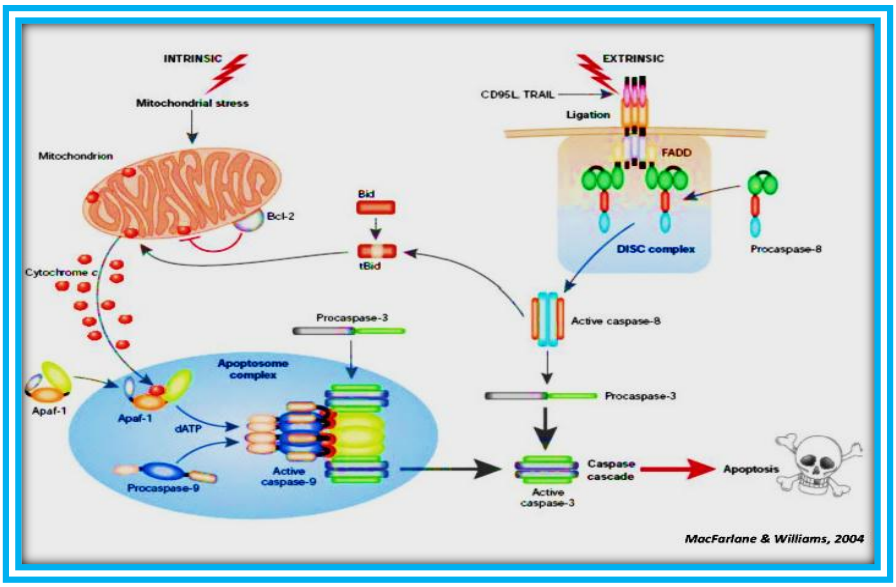


Figure 3.2. Apoptosis: The Extrinsic and Intrinsic Pathways
(Source: MacFarlane and Williams, 2004)

Apoptotic stimuli (cell injury, the release of certain steroids and growth factor withdrawal) trigger the release of apoptogenic factors such as cytochrome c (Gewies, 2003). Cytchrome c is an essential protein component of the mitochondrial electron transport chain that is a loosely bound to the outside of the inner mitochondrial membrane (Lieberman and Marks, 2009). The intrinsic apoptosis pathway initiates with the release of it from the inter-mitochondrial membrane space to the cytosol. Once released, cytochrome c binds to Apoptotic Protease-Activating Factor-1 (APAF-1) and procaspase-9 which bring about formation of the Apaf1–caspase-9 active complex called apoptosome and activation of the initiator caspase-9 (Denault and Salvesen, 2002). Then caspase-3 enzyme is activated by the activate caspase-9 (Salvesen and Renatus, 2002b; Adams, 2003; Danial and Korsmeyer, 2004).

Other apoptosis pathway (extrinsic) activated via death receptor activation by a ligand (usually a cell surface protein on another cell). Triggering of cell surface death

receptors of the Tumor Necrosis Factor (TNF) results in rapid activation of the initiator caspase-8 making a scaffold after its recruitment to a trimerized receptor-ligand complex (DISC) through the adaptor molecule Fas-Associated Death Domain Protein (FADD) (Sartorius et al., 2001). Then activated caspase-8 cleaves and thereby activates caspase-3 for the execution of apoptosis (Gewies, 2003).

Changes in caspase-3 enzyme activity of MM U-266 cells in response to Bortezomib were evaluated by using caspase-3 colorimetric assay kit (R&D Systems, USA) as described previously (Baran et al., 2007). This assay is based on spectrophotometric detection of the chromophor *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate DEVD- *p*NA . The *p*NA light emission can be quantified at 405 nm.

In short, $1x10^6$ cells were seeded in a 6-well plate in 2 ml growth medium in the absence or presence of increasing concentrations of Bortezomib (1 nM, 10 nM, 20 nM) at 37°C in 5% CO_2 for 72 hours. Untreated cells were used as control group.

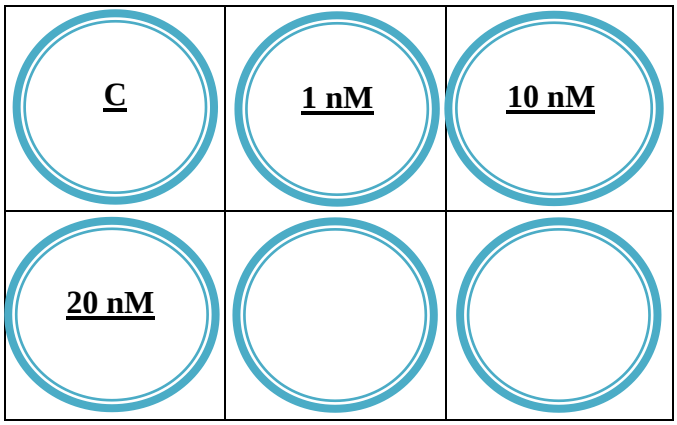


Figure 3.3. Aplied Bortezomib Doses on U-266 Cells for Caspase-3 Enzyme Activity

In Figure 3.3 ;

- ✓ C is correspond to control group ($1x10^6$ U-266 Cells / 2 ml / well),
- ✓ 1 nM is correspond to 1 nM Bortezomib concentration ($1x10^6$ U-266 Cells / 2 ml / well + 1 nM Bortezomib in 2 ml),
- ✓ 10 nM is correspond to 10 nM Bortezomib concentration ($1x10^6$ U-266 Cells / 2 ml / well + 10 nM Bortezomib in 2 ml),

- ✓ 20 nM is correspond to 20 nM Bortezomib concentration ($1x10^6$ U-266 Cells / 2 ml / well + 20 nM Bortezomib in 2 ml),

After incubation, the cells were taken to a falcon tube and centrifugated at 1000 rpm for 10 minutes. Then, the supernatant was gently removed and discarded, while the cell pellet was lysed by adding 50 µl of chilled Cell Lysis Buffer (1X) to get cell lysate. Then, the cell lysate was incubated on ice for 10 minutes before centrifugation at 10000g (~14000 rpm) for 1 minute. Next, the supernatants were transferred to new microcentrifuge tubes. So as to measure caspase-3 enzyme activity, reaction mixture was prepared in 96-well plates with the addition of 50 µl of Reaction Buffer (2X) (containing 10 mM DTT), 50 µl of sample and 5 µl of caspase-3 colorimetric substrate (DEVD-*p*NA) and incubated for 2 hours at 37°C in 5% CO_2. After that, absorbance of the sample was read under 405 nm wavelengths by Elisa reader (Thermo Electron Corporation Multiskan Spectrum, Finland).

3.2.6.2. Bradford Protein Assay for Protein Determination

Protein concentrations were measured by Bradford assay by using bovine serum albumin (BSA) as the standart. The Bradford protein assay is one of the spectroscopic analytical methods that are utilized to find out the total protein concentration of a sample (Bradford 1976). The method is also known as colorimetric assay because the addition of the sample causes color changes from brown that is cationic form to blue which is anionic form. Moreover the color of the sample becomes darker and the measured absorbance rises with the protein concentration increases. Basic principle of this method is absorption shift from 470 nm to 595 nm. When Coomassie Brilliant Blue G-250 (CBB G-250) dye binds to proteins through Van der Waals forces and hydrophobic interactions from its sulfonic groups, absorption occurs. In general, binding of dye to proteins becomes lysine, arginine and histidine residues, it also can bind tyrosine, tryptophan and phenylalanine weakly.

Caspase-3 activity levels were normalized to protein concentrations determined by Bradford protein assay. A series of standard protein solutions were prepared via serial dilution using BSA (Bovine Serum Albumine) diluted with 1X PBS (Phosphate Buffered Saline) (Table 3.1).

The preparation of stock BSA Standard was given in Appendix B.

Table 3.1. Preparation of BSA Standards and Test Sample for the Bradford Protein Assay

Serial Dilution	Test Sample	BSA Standards Volume, µl	PBS Volume, µl
	Blank	-	10
	50 µg/µl	200	200
	40 µg/µl	160	40
	20 µg/µl	100	100
	16 µg/µl	80	20
	8 µg/µl	50	50
	4 µg/µl	50	50
	2 µg/µl	50	50
	1 µg/µl	50	50

After the preparation of standard protein (BSA), protein samples were diluted by the ratios 1:100, 1:250, 1:500 and 1:1000) (Table 3.2).

Table 3.2. Dilution of Protein Sample

C	1/100	10 µl Sample from C + 90 µl PBS
	1/250	20 µl Sample from C + 480 µl PBS
	1/500	20 µl Sample from C + 980 µl PBS
	1/1000	10 µl Sample from C + 990 µl PBS
1 nM	1/100	10 µl Sample from 1 nM + 90 µl PBS
	1/250	20 µl Sample from 1 nM + 480 µl PBS
	1/500	20 µl Sample from 1 nM + 980 µl PBS
	1/1000	10 µl Sample from 1 nM + 990 µl PBS
10 nM	1/100	10 µl Sample from 10 nM + 90 µl PBS
	1/250	20 µl Sample from 10 nM + 480 µl PBS
	1/500	20 µl Sample from 10 nM + 980 µl PBS
	1/1000	10 µl Sample from 10 nM + 990 µl PBS
20 nM	1/100	10 µl Sample from 20 nM + 90 µl PBS
	1/250	20 µl Sample from 20 nM + 480 µl PBS
	1/500	20 µl Sample from 20 nM + 980 µl PBS
	1/1000	10 µl Sample from 20 nM + 990 µl PBS

Next, 90 µl of Bradford reagent, which is composed of 5 mg CBB G-250, 2.5 ml Absolut Ethanol and 5 ml Phosphoric Acid, were seeded in a 96-well plate (Figure 3.4), containing 10 µl of each sample. For Blank, we used 90 µl of Bradford reagent + 10 µl PBS. After that, the mixture in 96-well plates was allowed to incubate at room temperature for 10 minutes. Finally, the absorbance of dye within the plates was measured at 595 nm by Elisa reader (Thermo Electron Corporation Multiskan Spectrum, Finland) and the standard curve was constructed by plotting the reading of absorbance versus µg of protein in BSA standard samples. According to the equation (y = mx+n), the best straight line was acquired. In this equation ; x, which is correspond to protein concentration can be found with the help of y, which is the absorbance value read at 595 nm. Dilution of the sample is required for absorbance to fall within the linear range of the grafics.

Blank	**Blank**	**Blank**	**50 µg/µl**	**50 µg/µl**	**50 µg/µl**	**1/1000 (1 nM)**	**1/250 (10 nM)**
1 µg/µl	**1 µg/µl**	**1 µg/µl**	**1/100 (C)**	**1/100 (C)**	**1/100 (C)**	**1/1000 (1 nM)**	**1/250 (10 nM)**
2 µg/µl	**2 µg/µl**	**2 µg/µl**	**1/250 (C)**	**1/250 (C)**	**1/250 (C)**	**1/1000 (1 nM)**	**1/250 (10 nM)**
4 µg/µl	**4 µg/µl**	**4 µg/µl**	**1/500 (C)**	**1/500 (C)**	**1/500 (C)**		
8 µg/µl	**8 µg/µl**	**8 µg/µl**	**1/1000 (C)**	**1/1000 (C)**	**1/1000 (C)**		
16 µg/µl	**16 µg/µl**	**16 µg/µl**	**1/100 (1nM)**	**1/100 (1nM)**	**1/100 (1nM)**	**1/100 (10 nM)**	**1/500 (10 nM)**
20 µg/µl	**20 µg/µl**	**20 µg/µl**	**1/250 (1nM)**	**1/250 (1nM)**	**1/250 (1nM)**	**1/100 (10 nM)**	**1/500 (10 nM)**
40 µg/µl	**40 µg/µl**	**40 µg/µl**	**1/500 (1nM)**	**1/500 (1nM)**	**1/500 (1nM)**	**1/100 (10 nM)**	**1/500 (10 nM)**

1/1000 (10 nM)	**1/1000 (10 nM)**	**1/1000 (10 nM)**	**1/1000 (20 nM)**	**1/1000 (20 nM)**	**1/1000 (20 nM)**		
1/100 (20 nM)	**1/100 (20 nM)**	**1/100 (20 nM)**					
1/250 (20 nM)	**1/250 (20 nM)**	**1/250 (20 nM)**					
1/500 (20 nM)	**1/500 (20 nM)**	**1/500 (20 nM)**					

Figure 3.4. Bradford Assay

3.2.6.3. Detection of the Loss of Mitochondrial Membrane Potential (MMP)

Mitochondrion is responsible for mediating the release of cytochrome-c molecules. Thus, it has a vital function in the induction of intrinsic apoptosis. Loss of mitochondrial membrane potential (MMP), that is a hallmark for apoptosis, causes to form pores and these pores allows to release of cytochrome-c into the cytoplasm. This event known as the beginning of the apoptotic response. The decrease of MMP in MM U-266 cells was assessed by APO LOGIX JC-1 MMP Detection Kit (Cell Technology, USA). JC-1, which is a signal molecule to detected the changes in MMP, is a unique cationic dye. In non-apoptotic cells, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) exist as a monomer in the cytosol (green) and also accumulates as aggregates in the mitochondria which stain red. Whereas, in apoptotic cells or unhealthy cells, JC-1 exist in monomeric form and stains the cytosol to green. For this reason, apoptotic cells, showing only green fluorescence, are easily differentiated from healthy cells that show intense red fluorescence. This is the basic principle of the method.

Shortly, $1x10^6$ cells were seeded in a 6-well plate in 2 ml growth medium in the absence or presence of increasing concentrations of Bortezomib (1 nM, 10 nM, 20 nM) at 37°C in 5% CO_2 for 72 hours. Untreated cells were used as control group.

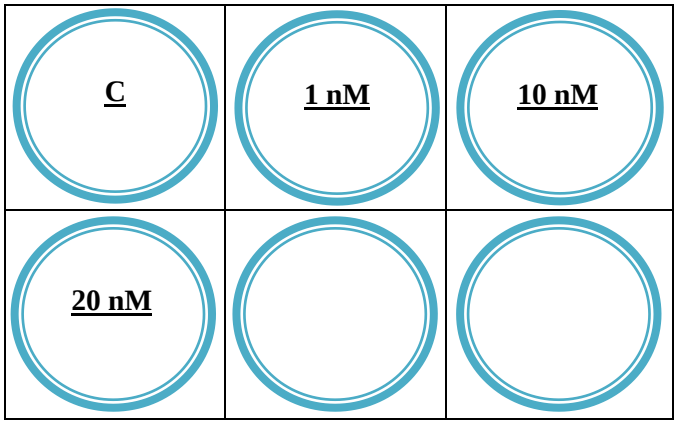


Figure 3.5. Aplied Bortezomib Doses on U-266 Cells for Dedection of Loss of MMP

In Figure 3.5 ;

- ✓ C is correspond to control group ($1x10^6$ U-266 Cells / 2 ml / well),
- ✓ 1 nM is correspond to 1 nM Bortezomib concentration ($1x10^6$ U-266 Cells / 2 ml / well + 1 nM Bortezomib in 2 ml),
- ✓ 10 nM is correspond to 10 nM Bortezomib concentration ($1x10^6$ U-266 Cells / 2 ml / well + 10 nM Bortezomib in 2 ml),
- ✓ 20 nM is correspond to 20 nM Bortezomib concentration ($1x10^6$ U-266 Cells / 2 ml / well + 20 nM Bortezomib in 2 ml),

After this period, all samples (control group and the cells induced to undergo apoptosis) are taken to the falcon tubes respectively and washed with 1X PBS before the centrifugation at 1000 rpm for 10 minutes. Supernatants were carefully removed from the pellet and discarded. Then the pellets were dissolved in 500 µl of JC-1 dye and the cells were incubated at 37˚C in 5% CO_2 for 15-30 minutes. Next, the mixtures were centrifuged at 400g (~1000 rpm) for 5 minutes and supernatants were carefully removed again. Subsequently, 2 ml of 1X Assay Buffer was added onto the pellets and vortexed till to be sure that it was homojenized. Immediately afterwards centrifugation was repeated with the same conditions (400g (~1000 rpm) for 5 minutes) to remove the excess dye. All the pellets were resuspended with 500 µl 1X Assay Buffer and 150 µl from each of them was seeded into black 96-well plate as triplicate. While the aggregate red form of the dye has absorption/emission maxima of 585/590 nm, the monomeric green form of the dye released to the cytoplasm because of the loss of MMP has absorption/emission maxima of 510/527 nm. The plate was read in these wavelengths by fluorescence Elisa reader (Thermo Varioskan Spectrum, Finland). Finally, green/red (510/585) values were calculated to determine the changes in MMP.

3.2.7. Total Protein Extraction from MM U-266 Cells

So as to extract the total proteins belonging to MM U-266 cells and 17 nM (which is the IC-50 value of Bortezomib on U-266 cells) Bortezomib applied U-266 cells, whole cell suspension was taken from tissue culture flask into a sterile falcon tube and then centrifuged at 1000 rpm for 10 minutes at room temperature, separately. After

centrifugation, the supernatant was removed and the pellet resuspended in 50 µl of chilled Cell Lysis Buffer (containing Sodium Chloride, Potassium Chloride, Sodium Phosphate Dibasic, Potassium Phosphate Monobasic,Triton X100 and Water) before incubated on ice for 10 minutes. Following the addition of Cell Lysis Buffer, samples taken to microcentrifugation tube from falcon and centrifugation was performed at maximum speed 14.000 rpm (~10.000g) for 1 minutes at 4˚C. Supernatant, which is the total protein of our samples, transferred to a fresh tube and used for further studies (2D-PAGE).

3.2.8. Determination of Protein Concentration

Protein concentrations of the samples were determined by using Bradford method as described above.

3.2.9. 2D-PAGE Analysis

3.2.9.1. Isoelectric Focusing (IEF)

Day One

The first dimension step of 2D PAGE (isoelectric focusing, (IEF)) was performed on 17 cm-immobiline strips which provided a linear gradient from pH 3 to 10 (Bio-Rad) by a PROTEAN IEF Cell (Bio-Rad).

Firstly, strips were taken out from -20°C freezer and waited in the room temperature till rehydration buffer is prepared (preparation of the buffer was given in Appendix B). Before the experiment, IEF rehydration tray was cleaned and dried. The passive rehydration was choosen for the rehydration step.

Table 3.3 indicates the amount of recommended protein sample to load per IPG strip.

Table 3.3. Required Protein Sample with Rehydration Buffer

Strip Length	**17 cm (pH 3 - 10)**
Sample Volume	300 - 600 µl
Protein Loaded	405 µg

- ✓ It is also suggested that the rehydration buffer should be equal or more than 300 µl.

Bradford results belonging to Bortezomib and control group are represented in Table 3.4.

Table 3.4. Bradford results for protein samples

Sample Name	**Protein Concentration**
Bortezomib (17 nM)	10.20 µg / µl
Control	10.36 µg / µl

By taking notice of the recommended value in Table 3.3 and the concentration of two protein samples (Bortezomib and control group) in Table 3.4, protein samples were reconstituted with rehydration buffer. The reorganized sample values can be seen in Table 3.5.

Table 3.5. Reconstituted sample values

Sample Name	**Sample Volume**	**Protein Loaded**
Bortezomib (17 nM)	40 µl + 360 µl Rehydration Buffer = 400 µl	408 µg
Control	40 µl + 360 µl Rehydration Buffer = 400 µl	414.4 µg

According to reorganized sample values, proteins were pipetted along the whole length of the rehydration tray channel except for about 1 cm at each end. For this process it was taken care not to introduce any bubbles that might interfere with the even distribution of protein samples in the strip. Channels of the rehydration tray was

selected symmetrically to load the protein samples. It is important for us to avoid confusing the samples. At the same time, during the IEF step, the weigh of the tray lid is evenly distributed over all the samples so contact between the IPGs and the electrode remains uniform. After all the protein samples loaded into the rehydration tray, the coversheet of the strips were peeled by using forceps. Then the strips gel side down were gently located onto the samples, respectively. And the tray was waited for an hour at the room temperature to allow to strip for soaking the sample. After that each of the strips were overlayed with 2 - 3 ml of mineral oil so as to hamper evaporation during the rehydration process. It was added slowly, by carefully dripping the oil onto the plastic backing of the strips while moving the pipet along the length of the strip. The final step of the day 1, the tray was covered with a plastic lid and lifted an incubator (20°C) to allow to rehydrate the IPG strips.

Day Two

In second day, the IEF focusing tray that has electrodes was cleaned and dried carefully. After that, paper wicks were placed at both ends of channels on the wire electrodes using forceps. It is crucial to prevent direct contact between IPG strips and wire electrodes, because it might cause to undesired burns on strips. Following, paper wicks were wetted with 8 µl of ultrapure water in order to enable electrical conductivity. Then, strips were taken from the rehydration tray by removing the cover and by the help of forceps; strips were held vertically for about 5 – 10 seconds to let the mineral oil to drain, carefully. Then they were transferred to related channels in the focusing tray by maintaining the gel side down position. Each IPG strips were covered with 2 – 3 ml of fresh mineral oil again and the lid was placed onto the focusing tray (positive to the left when the inclined portion of the tray is on the right). At the final step of isoelectric focusing, the focusing tray was put into the PROTEAN IEF cell and the cover of the tray was closed. Isoelectric focusing was performed at room temperature (~20°C) in three steps depending on the values in Table 3.6.

Table 3.6. PROTEAN IEF Cell Program

17 cm	Voltage	Time	Ramp	Conditions
Step 1	500 V	1 hour	Linear	20 °C 50 µA / Strip No Rehydration
Step 2	1000 V	1 hour	Linear	
Step 3	8000 V	8 hour	Rapid	
Total	64000 Volt - hour			

After the IEF run was complete, IPG strips were removed from IEF focusing tray and taken in a new and clean rehydration tray with the gel side up position. Before transferring the IPG strips, they were allowed to drain of mineral oil by holding vertically for 5 – 10 seconds. After they were either placed at -80ºC or immediately equilibrated with equilibration buffers for second dimension of the experiment.

3.2.9.2. SDS-PAGE

For second dimension of the experiment, Equilibration I and Equilibration II buffers were prepared (preparation of these two buffers were told in Appendix B). The strips taken from the focusing tray were equilibrated in equilibration buffers that contain SDS which is of great significance to transform the focused proteins taken part in the IPG strips into SDS-protein complexes. These complexes had unfolded structures and carried only negative charges. Basically, Equilibration Buffer I has DTT, which is necessary as a reducing agent for cleavage of sulfhydryl bonds between cysteine residues within a protein, Tris-HCl pH 8.8, glycerol and urea, in addition to SDS, while instead of DTT, Equilibration Buffer II has iodoacetamide which is used as an alkylation for reduced sulfhydryl groups in the protein.

Because SDS PAGE would carry out in the third day, the preparations began in second day. Related to, SDS-polyacrylamide gel was prepared. It contains only the separating gel but not the stacking gel. Instead of stacking gel overlay agarose was used to cover the strip completely. The composition of acrylamide for the separating gel was chosen as 12% so that components of the gel could be prepared according to this value. Two SDS-polyacrylamide gels were prepared for Bortezomib and control group samples in a comparative manner. As the volume of each gel was required

approximately 35 ml, the volume of each solution for two gels was adjusted to prepare 80 ml of 12% SDS-polyacrylamide gel. Preparation of the separating gel and its components were described in Appendix B.

After the preparation of separating gel components, they were mixed in a 100 ml erlenmayer flask in the order of dH_2O, acrylamide / biaacrylamide, Tris-HCl, and SDS, because the polymerization began as soon as the TEMED and APS were added. Thus, they were added to the mixture at the same time and final mixture was poured into the gap between glass plates without delay. After polymerization had been complete (~30 - 45 minutes), upper side of the gels were covered with tertamyl alcohol (~500 – 600 µl) and they were kept overnight at 4°C.

Day Three

For the equilibration, 6 ml of Equilibration buffer I was added on IPG strips which is in a clean rehydration tray with the gel side up position and the tray was carefully put on an orbital shaker for 10 minutes. Shaking was carried out in a slow speed to prevent the buffer from sloshing out of the tray. At the end of the incubation, equilibration buffer I was discarded by pouring the liquid cautiously from the tray. Subsequent to this, 6 ml of Equilibration buffer II containing iodoacetamide was added on each strip and again tray was placed to the orbital shaker for 10 minutes. End of the 10 minutes, equilibration buffer II was discarded as mentioned before.

At the end of the equilibration of IPG strip, a graduated cylinder (100 ml) was filled with 1X Tris - Glycine - SDS running buffer and each bubble on the surface of the buffer was removed by the help of a Pasteur pipette. Then, the strips were sopped in the graduated cylinder respectively to be rinsed in the buffer. Next, each strip which was positioned as gel side up was laid onto the glass plate of the SDS gel, and connected with the gel by paying attention not to be any air bubble at the interface. Meanwhile, the overlay agarose solution was melted in a microwave oven by loosening the cap of the bottle. The glass plates were then held vertically, and the necessary amount of overlay agarose solution was poured into the IPG well of each gel. After each strip were pushed carefully using a forceps, 5 minutes was enough for agarose to be solidified. Before began to electrophoresis, the reservoir of the gel box and the gap between the gels were filled with 1X Tris - Glycine - SDS running buffer. In order to control the cooling of

electrophoresis, water circulation was used and electrophoresis was started at constant current at 32 mA for 30 minutes and followed by 50 mA for 5 – 6 hours. To monitor the progress of the electrophoresis, Bromophenol Blue was used. When it reached the bottom of the gel, electrophoresis was stopped.

3.2.9.3. Staining of the Gel

A number of staining techniques have been developed to stain polypeptides with silver salts after separation by two-dimensional gel electrophoresis. In every case, the procedure based on differential reduction of silver ions that are bound to the side chains of amino acids (Switzer et al., 1979; Nielsen and Brown, 1984; Merril et al., 1984).

Table 3.7. Time Coarse for MALDI-TOF Compatible Mass Spectrometry (Source: Adapted from He et al., 2003; He et al., 2004; Lin et al., 2008)

STEP		SOLUTION	TIME OF TREATMENT	COMMENTS
1	**Fixing**	(Fixer Soln.) 40% EtOH, 10% HAc;	> 1 hour (overnight)	Overnight incubation is all right
2	**Washing**	-	-	-
3	**Sensitizing**	Pretreatment Soln. 30% EtOH, 4.1% NaAc, 0.2% $Na_2S_2O_3$;	30 minutes	Time should be exact
4	**Washing**	dH_2O	5 min. x 3	2^{nd} & 3^{rd} solution should always be fresh
5	**Impregnate**	Silver Nitrate Solution 0.1% $AgNO_3$, 0.02% formalin;	20 min.	-
6	**Washing**	dH_2O	1 min. x 2	Time should be exact 2^{nd} & 3^{rd} solution should always be fresh
7	**Developing**	Developing Solution 2.5% Na_2CO_3, 0.01% formalin	~ 10 min.	After a few minutes, add some dH_2O to proceed the reaction slowly. Time should be determined by observation of color development
8	**Stop**	Stop Solution 1.46% EDTA	> 10 minutes	The gels can be kept in this solution overnight
9	**Washing**	dH_2O	5 min. x 3	2^{nd} & 3^{rd} solution should always be fresh

After electrophoresis was finished, gels were taken out from two glass plates cautiously into a tray for staining process. A MALDI–TOF Mass Spectrometry compatible silver staining method was performed. The procedure of this mass spectrometry suitable silver staining technique was described in Table 3.7.

3.2.9.4. Image and Data Analysis of Gel

After staining process, gels were scanned and photographed by OLYMPUS-DP25 digital camera system. Then the image analysis, spot matching and determination of different protein spots were performed with the 7-day free licensed software package of DECODON Delta2D Version 4.3. The software superposed the gel photos and detected the differentiated protein spots.

According to this software the differentiated proteins were determined and cut from the gel to be identified by mass spectrometry.

3.2.9.5. In-Gel Digestion

Differentially expressed protein spots were in-gel digested before the mass spectrometry analysis. In 1996, Shevchenko and his co-workers defined a protocol that is well suited with further identification instruments(Shevchenko, et al. 1996). Protocol consists of three-day procedures which an initial washing of the protein spot and digestion reaction are performed overnight.

The preparation of in-gel digestion chemicals were described in Appendix B.

Before beginning the in-gel digestion process, if any spots which were in a large size, they were diminished by the help of pipette tip.

Day One: Excising and Destaining of Differentiated Protein Spots

- ✓ Protein spot was excised from the gel as closely as possible with a sharp scalpel. To extend the surface area they were divided into smaller pieces. The gel pieces were put in 1.5 ml of plastic micro centrifuge tubes, separately.

- ✓ After excising the differentiated spots from the gel, they should be destained before the in-gel digestion procedure. The destaining of silver stained gels were as follows:

- ✓ Following two stock solutions were prepared just prior to use.

    - ✶ Solution A: 2 ml of 100 mM Sodium Thiosulfate: 49.6 mg in 2 ml ultrapure water.
    - ✶ Solution B: 2 ml of 30 mM Potassium Ferricyanide: 19.75 mg in 2 ml ultrapure water.

- ✓ Solution A and Solution B were combined in a 1:1 ratio to make 4 ml working solution labeled as Reducing Solution.
- ✓ 200 µl Reducing Solution was added to gel tube, and destained until brownish color has disappeared, approximately 2 – 3 minutes.
- ✓ Then Reducing Solution was discarded quickly from the tube, and 500 µl ultrapure water was added to wash. Washing step was repeated 2 times, with 5 minutes each.
- ✓ Finally, samples were dried in speed vacuum until completely dry, approximately 15 minutes.

Day Two: Reduction, Alkylation, and Digestion	
Step	**Procedure**
1	✶ 200 µL of acetonitrile was added and the gel pieces were dehydrated for ~5 minutes at room temperature. When dehydrated, the gel pieces were an opaque white color and were smaller in size.
2	✶ Acetonitrile was carefully removed from the sample and discarded.
3	✶ The gel pieces were completely dried at ambient temperature in a vacuum centrifuge for 2-3 minutes.
4	✶ 30 µL of 10 mM DTT was added and the protein was reduced for 30 minutes at room temperature.
5	✶ Then, DTT was carefully removed from the sample and discarded.
6	✶ 30 µL of 100 mM iodoacetamide was added and the protein was alkylated for 30 minutes at room temperature.

cont. on next page

Day Two: Reduction, Alkylation, and Digestion (cont.)	
7	✶ Then, iodoacetamide was carefully removed from the sample and discarded.
8	✶ Step 1 was repeated
9	✶ Step 2 was repeated
10	✶ The gel pieces were rehydrated with 200 µL of 100 mM ammonium bicarbonate, incubating the samples for 10 minutes at room temperature.
11	✶ Then, ammonium bicarbonate was carefully removed from the sample and discarded.
12	✶ Step 1 was repeated
13	✶ Step 2 was repeated
14	✶ Step 3 was repeated
15	✶ 30 µL of the trypsin solution was added to the sample and the gel pieces were allowed to rehydrate on ice for 10 minutes with occasional vortex mixing.
16	✶ The gel pieces were driven to the bottom of the tube by centrifuging the sample for 30 seconds.
17	✶ 5 µL of 50 mM ammonium bicarbonate was added to the sample and the mixture was vortexed.
18	✶ The sample was driven to the bottom of the tube by centrifuging the sample for 30 seconds. Digestion was carried out overnight at 37ºC.
Day Three: Extraction of Peptides for Analysis	
19	✶ 30 µL of 50 mM ammonium bicarbonate was added to the digest and the sample was incubated for 10 minutes with occasional gentle vortex mixing.
20	✶ The digest was driven to the bottom of the tube by centrifuging the sample for 30 seconds.
21	✶ The supernatant was carefully collected and the sample was transferred to a 0.5 ml plastic micro centrifuge tube.
22	✶ 30 µL of extraction buffer was added to the tube containing the gel pieces and incubated for 10 minutes with occasional gentle vortex mixing.
23	✶ The extract was driven to the bottom of the tube by centrifuging the sample for 30 seconds.

cont. on next page

Day Three: Extraction of Peptides for Analysis (cont.)	
24	✶ The supernatant was carefully collected and combined the extract in the 0.5 ml plastic micro centrifuge tube.
25	✶ Step 22 was repeated
26	✶ Step 23 was repeated
27	✶ I was followed by carefully collection of supernatant and combination of the extract in the 0.5 ml plastic micro centrifuge tube.
28	✶ The volume of the extract was reduced to less than 20 µL by evaporation in avacuum centrifuge at ambient temperature. The extract must not be allowed to dry completely.
29	✶ The volume of the digest was adjusted to ~20 µL with acetic acid.
30	✶ Finally, the sample was ready for mass spectrometric analysis.

3.2.9.6. ZipTip

ZipTip is a small C-18 colon used for removing salts and detergents to obtain a better mass signal. Protocol of ZipTip can be summarized in this manner:

ZipTip				
1	**Wetting**	10 µl	%100 ACN (Acetonitrile)	10 times
2	**Equilibration**	10 µl	%0.1 TFA (Trifluoroacetic acid)	10 times
3	**Sample**	10 µl	Our Protein Samples	10-15 times
4	**Washing**	10 µl	%0.1 TFA	5-6 times
5	**Elution**	5 µl	%0.1 TFA/%50 ACN	2-3 times

3.2.9.7. Protein Identification and Mass Spectrometric Analysis

After in-gel digestion procedure, two samples (Bortezomib and Control group) were identified by MALDI-TOF-TOF Mass Spectrometry. The sequence of the diffentially expressed protein spots were found by using NCBInr (National Center for Biotechnology Information, Bethesda, USA) and SwissProt protein databases.

For mass analysis, α-cyano-4-hydroxycinnamic acid (HCCA) was used as a matrix. Its preparationwas described in Appendix B.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Cytotoxic Effects of Bortezomib on U-266 Cells

MM U-266 cells were treated with increasing concentrations of Bortezomib for 72 hours and XTT cell proliferation assay was carried out in order to determine the anti-proliferative effects of agent on these cells. The cells were exposed to Bortezomib from 0.1 to 70 nM and the results showed that there was a dose-dependent decrease in cell proliferation as compared to untreated controls. There were 3, 4, 15, 32, 57, 73 and 79% decrease of cell proliferation when the cells were exposed to 0.1, 1, 5, 10, 20, 50 and 70 nM Bortezomib, respectively (Figure 4.1). As a result, the inhibitory concentration (IC50) value of Bortezomib on MM U-266 cells was calculated from cell proliferation plots and found to be 17 nM. (Figure 4.1).

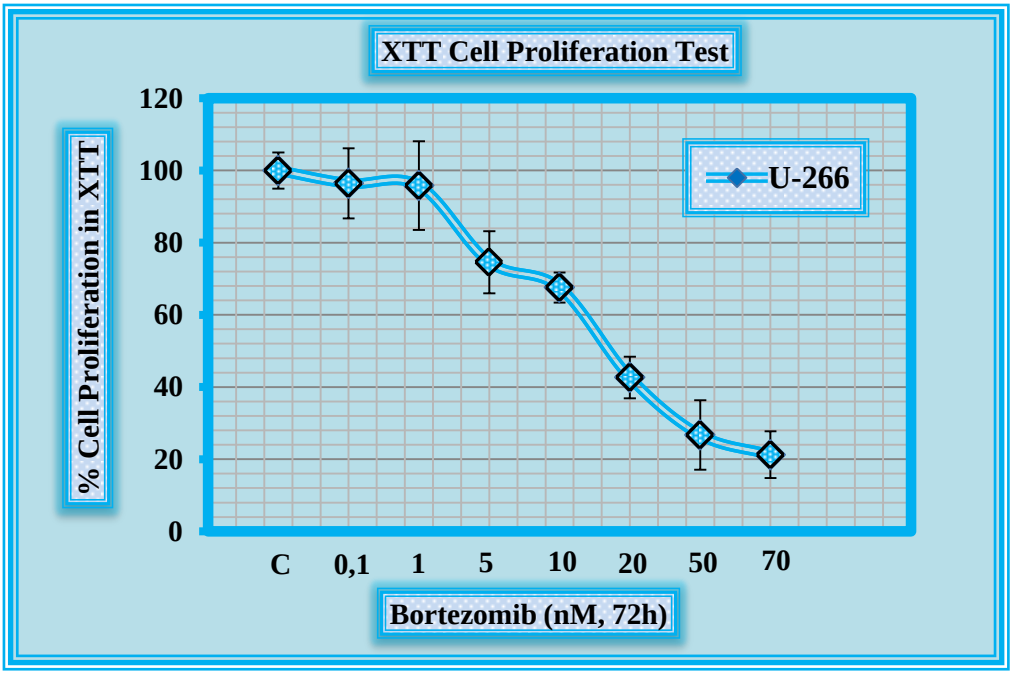


Figure 4.1. XTT Cell Proliferation Result

4.2. Changes in Caspase-3 Enzyme Activity and Loss of Mithocondrial Membrane Potantial) MMP

To evaluate the apoptotic effects of Bortezomib on U-266 cells, Caspase-3 enzyme activity and mitochondrial membrane potential were two important apoptotic indicators. Treatment of U-266 cells for 72 h with 1, 10 and 20 nM Bortezomib resulted in 1.06, 1.13 and 1.17-fold increases, respectively, in caspase-3 activity as compared to untreated controls (Figure 4.2).

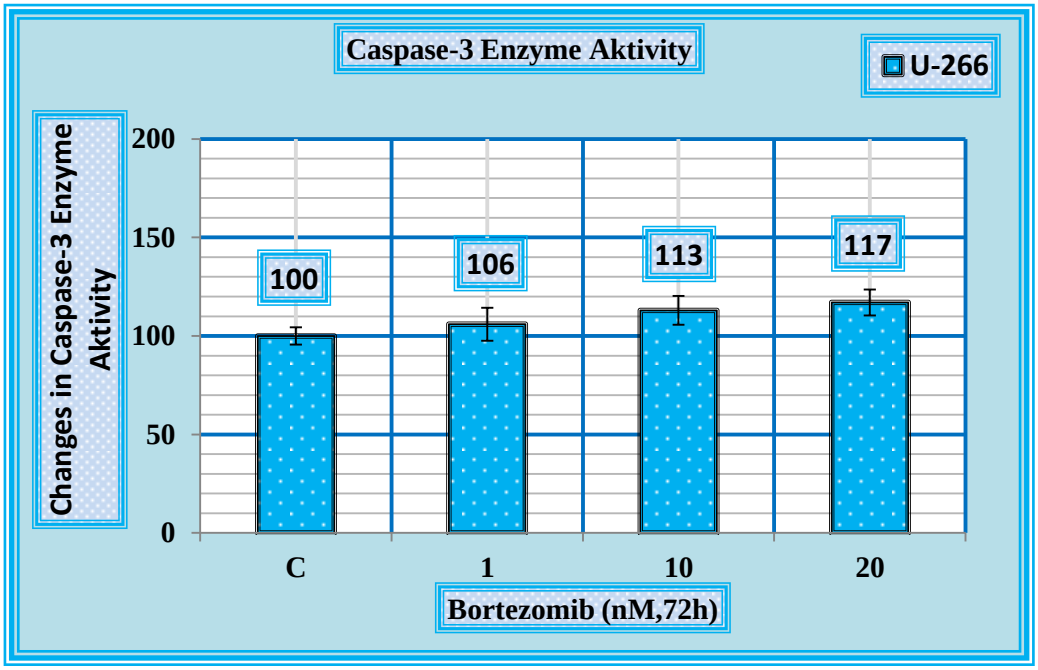


Figure 4.2. Changes in Caspase-3 Enzyme Activity

We also assessed the loss of mitochondrial membrane potential in order to confirm the caspase-3 enzyme activity results and examine the roles of mitochondria in Bortezomib-induced apoptosis. Treatment of U-266 cells for 72 h with 1, 10 and 20 nM Bortezomib caused a significant loss of MMP (about 1.34, 1.85 and 2.14-fold, respectively, when compared with untreated controls), as measured by increased accumulation of cytoplasmic mitochondrial JC-1 (Figure 4.3).

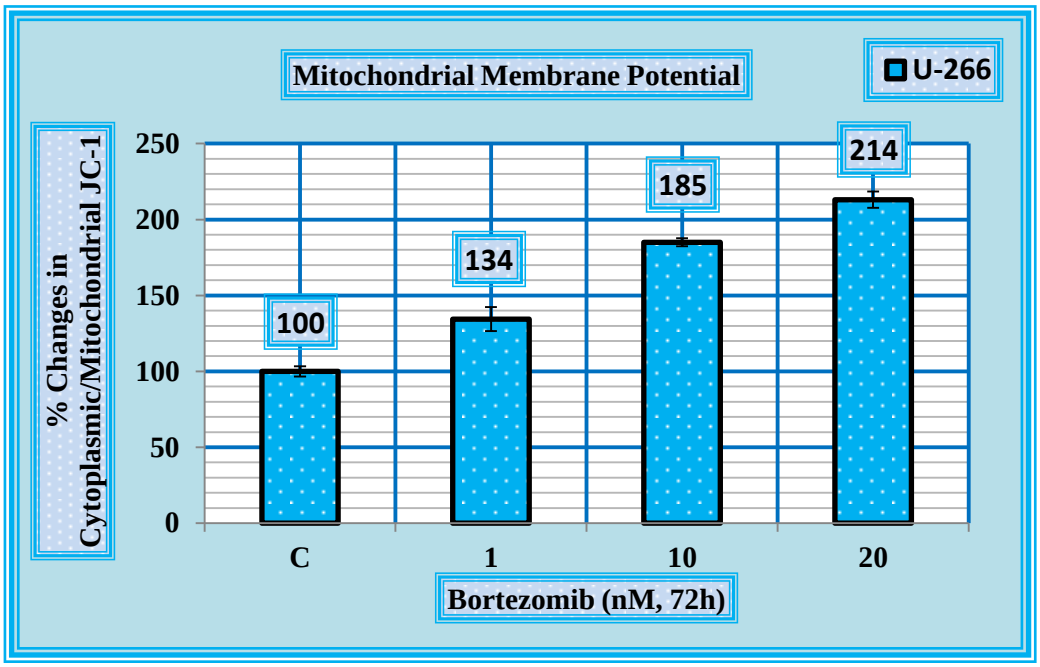


Figure 4.3. Changes in Cytoplasmic/Mitochondrial JC-1 in U-266 Cells Treated with Bortezomib

4.3. Two-Dimensional Polyacrylamide Gel Electrophoresis Results

After determining the cytotoxic and apoptotic effects of Bortezomib on MM U-266 cells, total protein isolation was performed from Bortezomib and control group separately. After that isolated proteins were analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Two-Dimensional Gel Electrophoresis, respectively to identify changes in protein profiles. Protein isolation and 2D PAGE experiments were repeated four times. Firstly, gels obtained from Bortezomib and control group were stained with CBB G-250, but the results were not satisfactory. Then CBB R-250 and R-350 was tried with another gels and with different staining procedures. Finally, a mass spectrometry compatible silver staining procedure was applied as explained in previous chapter to increase the sensitivity, because there was not enough protein that was visualized on the gels after Coomassie Brillant Blue Staining. The gel maps belonging to two different groups (Bortezomib (treated) and control (untreated)) indicated a broad distribution of spots in a pI range from 3.0 to 10.0 (Figure 4.4).

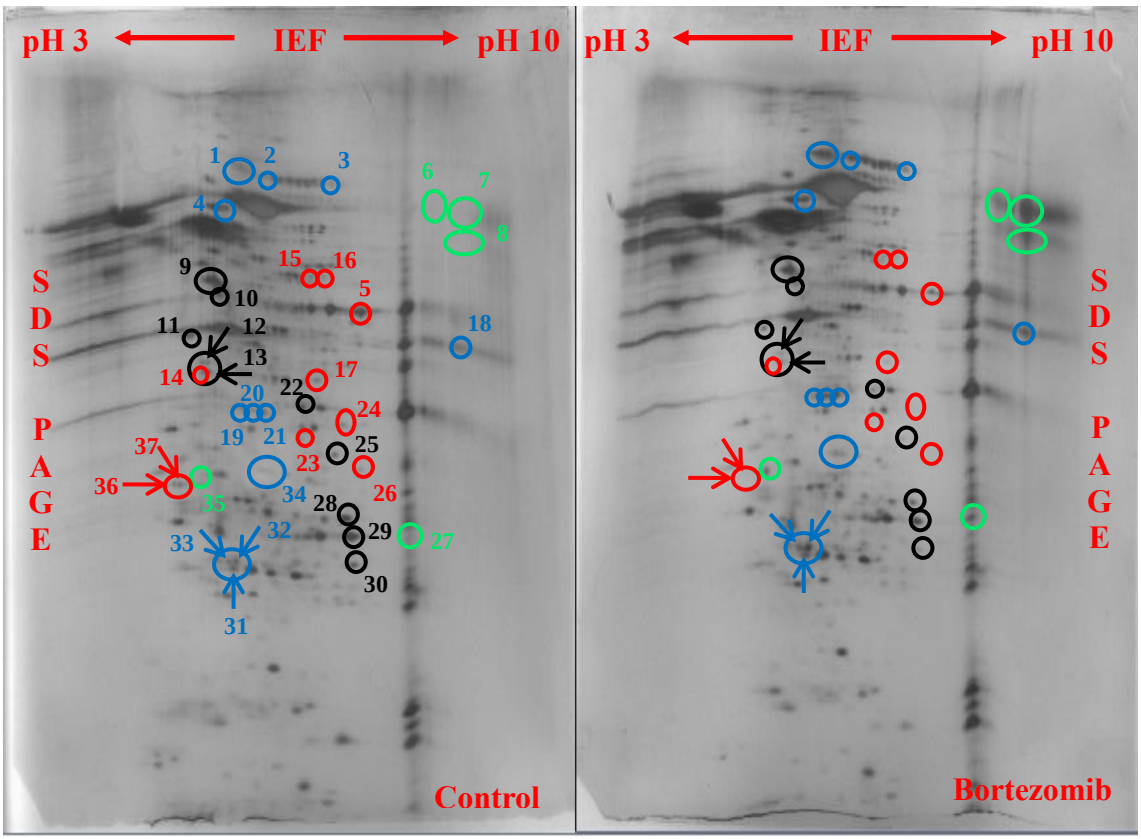


Figure 4.4. 2D PAGE Maps of Bortezomib (17 nM) and Control Group

In order to elucidate and compare protein profiles of two groups at issue, two individual gel images were imported into gel analysis software DECODON Delta2D Version 4.3. With the help of this software, these 2D PAGE gels were superposed and comparative detailed analysis was performed. According to the analysis, 37 protein spots were differentially expressed. Among them, 5 proteins were newly formed, 10 proteins lost, 12 proteins were up-regulated and 10 proteins were down-regulated as compared to control group (untreated cells) (Figure 4.4). These spots were numbered from 1 to 37, colored and labeled with a special name:

- ✓ Newly formed proteins were marked with the green color (Y)
- ✓ Lost proteins were marked with the red color (K)
- ✓ Upregulated proteins were marked with the blue color (M)
- ✓ Downregulated proteins were marked with the black color (S).

To observe differential expressed spots easily, gels were segmented and magnified (Table 4.1).

Table 4.1. Magnified Segments of Differentiated Protein Spots

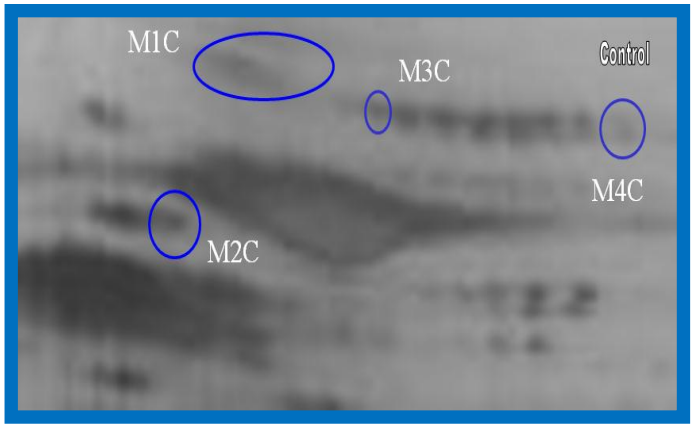
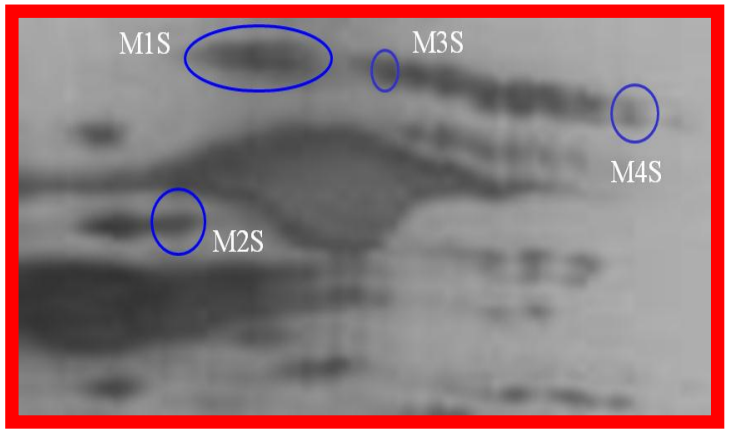
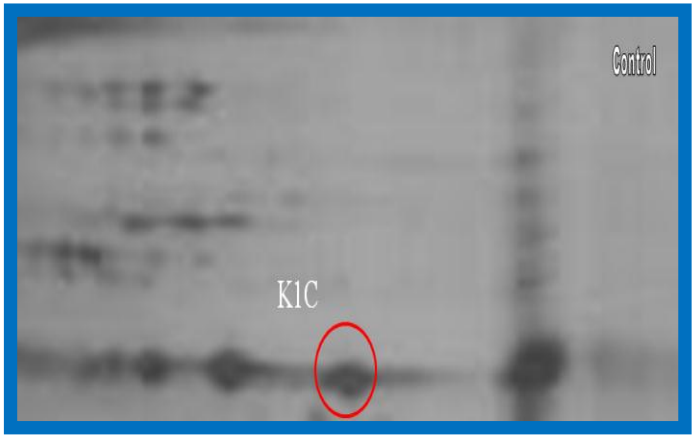
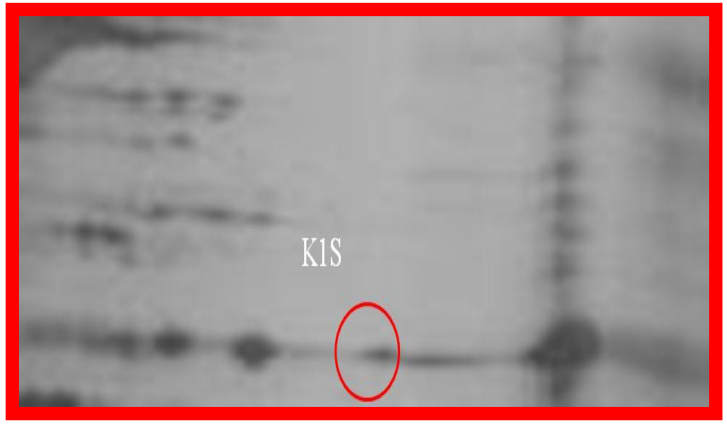
Spot No	Spot Name	CONTROL	BORTEZOMIB
1	M1S		
2	M2S		
3	M3S		
4	M4S		
5	K1C		

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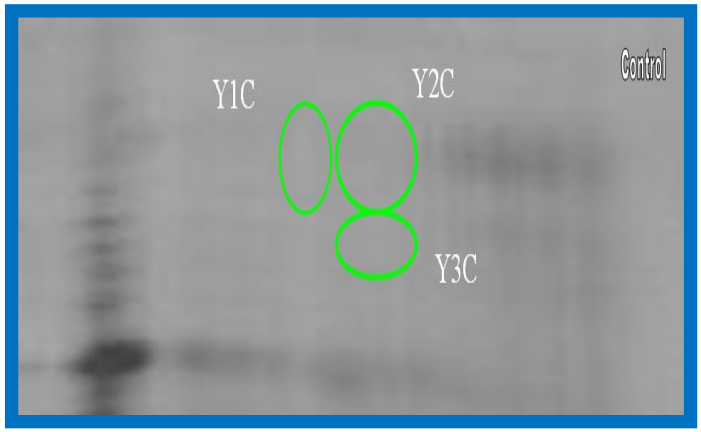
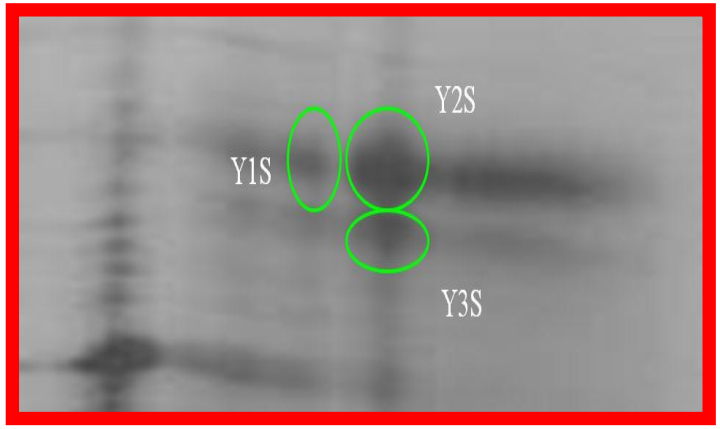
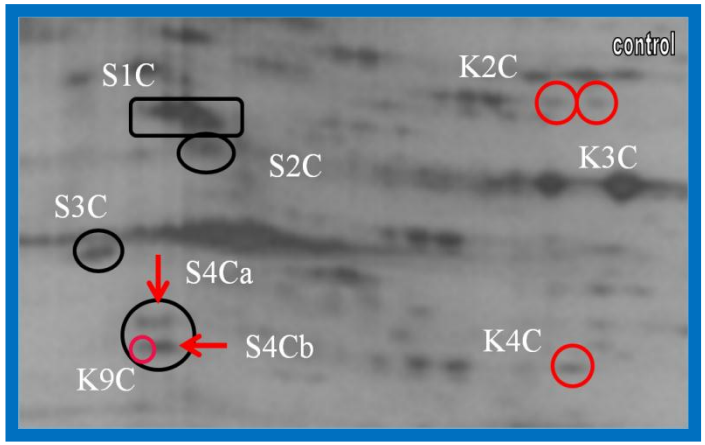
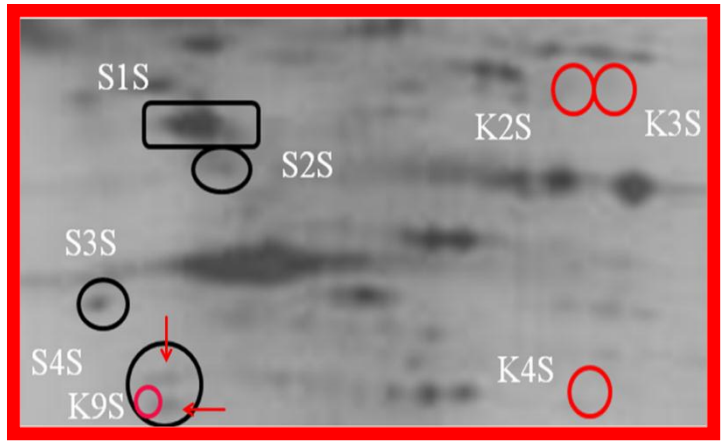
Table 4.1. Magnified Segments of Differentiated Protein Spots (cont)

Spot No	Spot Name	CONTROL	BORTEZOMIB
6	Y1S		
7	Y2S		
8	Y3S		
9	S1C		
10	S2C		
11	S3C		
12	S4Ca		
13	S4Cb		
14	K9C		
15	K2C		
16	K3C		
17	K4C		

cont. on next page

Table 4.1. Magnified Segments of Differentiated Protein Spots (cont.)

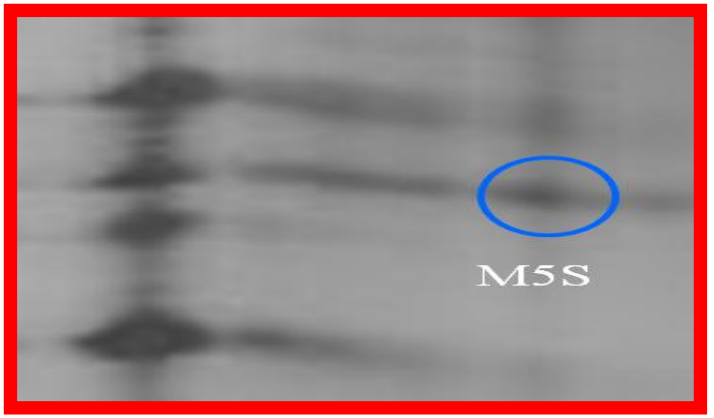
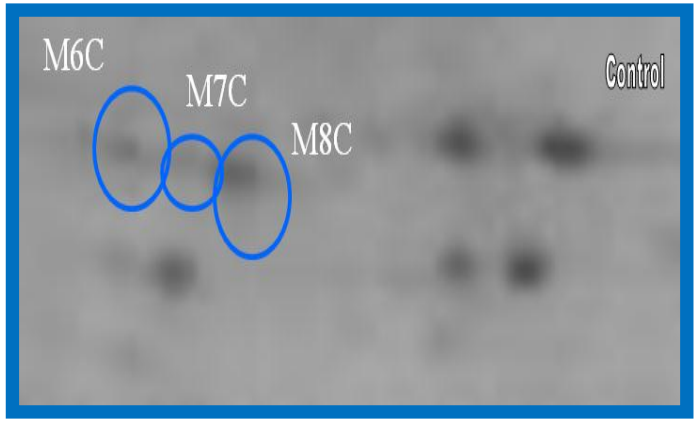
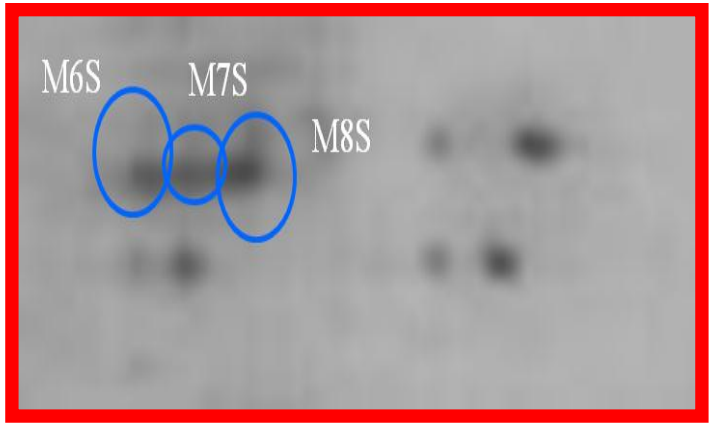
Spot No	Spot Name	CONTROL	BORTEZOMIB
18	M5S		
19	M6S		
20	M7S		
21	M8S		

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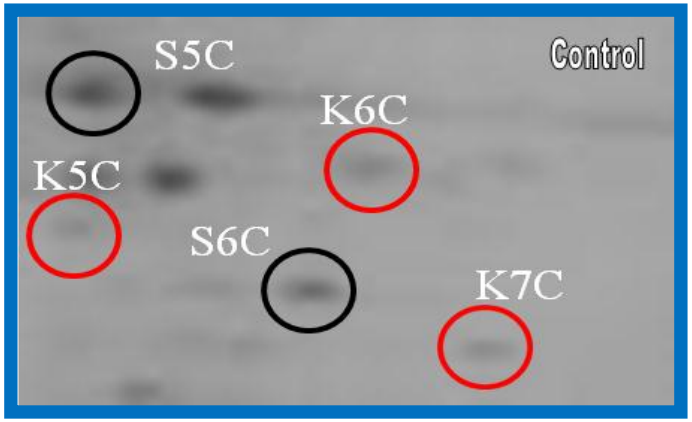
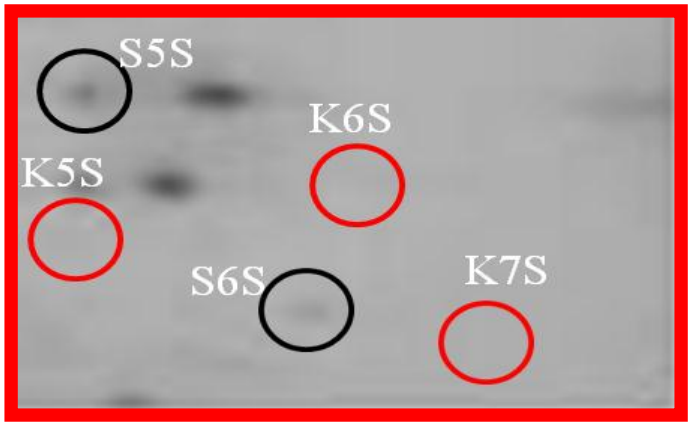
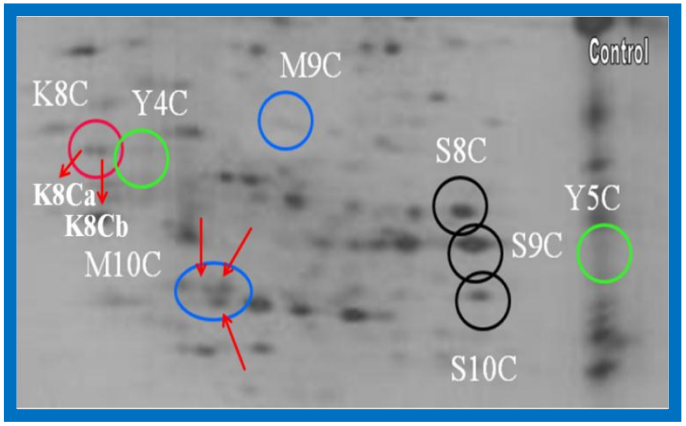
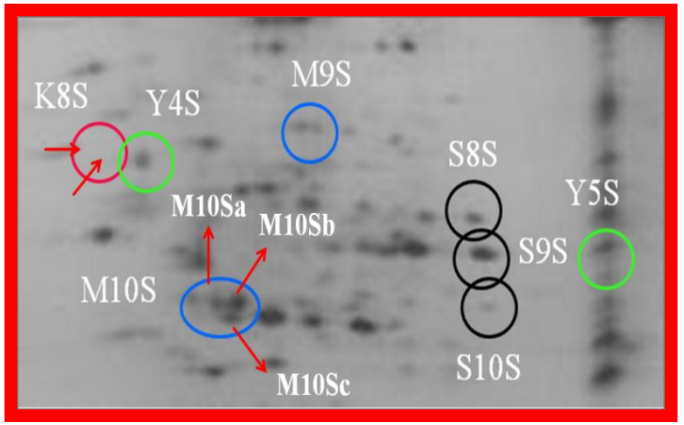
Table 4.1. Magnified Segments of Differentiated Protein Spots (cont.)

Spot No	Spot Name	CONTROL	BORTEZOMIB
22	S5C		
23	K5C		
24	K6C		
25	S6C		
26	K7C		
27	Y5S		
28	S8C		
29	S9C		
30	S10C		
31	M10Sc		
32	M10Sb		
33	M10Sa		
34	M9S		
35	Y4S		
36	K8Cb		
37	K8Ca		

After labeling, black and red colored spots were cut out from Control group while the others (green and blue ones) from Bortezomib applied group for enzymatic digestion by using trypsin. Then samples were analyzed by MALDI-TOF-TOF Mass Spectrometry and mass spectrometric data was compared to the protein database for sequence matches. If the amino acid sequence of a peptide could be identified, it would be used to find out the protein from which it was derived. In addition to this, in order to identify an unknown protein spot, it was necessary for a mass spectrometric analysis to match more than two peptide's sequences. In our experiments, protein spot determination was carried out thanks to parameters such as isoelectric point, molecular mass, sequence coverage, and mascot mowse score, but the desired peptide matches were not observed. Although we cannot say that the results obtained mass spectrometric analysis are definite, they are the most probable results because of their parameters and functions. These results were shown in Table 4.2.

Table 4.2 summarizes the 37 differentially expressed proteins that were characterized. The proteins were identified as follow:

Sixteen proteins were not identified due to some personal and instrumental errors during the experiment. These were Spot 1, 13, 16, 17, 19, 20, 21, 24, 26, 28, 30, 31, 32, 33, 36 and 37, namely M1S, S4Cb, K3C, K4C, M6S, M7S, M8S, K6C, S8C, S10C, M10Sc, M10Sb, M10Sa, K8Cb and K8Ca, respectively.

Spot 2, Caspase-3 is involved in the activation cascade of caspases responsible for apoptosis execution. At the onset of apoptosis, it proteolytically cleaves poly (ADP-ribose) polymerase (PARP) at a '216-Asp-|-Gly-217' bond and activates sterol regulatory element binding proteins (SREBPs) between the basic helix-loop-helix leucine zipper domain and the membrane attachment domain. Additionally, it cleaves and activates caspase-6, -7 and -9. Caspase-3 found in many cell lines, highest expression in cells of the immune system. Because of its function and role in apoptosis, after applying Bortezomib (17 nM) to MM U-266 cells, the increasing expression level of this protein was expected results.

Spot 3 were signed to uncharacterized protein C7orf46. Although its function does not know exactly, it is thought that this protein may be involved in alternative splicing.

Spot 18, C-type lectin domain family 5 member A has a function as a positive regulator of osteoclastogenesis. Thus, it was upregulated after the cells exposed to Bortezomib.

Table 4.2. Results of Differential Proteins Identified MALDI-TOF Mass Spectrometry

Spot No	Spot Name	Protein Identity	Matched Peptides	Sequence Coverage (%)	Mascot Mowse Score	Nominal Mass/pI (kDa)
1	M1S	Unidentified	-	-	-	-
2	M2S	CASP3 Caspase-3	MENTENSVDSKSIKNLEPKIIHGSESMDSGISLDNSYKMDYPEMGLCIIINNKNFHKST GMTSRSGDVDAANLRETFRNLKYEVRNKNDLTREEIVELMRDVSKEDHSKRSSFVCV LLSHGEEGIIFGTNGPVDLKKITNFFRGDRCRSLTGKPKLFIIQACRGTELDCGIETDS GVDDDMACHKIPVEADFLYAYSTAPGYYSWRNSKDGSWFIQSLCAMLKQYADKLEF MHILTRVNRKVATEFESFSFDATFHAKK QIPCIVSMLTKELYFYH	6%	20	31.5/6.09
3	M3S	CG046 Uncharacterized proteinC7orf46	MERLTLPLGGAAAVDEYLEYRRIVGEDDGGKLFTPEEYEEYKRKVLPLRLQNRLFV SWRSPTGMDCKLVGPETLCFCTHRYKQHKTDLEAIPQQCPIDLPCQVTGCQCRAYL YVPLNGSQPIRCRCKHFADQHSAAPGFTCNTCSKCSGFHSCFTCACGQPAYAHDTVV ETKQERLAQEKPVGQDIPYAAMGGLTGFSSLAEGYMRLDDSGIGVPSVEFLESPITAV DSPFLKAFQASSSSSPETLTDVGTSSQVSSLRRPEEDDMAFFERRYQERMKMEKAAK WKGKAPLPSATKPS	7%	21	33.0/6.33
4	M4S	UBE2C Ubiquitin-conjugating enzyme E2C	MASQNRDPAATSVAAARKGAEPSGGAARGPVGKRLQQELMTLMMSGDKGISAFPES DNLFKWVGTIHGAAGTVYEDLRYKLSLEFPSGYPYNAPTVKFLTPCYHPNVDTQGNI CLDILKEKWSALYDVRTILLSIQSLLGEPNIDSPLNTHAAELWKNPTAFKKYLQETYS KQVTSQEP	6%	21	19.6/6.82
5	K1C	PAXI (Paxillin)	MDDLDALLADLESTTSHISKRPVFLSEETPYSYPTGNHTYQEIAVPPPVPPPPSSEALN GTILDPLDQWQPSSSRFIHQQPQSSSPVYGSSAKTSSVSNPQDSVGSPCSRVGEEEHVY SFPNKQKSAEPSPTVMSTSLGSNLSELDRLLLELNAVQHNPPGFPADEANSSPPLPGAL SPLYGVPETNSPLGGKAGPLTKEKPKRNGGRGLEDVRPSVESLLDELESSVPSPVPAI TVNQGEMSSPQRVTSTQQQTRISASSATRELDELMASLSDFKIQGLEQRADGERCWA AGWPRDGGRSSPGGQDEGGFMAQGKTGSSSPPGGPPKPGSQLDSMLGSLQSDLNKL GVATVAKGVCGACKKPIAGQVVTAMGKTWHPEHFVCTHCQEEIGSRNFFERDGQP YCEKDYHNLFSPRCYYCNGPILDKVVTALDRTWHPEHFFCAQCGAFFGPEGFHEKD GKAYCRKDYFDMFAPKCGGCARAILENYISALNTLWHPECFVCRECFTPFVNGSFFE HDGQPYCEVHYHERRGSLCSGCQKPITGRCITAMAKKFHPEHFVCAFCLKQLNKGT FKEQNDKPYCQNCFLKLFC	2%	1	64.4/5.80
6	Y1S	GPX3 Glutathione peroxidase 3	MARLLQASCLLSLLLAGFVSQSRGQEKSKMDCHGGISGTIYEYGALTIDGEEYIPFKQ YAGKYVLFVNVASYUGLTGQYIELNALQEELAPFGLVILGFPCNQFGKQEPGENSEIL PTLKYVRPGGGFVPNFQLFEKGDVNGEKEQKFYTFLKNSCPPTSELLGTSDRLFWEP MKVHDIRWNFEKFLVGPDGIPIMRWHHRTTVSNVKMDILSYMRRQAALGVKRK	6%	21	25.5/8.26

cont. on next page

Table 4.2. Results of Differential Proteins Identified MALDI-TOF Mass Spectrometry (cont.)

7	Y2S	**M4K1 Mitogen-activated protein kinase kinase kinase kinase 1**	**MDVVDPDIFNRDPRDHYDLLQRLGGGTYGEVFKARDKVSGDLVALKMVKMEPDDD VSTLQKEILILKTCRHANIVAYHGSYLWLQKLWICMEFCGAGSLQDIYQVTGSLSEL QISYVCREVLQGLAYLHSQKKIHRDIKGANILINDAGEVRLADFGISAQIGATLARRLS FIGTPYWMAPEVAAVALKGGYNELCDIWSLGITAIELAELQPPLFDVHPLRVLFLMT KSGYQPPRLKEKGKWSAAFHNFIKVTLTKSPKKRPSATKMLSHQLVSQPGLNRGLIL DLLDKLKNPGKGPSIGDIEDEEPELPPAIPRRIRSTHRSSSLGIPDADCCRRHMEFRKL RGMETRPPANTARLQPPRDLRSSSPRKQLSESSDDDYDDVDIPTPAEDTPPPLPPKPKF RSPSDEGPGSMGDDGQLSPGVLVRCASGPPPNSPRPGPPPSTSSPHLTAHSEPSLWNPP SRELDKPPLLPPKKEKMKRKGCALLVKLFNGCPLRIHSTAAWTHPSTKDQHLLLGA EEGIFILNRNDQEATLEMLFPSRTTWVYSINNVLMSLSGKTPHLYSHSILGLLERKET RAGNPIAHISPHRLLARKNMVSTKIQDTKGCRACCVAEGASSGGPFLCGALETSVVL LQWYQPMNKFLLVRQVLFPLPTPLSVFALLTGPGSELPAVCIGVSPGRPGKSVLFHT VRFGALSCWLGEMSTEHRGPVQVTQVEEDMVMVLMDGSVKLVTPEGSPVRGLRTP EIPMTEAVEAVAMVGGQLQAFWKHGVQVWALGSDQLLQELRDPTLTFRLLGSPRL ECSGTISPHCNLLLPGSSNSPASASRVAGITGL**	1%	11	91.2/8.65
		SMAD7 Mothers against decapentaplegic homolog 7	**MFRTKRSALVRRLWRSRAPGGEDEEEGAGGGGGGGELRGEGATDSRAHGAGGGG PGRAGCCLGKAVRGAKGHHHPHPPAAGAGAAGGAEADLKALTHSVLKKLKERQLE LLLQAVESRGGTRTACLLLPGRLDCRLGPGAPAGAQPAQPPSSYSLPLLLCKVFRWP DLRHSSEVKRLCCCESYGKINPELVCCNPHHLSRLCELESPPPPYSRYPMDFLKPTAD CPDAVPSSAETGGTNYLAPGGLSDSQLLLEPGDRSHWCVVAYWEEKTRVGRLYCVQ EPSLDIFYDLPQGNGFCLGQLNSDNKSQLVQKVRSKIGCGIQLTREVDGVWVYNRSS YPIFIKSATLDNPDSRTLLVHKVFPGFSIKAFDYEKAYSLQRPNDHEFMQQPWTGFTV QISFVKGWGQCYTRQFISSCPCWLEVIFNSR**	3%	18	46.3/8.63
8	Y3S	**ST7 Suppressor of tumorigenicity 7 protein**	**MAEAATGFLEQLKSCIVWSWTYLWTVWFFIVLFLVYILRVPLKINDNLSTVSMFLNT LTPKFYVALTGTSSLISGLILIFEWWYFRKYGTSFIEQVSVSHLRPLLGGVDNNSSNNS NSSNGDSDSNRQSVSECKVWRNPLNLFRGAEYNRYTWVTGREPLTYYDMNLSAQDH QTFFTCDSDHLRPADAIMQKAWRERNPQARISAAHEALEINEIRSRVEVPLIASSTIWE IKLLPKCATAYILLAEEEATTIAEAEKLFKQALKAGDGCYRRSQQLQHHGSQYEAQH RRDTNVLVYIKRRLAMCARRLGRTREAVKMMRDLMKEFPLLSMFNIHENLLEALLE LQAYADVQAVLAKYDDISLPKSATICYTAALLKARAVSDKFSPEAASRRGLSTAEMN AVEAIHRAVEFNPHVPKYLLEMKSLILPPEHILKRGDSEAIAYAFFHLAHWKRVEGA LNLLHCTWEGTFRMIPYPLEKGHLFYPYPICTETADRELLPSFHEVSVYPKKELPFFI LFTAGLCSFTAMLALLTHQFPELMGVFAKAMIDIFCSAEFRDWNCKSIFMRVEDELE IPPAPQSQHFQN**	6%	24	67.1/6.82

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Table 4.2. Results of Differential Proteins Identified MALDI-TOF Mass Spectrometry (cont.)						
9	**S1C**	**BST2 Bone marrow stromal antigen 2**	**MASTSYDYCRVPMEDGDKRCKLLLGIGILVLLIIVILGVPLIIFTIKANSEACRDGLRA VMECRNVTHLLQQELTEAQKGFQDVEAQAATCNHTVMALMASLDAEKAQGQKKV EELEGEITTLNHKLQDASAEVERLRRENQVLSVRIADKKYYPSSQDSSSAAAPQLLIV LLGLSALLQ**	9%	22	19.7/5.43
		NFKB1 Nuclear factor NF-kappa-B p105 subunit	**MAEDDPYLGRPEQMFHLDPSLTHTIFNPEVFQPQMALPTDGPYLQILEQPKQRGFRF RYVCEGPSHGGLPGASSEKNKKSYPQVKICNYVGPAKVIVQLVTNGKNIHLHAHSLV GKHCEDGICTVTAGPKDMVVGFANLGILHVTKKKVFETLEARMTEACIRGYNPGLL VHPDLAYLQAEGGGDRQLGDREKELIRQAALQQTKEMDLSVVRLMFTAFLPDSTGS FTRRLEPVVSDAIYDSKAPNASNLKIVRMDRTAGCVTGGEEIYLLCDKVQKDDIQIRF YEEEENGGVWEGFGDFSPTDVHRQFAIVFKTPKYKDINITKPASVFVQLRRKSDLETS EPKPFLYYPEIKDKEEVQRKRQKLMPNFSDSFGGGSGAGAGGGGMFGSGGGGGGT GSTGPGYSFPHYGFPTYGGITFHPGTTKSNAGMKHGTMDTESKKDPEGCDKSDDKN TVNLFGKVIETTEQDQEPSEATVGNGEVTLTYATGTKEESAGVQDNLFLEKAMQLA KRHANALFDYAVTGDVKMLLAVQRHLTAVQDENGDSVLHLAIIHLHSQLVRDLLEV TSGLISDDIINMRNDLYQTPLHLAVITKQEDVVEDLLRAGADLSLLDRLGNSVLHLAA KEGHDKVLSILLKHKKAALLLDHPNGDGLNAIHLAMMSNSLPCLLLLVAAGADVNA QEQKSGRTALHLAVEHDNISLAGCLLLEGDAHVDSTTYDGTTPLHIAAGRGSTRLAA LLKAAGADPLVENFEPLYDLDDSWENAGEDEGVVPGTTPLDMATSWQVFDILNGKP YEPEFTSDDLLAQGDMKQLAEDVKLQLYKLLEIPDPDKNWATLAQKLGLGILNNAF RLSPAPSKTLMDNYEVSGGTVRELVEALRQMGYTEAIEVIQAASSPVKTTSQAHSLPL SPASTRQQIDELRDSDSVCDSGVETSFRKLSFTESLTSGASLLTLNKMPHDYGQEGPL EGKI**	1%	13	105.2/5.20
10	**S2C**	**MKNK1 MAP kinase-interacting serine/threonine-protein kinase 1**	**MVSSQKLEKPIEMGSSEPLPIADGDRRRKKKRRGRATDSLPGKFEDMYKLTSELLGE GAYAKVQGAVSLQNGKEYAVKIIEKQAGHSRSRVFREVETLYQCQGNKNILELIEFF EDDTRFYLVFEKLQGGSILAHIQKQKHFNEREASRVVRDVAAALDFLHTKDKVSLCH LGWSAMAPSGLTAAPTSLGSSDPPTSASQVAGTTGIAHRDLKPENILCESPEKVSPVKI CDFDLGSGMKLNNSCTPITTPELTTPCGSAEYMAPEVVEVFTDQATFYDKRCDLWSL GVVLYIMLSGYPPFVGHCGADCGWDRGEVCRVCQNKLFESIQEGKYEFPDKDWAHI SSEAKDLISKLLVRDAKQRLSAAQVLQHPWVQGQAPEKGLPTPQVLQRNSSTMDLT LFAAEAIALNRQLSQHEENELAEEPEALADGLCSMKLSPPCKSRLARRRALAQAGRG EDRSPPTAL**	2%	15	51.3/6.26
11	**S3C**	**SOCS3 Suppressor of cytokine signaling 3**	**MVTHSKFPAAGMSRPLDTSLRLKTFSSKSEYQLVVNAVRKLQESGFYWSAVTGGEA NLLLSAEPAGTFLIRDSSDQRHFFTLSVKTQSGTKNLRIQCEGGSFSLQSDPRSTQPVP RFDCVLKLVHHYMPPPGAPSFPSPPTEPSSEVPEQPSAQPLPGSPPRRAYYIYSGGEKI PLVLSRPLSSNVATLQHLCRKTVNGHLDSYEKVTQLPGPIREFLDQYDAPL**	7%	23	24.7/8.98

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Table 4.2. Results of Differential Proteins Identified MALDI-TOF Mass Spectrometry (cont.)

12	**S4Ca**	**TN13B Tumor necrosis factor ligand superfamily member 13B**	**MDDSTEREQSRLTSCLKKREEMKLKECVSILPRKESPSVRSSKDGKLLAATLLLALLS CCLTVVSFYQVAALQGDLASLRAELQGHHAEKLPAGAGAPKAGLEEAPAVTAGLKI FEPPAPGEGNSSQNSRNKRAVQGPEETVTQDCLQLIADSETPTIQKGSYTFVPWLLSF KRGSALEEKENKILVKETGYFFIYGQVLYTDKTYAMGHLIQRKKVHVFGDELSLVTL FRCIQNMPETLPNNSCYSAGIAKLEEGDELQLAIPRENAQISLDGDVTFFGALKLL**	2%	5	31.2/5.93
13	**S4Cb**	**Unidentified**	-	-	-	-
14	**K9C**	**SIT1 Signaling trashold-regulating transmembrane adapter 1**	**MNQADPRLRAVCLWTLTSAAMSRGDNCTDLLALGIPSITQAWGLWVLLGAVTLLFL ISLAAHLSQWTRGRSRSHPGQGRSGESVEEVPLYGNLHYLQTGRLSQDPEPDQQDPT LGGPARAAEEVMCYTSLQLRPPQGRIPGPGTPVKYSEVVLDSEPKSQASGPEPELYAS VCAQTRRARASFPDQAYAN SQPAAS**	9%	22	21.1/5.91
15	**K2C**	**CAR14 Caspase recruitment domain-containing protein 14**	**MGELCRRDSALTALDEETLWEMMESHRHRIVRCICPSRLTPYLRQAKVLCQLDEEE VLHSPRLTNSAMRAGHLLDLLKTRGKNGAIAFLESLKFHNPDVYTLVTGLQPDVDFS NFSGLMETSKLTECLAGAIGSLQEELNQEKGQKEVLLRRCQQLQEHLGLAETRAEG LHQLEADHSRMKREVSAHFHEVLRLKDEMLSLSLHYSNALQEKELAASRCRSLQEE LYLLKQELQRANMVSSCELELQEQSLRTASDQESGDEELNRLKEENEKLRSLTFSLA EKDILEQSLDEARGSRQELVERIHSLRERAVAAERQREQYWEEKEQTLLQFQKSKM ACQLYREKVNALQAQVCELQKERDQAYSARS AQREISQSLVEKDSLRRQVFELTDQVCELRTQLRQLQAEPPGVLKQEARTREPCPRE KQRLVRMHAICPRDDSDCSLVSSTESQLLSDLSATSSRELVDSFRSSSPAPPSQQSLYK RVAEDFGEEPWSFSSCLEIPEGDPGALPGAKAGDPHLDYELLDTADLPQLESSLQPVS PGRLDVSESGVLMRRRPARRILSQVTMLAFQGDALLEQISVIGGNLTGIFIHRVTPGS AADQMALRPGTQIVMVDYEASEPLFKAVLEDTTLEEAVGLLRRVDGFCCLSVKVNT DGYKRLLQDLEAKVATSGDSFYIRVNLAMEGRAKGELQVHCNEVLHVTDTMFQGC GCWHAHRVNSYTMKDTAAHGTIPNYSRAQQQLIALIQDMTQQCTVTRKPSSGGPQK LVRIVSMDKAKASPLRLSFDRGQLDPSRMEGSSTCFWAESCLTLVPYTLVRPHRPAR PRPVLLVPRAVGKILSEKLCLLQGFKKCLAEYLSQEEYEAWSQRGDIIQEGEVSGGR CWVTRHAVESLMEKNTHALLDVQLDSVCTLHRMDIFPIVIHVSVNEKMAKKLKKGL QRLGTSEEQLLEAARQEEGDLDRAPCLYSSLAPDGWSDLDGLLSCVRQAIADEQKK VVWTEQSPR**	1%	11	113.1/5.65
16	**K3C**	**Unidentified**	-	-	-	-
17	**K4C**	**Unidentified**	-	-	-	-

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Table 4.2. Results of Differential Proteins Identified MALDI-TOF Mass Spectrometry (cont.)

18	**M5S**	**CLC5A C-type lectin domain family 5 member A**	**MNWHMIISGLIVVVLKVVGMTLFLLYFPQIFNKSNDGFTTTRSYGTVSQIFGSSSPSPN GFITTRSYGTVCPKDWEFYQARCFFLSTSESSWNESRDFCKGKGSTLAIVNTPEKLKF LQDITDAEKYFIGLIYHREEKRWRWINNSVFNGNVTNQNQNFNCATIGLTKTFDAAS CDISYRRICEKNAK**	6%	21	21.5/9.04
19	**M6S**	**Unidentified**	-	-	-	-
20	**M7S**	**Unidentified**	-	-	-	-
21	**M8S**	**Unidentified**	-	-	-	
22	**S5C**	**CHIP E3 ubiquitin-protein ligase CHIP**	**MKGKEEKEGGARLGAGGGSPEKSPSAQELKEQGNRLFVGRKYPEAAACYGRAITRP LVAVYYTNRALCYLKQQHEQALADCRRALELDGQSVKAHFFLGQCQLEMESYDEAI ANLQRAYSLAKEQRLNFGDDIPSALRIAKKKRWNSIEERRIHQESELHSYLSRLIAAE RERELEECQRNHEGDEDDSHVRAQQACIEAKHDKYMADMDELFSQVDEKRKKRDI PDYLCGKISFELMREPCITPSGITYDRKDIEEHLQRVGHFDPVTRSPLTQEQLIPNLAM KEVIDAFISENGWVEDY**	7%	22	34.8/5.61
23	**K5C**	**IKBD NF-kappa-B inhibitor delta**	**MEAGPWRVSAPPSGPPQFPAVVPGPSLEVARAHMLALGPQQLLAQDEEGDTLLHLF AARGLRWAAYAAAEVLQVYRRLDIREHKGKTPLLVAAAANQPLIVEDLLNLGAEPN AADHQGRSVLHVAATYGLPGVLLAVLNSGVQVDLEARDFEGLTPLHTAILALNVAM RPSDLCPRVLSTQARDRLDCVHMLLQMGANHTSQEIKSNKTVLHLAVQAANPTLVQ LLLELPRGDLRTFVNMKAHGNTALHMAAALPPGPAQEAIVRHLLAAGADPTLRNLE NEQPVHLLRPGPGPEGLRQLLKRSRVAPPG LSS**	3%	3	33.4/6.90
24	**K6C**	**Unidentified**	-	-	-	-
25	**S6C**	**TNR17 Tumor necrosis factor receptor superfamily member 17**	**MLQMAGQCSQNEYFDSLLHACIPCQLRCSSNTPPLTCQRYCNASVTNSVKGTNAILW TCLGLSLIISLAVFVLMFLLRKINSEPLKDEFKNTGSGLLGMANIDLEKSRTGDEIILP RGLEYTVEECTCEDCIKSKPKVDSDHCFPLPAME EGATILVTTKTNDYCKSLPAALSATEIEKSISAR**	6%	21	20.1/5.24
26	**K7C**	**Unidentified**	-	-	-	-

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Table 4.2. Results of Differential Proteins Identified MALDI-TOF Mass Spectrometry (cont.)

27	Y5S	SIVA Apoptosis Regulatory Protein Siva	MPKRSCPFADVAPLQLKVRVSQRELSRGVCAERYSQEVFEKTKRLLFLGAQAYLDH VWDEGCAVVHLPESPKPGPTGAPRAARGQMLIGPDGRLIRSLGQASEADPSGVA SIACSSCVRAVDGKAVCGQCERALCGQCVRTCWGCGSVACTLCGLVDCSDMYEKV LCTSCAMFET	14%	25	18.6/7.86
28	S8C	Unidentified	-	-	-	-
29	S9C	PYDC1 Pyrin domain-containing protein 1	MGTKREAILKVLENLTPEELKKFKMKLGTVPLREGFERIPRGALGQLDIVDLTDKLV ASYYEDYAAELVVAVLRDMRMLEEAARLQRAA	17%	25	10.1/6.29
		PSB3 Proteasome subunit beta type-3	MSIMSYNGGAVMAMKGKNCVAIAADRRFGIQAQMVTTDFQKIFPMGDRLYIGLAG LATDVQTVAQRLKFRLNLYELKEGRQIKPYTLMSMVANLLYEKRFGPYYTEPVIA GLDPKTFKPFICSLDLIGCPMVTDDFVVSGTCAEQMYGMCESLWEPNMDPDHLFETI SQAMLNAVDRDAVSGMGVIVHIIEKDKITTRTLKARMD	12%	14	22.9/6.14
		LY6K Lymphocyte antigen 6K	MALLALLLVVALPRVWTDANLTARQRDPEDSQRTDEGDNRVWCHVCERENTFECQ NPRRCKWTEPYCVIAAVKIFPRFFMVAKQCSAGCAAMERPKPEEKRFLLEEPMPFFY LKCCKIRYCNLEGPPINSSVFKEYAGSMGESCGGLWLAILLLLASIAAGLSLS	10%	3	18.6/7.44
30	S10C	Unidentified	-	-	-	-
31	M10Sc	Unidentified	-	-	-	-
32	M10Sb	Unidentified	-	-	-	-
33	M10Sa	Unidentified	-	-	-	-
34	M9S	RAB25 (Ras-related protein Rab-25)	MGNGTEEDYNFVFKVVLIGESGVGKTNLLSRFTRNEFSHDSRTTIGVEFSTRTVMLG TAAVKAQIWDTAGLERYRAITSAYYRGAVGALLVFDLTKHQTYAVVERWLKELYD HAEATIVVMLVGNKSDLSQAREVPTEEARMFAENNGLLFLETSALDSTNVELAFETV LKEIFAKVSKQRQNSIRTNAITLGSAQAGQEPGPGEKRACCISL	4%	1	23.4/5.72

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Table 4.2. Results of Differential Proteins Identified MALDI-TOF Mass Spectrometry (cont.)						
35	Y4S	**RAB43 Ras-related protein Rab-43**	**MAGPGPGPGDPDEQYDFLFKLVLVGDASVGKTCVVQRFKTGAFSERQGSTIGVDFT MKTLEIQGKRVKLQIWDTAGQERFRTITQSYYRSANGAILAYDITKRSSFLSVPHWIE DVRKYAGSNIVQLLIGNKSDLSELREVSLAEAQSLAEHYDILCAIETSAKDSSNVEEAF LRVATELIMRHGGPLFS EKSPDHIQLNSKDIGEGWGCGC**	11%	24	23.3/5.44
		IL32 Interleukin-32	**MCFPKVLSDDMKKLKARMVMLLPTSAQGLGAWVSACDTEDTVGHLGPWRDKDPA LWCQLCLSSQHQAIERFYDKMQNAESGRGQVMSSLAELEDDFKEGYLETVAAYYEE QHPELTPLLEKERDGLRCRGNRSPVPDVEDPATEEPGESFCDKVMRWFQAMLQRLQ TWWHGVLAWVKEKVVALVHAVQALWKQFQSFCCSLSELFMSSFQSYGAPRGDKEE LTPQKCSEPQSSK**	10%	23	26.6/5.14
		MYL6 Myosin light polypeptide 6	**MCDFTEDQTAEFKEAFQLFDRTGDGKILYSQCGDVMRALGQNPTNAEVLKVLGNPK SDEMNVKVLDFEHFLPMLQTVAKNKDQGTYEDYVEGLRVFDKEGNGTVMGAEIRH VLVTLGEKMTEEEVEMLVAGHEDSNGCINYEAFVRHILSG**	15%	24	16.9/4.56
36	K8Cb	**Unidentified**	-	-	-	-
37	K8Ca	**Unidentified**	-	-	-	-

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Spot 4, Ubiquitin-conjugating enzyme E2C accepts ubiquitin from the E1 complex and catalyzes its covalent attachment to other proteins. It catalyzes 'Lys-11'- and 'Lys-48'-linked polyubiquitination in vitro. Morover this protein acts as an essential factor of the anaphase promoting complex/cyclosome (APC/C), a cell cycle-regulated ubiquitin ligase that controls progression through mitosis. Acts by initiating 'Lys-11'-linked polyubiquitin chains on APC/C substrates, leading to the degradation of APC/C substrates by the proteasome and promoting mitotic exit. As this protein is responsible for controlling progression and has a role in protein ubiquitination, its expression level is increasing with the Bortezomib effect.

Spot 34, Ras-related protein Rab-25 is involved in the regulation of cell survival. Similar to spot 2, 4, and 18, its expression degree increases in response to Bortezomib.

Spot 5, Paxillin is a cytoskeletal protein involved in actin-membrane attachment at sites of cell adhesion to the extracellular matrix (focal adhesion). With the applying anticancer agents to the cells, it was lost.

Spot 14, Signaling trashold-regulating transmembrane adapter 1 negatively regulates TCR (T-cell antigen receptor)-mediated signaling in T-cells. It is also involved in positive selection of T-cells. It is specifically expressed in T and B-cells and present in plasma cells but not in germinal center B-cells (at protein level). Furthermore, it is expressed in T- and B-cell lymphoma. Like spot 5, it was lost with the Bortezomib.

Spot 15, Caspase recruitment domain-containing protein 14 activates NF-κB via BCL10 and IKK and stimulates the phosphorylation of BCL10. The main goal of Bortezomib is inhibit the proteasomes for preventing the IκB degredation and accordingly NF-κB activation, so it is very required to lose of this protein as expected.

Spot 23, NF-κB inhibitor delta may regulate the expression of IL-2, IL-6, and other cytokines through regulation on NF-κB activity, similarly to spot 15.

Spot 6, Glutathione peroxidase 3 protects cells and enzymes from oxidative damage, by catalyzing the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxide, by glutathione. So, it is an example of newly formed protein after the Bortezomib effects upon MM U-266 cells.

For spot 7, we obtained two proteins that have the change to be the correct one after the mass spectrometric analysis. One of them was Mitogen-activated protein kinase kinase kinase kinase 1 which may play a role in the response to environmental stress. This protein appears to act upstream of the JUN N-terminal pathway and might play a role in hematopoietic lineage decisions and growth regulation. As well as, it is

expressed primarily in hematopoietic organs especially bone marrow. After the Bortezomib, the afore-mentioned protein came to light to carry out its function. The other protein was Mothers against decapentaplegic homolog 7 that is involved in cell signaling.

Spot 8, Suppressor of tumorigenicity 7 protein may act as a tumor suppressor. It is another newly formed protein.

Spot 27, Apoptosis Regulatory Protein Siva induces CD27-mediated apoptosis and inhibits BCL2L1 isoform Bcl-x(L) anti-apoptotic activity. It is also inhibits activation of NF-κB and promotes T-cell receptor-mediated apoptosis. So, generation of this protein after the Bortezomib stress is vital for cancerous cells.

Spot 35 has three opportunities since it did not determine exactly. One of them was Ras-related protein Rab-43. The others were Interleukin-32 and Myosin light polypeptide 6. IL-32 is a cytokine that may play a role in innate and adaptive immune responses. It induces various cytokines such as TNFA/TNF-alpha and IL8 and activates typical cytokine signal pathways of NF-κB and p38 MAPK.

Spot 9 has two alternatives: Bone marrow stromal antigen 2 and NF-κB p105 subunit. First protein may be involved in the sorting of secreted proteins and pre-B-cell growth. Also it may play a role in B-cell activation. NF-κB is a pleiotropic transcription factor which is present in almost all cell types and is involved in many biological processed such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. NF-κB is a homo- or heterodimeric complex formed by the Rel-like domain-containing proteins RELA/p65, RELB, NFKB1/p105, NF-κB1/p50, REL and NF-κB2/p52 and the heterodimeric p65-p50 complex appears to be most abundant one. The dimers bind at κB sites in the DNA of their target genes and the individual dimers have distinct preferences for different κB sites that they can bind with distinguishable affinity and specificity. Different dimer combinations act as transcriptional activators or repressors, respectively. NF-κB is controlled by various mechanisms of post-translational modification and subcellular compartmentalization as well as by interactions with other cofactors or corepressors. NF-κB complexes are held in the cytoplasm in an inactive state complexed with members of the NF-κB inhibitor (IκB) family. In a conventional activation pathway, IκB is phosphorylated by IκB kinases (IKKs) in response to different activators, subsequently degraded thus liberating the active NF-κB complex which translocates to the nucleus. NF-κB heterodimeric p65-p50 and RelB-p50 complexes are transcriptional activators. The NF-κB p50-p50 homodimer

is a transcriptional repressor, but can act as a transcriptional activator when associated with BCL3. NF-κB1 appears to have dual functions such as cytoplasmic retention of attached NF-κB proteins by p105 and generation of p50 by a cotranslational processing. The proteasome-mediated process ensures the production of both p50 and p105 and preserves their independent function, although processing of NF-κB1/p105 also appears to occur post-translationally. p50 binds to the κB consensus sequence 5'-GGRNNYYCC-3', located in the enhancer region of genes involved in immune response and acute phase reactions. In a complex with MAP3K8, NF-κB1/p105 represses MAP3K8-induced MAPK signaling; active MAP3K8 is released by proteasome-dependent degradation of NF-κB1/p105.

Spot 10, MAP kinase-interacting serine/threonine-protein kinase 1 may play a role in the response to environmental stress and cytokines.

Spot 11, Suppressor of cytokine signaling 3 is a SOCS family protein. It forms part of a classical negative feedback system that regulates cytokine signal transduction. SOCS3 is involved in negative regulation of cytokines that signal through the JAK/STAT pathway.

Spot 12, Tumor necrosis factor ligand superfamily member 13B promotes the survival of mature B-cells and the B-cell response.

Spot 22, E3 ubiquitin-protein ligase CHIP which targets misfolded chaperone substrates towards proteasomal degradation. It has a central role in protein modification and protein ubiquitination.

Spot 25, Tumor necrosis factor receptor superfamily member 17 promotes B-cell survival and plays a role in the regulation of humoral immunity and activates NF-κB and JNK. It is expressed in mature B-cells.

Spot 29 did not identified clearly. Pyrin domain-containing protein 1 associates with apoptosis-associated specklike protein containing a CARD domain and modulates its ability to collaborate with pyrin and cryopyrin in NF-κB and procaspase-1 activation. Suppresses kinase activity of NF-κB inhibitor kinase (IκK) complex, expression of NF-κB inducible genes and inhibits NF-κB activation by cytokines. Proteasome subunit beta type-3 is a multicatalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity. Lymphocyte antigen 6K may play a role in cell growth. After Bortezomib was applied onto MM U-266 cells, spot 9, 10, 11, 12, 22, 25, and 29 were down regulated.

CHAPTER 5

CONCLUSIONS

Multiple Myeloma is a malignant plasma cell disorder and the second most common hematological disorder (Dalton et al., 2001). Unfortunately, its incidence is increasing day by day. Chemical agents and natural products which may have strong anticancer potential always attract the attention of clinicians and basic science researchers. Bortezomib is one of them, known as proteasome inhibitor that has entered clinical trials for Multiple Myeloma (Mateos and Miguel., 2007). Until now, the effect mechanism of Bortezomib on Multiple Myeloma has been tried to explain by molecular level but, to the best of our knowledge, there is no any studies has been pursued about the changes in protein profiles of Bortezomib applied Multiple Myeloma cells (U-266) in the literature. It has a vital significance to investigate protein profiles in terms of understanding the dynamic alterations of cellular proteins for diagnostic and therapeutic options.

In this study, it was demonstrated anticancer potentials (both cytotoxic and apoptotic effects) of Bortezomib on human MM U-266 cells. In addition to this, we have identified the differentially expressed proteins of U-266 cells when they exposed to Bortezomib with an enough concentration, for the first time. Because the function of the differential expressed proteins are of great importance for new targeted teurapautic options both Multiple Myeloma and other cancer types.

In order to achieve our aims in the project, variety of multidisciplinary subjects came together. Cancer research techniques, biochemical studies and proteomics were combined. Protein profiling provides a much better understanding of an organism, in terms of structure and function. Use of protein profiling in the study of multiple proteins, protein forms, and protein families almost always by comparing two different states (diseased vs. healthy or treated vs. untreated) is expected to expand our understanding of molecular mechanisms. At the first experiment part, the cytotoxic effect was determined by measuring the IC50 value of Bortezomib at 72 h using XTT cell proliferation assay. It was calculated from cell proliferation plots and found to be 17 nM. Then, apoptosis was evaluated by measuring the changes in caspase-3 enzyme

activity and loss of mitochondrial membrane potential (MMP). Treatment of U-266 cells for 72 h with 20 nM Bortezomib caused a significant loss of MMP (about 2.14-fold), as measured by increased accumulation of cytoplasmic mitochondrial form of JC-1 and also resulted in 1.17-fold increase in caspase-3 activity.

After the 2D PAGE analysis, so as to elucidate and compare protein profiles of two groups at issue, two individual gel images were imported into gel analysis software DECODON Delta2D Version 4.3. With the help of software, these 2D PAGE gels were superposed and comparative detailed analysis was performed. According to the analysis, 37 protein spots were differentially expressed. Among them, 5 proteins were newly formed, 10 proteins lost, 12 proteins were up-regulated and 10 proteins were down-regulated as compared to control group (untreated cells). These differentiated proteins (increased and/or decreased or appear and/or disappear) were recovered from gel to be applied for MALDI-TOF Mass Spectrometry for protein identification and their function was evaluated. While sixteen proteins were not identified because of some personal and instrumental errors, twenty one of them were determined (it is considered that there were not seen two or more than peptide matches in the results). According to results, six proteins are related with cell signaling (Spot 7, 11, 14, 15, 23, 29), one protein has a role in cell survival (Spot 34), one is cell cycle regulator (Spot 4), two proteins are involved in apoptosis (Spot 2, 27), one protein is concerning tumor suppressor (Spot 8),five proteins take a key role in immunity and defense mechanism of the body (Spot 9, 12, 18, 25, 35), one protein is involved in protein modification and protein ubiquitination (Spot 22) and three of them has other functions (Spot5, 6, 10).

For the further step studies, the same anti-cancer agent can be tried to treat other cancer types, with the help of differentially expressed proteins. Additionally, the data obtained by this study can be used to improve new treatments for Multiple Myeloma by drug designers.

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APPENDIX A

MEDIAS

A.1. RPMI-1640 Growth Medium

RPMI-1640, penicillin-streptomycin and fetal bovine serum (FBS) were obtained from Invitrogen (Paisley, UK).

- ✓ 60 ml FBS (~10% of all volume) and
- ✓ 10 ml Penicillin-Streptomycin (~1%)

were added to

- ✓ 500 ml RPMI-1640 and mixed.

APPENDIX B

CHEMICALS, REAGENTS AND SOLUTIONS

B.1. Bortezomib

Bortezomib was obtained from LCLabs (USA). The stock solution of Bortezomib was prepared in dimethyl sulfoxide (DMSO) at a concentration of 2,6 mmol/ml (1,5 mg of Bortezomib was dissolved in 1 ml DMSO), stored at -20^{o}C and diluted in cell culture medium. Molecular weight of Bortezomib is 384.24 g/mol.

B.2. MM U-266 Cells

U-266 human MM cells were kindly obtained from the German Collection of Microorganisms and Cell Cultures (Germany).

Table B.1. Chemicals and Reagents Used in Experiments

NO	CHEMICALS	COMPANY
1	Dimethyl Sulfoxide (DMSO)	Sigma
2	Trypan Blue Dye	Sigma
3	Phosphate Buffered Saline (PBS)	Invitrogen
4	Penicillin-Streptomycin	Invitrogen
5	Fetal Bovine Serum (FBS)	Invitrogen
6*	XTT Reagent (should not be exposed to light)	Biological Industries
7*	Activation Reagent (should not be exposed to light)	Biological Industries
8**	*DEVD-p*NA (should not be exposed to light) (Stored at -20^{o}C)	BioVision
9**	Cell Lysis Buffer (Stored at +4^{o}C)	BioVision
10**	2X Reaction Buffer (Stored at -20^{o}C)	BioVision
11**	Dithiothreitol (DTT) (Stored at -20^{o}C)	BioVision

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Table B.1. Chemicals and Reagents Used in Experiments (cont.)		
12	Bovine Serum Albumine (BSA)	Sigma
13	Coomassie Brilliant Blue G-250 (CBB G-250) Dye	AppliChem
14	Absolute Ethanol	AppliChem
15	Phosphoric Acid	AppliChem
16***	JC-1 Reagent 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (lyophilized)	Cell Technology
17***	10X Assay Buffer	Cell Technology
18	Urea (8M)	AppliChem
19	Thiourea	AppliChem
20	Glycerol	AppliChem
21	Ampholites (3-10) (%0.2)	Fluka
22	Bromophenol Blue (%0.5)	AppliChem
23	CHAPS (%2)	AppliChem
24	Agarose	AppliChem
25	SDS	AppliChem
26	phosphoric acid	AppliChem
27	Iodoacetamide	Sigma
28	Acrylamide	AppliChem
29	Bisacrylamide	AppliChem
30	1.5 M Tris – HCl pH = 8.8	AppliChem
31	Ammoniun Persulfate (APS)	Sigma
32	Tetramethylethylenediamine (TEMED)	Sigma

- 6,7* are XTT Cell Proliferation Kit Components,
- 8, 9, 10, 11** are Caspase-3 Colorimetric Assay Kit Components,
- 16, 17*** are APO LOGIX JC-1 Mitochondrial Membrane Potential Detection Kit Components.

B.3. XTT Cell Proliferation Assay Kit

XTT Cell Proliferation Assay Kit was obtained from Biological Industries, Israel.

B.3.1. XTT Reaction Solution

In order to prepare a reaction solution sufficient for a 96-well plate, XTT reagent was defrosted and the Activation reagent was immediately defrosted prior to use in a 37^{o}C bath. Then 0,1 ml Activation reagent was added to 5 ml XTT reagent.

B.4. Caspase-3 Colorimetric Assay Kit

Caspase-3 colorimetric assay kit was obtained from BioVision, (USA).

B.4.1. 2X Reaction Solution

DTT was added to the 2X Reaction Buffer immediately before use.

- ✓ 10 mM final concentration :Add 10 µl of 1.0 M DTT stock per 1 ml of 2X Reaction Buffer.

Table B.2. Preparation of 2X Reaction Buffer

2X Reaction Solution ml	**2X Reaction Buffer µl**	**Dithiothreitol DTT µl**
1.0	990	10
0,6	594	6.0

B.4.2. Stock BSA Standard (1 mg/ml)

0.01 g of BSA was dissolved in 10 ml PBS.

B.4.3. Standard Curve for BSA

Table B.3. Absorbance Values for BSA Standards

Concentrations (µg/µl)	Absorbances (595 nm)
1	**0,1775**
2	**0,182**
4	**0,191**
8	**0,209**
20	**0,263**
40	**0,353**

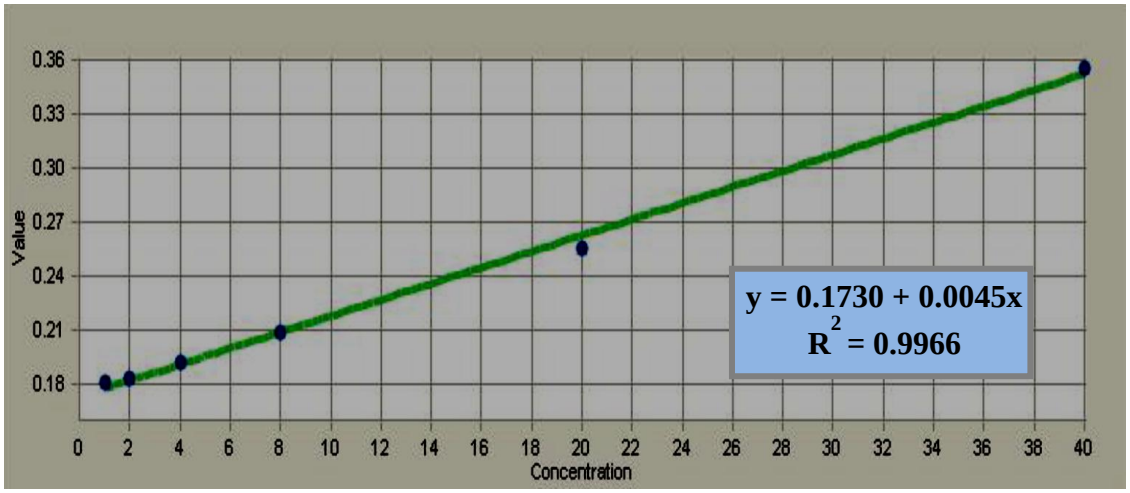

Figure B.1. Standard Curve for BSA

B.4.4. Coomassie Brilliant Blue G-250 (CBB G-250) Dye

- ✓ 100 mg Coomassie Brilliant Blue G-250 (CBB G-250)
- ✓ 50 ml Absolute Ethanol
- ✓ 100 ml 85% Phosphoric Acid

The above-mentioned mass of CBB G-250 was weighed and dissolved in 50 ml of absolute ethanol. Then, 100 ml of 85% Phosphoric Acid was added to this solution. After the overall solution was filtered through filter paper, the filtered solution (~15-20 ml) was diluted to 1 liter with ultrapure water and stored at 4^{o}C.

B.5. APO LOGIX JC-1 Mitochondrial Membrane Potential Detection Kit

APO LOGIX JC-1 Mitochondrial Membrane Potential Detection Kit was purchased from Cell Technology, USA.

B.5.1. JC-1 Reagent

The lyophilized vial was reconstituted with 500 µl DMSO to obtain a 100X stock solution. Following that, it was mixed by inverting several times at room temperature until contents were completely dissolved. Next, resuspended JC-1 reagent was aliquated in small amountssufficient for one day experimental work and stored at -20^{o}C. When it was used, it was diluated 1:100 in 1X Assay Buffer (100X JC-1 reagent to 1X) immediately prior to use.

B.5.2. 10X Assay Buffer

10X Assay Buffer were diluted 1:10 with ultrapure water (1 ml 10X Assay Buffer + 9 ml UP Water).

B.6. 2D PAGE

B.6.1. Isoelectric Focusing (IEF)

B.6.1.1. Preparation of Rehydration Buffer

- ✓ It was used for rehydration of strips.
- ✓ This protocol was for including proteins in buffer.

Preparation of Rehydration Buffer		
Urea (8M)	4.8 g	**Total Volume = 10 ml**
CHAPS (%2)	0.2 g	
DTT (50 mM)	0.077 g	
Ampholites (3-10) (%0.2)	100 µl	
Bromophenol Blue (%0.5)	1-2 µl	

- ✓ For urea was increasing the volume of liquid when dissolved, firstly, it was dissolved in 4ml ddH_2O. Then the other chemicals were added, total solution was measured to see its volume and the final volume was bringed to 10 ml.
- ✓ Ampholites was diluted to 1X from the 10X stock concentration. The ampholites concentration in total volume is %0.2.
- ✓ Nearly, 1-2 µl Bromophenol Blue (%0.5) was used just to have color in buffer.

B.6.1.2. Preparation of Equilibration Buffer I and II Stock

Equilibration Buffer I (20 ml)	Equilibration Buffer II (20 ml)
7.2 g / 20 ml Urea	7.2 g / 20 ml Urea
0.4 g / 20 ml SDS	0.4 g / 20 ml SDS
5 ml Ultrapura H_2O	5 ml Ultrapura H_2O
5 ml 1.5 M Tris – HCl pH = 8.8	5 ml 1.5 M Tris – HCl pH = 8.8
0.4 g / 20 ml DTT	-
4 ml Glycerol	4 ml Glycerol
-	0.5 g / 20 ml Iodoacetamide*

* Iodoacetamide should be added freshly. (in Day 3).

After preparation, Equilibration Buffer I was lifted to +4^{o}C while Equilibration Buffer II was kept at -20 ^{o}C.

B.6.2. SDS PAGE

B.6.2.1. Preparation of Separating Gel

Separating Gel (%12) for 2D PAGE		
Acrylamide / Bisacrylamide (%30)	32 ml	**Total** **80 ml**
dH_2O	26.8 ml	
1.5 M Tris – HCl pH = 8.8	20 ml	
SDS (%10)	800 µl	
Ammoniun Persulfate (APS) (%10)	400 µl	
Tetramethylethylenediamine (TEMED)	40 µl	

✓ 33 ml Separating Gel was loaded for each gel.

B.6.2.1.1. Preparation of Separating Gel Components

STOCK REAGENT PREPARATION		
A.	**Acrylamide / Bisacrylamide (30% T, 2.67% C)**	
	Acrylamide	146.0 g
	N, N'- Methylene-bis Acrylamide	4.0 g
	✶ (Preweighed Acrylamide/Bis 37.5:1 mixture may be substituted) ✶ Distilled water to 500ml. Filter and store at 4°C in the dark. Maximum shelf life under these conditions is 30 days.	
B.	**1.5 M Tris- HCl, pH 8.8**	
	✶ 54.45 g Tris base ✶ 150 ml. Distilled water ✶ Adjusted to pH 8.8 with HCl, Distilled water to 300ml. Store at 4°C	
C.	**0.5 M Tris- HCl, pH 6.8**	
	✶ 6 g Tris base ✶ 60 ml distilled water ✶ Adjusted to pH 6.8 with HCl, Distilled water to 100ml. Store at 4°C	
D.	**10% (w/v) SDS**	
	✶ Dissolve 10 g SDS in 60 ml water with gentle stirring. Distilled water to 100 ml.	

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STOCK REAGENT PREPARATION (cont.)		
E.	**10% Ammonium Persulfade (w/v)**	
	✶ Dissolve 100 mg ammonium persulfate in 1 ml distilled water	
F.	**Sample Buffer** (SDS reducing buffer: 62.5 Mm Tris- HCl, pH 6.8, 20% Glycerol, 2% SDS, 5% β- Mercaptoethanol)	
	Distilled water	3.0 ml
	0.5 M Tris- HCl, pH 6.8	1.0 ml
	Glycerol	1.6 ml
	10% SDS	1.6 ml
	β- Mercaptoethanol	0.4 ml
	0.5% (w/v) bromophenol blue (in water)	0.4 ml
	Total	8.0 ml
	✶ Dillute the sample at least 1:4 with sample buffer. Heat at 95°C for 4 minutes.	
G.	**5x Running Buffer (1x= 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3)**	
	Tris base	45.0 g
	Glycine	216.0 g
	SDS	15.0 g
	✶ Distilled water to 3 L. Do not adjust the pH with acid or base. Store at 4°C. Warm to 37°C before use if precipitation occurs. Dillute 300 ml 5x stock with 1.2 L distilled water for one electrophoretic run. ✶ To prepare 100 ml of 1X TGS buffer, 20 ml of 5X TGS buffer was diluted to a final volume of 100 ml with ultra pure water.	

B.6.2.2. Preparation of Overlay Agarose

SDS Electrophoresis Buffer		
Tris Base	3.03 g	
Glycine	14.40 g	
SDS	1.0 g	
✶ were dissolved in 1000 ml of distilled water		
Overlay Agarose		
Agarose	0.50 g	✶ stored at 4°C
Bromophenol Blue	2.0 mg	
SDS Electrophoresis Buffer	100 ml	

B.6.2.3. Preparation of In-Gel Digestion Chemicals

- ✓ Ammonium Bicarbonate (100 mM) : 0.2 g of ammonium bicarbonate was dissolved in 20 ml of water.
- ✓ Ammonium Bicarbonate (50 mM) : 2 ml of 100 mM ammonium bicarbonate was mixed with 2 ml of water.
- ✓ DTT (10 mM) : 1.5 mg of dithiothreitol was placed in a 1.5 ml plastic centrifuge tube followed by addition of 1 ml of 100 mM ammonium bicarbonate for complete dissolving of DTT.
- ✓ Iodoacetamide (100 mM) : 18 mg of iodoacetamide was placed in a 1.5 ml plastic centrifuge tube followed by addition of 1 ml of 100 mM ammonium bicarbonate for complete dissolving of iodoacetamide.
- ✓ Trypsin Solution : 1 ml of ice cold 50 mM ammonium bicarbonate was added to 20 µg of sequencing-grade modified trypsin (V5111; Promega) and dissolved by drawing the solution into and out of the pipette. The trypsin solution was kept on ice until use. The final concentration is 20 ng/ml trypsin.
- ✓ Extraction Buffer : 10 ml of acetonitrile (Merck) was added to 5 ml of water followed by addition of 1 ml of formic acid (Merck) and the final volume was adjusted to 20 ml with water. The final concentrations were 50 % (v/v) acetonitrile and 5 % (v/v) formic acid.

B.6.2.4. Preparation of MALDI Matrix

α-cyano-4-hydroxycinnamic acid (HCCA) was used as a matrix in proteomics studies and it was prepared with two layer method.

First Layer : Approximately 3–6 mg of HCCA was weighted in 100 µl methanol (MeOH). Then 400 µl acetone was added to the mixture and dissolved all of them.

Increase MeOH part on very dry days and lower it on very humid days.

Second Layer : Approximately 5 mg of HCCA was weighted into a plastic vial and 200 µl MeOH was added. Firstly, all the matrices into MeOH were dissolved and than, 300 µl deionized water which contains 0.1% TFA was added. HCCA started to precipitate out. Finally, it was centrifugated at maximum speed for nearly 8 minutes.