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Original Article

Apoptotic effect of Belinostat (PXD101) on MCF-7 cancer cells

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Abstract

Aim: Breast cancer continues to be the most common type of cancer among women. Recent development in epigenomics has highlighted key mechanisms in which epigenetic regulation contributes to cancer treatment. Epigenetic modulating drugs promises novel approaches targeting cancer treatment. Belinostat is a histone deacetylase inhibitor approved by the U.S. Food and Drug Administration (FDA) in 2014 for the T-cell lymphoma. This study aimed to investigate the apoptotic effects of Belinostat on MCF-7 cells.

Materials and Methods: For this purpose, The IC_{50} value of Belinostat was determined by XTT assay and the apoptotic effect of Belinostat on MCF-7 cells was evaluated. Expression of apoptosis-related genes including Caspase 3 (CASP3), CASP9, apoptotic protease activating factor-1 (APAF-1) and tumor protein P53 (P53) were evaluated by quantitative Real-Time PCR.

Results: The IC_{50} dose of Belinostat was determined as 5 µM for 48 h. The results of study showed that Belinostat administration decreased the number of cancer cells in the MCF-7 cell population and down regulated the gene expression of apoptosis-related genes.

Conclusion: Results indicated that Belinostat, can be considered as an option in the treatment of breast cancer. It is considered that it would be more beneficial to perform more sophisticated *in vivo* and *in vitro* studies about apoptotic effect of Belinostat.

Keywords: Apoptosis; Belinostat; epigenetics drug; MCF-7 cell

INTRODUCTION

Radiation, chemical carcinogens, tumor viruses, oncogenes, loss of the function of tumor suppressor genes and the deterioration of mechanisms regulating the normal behavior of the cell result in abnormal cell proliferation that is called cancer (1). Breast cancer is the most frequent cancer among women in the world as well as in Turkey and also causes the greatest number of cancer related deaths among women (2,3). Genes (BRCA1 and BRCA2) related to breast cancer, aging, family history, reproductive factors, estrogens and lifestyle are the risk of breast cancer. Great advances of clinical studies of breast cancer have been accomplished using prevention methods including chemoprevention, hormone prevention etc. In this way, the mortality of breast cancer has decreased; however, it is still the most common cancer among females (4).

Epigenetic modifications regulating transcriptional activity are dysregulated in many diseases, including cancer. Aberrant DNA methylation and histone modifications are key epigenetic mechanisms associated with tumor initiation, cancer progression and metastasis. Therefore, within the past decade novel epigenetics drugs have been developed as a therapeutic target for cancer treatment (5). Epigenetic drugs including DNA methyltransferase inhibitors (DNMTi), histone acetyltransferase inhibitors (HATi), histone deacetylase inhibitors (HDACi) and histone demethylase inhibitors (HDMi) have been used in cancer research (6). In many cancer types, unbalanced HDAC activities were determined. This can cause aberrant histone acetylation, which thereby can result in tumorigenesis and cancer progression. Different HDACi compounds have been developed and used as a therapeutic agent for cancer treatment (7). Since HDCAi opens condensed chromatin structure, it increases the accessibility of chemotherapeutics targeting DNA (8). HDACi can be used also in various types of neurodegenerative diseases, inflammation disorders and cardio vascular diseases (9).

Belinostat (PXD101) is a histone deacetylase inhibitor which has $C_{15}H_{14}N_2O_4S$ molecular formula (10). Belinostat was developed by TopoTarget for hematological malignancy and solid tumors. Belinostat was approved

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by the U.S. Food and Drug Administration (FDA) in 2014 for the treatment of peripheral T cell lymphoma (11,12). Effects of Belinostat have been evaluated on different types of cancer and non-malignant diseases (13).

Apoptosis is known as programmed cell death that requires energy-dependent biochemical process (14). The proteins that are effective in the apoptotic pathway are named caspases. Because, they carry cysteine (C) residues in their catalytic regions and cleave their target proteins on the C-terminal side of the aspartate (asp) and act as enzymes. When a death signal comes to the cell, procaspases turn into active caspases through a proteolytic process (15). Apoptosis is triggered by activation of caspase proteins (16,17). Apoptosis is mediated by either mitochondria/cytochrome C mediated cell death (intrinsic pathway) or cell death through death signals (extrinsic pathway). In extrinsic pathway, specific external stimuli FAS or TNFR ligands bind at death receptors on the cell surface, which leads downstream caspase activation. The intrinsic pathway is modulated by Bcl-2 and Bax. The aim of this study was to investigate the Belinostat's apoptotic effects on MCF-7 breast cancer cells.

MATERIALS and METHODS

Cell and Reagents

The breast cancer MCF-7 cell line was purchased from American Type Culture Collection (ATCC, Rockville, Maryland 20852, USA). MCF-7 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (Sigma-Alrich, St. Louis, MO) and 1% antibiotic-penicillin solution (Sigma-Alrich, St. Louis, MO) at 37°C in a humidified atmosphere of 5% CO_2. Cells were harvested with 0.25% trypsin (Sigma- Aldrich, St. Louis, USA) after the cell culture reached 90% confluence. Belinostat was purchased from (MCE MedChem Express), and dissolved in DMSO. MCF-7 cells were treated with various concentrations of Belinostat in different time durations.

The IC50 of Belinostat determined by XTT assay

To determine the IC_{50} dose of Belinostat, approximately 10^4 cells were seeded into the 96 well-plate and cultured for 24 hours. Then, the predetermined doses of Belinostat (0.25, 0.5, 0.75, 1, 3, 5, 15 and 25 µM) were applied to the wells and the cells were cultured for 24, 48 and 72 h. In each dose group, a well was cultured under the same conditions as the control group without Belinostat administration. After 24, 48 and 72 h of the application, the cells in each well subjected to a XTT test using Cell Proliferation Kit (Biological Industries) according to the manufacturer's instructions and absorbance values were quantified at 450 nm in a microplate reader.

RNA isolation and the quantitative real-time polymerase chain reaction

Total RNAs were isolated from the Belinostat-treated (5 µM for 48 h.) and untreated cells using TRIzol (Sigma Aldrich®) according to the manufacturer's instructions. RNA was precipitated with 75% ethyl alcohol and dissolved in RNase-free water. Concentrations of RNAs were measured at 260 and 280 nm using Nano Drop 2000 (Thermo Fisher Scientific). cDNAs were synthesized by from 1 µg of total RNA using iScript™ cDNA Synthesis Kit (Bio-Rad) and the protocol suggested by the manufacturer. Quantitative RT-PCR (qPCR) mix for each gene was set up in 10 µl final volume containing 5 µl iTaq Universal SYBR Green Supermix, 12.5 pMol each primer and 3 µl cDNA. The nucleotide sequences of the primer pairs were given in Table 1. PCR profile was consist of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. PCR analysis was performed in triplicate using a CFX Connect™ Real-Time PCR instrument. The resulting PCR products were run on 2% agarose gel for 40 minutes at 110 volts to confirm the accuracy of the products obtained at the end of the reaction (Figure 1).

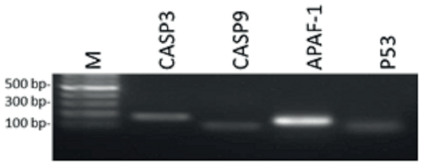

Figure 1. Agarose gel electrophoresis of apoptosis-related genes (M: 100 bp DNA standard)

Table 1. The nucleotide sequences of the primer pairs use for quantitative gene expression

Gene	Oligonucleotide sequence	(bp)
CASP3	GAAATTGTGGAATTGATGCGTGA	164
	CTACAACGATCCCCTCTGAAAAA	
CASP9	CTGTCTACGGCACAGATGGA	75
	GGGACTCGTCTTCAGGGA	
APAF-1	AAGGTGGAGTACCACAGA	116
	TCCATGTATGGTGACCCATCC	
P53	CAGCACATGACGGAGGTTG	125
	TCATCCAAATACTCCACACGC	
GAPDH	GGAGCGAGATCCCTCCAAAAT	197
	GGCTGTTGTCATACTTCTCAT	
CYPA	TATCTGCACTGCCAAGACTGAGTG	124
	CTTCTTGCTGCTGGTCTTGCATTCC	

Statistical Analysis

GAPDH and CYPA reference genes were used for normalization. The comparative ΔΔCT method was used to calculate the relative gene expression. Statistical analyses were performed by using "RT² Profiler™ PCR Array Data Analysis" software. Comparisons between treated groups and untreated controls were carried out using the student *t*-test. The tests considered a basic significance level of $p < 0.05$.

RESULTS

Effect of Belinostat on MCF-7 cells and determination of IC_{50}

Effect of Belinostat on MCF-7 cells was determined by the administration of various concentrations (0.25, 0.5, 0.75,

1, 3, 5, 15 and 25 µM) for 24, 48 and 72 h. Cell viability was assessed by the XTT assay. Results indicated that Belinostat inhibited the cell proliferation of MCF-7 cells. The IC_{50} value of Belinostat was determined as 5 µM for 48 h. (Figure 2).

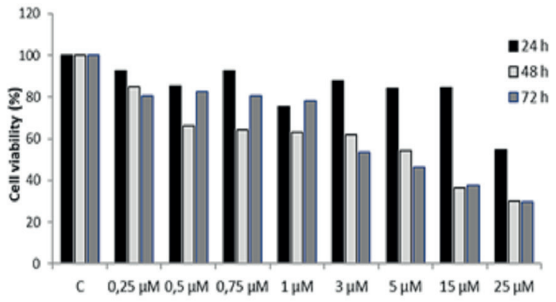

Figure 2. The effect of Belinostat on MCF-7 cell viability

Quantitative gene expression results of MCF-7 cells

To assess the effect of Belinostat on apoptosis pathway, the gene expression levels of apoptosis-related genes including CASP3, CASP9, APAF-1 and P53 were determined in MCF-7 cells. Relative expression levels were determined after the normalization with GAPDH and CYPA. According to the results, Belinostat administration significantly down regulated the expression of CASP3, CASP9 and P53 in MCF-7 cells. There were statistically significant difference between the gene expression ($p<0.05$). APAF-1 expression was also decreased, but this difference was not significant ($p=0,063$, Figure 3).

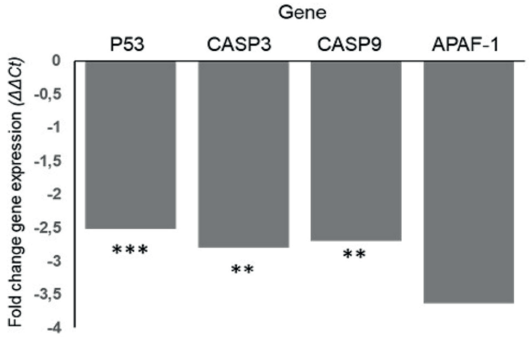

Figure 3. Fold change expression of apoptosis-related genes (**p>0,01, ***p>0,01)

DISCUSSION

Cancer is a disease characterized by abnormal differentiation and uncontrolled proliferation of cells and remains the most common cause of death after cardiovascular diseases. The incidence of cancer worldwide has increased by 4 million in the last 6 years and reached to 18 million reported by the World Health Organization in 2018 (18). Breast cancer is the most common type of cancer among women in the worldwide as well as in Turkey (2). In addition to many mechanisms that are effective in cancer formation, epigenetic mechanisms including DNA methylation, histone modifications and nucleosome rearrangement have been recently found to be effective (19). Therefore, different epigenetic compounds and drugs were developed to target cancer including DNA methyl transferase inhibitors (DNMTi), histone acetyltransferase inhibitors (HATi), histone deacetylase inhibitors (HDACi) and histone demethylase inhibitors (HDMi) (20). Clinical studies of Belinostat, a histone deacetylase inhibitor, continue in many cancer and non-malignant diseases (11). In this study, the apoptotic effect of Belinostat on MCF-7 cells was investigated.

In this study, the cytotoxic effect of Belinostat on MCF-7 cells was determined as 5 µM for 48 h according to XTT assay. There are several studies investigating the cytotoxic effect of Belinostat on MCF-7 cells. Dejligbjerg et al. used a panel of 18 wild-type cancer cell lines to investigate the Belinostat's effect on cell viability by Cell Titer-Gloluminescent viability assay and found that Belinostat IC_{50} value change from 0,23 µM to 5,92 µM according to cancer cell type (21). Different studies reported that Belinostat's IC_{50} values were 0,4 µM and 0,54 µM for 48 h in MCF-7 cells (21,22). These different IC_{50} values may be due to the different cell lines and cytotoxicity tests used. According to a literature, different HDACs and Belinostat exhibit cytotoxic effect generally at lower doses (23).

Caspases, known as molecular guillotines, play a critical role in the programmed cell death process. Caspases and their actives are widely used as biomarkers in the investigation of the balance between cell proliferation and cell death in cancer research including breast cancer. Effect of Belinostat on an effector (CASP3) and an initiator (CASP9) caspases expression were evaluated in this study. After the application of Belinostat on MCF-7 cells, CASP3 and CASP9 expressions were down regulated. Lu et al. (24) reported that Belinostat suppressed cell proliferation and promoted apoptosis via Wnt-β and PKC pathway in MCF-7 cells. Belinostat showed this antitumor effect by dose- and time-dependent manner. Similar to this study, Lu et al. also showed that Belinostat treatment down regulated CASP3 expression at the protein level using western blot analysis.

It is well known that P53 is involved different cellular functions depending on environmental conditions and cellular status. These tasks include gene expression, control of cell cycle, DNA repair, cell differentiation, genomic plasticity and programmed cell death. Therefore, wild type P53 expression level is also often used as an apoptotic marker. Lu et al. indicated that both mRNA and protein level expressions of P53 were increased when 50 and 100 µM of Belinostat was used (25). In the presenting study, however, we found that P53 expression was significantly down regulated comparing to the control group. This difference may be due to the Belinostat dose used. Because, Lu et al. used a relatively higher Belinostat dose in MCF-7 cells. It is stated that the mechanisms of HDCAi action may vary depending on the type of cancer, dose and duration of administration (25,26).

These results suggested that the intrinsic apoptosis pathway did not cause the decrease in cancer cell

proliferation. Therefore, to investigate the cause of cytotoxic effect of Belinostat in MCF-7 cells, expression of genes effective in cell cycle check points, extrinsic pathway of apoptosis and autophagy should also be considered. Lee et al. reported that the HDACi administration on hepatocellular carcinoma cells caused G2/M cell cycle arrest (27). Yang et al. also accomplished a study that supports this finding. It was reported that HDACi could cause a significant induction of the G2/M phase cell cycle arrest on cancer cells and inhibited proliferation (28).

Several studies reported that HDACi promotes the apoptotic effect on cancer cells by different mechanism such as restoring of cellular sensitivity to cancer cells with TRAIL and increasing gene expression of pro-apoptotic genes (29-31). In conclusion, we found that administration of Belinostat reduced the number of MCF-7 cancer cell population. HDACi can restore the blocked or suppressed apoptotic pathways in cancer (32). However, in this study, expression of apoptosis genes was decreased after Belinostat application. As a result of Belinostat treatment, acetylation may not been occurred for H3 and H4 histones of apoptosis genes (CASP3, CASP9 and P53).

CONCLUSION

As a conclusion, Belinostat decreased the expression of apoptosis-related genes. These results suggested that effect of Belinostat on MCF-7 cells can be a different biological pathway other than apoptosis-related genes. Further studies are needed to investigate the relationship between cycling dependent kinases (P21, etc.) and to measure enzyme inhibitory activities of Belinostat.

Competing interests: The authors declare that they have no competing interest.

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