Proteasome and HDAC Inhibition Changes the Expression Levels of Bcl-2, Bcl-XL, Bim and Bik Proteins in Androgen-Independent PC-3 Cell Line

Proteazom ve HDAC İnhibisyonunun Androjenden-Bağımsız PC-3 Hücre Hattında Bcl-2, Bcl-XL, Bim ve Bik Proteinlerinin İfadelenme Düzeylerini Değiştirmesi

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ABSTRACT

Objective: Prostate cancer is the most common cancer observed and it is estimated to have caused 10% of all cancer-related deaths in men in western countries. Transition from androgen-dependent form to metastatic androgen-independent phase is a consequence of deranged apoptotic response by the cancer cells to therapy. Bcl-2 family members are important modulators of apoptotic response. In this study, we investigated the effects of proteasome and histone deacetylase (HDAC) inhibitor treatment on the expression changes in some Bcl-2 family genes at protein level.
Methods: PC-3 cells were cultured in plate before being exposed to different concentrations of unaccompanied bortezomib and TSA as well as bortezomib – TSA combination. After the protein isolation from control and treated cells, whole cell lysate was used for determining Bcl-2, Bcl-XL, Bim and Bik proteins with western blotting method.
Results: We observed that after the drug treatment, the expression levels of pro-apoptotic Bim and Bik proteins have increased and those of anti-apoptotic Bcl-2 and Bcl-XL proteins have decreased. Synergy between bortezomib and TSA induces apoptosis.
Conclusion: Our results showed that application of low doses of bortezomib and TSA combination may be sufficient for apoptotic response. Our findings may come in handy for the advanced prostate cancer research.

Key Words: Bcl-2 family, histone deacetylase and proteasome inhibitors, prostate cancer cells.

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ÖZET

Amaç: Prostat kanseri, batı ülkelerindeki erkeklerde kanser-ilişkili ölümlerin % 10'unu oluşturduğu tahmin edilen, en sık görülen kanserdir. Androjen-bağımlı formdan metastatik androjenden-bağımsız faza geçiş, kanser hücreleri tarafından terapiye verilen bozulmuş apoptotik yanıtın bir sonucudur. Bcl-2 ailesi üyeleri, apoptotik yanıtın en önemli düzenleyicileridir. Bu çalışmada, proteazom ve Histon Deasetilaz (HDAC) inhibitörlerinin, bazı Bcl-2 ailesi genlerinin ifade değişiklikleri üzerine etkisini protein düzeyinde araştırdık.
Yöntem: PC-3 hücrelerine, bortezomib ve TSA'nın tekli ve kombine farklı konsantrasyonları uygulanmadan önce kültüre edildi. Kontrol ve ilaç uygulanmış hücrelerden protein izolasyonu sonrasında, tüm hücre lizatı Bcl-2, Bcl-XL, Bim ve Bik proteinlerini western blot yöntemi ile belirlemek için kullanılmıştır.
Bulgular: Hücrelere ilaç uygulamasının ardından pro-apoptotik Bim ve Bik proteinlerinin ekspresyon seviyelerinin arttığını ve anti-apoptotik Bcl-2 ve Bcl-XL proteinlerinin ekspresyon seviyelerinin azaldığı gözlemlenmiştir. Bu ilaçlar arasındaki sinerji, apoptozisin indüksiyonuna yol açmıştır.
Sonuç: Bulgularımız, bortezomib ve TSA'nın düşük doz kombinasyonlarının uygulanması, apoptotik yanıtta yeterli olabileceğini göstermiş olup; ileri evre prostat kanseri araştırmaları için önemli olabilir.

Anahtar Sözcükler: Bcl-2 ailesi, histon deasetilaz ve proteazom inhibitörleri, prostat kanser hücreleri

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INTRODUCTION

Prostate cancer is the most common cancer type in males and the second leading cause of cancer-related deaths in developed countries. Despite remarkable advances in prostate cancer research, this disease is still associated with a significant mortality rate especially when it is resistant to treatment (1, 2).

Bortezomib (PS-341, Velcade) is a dipeptidyl boronic acid inhibitor of the 26S proteasome that was developed as a therapeutic agent for various cancers (3). Bortezomib selectively and reversibly inhibits the chymotrypsin-like activity of the 26S proteasome and is an agent used quite common in investigations. The main purposes of the use of bortezomib and other proteasome inhibitors are accumulating tumor suppressors such as p53, p21 and p27 in the cell, inhibition of anti-apoptotic NF-κβ pathway, and generating ER stres by accumulation of unfolded and damaged proteins in the cell. In this way, apoptosis is triggered to create an effect that stops the formation of tumorigenesis (4). In recent years, researchers have studied the combination of proteasome and histone deacetylase (HDAC) inhibitors as therapeutic agents in various malignancies. Combination of HDAC inhibitors with the proteasome inhibitors induces a marked increase in mitochondrial injury and apoptosis in many cancer cells and also generates reactive oxygen species (ROS), hence triggers apoptosis (5-7). TSA is a classic potent inhibitor of HDAC and a hydroxamic acid–derived compound from the metabolic product of streptomyces. This drug has been widely studied as a therapeutic agent for various cancers and has been shown to induce cell cycle arrest, apoptosis and inhibit metastasis by blocking HDAC activity on promoters of tumor suppressor genes (8).

In the mechanism of apoptosis, caspase activation plays a pivotal role and it is mainly controlled by Bcl-2 family proteins (9). In living cells, bax and bak proteins exist as monomer under normal conditions. When the death signal is received, bax and bak proteins are oligomerized. This makes the mitochondrial outer membrane permeable and apoptotic molecules such as cytochrome-c are released. Cytochrome-c binds to Apaf-1 molecule and caspase-9 is activated. After this formation, called apoptosome, activation of caspase-9 occurs and this triggers activation of effector caspase-3, 6 and 7. On the other hand, anti-apoptotic Bcl-2 protein inactivates all pro-apoptotic proteins such as Bim, Bik, Bak, Bax by binding to their active sides. However, the balance of cell survival and death is determined by controlling Bcl-2 family proteins at transcriptional level (9, 10). In all these processes, the ubiquitin proteasome system has important roles (4, 10).

In this study, we examined the anti-apoptotic effects of unaccompanied bortezomib and TSA as well as bortezomib – TSA combination on the human PC3 prostate cancer cell line and investigated the expression levels of anti-apoptotic Bcl-2, Bcl-XL and pro-apoptotic Bim and Bik proteins.

METHODS

Cell Culture and Reagents

The human prostate cancer cell line PC-3 was provided by Prof. Levent Türkeri (Department of Urology, Faculty of Medicine, Marmara University, Istanbul, Turkey).

Cells were cultured in RPMI medium containing 10% fetal bovine serum and 1% penicillin-streptomycin and maintained at 37°C with 5% carbon dioxide. Bortezomib was purchased from BioVision (Milpitas, CA, USA). It was dissolved in dimethylsulfoxide at a concentration of 1mM and stored at -20°C. TSA was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). It was dissolved in ethanol at a 1mM stock solution and stored at -20°C. Stock solutions were diluted to their working concentrations with growth medium just before use.

Protein Isolation and Western Blot Analysis

Cells (2 x 10^6) were plated on 25cm^2 cell culture dishes one day before bortezomib and/or TSA treatment. Whole cell lysate was obtained using RIPA buffer (Thermo Fisher Scientific, Waltham, MA USA). Next, 35µg total protein lysate from each sample was loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane using a Bio-Rad wet-blot transfer apparatus (Bio-Rad, Hercules, CA, USA). The membrane was blotted with 5% non-fat milk powder at room temperature for 1h and probed with primary antibodies at 4°C overnight. The primary antibodies are Bcl-2, Bcl-XL, Bim and Bik (Thermo Scientific, Waltham, MA, USA) and β-actin (Cell Signaling Technology, Danvers, MA, USA). They were then incubated with the secondary antibody anti-rabbit IgG-HRP (Cell Signaling Technology, Danvers, MA, USA) for 1h to detect the primary antibodies. Proteins were visualized by Kodak Gel Logic 2200 using Lumina Crescendo Western HRP substrate (Millipore, MA, USA).

RESULTS

Bortezomib and TSA Induce Apoptotic Effect by Inhibiting Anti-apoptotoic Proteins and Activating Pro-apoptotic Proteins

To identify the changes in the expression levels of anti-apoptotic Bcl-2, Bcl-XL and pro-apoptotic Bim and Bik, protein lysates were obtained from PC-3 cells which underwent unaccompanied bortezomib (10nM and 50nM) and TSA (400nM) as well as combination (5nM Bortezomib and 300nM TSA) treatment. Expression level of Bcl-2 protein only increased 24h after combination treatment. However, combination treatment at 48h showed a decreased expression compared to control dose (Figure 1A). Bcl-XL did not decidedly change following treatment of PC-3 cells with unaccompanied bortezomib or TSA compared to the control. However, combination treatment resulted in decreased expression of Bcl-XL (Figure 1A). This result showed that there is a synergy between the two drugs for enhancing apoptotic and anti-proliferative effect. Also, it was demonstrated that after unaccompanied and combination treatment, the expression of pro-apoptotic proteins Bim and Bik increased (Figure 1B). Increased expression of Bim and Bik may be acting to mediate proteosome and HDAC inhibition-induced apoptosis in PC-3 cells. These data showed us that combination of proteosome and HDAC inhibition led to increased expression of both pro-apoptotic proteins while reduced expression of both anti-apoptotic proteins.

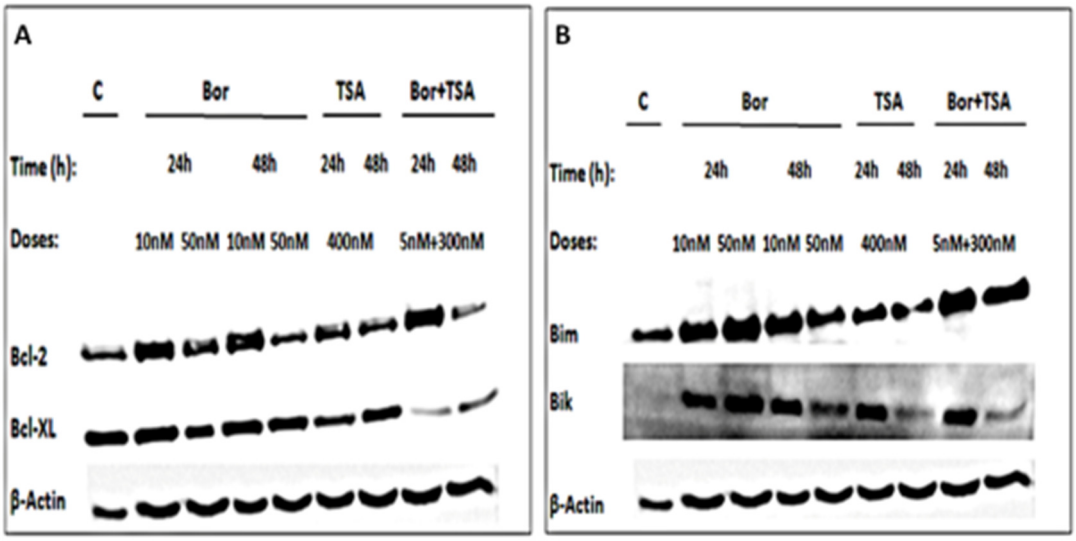


Figure 1. Effects of Bortezomib and Trichostatin A (TSA) treatment on changes in expression levels of Bcl-2, Bcl-XL (**A**) and Bim and Bik (**B**) proteins in PC-3 prostate cancer cell line analyzed by Western Blot. After combination treatment with 5nM bortezomib plus 300nM TSA, both Bcl-XL and Bcl-2 expression levels declined at 48h (**A**). After treatment with specified concentrations of unaccompanied and combined Bortezomib and Trichostatin A (TSA), the expression levels of pro-apoptotic proteins Bim and Bik increased (**B**) (Bor = bortezomib, TSA= Trichostatin A, C = control).

DISCUSSION

Bcl-2 family proteins play an essential role in apoptosis control points. The most important member of this family is Bcl-2. It is known that Bcl-2 inhibits apoptosis and accelerates the process of carcinogenesis. Bcl-2 protein family members are apoptosis-inhibiting (Bcl-2, Bcl-XL, MCL-1, Bcl-w) or activating (Bax, Bak, Bad, Bim, Bik, Noxa, etc.), each highly showing homology of protein family (11, 12). Expression levels of pro-apoptotic and anti-apoptotic Bcl-2 family proteins are directly regulated by the ubiquitin-proteasome system. This protein family, its kinases and various transcription factors are proteasome main substrates. Various studies showed that the expression level of Bcl-2 protein family may change in response to inhibitors of the ubiquitin proteasome system (10, 12).

Some members of the Bcl-2 family, especially Bcl-2 and Bcl-XL, often play a role in development of metastatic prostate cancer. Overexpression of Bcl-2 proto-oncogene is known to result in malignant transformation in various cancers. Many studies showed that, in initiation and progression phases of androgen-independent metastatic prostate cancer, Bcl-2 and Bcl-XL play an important role (13-15).

Western Blot analyses performed in our study showed that, expression level of Bcl-2 protein increased only after 24h of combination treatment. This result may point to the resistance of androgen-independent PC-3 cells which have high metastatic potential for treatment periods < 24h. (16). Bortezomib-TSA combination treatment for 48h resulted in a remarkable decrease in the expression level of Bcl-2 protein. Also we identified no noticeable alterations in the expression levels of Bcl-XL following unaccompanied bortezomib and TSA treatment when compared with those of the control. However, the expression levels of Bcl-2 and especially Bcl-XL markedly decreased after combination treatment. Furthermore, we demonstrated that both unaccompanied and combination treatments resulted in increased expression levels of the two pro-apoptotic proteins, Bik and Bim. This accumulation of Bik and Bim indicated the synergistic action of low dose bortezomib and TSA targeting the induction of apoptosis. Our results are in agreement with studies performed on effects of these two drugs on other cell lines (17, 18).

Moreover, taking into account the metastatic characteristics of PC-3 cells, the expression levels of Bcl-2 and Bcl-XL can be further reduced by increasing the dose and the duration of combination treatment. Also, the use of bortezomib with Bcl-2 antagonists may help to reduce the expression of these anti-apoptotic proteins (19, 20).

The HDAC inhibition-enhanced proteasome inhibition promotes the degradation of anti-apoptotic proteins while preventing the degradation of pro-apoptotic proteins. This results in increased accumulation of pro-apoptotic proteins and tumor suppressors in the cells, subsequently leading to cell growth inhibition and apoptotic response in numerous malignant cell types (21, 22).

CONCLUSION

These results show us that the combination of HDAC and proteasome inhibition would induce synergistic inhibition of anti-apoptotic proteins and induction of apoptosis by inducing stabilization of pro-apoptotic proteins and tumor suppressors. The combination of both low-dose HDAC and proteasome inhibitors may activate pro-apoptotic signals by causing histone acetylation and ubiquitinated protein accumulation. Although there exists a synergistic effect between bortezomib and TSA, the further evaluation of their combined use should be undertaken in animal models to come up with new approaches regarding advanced prostate cancer.

Conflict of Interest

No conflict of interest was declared by the authors.

REFERENCES

1. Shen MM, Abate-Shen C. Molecular genetics of prostate cancer: new prospects for old challenges. Genes Dev. 2010; 24: 1967-2000.
2. Rider JR, Sandin F, Andrén O, Wiklund P, Hugosson J, Stattin P. Long-term outcomes among noncuratively treated men according to prostate cancer risk category in a nationwide, population-based study. Eur Urol. 2013; 63:88-96.
3. Chen D, Frezza M, Schmitt S, Kanwar J, Dou QP. Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. Curr Cancer Drug Targets. 2011; 11: 239-53.
4. Frankland-Searby S, Bhaumik SR. The 26S proteasome complex: an attractive target for cancer therapy. Biochim Biophys Acta. 2012; 1825: 64-76.
5. Carew JS, Giles FJ, Nawrocki ST. Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy. Cancer Letters. 2008; 269: 7-17.
6. Lin Z, Bazzaro M, Wang MC, Chan KC, Peng S, Roden R. Combination of proteasome and HDAC inhibitors for uterine cervical cancer treatment. Clin Cancer Res. 2009; 15: 570–7.
7. Heider U, Rademacher J, Lamottke B, Mieth M, Moebs M, von Metzler I, et al. Synergistic interaction of the histone deacetylase inhibitor SAHA with the proteasome inhibitor bortezomib in cutaneous T cell lymphoma. Eur J Haematol. 2009; 82: 440–9.
8. Ellis L, Pili R. Histone deacetylase inhibitors: advancing therapeutic strategies in hematological and solid malignancies. Pharmaceuticals 2010; 3(8): 2411-69.
9. Brunelle JK, Leatai A. Control of mitochondrial apoptosis by the Bcl-2 family. J Cell Sci. 2009; 122 (Pt 4): 437-41.
10. Fennell DA, Chacko A, Mutti L. Bcl-2 family regulation by the 20S proteasome inhibitor bortezomib. Oncogene 2008; 27: 1189–97.
11. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol. 2008; 9:47-59.
12. Brancolini C. Inhibitors of the ubiquitin-proteasome system and the cell death machinery: how many pathways are activated? Curr Mol Pharmacol. 2008; 1: 24-37.
13. Yip KW, Reed JC. Bcl-2 family proteins and cancer. Oncogene 2008; 27: 6398-406.
14. Gurumurthy S, Vasudevan KM, Rangnekar VM. Regulation of apoptosis in prostate cancer. Cancer Metastasis Rev. 2001; 20: 225-43.
15. Vela L, Marzo I. Bcl-2 family of proteins as drug targets for cancer chemotherapy: the long way of BH3 mimetics from bench to bedside. Curr Opin Pharmacol. 2015; 23:74-81.
16. McConkey DJ, Zhu K. Mechanisms of proteasome inhibitor action and resistance in cancer. Drug Resist Updat. 2008; 11: 164-79.
17. Marshansky V, Wang X, Bertrand R, Luo H, Duguid W, Chinnadurai G, et al. Proteasomes modulate balance among proapoptotic and antiapoptotic Bcl-2 family members and compromise functioning of the electron transportchain in leukemic cells. J Immunol. 2001; 166: 3130-42.
18. Nikrad M, Johnson T, Puthalalath H, Coultas L, Adams J, Kraft AS. The proteasome inhibitor bortezomib sensitizes cells to killing by death receptor ligand TRAIL via BH3-only proteins Bik and Bim. Mol Cancer Ther. 2005;4:443-9.
19. Fang Y, Hu Y, Wu P, Wang B, Tian Y, Xia X, et al. Synergistic efficacy in human ovarian cancer cells by histone deacetylase inhibitor TSA and proteasome inhibitor PS-341. Cancer Invest. 2011; 29: 247-52.
20. Pei XY, Dai Y, Grant S. Synergistic induction of oxidative injury and apoptosis in human multiple myeloma cells by the proteasome inhibitor bortezomib and histone deacetylase inhibitors. Clinical Cancer Research 2004; 10: 3839-52.
21. Denlinger CE, Keller MD, Mayo MW, Broad RM, Jones DR. Combined proteasome and histone deacetylase inhibition in non-small cell lung cancer. J Thorac Cardiovasc Surg. 2004; 127: 1078-86.
22. Kim J, Guan J, Chang I, Chen X, Han D, Wang CY. PS-341 and histone deacetylase inhibitor synergistically induce apoptosis in head and neck squamous cell carcinoma cells. Mol Cancer Ther. 2010; 9: 1977-84.