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The Apoptotic Effects of a Proteasome Inhibitor on Caco-2 Colon Carcinoma Cells

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Abstract

The first and reversible inhibitor of the 26S proteasome (Velcade, formerly PS-341) has a crucial role with its antitumor activity against a number of malignancies and acts as a prominent inducer of apoptosis. In the present work, it was demonstrated the enhanced anticancer activity of a proteasome inhibitor by evaluating the levels of Bcl-2 protein family members on Caco-2 cell line. For this reason, Caco-2 colon carcinoma cells were treated with Velcade at four different doses (0, 5, 10, and 20nM). Western blotting was performed to detect the expression levels of apoptotic protein. Additionally, apoptotic cell death was demonstrated with in situ TUNEL staining by immunohistochemistry (IHC). An increase in the expression levels of both pro-apoptotic proteins was detected in a dose-dependent manner; however, the effective dose was detected as 10 nM. According to the IHC results, it can be said that a serious increase in the amount of apoptotic cells was determined. Taking everything into account, this proteasome inhibitor has a potential to be effective in inducing apoptotic mechanisms on colon carcinoma cells.

Keywords: Pro-apoptotic proteins, Bax, Bcl-2, Velcade, Caco-2, Colon carcinoma

Introduction

Colon carcinoma is one of the most common and aggressive cancer types in both men and women all around the world (1, 2). In recent years, approximately two million new cases and one million deaths were reported; however, this high mortality rate has decreased year by year, because of earlier diagnosis through screening and better treatment possibilities. At that point, standard therapies for colon carcinoma contain firstly chemotherapy and then biological therapy followed by tumor resection (3-8). It is known that standard chemotherapy in this disease is 5-fluorouracil plus either oxaliplatin or irinotecan (3, 5, 6, 9). Although large clinical experiments are still trying to find the best way to combine these therapeutics, unfortunately advanced unresectable colorectal cancer remains incurable and new targets and new approaches to treatment are needed (10).

Bortezomib (Velcade, PS-341), a dipeptide boronic acid derivative and the first 26S proteasome inhibitor, shows antitumor and anticarcinogenic activity in most human cancers (11-15). It is mediated through

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reversible binding to the N-terminus threonine residue in the β-1 subunit of the catalytic core complex of the 26S proteasome, leading to reversible inhibition of the proteolytic activity of the proteasome. Bortezomib is also a primary subcellular component of the protein degradation pathway that regulates the turnover of proteins involved in cell cycle arrest and apoptosis, such as the p21 cyclin-dependent kinase inhibitor, cyclins, and IKB, a regulator of nuclear factor-KB transcriptional activity (1, 10).

For the apoptotic cellular death, two important groups of proteins have a key role: the Bcl-2 family and a class of cysteine proteases known as caspases (16). The Bcl-2 family proteins have key functions especially in the regulation of a cellular mechanism, apoptosis, by targeting the mitochondria to show their pro- or anti-apoptotic activities. Bcl-2, an anti-apoptotic protein, is known to regulate apoptotic pathways and promote cell survival. Bax, a pro-apoptotic molecule of that family, is expressed abundantly and selectively during apoptosis and essential to mitochondrial release of apoptogenic factors (17). Increasing the ratio of Bcl-2 to Bax has commonly been used to determine the induction of apoptosis in several tissues. On the other hand, lack of Bax markedly decreases the cytotoxicity of proteasome inhibitors (18). It is still unclear the molecular mechanisms of Bax activation by proteasome inhibition; however, most scientific studies are focused on this issue (19).

In the present work, it was demonstrated the enhanced anticancer activity of a proteasome inhibitor by evaluating the levels of Bcl-2 protein family members (Bcl-2 and Bax) on Caco-2 cell line.

Materials and Methods

Culture of Caco-2 Colon Carcinoma Cells: A kind of human colon cancer cell line (Caco-2) was purchased from ATCC by the special code of HTB-37. The Caco-2 cells were incubated with a mixture containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin-streptomycin-gentamicin antibiotic solution. Caco-2 cells were cultured in T75 culture flasks (Corning) in a special CO_2 incubator at 37 ^{o}C. Subconfluent cultures were detached by using trypsin and counted with firstly trypan blue and then automated cell counting machine, and cells were then seeded in 6-well plates in DMEM (Dulbecco's Modified Eagle Medium). Then, bortezomib (Velcade, PS-341; Millennium Pharmaceuticals) application was performed with the following doses; 5 nM, 10 nM and 20 nM. After co-incubation, the media were collected; then the cells were washed with PBS and finally, MTT cell proliferation assay were performed.

MTT Cell Viability Assay: The viability of incubated cells was measured by using MTT. Firstly, incubated cells were inoculated into the 96-well plate at a density of 106 cells in each well. Then, colon cells were cultured with 3-different concentrations of Velcade (5nM, 10 nM and 20 nM), and then incubated with 1 ml of MTT solution for 4 h at 37oC. Then, the culture medium was removed and 1 ml of 0.1 mol/l HCl in absolute isopropanol was added into the plate. The absorbance was measured at 490 nm with an automatic microplate reader (Thermo, Multiskan GO, USA). The final calculation for cell viability was as follows: cell viability (fold) ¼ the absorbance of treated group/the average absorbance of untreated group

TUNEL Assay: For detection of DNA breakage, cells were air dried and fixed in 4% paraformaldehyde for 1 h at room temperature. In Situ Cell Death Kit from Roche (California, USA) was used for TUNEL assay according to the manufacturer's protocol. Briefly, fixed cells were rinsed in PBS, blocked, and permeabilized. TUNEL reaction mixture was then added to the cells and incubated at 37 C for 1 h. Apoptotic signals were converted with peroxidase and stained with 3,3′-diaminobenzidine tetrahydrochloride. Apoptotic cells were detected and scored under fluorescence microscopy with 4′,6-diamidino-2-phenylindole counter staining. The average of the TUNEL-positive nuclei ratio in at least 10 representative microscopic

fields was calculated to compare the apoptosis ratio within the different groups.

Western Blotting and analysis: After treatment, cells were collected using a cell scraper at 4°C and lysed in a whole-cell lysis buffer. Approximately 25 µg total cellular protein from each sample was subjected to SDS-PAGE, proteins were transferred to nitrocellulose membranes, and the membranes were blocked with 5% nonfat milk in a TBS solution containing 0.1 % Tween 20 for 1 h. The blots were then probed overnight with anti-Bax and anti-Bcl-2 antibodies, washed, and probed with specific secondary antibodies coupled to HRP. Immuno-reactive material was detected by enhanced chemiluminescence (Fusion Fx, Fisher Biotek, Australia).

Results

The anti-proliferative effect of Velcade/bortezomib (in 5, 10, and 20 nM doses) was evaluated by cell viability MTT method in Caco-2 cells in both 24 and 48 h. It was seen that bortezomib caused both time- and dose-dependent strong decreases in the viability of Caco-2 cells. According to the MTT findings, a decrease was detected in the viability of Caco-2 cells in 24-h evaluation. The higher doses of Velcade/bortezomib (10 and 20 nM) showed expected results in both 24 and 48-hour as seen in Figure 1. According to Figure 1, a time- and dose-dependent inhibition in cell viability of colon cancer Caco-2 cells were detected.

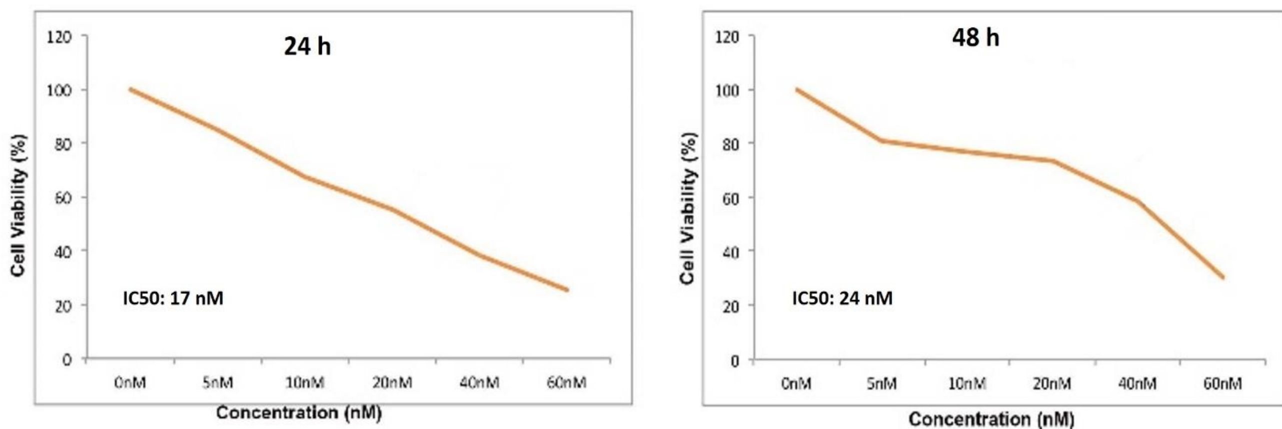

Figure 1. MTT analysis of Caco-2 colon cancer cells treated with bortezomib for 24 and 48 h., inhibitory concentration 50 (IC50) values were calculated by obtained 2 results of MTT.

The effect of bortezomib on apoptotic pathways in Caco-2 cell line: In the analysis of protein densities, bortezomib was increased pro-apoptotic Bax level in the 24 h period of colon cancer cells (Caco-2) and higher concentration of bortezomib markedly increased Bax protein level. Besides treatment of bortezomib, a particually higher dose of bortezomid decreased the Bcl-2 level in the both 24 and 48 h periods in colon cancer cells. Bax and Bcl-2 protein densities were demonstrated in Fig 2.

According to the analyses of TUNEL results, it was seen that there were increased apoptotic cells in 24 and the 48 h periods of bortezomib treated Caco-2 colon carcinoma cells. Also, 48 h period of bortezomib increased apoptotic cells more than the 24 h period of bortezomib treatment in colon cancer cells. The results of TUNEL positive cell densities for all groups were presented in Fig 3.

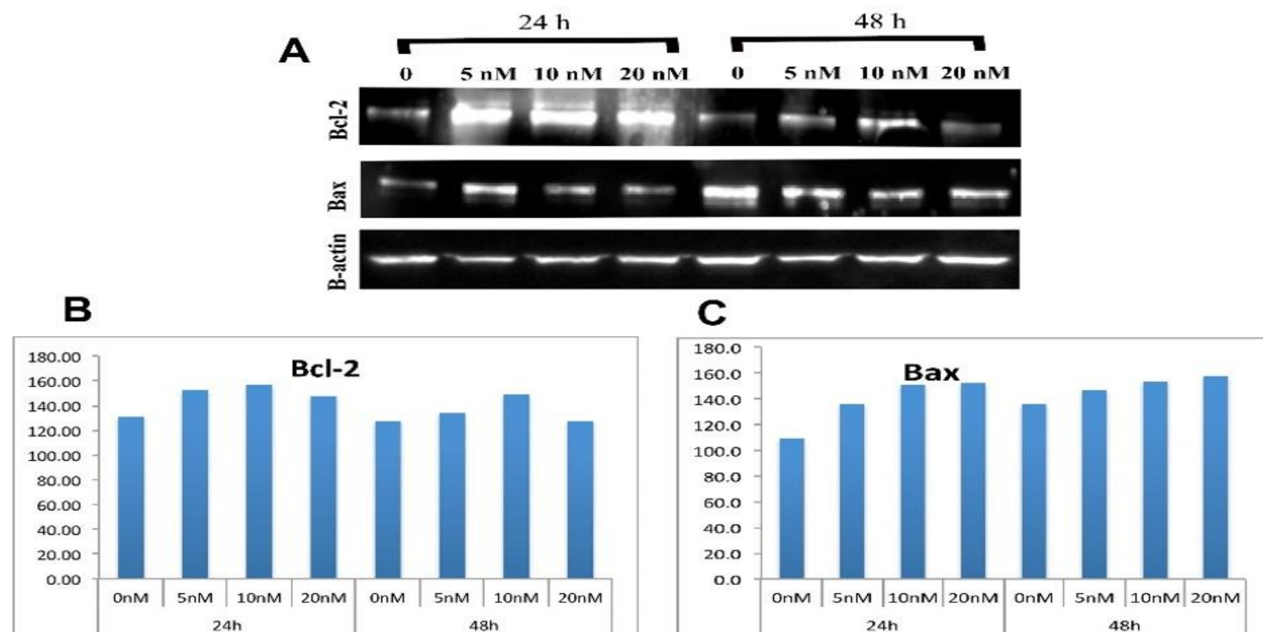

Figure 2. Western blot analysis of Caco-2 cells lysates, treated with 5, 10, 20 nM bortezomib for 24 and 48 h periods, A: Western blot reveals expression levels of Bcl-2 and Bax in Caco-2 cells, B: western blot analysis of Bcl-2 protein levels, and C: Bax protein levels using ECL Western blotting detection system (described in material and method).

Discussion

Bortezomib, a first and specific inhibitor of a proteasome (26S), (20) is generally used for the treatment of some human cancer types especially multiple myeloma and known as acting as a prominent inducer of apoptosis (21). In current literature, most scientific studies reported and suggested that Bortezomib (Velcade) might also be applied for the treatment of a variety of additional cancer types (22). Colon cancer therapy is based on the surgical removal of solid tumor masses, usually combined with a series of chemical treatments. Most drugs, especially bortezomib are thought to ultimately induce apoptosis of tumor cells through mitochondrial or death-receptor pathways. Recently, other cell death mechanisms are being considered for therapeutic application, such as autophagy and necrosis (1, 23, 24).

Several conclusions can be come out upon consideration of the data presented here. First, our data supported that a treatment with bortezomib may induce apoptosis. Second, the Bcl-Bax pathway plays an important role in the apoptosis through activating

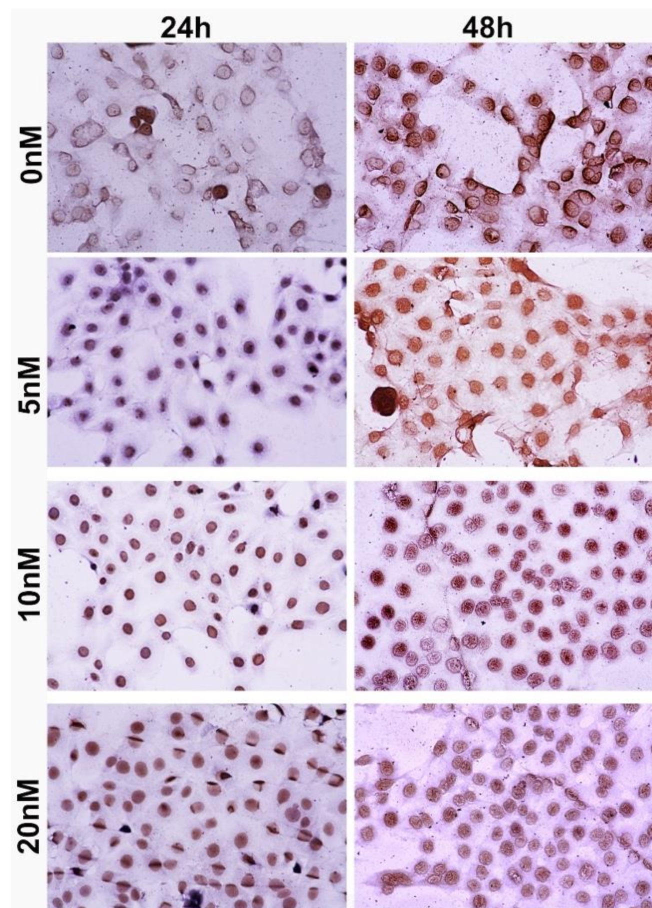

Figure 3. Tunnel staining of Caco-2 colon cancer cells for all groups, brown color shows tunnel positive cells.

the mitochondria-dependent pathway. Finally, using 26S proteasome inhibitor in colon carcinoma in vitro model shows an apoptotic effect by inducing some pro-apoptotic proteins including Bcl-2 and Bax.

In the present study, we chose the human colon carcinoma cell line Caco-2 as a model to explore the ability of bortezomib to modulate the main proteolytic cellular pathways: especially apoptosis. The importance of the mitochondrial component of the apoptotic pathway is perhaps best approached by the overexpression of the anti-apoptotic proteins Bcl-2 to block the mitochondrial contribution to the intrinsic apoptotic pathway. Our results showed that bortezomib was suppressing the Bcl-2 expression level whereas it was increasing the Bax level. Some scientific studies in current literature showed that treatment with bortezomib has been reported to cause alterations in the levels of apoptosis-regulating proteins including Bcl-xL (25), Bcl-2, and other Bcl-2-related proteins (26, 27). Kretowski et al. (28) pointed out that bortezomib has a potential to induce apoptosis via anti-apoptotic Bcl-2 family members in DLD-1 colon cancer cell line. In another study, (1) it was claimed that apoptosis was induced by Bcl-2/Bcl-xL phosphorylation in response to treatment with bortezomib. Pitts et al. also reported that bortezomib had anti-proliferative and pro-apoptotic effects against colon cancer cell lines by effecting Bax, Bcl-2, caspase 3, and caspase 7 levels (10).

As a conclusion, the proteasome is involved in the proteolysis of cell cycle regulators and damaged proteins, therefore the identification of proteasome modulators is a promising approach in a number of pathologies, such as cancer, neurodegenerative, cardiovascular, and metabolic diseases (29). On the other side, autophagy can be involved in cell defense mechanisms or in type II programmed cell death, in response to cellular stress conditions (30, 31).

Proteasome inhibitor bortezomib promotes cell death in colon cancer cell line Caco-2 through apoptosis. In conclusion, we suggest that bortezomib may be candidates for further evaluation as chemotherapeutic agents for human colon cancer. On the other hand, future studies will be needed to investigate other cellular mechanisms including autophagy and related parameters.

Declaration of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

Author Contribution

SAK contributed all parts of the study.

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