BIOMEDICAL

Synergistic Apoptotic Effects of Bortezomib and Methylstat on Multiple Myeloma Cells

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Background. In this study, we aimed to determine synergistic apoptotic and cytotoxic effects of methylstat and bortezomib on U266 and ARH77 multiple myeloma (MM) cells.

Methods. Cytotoxic effects of the drugs were demonstrated by MTT cell proliferation assay while apoptotic effects were examined by loss of mitochondrial membrane potential (MMP) by JC-1 MMP detection kit, changes in caspase-3 enzyme activity and Annexin-V apoptosis assay by flow cytometry. Expression levels of apoptotic and antiapoptotic genes were examined by qRT-PCR.

Results. Our results showed that combination of methylstat and bortezomib have synergistic antiproliferative effect on MM cells as compared to either agent alone. These results were also confirmed by showing synergistic apoptotic effects determined by increased loss of mitochondrial membrane potential and increased caspase-3 enzyme activity and relocation of phosphotidylserine on the cell membrane by Annexin-V/PI double staining. Combination of bortezomib with methylstat arrested cells at the S phase of the cell cycle. Methylstat treatment caused upregulation of FASLG, NGFR, TNF, TNFRS10B and TNFRS1B apoptotic genes and downregulation of AKT1, AVEN, BAG1 BCL2L2 and RELA antiapoptotic genes in a dose and time dependent manner.

Conclusion. In conclusion, our data suggested that bortezomib in combination with methylstat decreased cell proliferation and induced apoptosis significantly in U266 and ARH77 cells. When supported with in vivo analyses, methylstat might be considered as a potential new agent for the treatment of MM. © 2020 IMSS. Published by Elsevier Inc.

Key Words: Multiple myeloma, Bortezomib, Methylstat, Apoptosis.

Introduction

Multiple myeloma (MM) is a B-cell hematological malignancy characterized by abnormal proliferation of plasma cells in the bone marrow (1). MM is the second most common hematological malignancy (1) and is observed in people over the age of 50 (2). Ageing increases the incidence rate of the disease. MM is responsible for 10% of all blood cancers (2). Genetic and environmental factors are important for the development of multiple myeloma (3,4).

Several strategies are used for the treatment of MM. Because of genetic heterogeneity of MM (1,5), it is still an incurable and the patients show different clinical response against the drugs and used chemotherapeutics (6). Bortezomib is the first proteasome inhibitor used for the treatment of MM (7–10). It binds to the catalytic site of 26S proteasome and prevents its function (11–13). Proteasome is responsible for degradation of misfolded, unfolded, nonfunctional proteins and some other molecules in the cell. Previous studies showed that proteasome inhibition triggers apoptosis in neoplastic cells and inhibits degradation of pro-apoptotic factors, angiogenesis and metastasis of cancer cells (14). Bortezomib application regulates the nuclear factor–κB (NF-κB) signaling pathway (15). NF-κB is a transcription factor that is found as an inactive form stuck to its inhibitory partner protein IκB in the...
cytoplasm. After IκB is ubiquitinated or phosphorylated by cytokines or by other cellular processes, it is degraded by proteasomes resulting in activation of NF-κB. NF-κB activation stimulates the transcription of several genes whose protein products block the apoptotic pathways and promote cell proliferation (16,17).

Methylstat is a new drug selectively inhibiting Jumonji C domain-containing histone demethylases in cells (18). Jumonji C (JMJC) is a transcription factor and is amplified in human cancers under the hypoxic conditions. Thus, there is an overexpression of JMJC domain containing proteins in different types of cancers. Recently, many experimental studies revealed that Methylstat inhibits angiogenesis and cell proliferation in human umbilical vascular endothelial cells (HUVECs) and oesophageal cancer types (18,19). However, there is no study examining therapeutic potential of Methylstat on MM cells.

In this study, we investigated therapeutic potential of Methylstat alone and in combination with bortezomib on U266 and ARH77 multiple myeloma cells.

Material and Methods

Cell Lines and Culture Conditions

Human U266 and ARH77 multiple myeloma cells were obtained from German Collection of Microorganisms and Cell Cultures (Germany). The cells were grown and maintained in RPMI-1640 growth medium containing 1% L-glutamine, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO₂.

Measurement of Cell Proliferation by MTT Assay

Antiproliferative effects of Bortezomib and Methylstat alone and in combination on U-266 and ARH-77 cells were determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide) cell proliferation assay. In brief, 1 × 10⁴ MM cells were inoculated in each well of 96 well plate containing 100 μL of the growth medium. The cells were incubated in the absence or presence of increased concentrations of Bortezomib (from 1–500 nM) or Methylstat (from 1–40 mmol). After 48 and 72 h incubation periods, 20 μL of MTT solution is added to the cells and incubated for 3 h. Then, the plates were read at 570 nm by a spectrophotometer (Thermo Electron Corporation Multiskan Spectrum, Finland).

In order to detect the possible synergistic effects of Bortezomib in combination with Methylstat, 1 × 10⁴ MM cells were seeded into each well of 96 well plate and exposed to increased concentrations (from 1–160 nM) of Bortezomib together with constant Methylstat (4.2 mmol) concentration. After the incubation time, the same procedure, as explained above, is applied for this set of experiments. According to the cell proliferation plots, IC₅₀ (the drug concentration that inhibits cell proliferation by 50% as compared to untreated control group) values of the drugs were calculated as follows: The MTT data is plotted as a scatter plot with logarithmic Y-axis. Then, the trend line fort he graph is created and by using the equation given, the concentration corresponds to 50% of death is calculated.

Detection of Caspase-3 Enzyme Activity

Caspase-3 colorometric assay kit (BioVision Research products, USA) was used to detect the changes in caspase-3 enzyme activity in MM cells. This assay is based on the spectrophotometric emmision of chromophore p-nitroanilide (pNA) after cleaving from DEVD-pNA which is a target for Caspase-3 enzyme.

Briefly, 1 × 10⁶ cells were inoculated in 2 mL complete medium in a 6 well plate and then the cells were exposed to increasing concentrations of Methylstat and Bortezomib. After incubation period, the cells were collected into falcon tubes and centrifuged at 1000 rpm for 10 min. Then, the cell pellet was lysed by adding 100 μL of cell lysis buffer and they were left on ice for 10 min. After that, the samples were centrifuged at 10,000 g for 1 min. and supernatants were transferred into other centrifuge tubes. Halves of the supernatants were separated for Bradford assay. 50 μL of samples were transferred to each well of 96 well plate. Dithiothreitol (DTT) was added to 2X reaction buffer (10 mmol DTT in 50 μL reaction buffer) and 5 μL DEVD-pNA added to each sample in the dark. Plates were incubated at 37°C for 2 h in CO₂ incubator. With the help of spectrophotometer, their absorbances were measured at 405 nm. Protein concentrations were measured by Bradford assay by using bovine serum albumin (BSA) as a standard. The results of measurements are normalized to protein concentrations, as determined by BSA, for each sample.

Detection of the Loss of Mitochondrial Membrane Potential

To confirm previous results, we have also examined the changes in loss of mitochondrial membrane potential (MMP), another important marker of apoptosis, by using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide) MMP detection kit. JC-1 is a cytofluorimetric, lipophilic and cationic dye. In healthy cells, it aggregates in mitochondria and gives red fluorescence whereas in apoptotic or dead cells, dye cannot pass through mitochondria and stay in cytoplasm as monomer form. This monomer form of dye gives green fluorescence.

In sum, 1 × 10⁶ cells/2 mL cells were plated into each well of 6 well plates and increasing concentrations of Bortezomib and Methylstat were applied to MM cells. After incubation, the cells were collected and centrifuged at 1000 rpm for 10 min. Pellets were dissolved in 300 μL complete medium and 30 μL of JC-1 dye was added to each
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Detection of the Apoptotic Cell Population by Annexin-V/PI Double Staining

Location of phosphatidylserine (PS) on the cell membrane is an important sign of apoptotic cells. In healthy cells, phosphatidylserines stay in the inner layer of the membrane while in apoptotic cells, PSs flip and face the extracellular matrix of cells. Annexin-V has high affinity for PSs with Ca\(^{2+}\) co-factor. Thus, apoptosis can be detected with help of binding of Annexin-V to PSs. Propidium iodide (PI) is used as a second staining dye. While living cells are impermeable to PI, membranes of dead cells are permeable to PI. With the analyses by flow cytometry Annexin V + PI + cells considered as late apoptotic; Annexin V + PI - cells were early apoptotic and Annexin V - PI + cells were analyzed as necrotic; while Annexin V - PI - cells were with no positive staining were the healthy cells.

Firstly, 1 \times 10^6 cells were inoculated in 2 mL complete medium to each well of 6 well plate and the cells were exposed to increasing concentrations of Bortezomib and Methylstat for 72 h. After the incubation, cells were collected, transferred to falcons and were centrifuged at 800 rpm for 5 min to obtain pellet. The cells were washed with cold PBS for two times. Obtained pellets were resuspended with 1X Annexin binding buffer and transferred to properly labelled FACS tubes. 5 \mu L FITC and 5 \mu L PI dye were added to each sample. Tubes were incubated for 15 min at RT in the dark. At the end of incubation, 400 \mu L 1X Annexin binding buffer were added and results were obtained with the help of flow cytometry (FACSCanto, BD).

Detection of Cell Cycle Progression

Determination of DNA contents of cells were carried out with the help of propidium iodide dye. This dye binds to the DNA directly and amount of fragmented DNA could be detected at different stages of cell cycle. 1 \times 10^6 cells were inoculated to each well of 6 well plate in 2 mL and were exposed to increasing concentrations of Bortezomib and Methylstat. The cells were collected by centrifugation at 1200 rpm for 5 min. In order to fix the cells, ice-cold 70% ethanol (at \(-20^\circ\text{C}\)) was added on pellets and cells were incubated at \(-20^\circ\text{C}\) overnight. The next day day, the cells were centrifuged at 1200 rpm for 10 min at 4\(^\circ\text{C}\) and washed with cold PBS. Pellets were re-suspended with 200 \mu L PBS with 0.1% triton X 100. 20 \mu L (200 \mu g/mL in dH\(_2\)O) RNase-A enzyme was added to each sample and they were incubated at 37\(^\circ\text{C}\) for 30 min. After incubation, cells were stained with 20 \mu L (1 \mu g/mL in dH\(_2\)O) PI dye and incubated for 10 min at RT. They were then analysed by flow cytometer. PI is used as a staining dye to detect the DNA amount in the cells. When cells are going through the usual cycle, the DNA amount doubles during S phase which cause taking up more dye and higher fluorescence detection. The cells can be found in one of 3 distinct groups which are G0/G1 having 2n DNA amount, S phase having 2n-4n DNA amount, and G2/M having 4n DNA amount. Therefore, they can be distinguished by flow cytometry with using PI staining.

Total RNA Isolation, cDNA Synthesis and PCR Array

1 \times 10^6 MM cells/2 mL were incubated with Methylstat for 72 h and total RNAs were isolated by NucleoSpin RNA II Purification Kit. Total RNAs was used to synthesize cDNA by reverse transcriptase enzyme. Then, total cDNA was used to indicate the molecular mechanisms and apoptosis related gene expression profiles underlying Methylstat-induced cell deaths. Human Cancer Pathway Finder RT\(^2\) Profiler PCR Array System (SABiosciences Corporation, USA) was used to determined the changes in expression profiles of 84 genes involved in cancer initiation, promotion and progression.

Results

Bortezomib and Methylstat combination a dose and time dependent manner antiproliferative effects on human U266 and ARH77 multiple myeloma cells.

In order to identify antiproliferative effects of bortezomib and methylstat, U266 and ARH77 cells were treated with increasing concentrations of the agents for 48 and 72 h and the MTT cell proliferation assay was performed. The results showed that there were a dose and time dependent decreases in cell proliferation as compared to untreated controls. IC\(_{50}\) values of bortezomib for U266 and ARH77 cells were calculated and found as 41, and 19 nM, respectively while IC\(_{50}\) values for Methylstat were found to be 2.2 and 4.2 mmol for U266 and ARH77 cells, respectively.

Bortezomib and Methylstat combination has synergistic cytotoxic effects on human U266 and ARH77 multiple myeloma cells U266 cells were exposed to increasing concentrations of Bortezomib (from 1–160 nM) in combination with 2.2 mmol Methylstat while ARH-77 cells were exposed to 4.2 mmol of Methylstat. Starting from the lowest concentration of Bortezomib, Methylstat increased antiproliferative effects of Bortezomib, significantly. The IC\(_{50}\) value of Bortezomib in combination with Methylstat
were calculated from cell proliferation plots and found to be 0.6 nM for 48 h and 0.5 nM for 72 h both for U266 (Figure 1A) and ARH-77 cells (Figure 1B).

The combination of these agents resulted in significant increases of cytotoxic effects of Bortezomib.

Bortezomib in combination with Methylstat increased caspase-3 enzyme activity significantly in MM cells.

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

In other respects, treatment of ARH77 cells for 72 h with 5 nM bortezomib, 4.2 mmol methylstat and combination of them resulted in 1, 1.2, and 8.5 fold increases in caspase-3 activity, respectively, as compared to untreated controls (p < 0.05) (Figure 2B).

Loss of mitochondrial membrane potential in response to combination of bortezomib and methylstat on U266 and ARH77 cells.

In order to confirm the increase in caspase-3 enzyme activity, U266 cells were treated with the two different concentrations of bortezomib (10 and 20 nM), 2.2 mmol methylstat and combination of them. There were 29, 43.2, 18.6, 111.6 and 180.3 fold increases in loss of MMP of U266 cells, respectively (p < 0.05) (Figure 3A).

Also three different concentration (5.10m and 20 nM) of bortezomib, 4.2 mmol methylstat and combination of them applied for ARH77 cells and resulted in 1.2, 1.2, 2.2, 2.5, 3.6, 26 and 26.3 fold increases in loss of MMP as compared with untreated cells, respectively (p < 0.05) (Figure 3B).

Apoptotic Effects of Combination of Bortezomib with Methylstat on MM Cells

In order to detect apoptotic effects of drugs, we measured apoptosis and cell viability of MM cells by FITC AnnexinV/PI double staining by flow cytometry. The results indicated that 4, and 10 fold increases in the percentage of apoptotic cell populations in U266 cells, in response to 10 nM bortezomib and 2.2 mmol methylstat alone, respectively, while combination of both increased 12.5 fold apoptotic cell population as compared to untreated control cells (p < 0.05) (Figure 4A).

Also, the same experiment was done for ARH77 cells. There were 1, 3.2, 9.3, 10, and 11.3 fold increasing apoptotic cell populations, exposed to 5 and 10 nM bortezomib, 4.2 mmol methylstat and combination of them, respectively (p < 0.05) (Figure 4B).
Changes in Gene Expression Levels in Response to Methylstat

In order to analyse the genes regulated by methylstat-induced apoptosis, U266 and ARH77 cells were treated with increasing concentrations of methylstat (1.1, and 2.2 mmol for U266 cells and 2.1, and 4.2 mmol for ARH77 cells) and expression levels of 84 genes were determined by Human Cancer Pathway Finder PCR Array. Methylstat treatment induced overexpression of FASLG, NGFR, TNF, TNFRS10B and TNFRS1B apoptosis-triggering genes in U266 cells (Figure 5A) and BCL2L11, CASP7, TNFRSF21 and TNFSF8 apoptosis-triggering genes in ARH77 cells in a dose-dependent manner (Figure 6A). Furthermore, there were significant decreases in the expression levels of AKT1, AVEN, BAG1, BCL2L2 and RELA anti-apoptotic genes in U266 cells (Figure 5B) and NFKB1 anti-apoptotic gene in ARH77 cells (Figure 6B) in response to increasing concentrations of methylstat.

Discussion

The effect of bortezomib has been reported before by various in vitro and clinical studies which led to use of bortezomib in the treatment of MM patients (20). On the other hand, bortezomib has been shown to induce responses when applied in combinatory with different agents. In a study by Hideshima et al. bortezomib and dezamethasone treatment was shown to increase cell death in MM cells (12). Other studies also investigated the effects of bortezomib together with other chemotherapeutic agents such as cyclophosphamide, prednisone, thalidomide by phase I and II studies (21,22). All these studies showed better overall survival rates and reduced toxicity. Therefore, our study is in line with the idea of using bortezomib as a combinatory treatment with other agents to receive better outcome. Here, we investigated cytotoxic and apoptotic effects of bortezomib and methylstat on U266 and ARH77 multiple myeloma cells. For this purpose, U266 and ARH77 MM cell lines were exposed to increasing concentrations of bortezomib and methylstat alone or in combination of both. IC_{50} values of drugs alone and in combination revealed that bortezomib has a synergistic apoptotic effects as applied together with methylstat and even very low concentrations of bortezomib.
has significant cytotoxic effects as applied together with methylstat on U266 and ARH77 cells.

Moreover, histone demethylation has been highlighted in cancer treatment by various studies (23). Methylstat, a Ju- monji C domain that inhibits histone demethylation has been tested in different models. In a study by Cho et al. methylstat was reported to inhibit angiogenesis in vitro and in vivo (19). In our study, we showed anti-proliferative effects of methylstat which is again in line with the studies in the literature.

Then, apoptotic effects of drugs were evaluated by measuring the changes in caspase-3 enzyme activity and loss of mitochondrial membrane potential (MMP). In order to detect possible synergistic effects of bortezomib and methylstat, we assessed combinational treatments of bortezomib with methylstat. There were synergistic apoptotic effects of bortezomib in combination with methylstat on ARG77 and U266 cells as compared to any agent alone and untreated controls.

These results confirmed the results obtained in MTT analyses and revealed that effectiveness of bortezomib was increased synergistically in combination with methylstat.

In order to confirm apoptotic effects of drugs, we also determined the loss of mitochondrial membrane potential (MMP). The results were in agreement with our previous results and have shown that bortezomib and methylstat induce apoptosis through increasing loss of MMP while combination of both increases loss of MMP synergistically as compared to any agent alone. The results of Anexin V assay also confirmed the results obtained by caspase-3 enzyme activity assay and MMP. Interestingly, cell cycle analysis showed that combination of bortezomib and methylstat arrested cells at the S phase.

In previous studies, bortezomib was shown to function through inhibition of inhibitory kappa B (IkB) which causes stabilization of nuclear factor kappa B (NFkB) complex in multiple myeloma (20,24). To further investigate the mechanism in our setting we examined the expression levels of 84 genes related with apoptosis, cell cycle, angiogenesis, invasion and metastasis by human cancer pathway finder PCR array in order to determine the genes involved in methylstat induced cytotoxic, apoptotic and cytostatic effects on MM cells. The results showed for the first time that methylstat treatment resulted in upregulation of apoptotic

![Figure 5](image5.png)

**Figure 5.** Changes in expression levels of (A) apoptotic and (B) anti-apoptotic genes in response to methylstat in U266 cells (Error bars represents SD).

![Figure 6](image6.png)

**Figure 6.** Changes in expression levels of (A) 4 apoptotic genes (B) NFKB1 gene in response to methylstat in ARH77 cells (Error bars represents SD).
FASLG, NGFR, TNF, TNFRS10B and TNFRS1B genes in U266 cells and BCL2L11, CSP7, TNFRSF21 and TNFSF8 genes in ARH77 cells in a dose-dependent manner. Furthermore, there were significant decreases in expression levels of anti-apoptotic AKT1, AVEN, BAG1 BCL2L2 and RELA genes in U266 cells and anti-apoptotic NFkB1 gene in ARH77 cells in response to increasing concentrations of methylstat which is similar to what was reported in the literature. These results demonstrated which genes are involved in therapeutic effects of methylstat in MM.

Although, this study is preliminary and more needed to fully clarify the mechanism, it still shows the potential use of an epigenetic modulator together with a chemotherapeutic agent in MM. As a part of future studies, the genes that we showed to change by methylstat and bortezomib will be investigated and their protein levels will be tested in order to fully enlighten the mechanism. Also, these study can be lifted to a study with animal models to see the reflection of the effects in vivo.

In conclusion, our results showed in agreement and for the first time that methylstat has antiproliferative and apoptotic effects on MM cells. Methylstat and bortezomib combination has synergistic antiproliferative and apoptotic effects on MM cells.

Conflict of Interest

Authors declares that they do not have any conflict of interest.

References