



Belinostat effects on expression Of RBM5 tumor suppressor gene and inhibits prostate cancer cell line (PC3) proliferation

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ABSTRACT

Prostate cancer as the second most prevalent cancer and the sixth most common cause of cancer death among men appears with increasing mortality incidence rate amongst Asian population. Although, surgery, chemotherapy and radiotherapy are investigated as potent therapeutic strategies for the treatment of localized prostate cancer, advanced prostate cancer does not completely respond to these methods. According to the ambiguous molecular mechanism of belinostat, the present study was conducted to determine the effect of belinostat on an advanced prostate cancer cell line (PC3). The Flowcytometry assay was done in a triplicate repeat to measure the viability and the percent of apoptotic cells. Real-time PCR was done to evaluate mRNA expression changes in *RBM5* tumor suppressor gene and growth inhibition of PC3. The results indicated that PC3 that treated with 1 μ M belinostat showed significant increase in expression of *RBM5* mRNA and also a significant increase in apoptosis. In conclusion, this study demonstrated that belinostat increases *RBM5* expression and this may be responsible for some part of belinostat apoptosis inducing effect.

Keywords: prostate cancer, Belinostat, *RBM5*, PC3, HDACI

INTRODUCTION

Prostate cancer that is a worldwide significant health problem as the second most prevalent cancer, and the sixth most common cause of cancer death among men appears with increasing mortality incidence rate amongst Asian population (1-4). Although, surgery, chemotherapy and radiotherapy are investigated as potent therapeutic strategies for the treatment of localized prostate cancer, advanced prostate cancer does not completely respond to these methods. The limitations of chemotherapy could be characterized by dose-related toxicity, the occurrence of tumor cell resistance and also a poor extending life time of advanced prostate cancer patients (5). Understanding molecular pathways involved in prostate cancer regulation, present new insights in prostate cancer management (6).

The gene of RNA-binding motif protein 5 (*RBM5*), as a tumor suppressor gene (TSG), is located on human chromosome 3p21.3 (7, 8). *RBM5* takes part in modulation of apoptosis, cell proliferation, and oncogenesis of the cells (9, 10). The mRNA and protein expression of *RBM5* gene reduces mostly in ras-transformed Rat-1 embryonic fibroblasts (11), human vestibular schwannoma (12), breast cancer (13) and primary lung cancer specimens (14, 15). The *RBM5* gene has a correlation with prostate cancer too. It can induce apoptosis of human prostate cancer PC3 cells by modulating the mitochondrial apoptotic pathway, so *RBM5* can be a promising target for gene therapy on prostate cancer (6). In addition the ectopic expression of *RBM5* inhibits the progression of human lung (16, 17), breast (18), and renal cancers (19), fibrosarcoma (13) and hematopoietic cells (11, 20, 21).

Histone deacetylation is one of the epigenetic conditions that dysregulates TSGs. In this regards, histone deacetylase inhibitors (HDACIs) are developed as a new class of anti-cancer drugs. However, the anti-cancer mechanism of HDACIs is not completely understood mainly because they enhance acetylation in both histone and non-histone proteins that provide an active field for further investigations about the function of HDACIs (22).

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Belinostat (PXD101, trade name Beleodaq), a histone deacetylase inhibitor is a developing drug for the treatment of hematological malignancies and solid tumors (23).

The anti-cancer efficacy of belinostat in several urothelial tumor cell lines in sub- to low micromolar doses has been established that introduce this agent as a potent anti-neoplastic drug (22). Further, successful investigations in animal models (24, 25) and also in phase I clinical studies, introduce belinostat as a tumor growth inhibitor at non-toxic concentrations (23, 26, 27). In some studies, it was found that human prostate cancer cell lines including: LNCaP, DU145, LAPC4, and PC3, were differ in sensitivity to HDACI induced cell death, as DU145 was very sensitive and PC3 was resistant (28-33). In many studies PC3 cell line was used as relatively HDACI-resistant cell line, also this cell line was used to determine the apoptotic function of *RBM5* *in vitro* (6, 34-36).

According to the ambiguous molecular mechanism of belinostat, the present study was conducted to determine the effect of belinostat on PC3.

MATERIALS AND METHODS

Cell Culture and Treatments

Human prostate cancer cell line (PC3) was purchased from the National Cell Bank of Iran-Pasteur Institute. It was cultured and allowed attaching in tissue culture flasks T75 containing Dulbecco's Modified Eagle's Medium (DMEM) (sigma) supplemented by 10% Fetal Bovine Serum (FBS) (Invitrogen), 1 % penicillin and streptomycin and the cells incubated at 37°C, 5% CO₂, 95% humidity as bringing up condition to confluent rate more than 80%. Belinostat drug (Toronto Research ;B131400) was prepared as a 10 mM stock dissolved in DMSO/PBS(22) and PC3 cells were exposed to the doses of 1 to 10 µM for 24 hours for IC50 assay and, 24-72 hours for another MTT, Flowcytometry and Real-Time PCR analyses, respectively.

MTT Assay and IC50 Evaluation

About 5×10^4 PC3 cells were seeded into each well of 24-well plates (Becton-Dickinson) to allow attaching and growing for 24 hours before treatment with belinostat. PC3 cells were treated by belinostat for one day and MTT assay (Chemicon; Temecula, CA) was done for determining the IC50 value for belinostat. MTT assay is a spectrophotometrical method, that measures the diminished yellow color of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) induced by mitochondrial succinate dehydrogenase. The emerging MTT dye subsides into the mitochondria, as an insoluble, dark purple colored formazan product, which could be detectable due to solving purple formazan crystals with DMSO, at 570 nm of spectrophotometer. The experiment was conducted in a triplicate repeat. MTT assay was either executed to confirm the data of IC50. In this regard, the cells in 24-well plates were treated for 24, 48 and 72 hours with that dose of belinostat achieved from IC50 assay.

Flowcytometry Assay

The flowcytometry assay was done in a triplicate repeat to measure the number of apoptotic cells. Three 24-well plates of 24- hours cultured PC3 cells received a single selected dose of belinostat and allowed to further grow in 24, 48 and 72 hours as experimental groups, while three other wells did not treat with any reagent, as control group. At the end of the experiments, the cells were harvested with 0.05% trypsin, washed with cold phosphate-buffered saline (PBS) and then binding buffer (1x). Then, the cells were stained by Annexin V-FITC and propidium iodide (PI) (Becton-Dickinson, San Diego, CA) based on the manufacturer's protocol (BMS500F1/100CE AnnexinV-FITC, eBioscience, USA) and the apoptotic cells were counted by FACS flowcytometry (Becton Dickinson, Heidelberg, Germany).

RNA Extraction and cDNA Synthesis

Total RNA was isolated using YTA Total RNA Purification Mini kit (Yekta Tajhiz Azma, Iran), according to the manufacturer's instructions. For removing genomic DNA from extracted RNA, DNaseI treatment was performed using DNaseI (Cinagen, Iran) according to manufacturer's instruction. All RNA extracted samples were stored at -80°C for further experiments.

Five hundred nanograms of extracted mRNA were reverse transcribed into cDNA using the cDNA synthesis kit RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. The resulting cDNA kept at -20°C until use.

Table 1: Primer sequences used for real-time PCR

Gene		Primer sequence (5' to 3')
GAPDH	F	AAGCTCATTTCCTGGTATG
GAPDH	R	CTTCCTCTTGCTCTTG
RBM5	F	GCTGCTGCTAGTGGTCATC
RBM5	R	GTGCTTCCTCAGTCGGAGAG

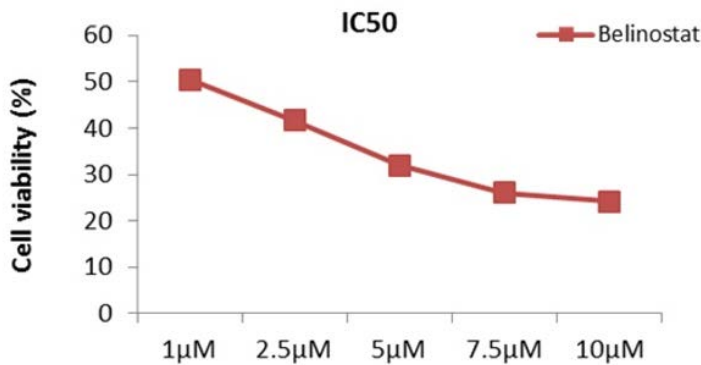


Figure 1: Inhibitory concentration (IC₅₀) assay for half-maximal IC analysis of belinostat. In PC3 cell line after 24 h of treatment. Cells were incubated with or without the belinostat using 1, 2.5, 5, 7.5 and 10 µM doses and the relative amount of viable cells were estimated by measuring the absorbance of the cell suspension after incubation with MTT assay was carried out and a graph of viability versus drug concentration was used to calculate IC₅₀ values for PC3 cell line.

Real-Time PCR

Real time PCR was carried out using Applied Biosystems™ Real-Time PCR instruments. The Maxima SYBR Green/RoxqPCRMaster Mix kit (Fermentas) was used for real time PCR and Primer sequences are shown in **Table 1**. Thermal cycle parameters included the initial denaturation; 95°C for 1 minute, and 40 cycles at 95°C for 20 seconds, 58°C for 15 seconds and 72°C for 15 seconds. Data were analyzed using the comparative C_t (2^{-ΔΔC_t}) method. The relative expression ratio between the amount of *RBM5* gene and the endogenous control was calculated and melting temperature curves of specific amplification products and primer dimmers were drawn. GAPDH was used as a reference gene for internal control.

Statistics

Chi-square test was used to analyze the difference in *RBM5* expression between treated and untreated PC3 cells. Data were presented as mean ± standard deviation (SD) when appropriate. The regression analysis was performed with SPSS software version 17.0. Comparisons between treatments were made using a paired Student's t-test, or one-way ANOVA for multiple group comparisons. A P-value of <0.05 was considered statistically significant.

RESULTS

IC₅₀ Calculation

After the treatment of PC-3 cells with MTT solution, the dark purple formazan crystals were seen in the cells, which indicated their metabolic activity. The reduction in the number of cells was dependent on the drug concentration as shown by the half-maximal IC₅₀ index. The results showed that the IC₅₀ of belinostat on PC-3 cells at 24 h was 1 µM (**Figure 1**).

Flowcytometry

To establish the apoptotic induction potential of the belinostat, we first investigated the effects of this drug on the proliferation of the PC3 cell line. The flowcytometry results showed that belinostat at different time course (24, 48 and 72 h) could significantly induce apoptosis in PC3 cells and it was increased with ascending time. At different time course (24, 48 and 72 h) the percent of apoptosis in treated cells was significantly more than untreated ones and PV in all three groups were less than 0.001 (PV *P* < 0.001) and the greatest effect was shown at 48 hours group (**Figure 2**).

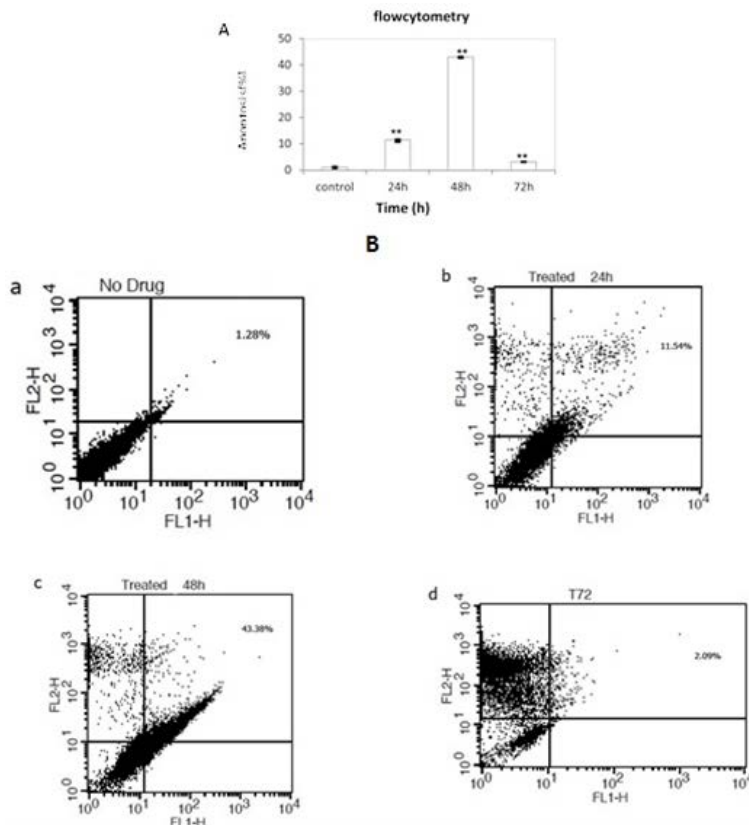


Figure 2: Relative levels of apoptotic cells in Human prostate cancer cell line (PC3) treated with belinostat drug for different times A and B. Cells incubated with dimethyl sulfoxide (DMSO) were used as a control. (a and b) The percentage of apoptotic cells was measured using the AnnexinV FITC (FL1-H) and propidium iodide (PI) (FL2-H) assay. The representative data of FCM with Annexin V and PI staining for detecting apoptotic cells were shown in control and belinostat treated groups. LL represents normal cells, LR and UR represent early and late apoptotic cells, respectively, UL represents necrotic cells. Control group (a), treated cells with belinostat for 24 hours (b), treated cells with belinostat for 48 hours (c) and treated cells with belinostat for 72 hours (d).

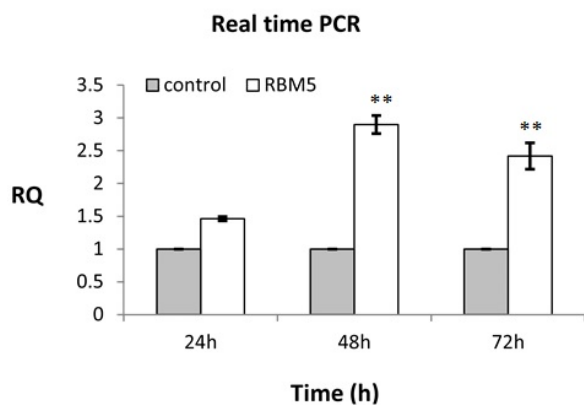


Figure 3: Changes in the RBM5 ratio of the mRNA levels in 1 μ M belinostat at different times (24, 48 and 72 h) treated prostate cancer cell line (PC3) in comparison with control cells. ** $p < 0.01$

Real-Time PCR

The effects of belinostat, on the RBM5 mRNA expression levels in PC3 were studied. Real-Time PCR analysis revealed the up-regulation of RBM5 gene expression after treatment with 1 μ M belinostat at different time durations (24, 48 and 72 h). RBM5 gene expression was not raised significantly at 24 hours treatment. The strongest effect was induced by belinostat at 48 hours that belinostat induces significant increase in RBM5 gene expression compared to control group (Figure 3, $P < 0.01$); its expression was significantly raised at 72 hours treatment too (Figure 3, $P < 0.01$).

DISCUSSION

According to the importance of HDACI family as potential therapeutic agents for the treatment of high metastatic cancerous cells, the present study was conducted to determine the effects of belinostat on an advanced PC3. The results indicated that PC3 that treated with 1 μ M belinostat showed significant increase in expression of *RBM5* mRNA and also a significant increase in apoptosis and the strongest effect was induced after 48 hours.

Prostate cancer is the most commonly diagnosed cancer and second leading cause of mortality in males in industrialized countries (37). The incidence of prostate cancer in Asian countries is lower than that in western countries; however, the mortality of prostate cancer is increasing rapidly in Asian males due to westernization of dietary life style (1).

It has been reported that hydroxamic acid-based inhibitors, such as belinostat, are potent inducers of differentiation and apoptosis in transformed cells like PC3 cell line (27, 38, 39). Belinostat is currently being evaluated as an anticancer agent in clinical trials (40).

RBM5 is a known putative tumor suppressor gene that encodes a number of alternative RNA splice variants with different abilities to enhance, sensitize or suppress apoptosis (10, 20, 21, 41, 42). Previous reports showed that *RBM5* inhibited cell proliferation by promoting apoptosis in human lung cancer cells (16, 43). Results reported by Zhang L *et al.*, suggested that *RBM5* could induce *caspase-3*, *caspase-9*, PAPP cleavage by decreasing *Bcl-2* expression and promoted apoptosis (44). Welling *et al* (2002) reported that, *RBM5* gene is down regulated in spontaneously developing tumors, human schwannomas (12) and ras-transformed Rat-1 rat embryonic fibroblastic cells, respectively (13). Zhao *et al* (2012) showed that *RBM5* may induce the apoptosis of prostate cancer cells by modulating the mitochondrial apoptotic pathway and concluded that *RBM5* can be used as a promising target for gene therapy on prostate cancer (6).

The slow-down of cell proliferation was associated with a significant increase of *P21* and *P27* and with the preferential accumulation of tumor cells in the G2/M phase of the cell cycle. The cell cycle block was followed by apoptosis, as described for other HDAC inhibitors, and was associated to both intrinsic and extrinsic apoptotic pathway activation (45). Frønsdal and Saatcioglu (2005) showed that three HDAC inhibitors including TSA, FR901228, and Na-But induce apoptosis in both LNCaP and DU-145 prostate cancer cell lines, while PC-3 cells were largely resistant to the three HDAC inhibitors under same conditions (33). For the first time Gleave *et al* (1998) found that treatment with a fatty acid inhibitor of HDACs like butyrate analog isobutyrate inhibits growth of prostate tumor in vivo by inducing cell cycle arrest (46). SAHA is one of HDAC inhibitors could decrease the expression of *HIF-1 α* , *VEGF*, and *CD31* in murine B cell lymphoma A20 cells by anti-angiogenesis effect (47). Using of 4SC-202 as a novel class I HDACI, potently suppresses colorectal cancer cells both in vivo and in vitro (48). Treating LNCaP cells with Valproic acid as HDAC had effects on the expression of genes relevant in proliferation and apoptosis for example *caspase-3* was up-regulated in LNCaP of tumor cells and resulting in apoptosis (49). Inducing apoptosis by belinostat was associated with the expression of many genes related to this event. In the belinostat-treated tumor cells, expression of *Bax* and *caspase-3* were increased with a contextual decrease of the anti-apoptotic gene *Bcl-2* (40). In our study, for the first time, it is demonstrated that belinostat induces apoptosis in PC3 cells via up-regulation of *RBM5* gene. Gravina *et al* confirmed that belinostat suppresses, both *in vitro* and *in vivo*, the growth of a wide panel of prostate tumor cells with graded androgen dependence at micro-molar concentrations (40).

CONCLUSION

The results of the present study demonstrated the role of *RBM5* in belinostat apoptosis induction of human prostate cancer cell line (PC3). These findings can be useful for further studies that evaluate the effects of belinostat on prostate cancer.

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