

ORIGINAL ARTICLE: RESEARCH

## Changes in protein profiles of multiple myeloma cells in response to bortezomib

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### Abstract

The objective of this study was to determine the changes in protein profiles of U-266 multiple myeloma cells in response to bortezomib. Bortezomib inhibited cell proliferation and increased the loss of mitochondrial membrane potential and caspase-3 activity in a dose-dependent manner. DECODON Delta2D Version 4.3 software demonstrated 37 differentially expressed protein spots: five proteins were newly formed, 10 proteins were lost, 12 proteins were up-regulated and 10 proteins were down-regulated in bortezomib-treated cells as compared to untreated cells. Some of the identified proteins after mass spectrometric analysis were as follows: apoptosis regulatory protein Siva (newly formed), caspase recruitment domain-containing protein 14 (lost), Ras-related protein Rab-25 (up-regulated), nuclear factor  $\kappa$ B (NF- $\kappa$ B) p105 subunit (down-regulated). In summary, differentially expressed proteins of MM U-266 cells in response to bortezomib were analyzed and identified. The data obtained from this study may indicate the use of bortezomib for the treatment of various diseases.

**Keywords:** Bortezomib, multiple myeloma, proteomics, MALDI-TOF-TOF, mass spectrometry

### Introduction

Multiple myeloma (MM) is a malignant B-cell neoplasm characterized by the accumulation of malignant plasma cells in the bone marrow. It is the second most common hematological disorder [1], accounting for 2% of cancer deaths [2], with median survival times of 4–7 years [3]. Recently, an increasing number of people are being diagnosed with MM. It has a yearly incidence number of nearly 14–15 thousand that accounts for approximately 10% of all hematologic cancers in the United States [4,5].

The treatment strategy for MM is an important step for patients since MM is currently still accepted as an incurable disease [6,7]. Bortezomib (Velcade<sup>®</sup>) is known to be effective in more than 200 types of cancer cell lines [8], and is the first

of a new class of anticancer agents known as proteasome inhibitors [8]. It is a dipeptide boronic acid derivative, 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 [9–14]. The 26S proteasome appears to play an essential role in the targeted degradation of intracellular proteins that are responsible for regulating essential cellular functions, such as cell-cycle control, signal transduction, cellular adhesion, proliferation, development, survival and apoptosis [15,16]. Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a transcription factor that has a central role in bortezomib-induced apoptosis, since it regulates genes involved in apoptosis and cell-cycle control [17]. Up to 80% of patients with MM have specific mutations in certain genes which regulate the NF- $\kappa$ B pathway [18,19]. In MM, tumor cells and bone marrow stromal cells (BMSCs) indicate enhanced NF- $\kappa$ B activity as compared to healthy cells [20–22]. Proteasome-mediated activation of NF- $\kappa$ B brings about the expression of multiple enzymes and cell cycle regulators, cytokines, cell adhesion molecules and anti-apoptotic factors inducing cell growth and survival [11,12,23]. Therefore, it is important to both understand what is happening at the protein level underlying the NF- $\kappa$ B pathway and solve the roles of differentially expressed proteins. Although many studies from several research groups have been conducted regarding the molecular pathways of MM, studies of changes in protein profiles in response to bortezomib in MM cells have not been reported, to the best of our knowledge.

In this study, our results demonstrated that bortezomib has anti-proliferative and anti-apoptotic effects on U-266 MM cells. More importantly, proteins involved in the NF- $\kappa$ B pathway were determined by matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF-TOF) mass spectrometry for the first time. According to the mass spectrometric analysis, 20 protein spots were differentially expressed. Six proteins are related to cell signaling (spots 7, 11, 14, 15, 23, 29), one protein has a role in survival (spot 34), one is a cell-cycle regulator (spot 4), two proteins are involved

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in apoptosis (spots 2, 27), one protein is a tumor suppressor (spot 8), five proteins take a key role in the immune defense mechanism (spots 9, 12, 18, 25, 35), one protein is involved in protein modification and ubiquitination (spot 22) and three have other functions (spots 5, 6, 10). Among them, five proteins were newly formed, four proteins were lost, four proteins were up-regulated and seven proteins were down-regulated in response to bortezomib as compared to untreated control cells.

## Materials and methods

### Cell culture

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 (Gibco Industries Inc., Los Angeles, USA) growth medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, USA) at 37°C under a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The medium was refreshed every 3 days.

### Cell proliferation assay

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, stored in aliquots at -20°C and diluted in cell culture medium.

Anti-proliferative effects of bortezomib on U-266 MM cells were determined by XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] cell proliferation assay. U-266 cells (2 × 10<sup>4</sup>/200 µL) were seeded into 96-well plates containing 200 µL growth medium in the absence and presence of increasing concentrations of bortezomib. After a 72 h incubation period, 50 µL XTT reagent was added to each well. Plates were then incubated for 4 h at 37°C in 5% CO<sub>2</sub>, and growth medium and XTT reagent were removed. The absorbances of the samples were measured under 492 nm wavelength of light by enzyme-linked immunosorbent assay (ELISA) reader (Multiskan Spectrum; Thermo Electron Corporation, Finland) and the IC<sub>50</sub> value (concentration of drug that inhibits 50% of cell proliferation as compared to untreated control) of bortezomib was calculated from cell proliferation plots.

### Caspase-3 activity

Changes in caspase-3 enzyme activity of U-266 MM cells in response to bortezomib were evaluated using a caspase-3 colorimetric assay kit (R&D Systems, USA) as described previously [24]. In short, 1 × 10<sup>6</sup> cells were seeded in a six-well plate in 2 mL growth medium in the absence or presence of increasing concentrations of bortezomib at 37°C in 5% CO<sub>2</sub> for 72 h. Untreated cells were used as the control group. After incubation, the cells were centrifuged at 1000 rpm for 10 min and the cell pellet was lysed by adding 50 µL of chilled Cell Lysis Buffer (1×). Then, the cell lysate was incubated on ice for 10 min before centrifugation at 14 000 rpm for 1 min. The supernatants were used to measure caspase-3 enzyme activity, and the reaction mixture was prepared in 96-well plates with the addition of 50 µL

of Reaction Buffer (2×, containing 10 mM dithiothreitol [DTT]), 50 µL of sample and 5 µL of caspase-3 colorimetric substrate (DEVD-pNA) and incubated for 2 h at 37°C in 5% CO<sub>2</sub>. The absorbance of the reaction mixture was measured under 405 nm wavelength by ELISA reader (Multiskan Spectrum; Thermo Electron Corporation). Caspase-3 activity levels were normalized to protein concentrations determined by the Bradford protein assay [25].

### Loss of mitochondrial membrane potential

The loss of mitochondrial membrane potential (MMP) in U-266 MM cells in response to bortezomib was assessed by an APO LOGIX JC-1 MMP Detection Kit (Cell Technology, USA). Briefly, 1 × 10<sup>6</sup> cells were seeded in a six-well plate in 2 mL growth medium in the absence or presence of increasing concentrations of bortezomib at 37°C in 5% CO<sub>2</sub> for 72 h. Untreated cells were used as the control group. After this period, all samples were washed with 1 × phosphate buffered saline (PBS) before centrifugation at 1000 rpm for 10 min. Supernatants were carefully removed from the pellets and discarded. Then the pellets were dissolved in 500 µL of JC-1 dye (1%) and the cells were incubated at 37°C in 5% CO<sub>2</sub> for 30 min. Next, the mixtures were centrifuged at 1000 rpm for 5 min and supernatants were carefully removed again. Subsequently, 2 mL of 1 × Assay Buffer was added to the pellets and vortexed for homogenization. The centrifugation step was repeated with the same conditions to remove the excess dye. All the pellets were resuspended with 500 µL 1 × Assay Buffer and 150 µL from each of them was seeded into black 96-well plates. 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 using these wavelengths by fluorescence ELISA reader. Finally, green/red (510/585) values were calculated to determine the changes in MMP.

### Total protein extraction

In order to extract the total proteins from untreated and bortezomib treated U-266 cells, whole cell suspension was centrifuged at 1000 rpm for 10 min at room temperature. After centrifugation, the supernatant was removed and the pellet was resuspended in 50 µL of chilled Cell Lysis Buffer (containing sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, Triton X100 and water) and incubated on ice for 10 min. Following the addition of Cell Lysis Buffer, samples were centrifuged at 14 000 rpm for 1 min at 4°C. The supernatant, which was the total protein of our samples, was transferred to a fresh tube and used for further studies (two-dimensional [2D]-polyacrylamide gel electrophoresis [PAGE]). Protein concentrations of the samples were determined using the Bradford protein assay as described previously [25].

### Two-dimensional PAGE

All 2D-PAGE equipment and immobilized pH gradient (IPG) strips (3-10 NL, 17 cm) were obtained from Bio-Rad (USA). Chemicals used in 2D-PAGE experiments were

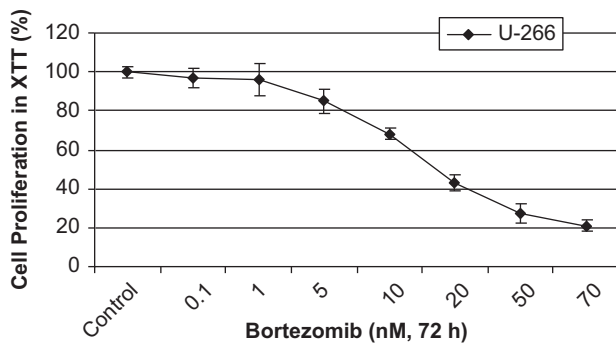


Figure 1. Antiproliferative effects of bortezomib on U-266 cells. The  $IC_{50}$  concentration of bortezomib was determined by XTT assay. The XTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the standard deviations.

purchased from Sigma, AppliChem and Amersham Biosciences (Piscataway, USA).

### Isoelectric focusing

Passive rehydration was performed for the rehydration of strips. The procedure was carried out in accordance with the user's manual for the IPG system. Protein samples were reconstituted with rehydration buffer which contained urea (4.8 g, 8 M), CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate) (0.2 g, 2%), DTT (0.077 g, 50 mM), ampholytes (pH 3–10) (100  $\mu$ L, 0.2%) and Bromophenol Blue (1–2  $\mu$ L, 0.5%). The reorganized protein applied to the strips for bortezomib treated U-266 cells was 408  $\mu$ g, and 414.4  $\mu$ g protein for untreated U-266 cells as control.

Isoelectric focusing (IEF) was performed at room temperature and 50  $\mu$ A in three steps with both different voltages and times. In the first step, 500 V was applied for 1 h, 1000 V was applied for 1 h in the second step, and 8000 V was applied for 8 h as a final step.

### Sodium dodecyl sulfate

Prior to sodium dodecyl sulfate (SDS)-PAGE, focused IPG strips were equilibrated in Equilibration Buffers (I and II). Equilibration Buffer I contains DTT (0.4 g/20 mL), Tris-HCl pH 8.8 (5 mL, 1.5 M), glycerol (4 mL), urea (7.2 g/20 mL), SDS (0.4 g/20 mL) and ultrapure  $H_2O$  (5 mL). Equilibration

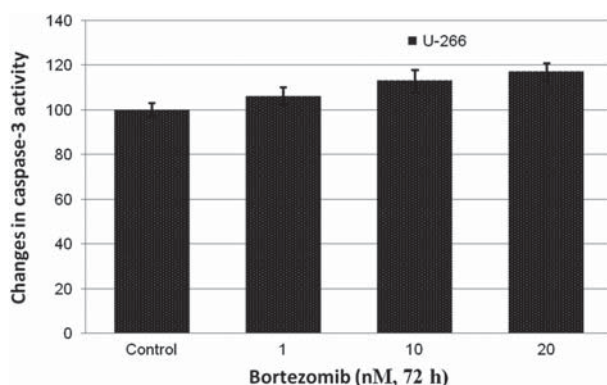


Figure 2. Percent changes in caspase-3 activity in response to bortezomib in U-266 cells. The results are the means of two independent experiments. The error bars represent the standard deviations.

Buffer II contains iodoacetamide (0.5 g/20 mL) instead of DTT. Strips were loaded onto a 12% SDS-polyacrylamide gel. In order to control electrophoresis cooling, water circulation was used, and electrophoresis was started at a constant current of 32 mA for 30 min, followed by 50 mA for 5–6 h. To monitor the progress of electrophoresis, Bromophenol Blue was used.

### Gel staining and image analysis

After electrophoresis, a MALDI-TOF mass spectrometry-compatible silver staining method was performed, as described previously [26].

After the staining process, gels were scanned, and photographed by an Olympus DP25 digital camera system. Then image analysis, spot matching and determination of different protein spots were performed using DECODON Delta2D Version 4.3 software, and the differentiated proteins were determined and cut from the gel to be identified by mass spectrometry.

### In-gel digestion and peptide sequencing

Differentially expressed protein spots were in-gel digested overnight before mass spectrometry analysis, according to the procedure previously described by Shevchenko, with some modifications [27]. Sequencing grade modified trypsin was from Promega Corporation (USA).

ZipTip<sup>®</sup> pipette tips were obtained from Millipore Corporation (USA). Peptides were desalted and concentrated using C18 micro-columns (Eppendorf PerfectPure; Millipore) according to the manufacturer's protocol.

### Protein identification by mass spectrometry

After the in-gel digestion procedure, peptides were eluted with 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) and mixed 1:1 with 10 mg/mL  $\alpha$ -cyano 4-hydroxy cinnamic acid (used as a matrix) in acetonitrile prior to spotting onto a stainless steel MALDI target plate. The peptide masses from two samples (bortezomib and control group) were analyzed by a MALDI-TOF-TOF mass spectrometer (Bruker, Bremen, Germany). The sequence of the differentially expressed protein spots was found using NCBIInr (National Center for Biotechnology Information, Bethesda, USA) and SwissProt protein databases.

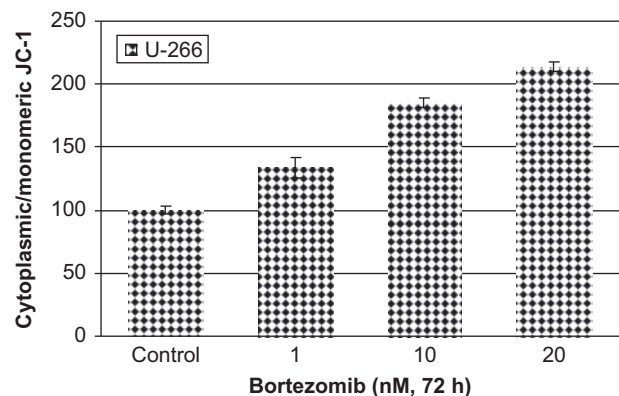


Figure 3. Percent changes in cytoplasmic/mitochondrial JC-1 in bortezomib treated U-266 cells. The results are the means of two independent experiments. The error bars represent the standard deviations.

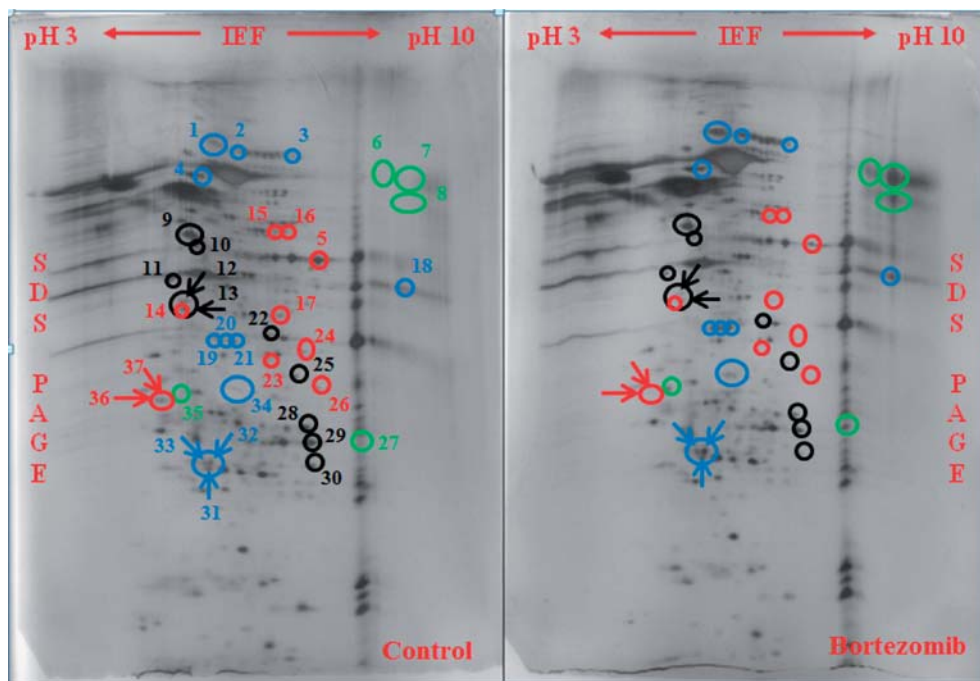


Figure 4. Representative overview of 2D PAGE maps of untreated and 17 nM bortezomib treated human U-266 cells. IEF as the first dimension separation was over a pH range 3–10, while the second dimension separation was carried out by SDS-PAGE in a 12% polyacrylamide gel and gels were silver stained. The circles indicate the differentiated protein spots.

## Results

### Bortezomib decreased proliferation of U-266 cells in a dose-dependent manner

U-266 cells were treated with increasing concentrations of bortezomib for 72 h, and the XTT cell proliferation assay was carried out in order to determine the anti-proliferative effects of bortezomib. There were 3, 4, 15, 32, 57, 73 and 79% decreases in cell proliferation in response to 0.1, 1, 5, 10, 20, 50 and 70 nM bortezomib, respectively, as compared to untreated cells (Figure 1). The  $IC_{50}$  value of bortezomib was calculated from the cell proliferation plots and found to be 17 nM (Figure 1).

### Bortezomib induced apoptosis through loss of MMP and increased caspase-3 activity in U-266 cells

In order to evaluate the apoptotic effects of bortezomib on U-266 cells, U-266 cells were treated with three different concentrations of bortezomib, and loss of mitochondrial membrane potential and changes in caspase-3 enzyme activity were determined. As shown in Figure 2, caspase-3 activity was increased 1.06-, 1.13- and 1.17-fold in response to 1, 10 and 20 nM bortezomib, respectively, as compared to the untreated control group.

In order to confirm the caspase-3 activity results, the same set of experiments were done and changes in mitochondrial membrane potential were determined. The results revealed that there were 1.34-, 1.85- and 2.14-fold increases in loss of MMP in response to the same concentrations of bortezomib, respectively, as compared with untreated cells (Figure 3).

### Comparison of protein profiles between untreated and bortezomib-treated U-266 cells

In order to identify changes in protein profiles, isolated proteins were analyzed by 2D-PAGE. Protein isolation and 2D-PAGE experiments were repeated four times. A mass spectrometry-compatible silver staining procedure was applied to increase the sensitivity [26]. The gel maps derived from untreated and bortezomib treated groups indicated a broad distribution of spots in a pI range from 3.0 to 10.0 (Figure 4).

In order to elucidate and compare protein profiles of untreated and bortezomib-treated U-266 cells, two individual gel images were imported into gel analysis software DECODON Delta2D Version 4.3. With the help of this software, the 2D-PAGE gels were superposed and comparative detailed analysis was performed. According to the analysis, 37 protein spots were differentially expressed. Among them, five proteins were newly formed (Figure 5a), 10 proteins lost (Figure 5b), 12 proteins increased (Figure 5c) and 10 proteins decreased (Figure 5d) as compared to the control group (untreated cells) (Figure 5). These spots were numbered from 1 to 37, colored and labeled: newly formed proteins were marked with green (Y), lost proteins were marked with red (K), increased proteins were marked with blue (M) and decreased proteins were marked with black (S).

### Identification and categorization of differentially expressed proteins in response to bortezomib

After labeling, black and red colored spots were cut from control group gels and others (green and blue) were cut from bortezomib treated group gels for enzymatic digestion using

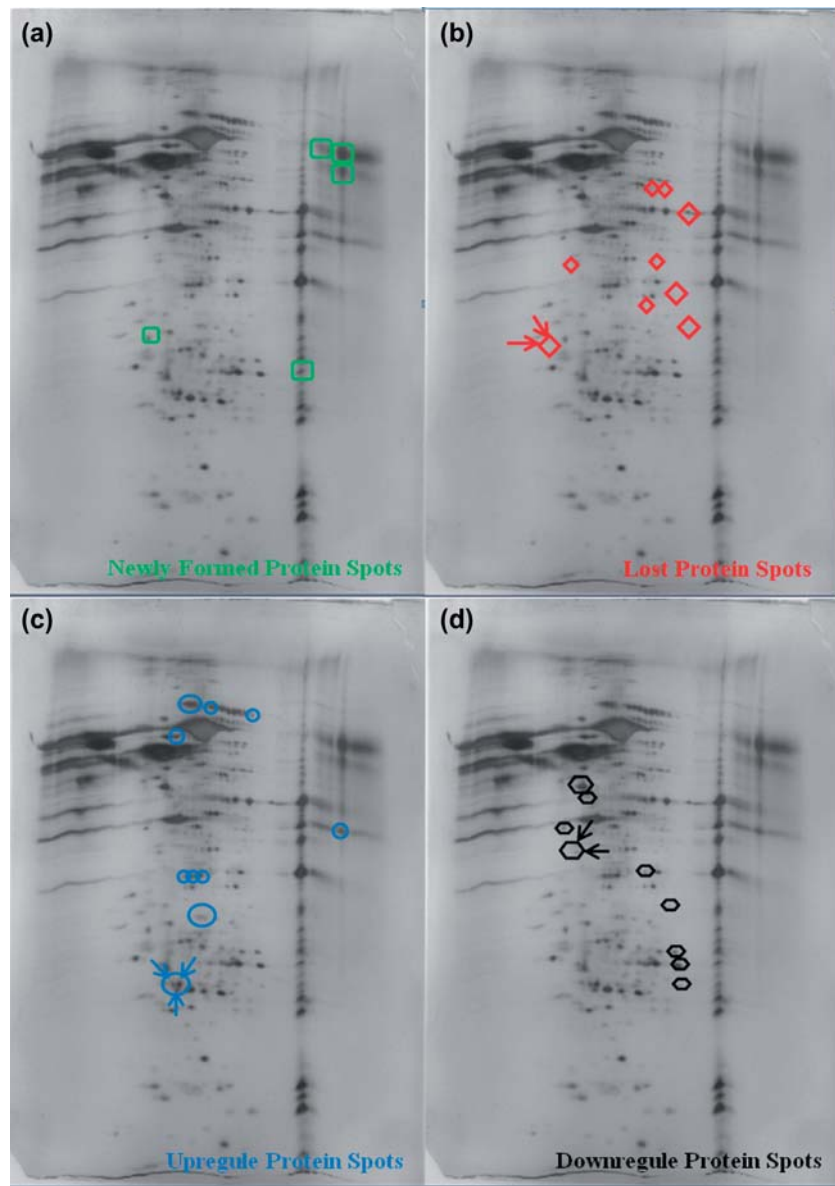


Figure 5. 2-D PAGE images of U-266 cells in response to bortezomib: (a) newly formed (rectangles), (b) lost (diamonds), (c) increased (circles) and (d) decreased protein spots (hexagons). All labeled spots were identified by mass spectrometry and database search.

trypsin. Then samples were analyzed by MALDI-TOF-TOF mass spectrometry and mass spectrometric data were compared to the protein database for sequence matches.

In our experiments, protein spot determination was carried out according to isoelectric point, molecular mass, sequence coverage and Mascot Mowse score. According to these parameters and mass spectrometric analysis, the most important 20 proteins related to the mechanism of the effect of the anticancer agent on the cells were listed. Table I shows these proteins, which have functions ranging from cell signaling transduction, cell cycle regulation and apoptosis to immune defense mechanism in response to bortezomib. Figure 6 illustrates categorization of the biological functions of the differentially expressed proteins.

## Discussion

Bortezomib is the first of a new class of anticancer agents known as proteasome inhibitors. Although bortezomib

is antiproliferative and apoptotic to more than 200 different cancer cell lines, significant problems have arisen in response to bortezomib in patients with MM. Thus, it is of vital importance to determine the changes in total protein levels in response to bortezomib. In this study, we demonstrated 37 differentially expressed protein spots (five proteins newly formed, 10 proteins lost, 12 proteins up-regulated and 10 proteins down-regulated) in bortezomib-treated MM cells as compared to untreated cells.

The lost proteins were signaling threshold-regulating transmembrane adapter 1, paxillin, caspase recruitment domain-containing protein 14 and NF- $\kappa$ B inhibitor delta. The basic function of signaling threshold-regulating transmembrane adapter 1 is to regulate TCR (T-cell antigen receptor)-mediated signaling negatively in T-cells [28]. Furthermore, it is specifically expressed in T- and B-cell lymphomas and present in plasma cells [29,30]. Paxillin is an important cytoskeletal protein with function in actin-membrane attachment at sites of cell adhesion to the extracellular

Table I. Differentially expressed proteins and their functions.

	Spot name	Protein name	Function
Lost proteins	Spot 14	SIT1 - signaling threshold-regulating transmembrane adapter 1	Cell signaling (regulation of TCR [T-cell antigen receptor]-mediated signaling)
	Spot 15	CAR14 - caspase recruitment domain-containing protein 14	Cell signaling (activation of nuclear factor- $\kappa$ B [NF- $\kappa$ B] via Bcl-10)
	Spot 23	IKBD - NF- $\kappa$ B inhibitor delta	Cell signaling (regulation of NF- $\kappa$ B activity)
	Spot 5	PAXI - (paxillin)	Other function (involved in actin-membrane attachment at sites of cell adhesion to the extracellular matrix)
Newly formed proteins	Spot 27	Siva - apoptosis regulatory protein Siva	Apoptosis (inducing CD27-mediated apoptosis and inhibiting the activation of NF- $\kappa$ B)
	Spot 8	ST7 - suppressor of tumorigenicity 7 protein	Tumor suppressor
	Spot 7	Mitogen-activated protein kinase kinase kinase 1	Cell signaling (upstream of the Jun N-terminal pathway) and growth regulation
	Spot 35	IL-32 - interleukin-32	Immune defense mechanism (inducing various cytokines such as TNF- $\alpha$ and IL-8 and activating typical cytokine signal pathways of NF- $\kappa$ B and p38 MAPK)
	Spot 6	GPX3 - glutathione peroxidase 3	Other function (protection of cells and enzymes from oxidative damage)
Up-regulated proteins	Spot 2	CASP3 - caspase-3	Apoptosis (activation cascade of caspases responsible for apoptosis execution)
	Spot 4	UBE2C - ubiquitin-conjugating enzyme E2C	Cell cycle regulator (acting as essential factor of the anaphase promoting complex/cyclosome [APC/C], a cell cycle-regulated ubiquitin ligase that controls progression through mitosis)
	Spot 34	RAB25 - Ras-related protein Rab-25	Cell survival (regulation of cell survival)
	Spot 18	CLC5A - C-type lectin domain family 5 member A	Immune defense mechanism (positive regulator of osteoclastogenesis)
Down-regulated proteins	Spot 11	SOCS3 - suppressor of cytokine signaling 3	Cell signaling (part of a classical negative feedback system that regulates cytokine signal transduction)
	Spot 29	PYDC1 - pyrin domain-containing protein 1	Cell signaling (suppresses kinase activity of NF- $\kappa$ B inhibitor kinase [IKK] complex, expression of NF- $\kappa$ B inducible genes and inhibits NF- $\kappa$ B activation by cytokines)
	Spot 22	CHIP - E3 ubiquitin-protein ligase CHIP	Protein modification and protein ubiquitination (targeting misfolded chaperone substrates toward proteasomal degradation)
	Spot 9	BST2 - bone marrow stromal antigen 2	Immune defense mechanism (involved in pre-B-cell growth)
	Spot 12	TN13B - tumor necrosis factor ligand superfamily member 13B	Immune defense mechanism (promoting the survival of mature B-cells and the B-cell response)
	Spot 25	TNR17 - tumor necrosis factor receptor superfamily member 17	Immune defense mechanism (promoting B-cell survival and playing a role in the regulation of humoral immunity and activating NF- $\kappa$ B and JNK)
	Spot 10	MKNK1 - MAP kinase-interacting serine/threonine-protein kinase 1	Other function (response to environmental stress and cytokines)

TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-8, interleukin-8; MAPK, mitogen-activated protein kinase.

matrix [31]. Caspase recruitment domain-containing protein 14 activates NF- $\kappa$ B via Bcl-10 and stimulates the phosphorylation of Bcl-10 [32]. NF- $\kappa$ B inhibitor delta is responsible for regulating the expression of interleukins IL-2, IL-6 and other cytokines through regulation of NF- $\kappa$ B activity, similarly to caspase recruitment domain-containing protein 14 [33]. NF- $\kappa$ B is a transcription factor which is present in almost all cell types and is involved in many biological process such as

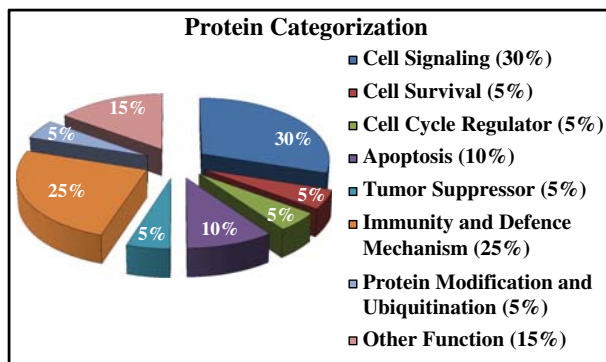


Figure 6. Categorization of biological functions of differentially expressed proteins.

inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis [34,35]. These proteins were lost in response to bortezomib, and these findings are in agreement with the literature. It has been shown that bortezomib treatment induces apoptosis through the NF- $\kappa$ B pathway [17].

The newly formed proteins were apoptosis regulatory protein Siva, suppressor of tumorigenicity 7 protein, mitogen-activated protein kinase kinase kinase 1, interleukin-32 and glutathione peroxidase 3. Apoptosis is a significant part of cell cycle regulation, especially in cancerous cells. Apoptosis regulatory protein Siva induces CD27-mediated apoptosis and also inhibits Bcl-2L1 isoform Bcl-x(L) anti-apoptotic activity [36]. It is also inhibits activation of NF- $\kappa$ B and promotes T-cell receptor-mediated apoptosis [37]. Suppressor of tumorigenicity 7 protein acts as a tumor suppressor [38]. It has been shown that mitogen-activated protein kinase kinase kinase 1 acts upstream of the Jun N-terminal pathway [39,40]. It also plays a key role in hematopoietic lineage decisions and growth regulation. As well, it is expressed primarily in hematopoietic organs, especially bone marrow [41]. Interleukin-32 is a cytokine and is responsible for innate and adaptive immune responses [42]. Moreover, it induces

various cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-8 and activates typical cytokine signal pathways of NF- $\kappa$ B. By catalyzing the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxide, glutathione peroxidase 3 protects cells and enzymes from oxidative damage [43].

The up-regulated proteins were caspase-3, ubiquitin-conjugating enzyme E2C, Ras-related protein Rab-25 and C-type lectin domain family 5 member A. Caspase-3 is involved in the activation cascade of caspases responsible for apoptosis execution [44]. Additionally, it cleaves and activates caspase-6, -7 and -9. The highest expression level of this protein is expected in cells of the immune system, because of its function and role in apoptosis [44]. Ubiquitin-conjugating enzyme E2C acts as an essential factor of the anaphase promoting complex/cyclosome (APC/C), a cell cycle-regulated ubiquitin ligase that controls progression through mitosis [45]. As this protein is responsible for controlling progression and has a role in protein ubiquitination, its expression level increased with the bortezomib effect. Ras-related protein Rab-25 is involved in the regulation of cell survival [46]. C-type lectin domain family 5 member A has a function as a positive regulator of osteoclastogenesis [47]. These up-regulated proteins help the cells balance the regulation of cell survival and apoptosis.

The down-regulated proteins were suppressor of cytokine signaling 3, pyrin domain-containing protein 1, E3 ubiquitin-protein ligase CHIP, bone marrow stromal antigen 2, tumor necrosis factor ligand superfamily member 13B, tumor necrosis factor receptor superfamily member 17 and MAP kinase-interacting serine/threonine-protein kinase 1. Suppressor of cytokine signaling 3 (SOCS3) is a part of a classical negative feedback system that regulates cytokine signal transduction [48]. SOCS3 is responsible for negative regulation of cytokines through the JAK/STAT pathway [49]. Pyrin domain-containing protein 1 is associated with apoptosis-related specklike protein [50]. It also modulates its ability to collaborate with pyrin and cryopyrin in NF- $\kappa$ B and procaspase-1 activation [50]. This protein has vital importance in that it suppresses the kinase activity of NF- $\kappa$ B inhibitor kinase (I $\kappa$ K) complex and expression of NF- $\kappa$ B inducible genes [50]. E3 ubiquitin-protein ligase CHIP is involved in targeting misfolded chaperone substrates toward proteasomal degradation [51]. Bone marrow stromal antigen 2 is involved in pre-B-cell growth and B-cell activation [52]. Tumor necrosis factor ligand superfamily member 13B promotes the survival of mature B-cells and the B-cell response [53]. Tumor necrosis factor receptor superfamily member 17 promotes B-cell survival and plays a role in the regulation of humoral immunity, and it activates NF- $\kappa$ B and JNK [54]. MAP kinase-interacting serine/threonine-protein kinase 1 plays a role in response to environmental stress and cytokines [55,56].

In conclusion, the data obtained from this study demonstrated that in addition to inhibition of the proteasome, bortezomib treatment also induces significant changes in proteins involved in cell fate. The results of this study may suggest potential use of bortezomib for the treatment of different diseases resulting from these proteins. On the

other hand, severe side effects of bortezomib may also be explained by the changes in levels of these proteins. The changes in total proteins of U-266 cells in response to bortezomib were determined by MALDI-TOF-TOF mass spectrometry for the first time.

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**Potential conflict of interest:** Disclosure forms provided by the authors are available with the full text of this article at [www.informahealthcare.com/lal](http://www.informahealthcare.com/lal).

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