Effect of Bortezomib administration on autophagic cell death in colorectal cancer cells

Elif Erbaş,¹ Nevra Aydemir Celep¹ and Kader Çiftci²

¹Atatürk University, Faculty of Veterinary Medicine, Department of Histology and Embriology, 25240, Erzurum, Turkey
²Atatürk University, Faculty of Veterinary Medicine, 25240, Erzurum, Turkey

Received: 28 December 2021, Accepted: 10 February 2022.

Abstract

Colon cancer is the most common cancer type after breast and prostate cancer in humans. Bortezomib is a proteasome inhibitor and is commonly preferred for the treatment of some types of cancer due to its efficiency and lower side effects. This study has investigated the impact of Bortezomib on cell death regarding the stimulation of autophagy. Bortezomib (Velcade) was treated to colorectal cancer cells (HT-29) for 24 hours at different concentrations (10 nM, 20 nM, and 40 nM). MTT analysis was used to determine the viability of Bortezomib-treated HT-29 cells, and immunocytochemical methods were used to determine bortezomib’s effects on the expression of Beclin-1 and LC3 levels in the HT-29 cells. In MTT analysis, viability was decreased with an increase in bortezomib concentration and the lowest viability was found at 40 nM concentration. In the study, Beclin-1 immune reactive cells were seen as higher in 10nM and 40 nM concentrations of Bortezomib than other groups. Additionally, in LC3 evaluation, the immune reactive cell density was the highest at 40 nM concentration of Bortezomib (p<0.05). However, the LC3 immune reactivity was higher at 20nM and 40 nM concentrations of bortezomib groups (p<0.05). The findings revealed that the treatment of Bortezomib leads to an increase in levels of LC3 and Beclin-1 and activate the autophagy in colon cancer cells.

Keywords: Cancer, Autophagy, Bortezomib, Beclin-1, LC3.

Introduction

Colon cancer is one of the most common cancers in men and women especially in developed western countries [1]. The mortality rate of colon cancer has decreased in recent years because of the better treatment possibilities. Current standard treatment of colon cancer includes tumor resection after chemotherapy and biologic therapy [2-7]. The standard chemo-therapy for colorectal cancer patients is 5-fluorouracil combined with either oxaliplatin or irinotecan [2,4,5,8]. Although large number of clinical studies focused on finding the most effective approach including these drugs, unfortunately advanced colorectal cancer remains unresectable and incurable. However, studies on new targets and new treatment approaches are continued [9].

Bortezomib is the first clinically available 26S proteasome inhibitor and is used in various cancer treatments [10-13]. Bortezomib mediates reversible binding of the catalytic core complex to the N-terminal
threonine residue in the β-1 subunit. It also leads to reversible inhibition of the proteolytic activity of the proteasome. This triggers various biological changes such as cell cycle arrest as well as induction of autophagy and apoptosis [1].

Bortezomib is the first clinically available 26S proteasome inhibitor and is used in various cancer treatments [10-13]. Bortezomib mediates reversible binding of the catalytic core complex to the N-terminal threonine residue in the β-1 subunit. It also leads to reversible inhibition of the proteolytic activity of the proteasome. This triggers various biological changes such as cell cycle arrest as well as induction of autophagy and apoptosis [1].

**Materials and Methods**

**Cell culture:** Human colon cancer cell line HT-29 was obtained from Ankara Şap Enstitüsü. The cells were incubated with high-glucose DMEM (Gibco) supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml). HT-29 cells were cultured in 25 cm² cell culture flasks (Corning) in a 5% CO₂ incubator (Esco) at 37°C. Subcultures were detached with trypsin and then counted with the trypan blue counting method. The HT-29 cells were then seeded in 96 well plates with 100 µm DMEM for cytotoxicity analysis and seeded on the coverslips in the six-well plates with 2000 µm of DMEM. After 24h incubation, the medium was removed and 1 ml of 0.1 mol/l HCl in absolute isopropanol was added. The absorbance was measured by a spectrophotometer at the wavelength of 570 nm. The absorbance measurements were done two times.

**Drug administration:** Bortezomib application was performed with the 0, 5, 10, 20 and 40 nanomolar (nM) concentrations for MTT analysis and 0, 10, 20 and 40nM concentrations for immunocytochemical analysis. Human Colorectal cancer cells (HT-29) were seeded in a six-well plate at a density of 20000 cells/well on a coverslip with 2 ml of complete culture medium and overnighted to attach. After 24 h, bortezomib was diluted with DMEM solution and added to cell’s media at the concentration of 0nM, 10nM, 20nM, and 40nM (Velcade by Millennium Pharmaceuticals Inc. USA). After 24 h incubation, the coverslips were taken, and the cells were fixed with methanol solution at -20 oC for 10 min and then washed with PBS in a six-well plate. After fixation immunocytochemical staining was performed in coverslips mounted to the slides.

**Cell viability:** Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Co. USA, Cat No: M5655). For MTT analysis, the cells were seeded in a 96-well plate at a density of 100,000 per well in the 100 µm growth media for 24h. The cells were incubated with different concentrations of bortezomib (5nM, 10nM, 20nM, and 40nM) for 24h, then the cells were washed with PBS and then incubated with 1 ml of MTT solution (0.25 mg/ml in PBS) at 37°C for 4h. After incubation, the medium was removed and 1 ml of 0.1 mol/l HCl in absolute isopropanol was added. The absorbance was measured by a spectrophotometer at the wavelength of 570 nm. The absorbance measurements were done two times.

**Stereological estimation of anti-Beclin-1 and LC-3 immune reactive cell densities:** The “Stereological Optic Fractionator Frame” method was used to estimation of anti-Beclin-1 and LC-3 immune reactive cell densities. These analyzes were performed under a stereology workstation system (BioPrecision MAC 5000controller system) and stereology software (Stereo Investigator version 9.0, Microbrightfield, Colchester, VT) attached to the light microscope (Leica DM4000 B, Tokyo, Japan).

In our study, anti-Beclin-1 and anti-LC-3 immune reactive cell densities in HT-29 cell preparations were calculated using the “Unbiased Counting Frame and Fractionator” method, and the positive cell density in each preparation belonging to all groups was calculated according to the following formula:
PHY = PHS/(CA x RS),

PHY; positive cell density per μm² area, PHS; positive cell count, CA; frame area (μm²) and RS; the number of frames. The data obtained are based on duplicate measurements for each group, and 4 parallel preparations from each group were stained. The results are expressed as immune reactive cells / 1000 μm².

**Statistical analysis:** Normally distributed values from immunocytochemical analyses were evaluated with Duncan Post hoc test after one-way analysis of variance (ANOVA) using SPSS statistical software version 20.0 (SPSS Inc., Chicago, IL, USA). The significant value (p) was accepted as 0.05.

**Results**

**Cell Viability:** MTT analysis revealed that cell viability was decreased with the increase of bortezomib concentration treated to HT-29 cells. The lowest toxicity was found at 5nM concentration treated HT-29 cells, the highest toxicity was found in 40nM concentration treated HT-29 cells (Figure 1).

![Figure 1](image_url)

Figure 1. Cell viability values of HT-29 colon cancer cells treated with bortezomib for 24h using MTT analysis.

**The effect of bortezomib on the anti-Beclin-1 and LC-3 immune reactive cells in HT-29 cell line:** In the stereological estimation of immune reactive cell analysis, there were significantly increased levels of Beclin-1 proteins after bortezomib treatment as 10nM, 20nM, and 40nM in HT-29 cells compared with untreated HT-29 cells (p<0.05). In addition, 40nM concentration of bortezomib was markedly increased in Beclin-1 immune reactive cells at 24 h incubation in HT-29 cells (p<0.05) (Table 1 and Figure 2).

On the other hand, in LC-3 immune cells, the low dose of bortezomib (10nM) did not induce a significant difference (p>0.05). However, 20nM and 40nM concentrations of bortezomib increased the immune reactive cell density compared to onM and 10nM concentrations in the HT-29 cells. Beclin-1 and LC3 immune reactive cell densities are presented in Table 1 and demonstrated in Figure 2.

**Discussion**

Bortezomib is a proteasome inhibitor and is widely used in many tumoral malignancies. Despite abundant evidence of the therapeutic potential of this drug, the relevant signaling pathways leading to autophagy in cancer cells were not clear. In this study, we demonstrated that bortezomib could induce autophagy via Beclin-1 and LC3 proteins in the HT-29 cell line. These results suggest that bortezomib could be a potentially significant chemotherapeutic agent for the treatment of colorectal cancers.

**Table 1.** The Stereological estimation of anti-Beclin-1 and anti-LC3 immune reactive cells in HT-29 colon cancer cells incubated with 0nM, 10nM, 20nM and 40nM concentration for 24h.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Anti-Beclin-1 positive cell density (n/1000 μm²)</th>
<th>Anti-LC3 positive cell density (n/1000 μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.192±0.0032b</td>
<td>0.211±0.0038b</td>
</tr>
<tr>
<td>10-nM</td>
<td>0.342±0.0023b</td>
<td>0.292±0.0061a</td>
</tr>
<tr>
<td>20-nM</td>
<td>0.363±0.0062b</td>
<td>0.603±0.0042b</td>
</tr>
<tr>
<td>40-nM</td>
<td>0.447±0.0083b</td>
<td>0.641±0.0019b</td>
</tr>
</tbody>
</table>

Bortezomib is a specific inhibitor of the 26S proteasome [22] and it is an approved product for the treatment of multiple myeloma and acts as a prominent apoptosis inducer [23]. Most researchers report that
bortezomib has anti-tumor properties that can be used in the treatment of many types of cancer [24]. In the treatment of colon cancer, surgical removal of solid tumor masses is usually required with appropriate chemical therapy. Chemotherapeutics such as bortezomib are triggered with the autophagy cell death via cell death receptor induction or disturbance of mitochondrial balance. Recently, the cell death mechanisms such as autophagy and necrosis are desired targets for therapeutic applications [1,25,26].

Despite its increasing therapeutic use for the treatment of many cancers, little is known about cytotoxicity-related mechanism of colon cancers [27]. Lou et al., suggested that the bortezomib may inhibit HOS cell proliferation through inhibition of ERK phosphorylation [14]. Hong et al., reported that bortezomib trigger G2/M arrest through intracellular reactive oxygen species-inducible ataxia telangiectasia mutated phosphorylation in colon cancer cells [28]. In our study, it was determined that increasing concentrations of bortezomib treatments decreased cell viability in the HT-29 cell line.

In this study, we examined the effectiveness of bortezomib to determine its potential effects on tumoricidal activity in colon cancer. Our data shown in Figure 2 presented that Beclin-1 and LC3 expression levels increased in a higher concentration of bortezomib treated groups (especially 10nM, 20nM, and 40nM) in a dose-dependent manner, once again confirming autophagy induction. Song et al., found that Beclin-1 and LC3-I expressions increased after 12 h from treatment [12]. On the other hand, bortezomib has also the potential to increase the Beclin-1, LC3-I, and LC3-II expression levels in different carcinoma cells such as hepatocellular carcinoma and multiple myeloma [17,29].

Several conclusions can be taken out from the data presented here. First, it was understood that the treatment of bortezomib induces autophagy and secondly, LC-3 and Beclin-1 play an important role in the autophagy activating the mitochondria-dependent pathway. Third, Beclin-1 and LC3 levels increased in bortezomib treated colon cancer cells. Therefore, it can be said that bortezomib treatment induced the Beclin-1 mediated autophagy. Finally, the bortezomib treatment significantly inhibits colon cancer tumor growth.

In the light of the findings obtained in the study, it was concluded that bortezomib administration may induce autophagy activation by causing an increase in LC3 and Beclin-1 levels in colon cancer cells, and thus cause cell death.

**Declaration of Interest:** No potential conflict of interest relevant to this article was reported.
References


