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Research Article

The histone deacetylase inhibitor belinostat effects on expression of HDAC1 and p21 and inhibits prostate cancer cell line (PC3) proliferation

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ABSTRACT

Prostate cancer, the second most common diagnosed cancer among men and the sixth most prevalent cause of cancer death in men. Though, radiotherapy, chemotherapy and surgery are potent therapeutic strategies for the treatment of localized prostate cancer, advanced prostate cancer does not fully respond to these therapeutic 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). Real-time PCR was done to identify mRNA expression changes in P21 and HDAC1 mRNA expression levels in PC3 cells. Real-Time PCR analysis revealed the significant up-regulation of P21 and down-regulation of HDAC1 mRNA expression after treatment with 1 µM belinostat at 48h. Flowcytometric analysis showed that belinostat induced apoptosis in PC3 cells as greatest effect observed at 48 hours. In conclusion, this study shows belinostat can act as HDAC inhibitor that's according to the results can suggest the belinostat as a potential adjuvant treatment for prostate cancer.

Key words: Prostate cancer, Belinostat, P21, HDAC1, PC3

INTRODUCTION

Prostate cancer, the second most common diagnosed cancer among men and the sixth most prevalent cause of cancer death in men (1, 2). Though, radiotherapy, chemotherapy and surgery are potent therapeutic strategies for the treatment of localized prostate cancer, advanced prostate cancer does not fully respond to these therapeutic methods. (3).

Prostate cancer begins in the form of hormone sensitive tumor, and then androgen receptor (AR) in the association with proteins, such as histone deacetylases, stimulated its growth (4, 5). Determination molecular pathways involved

in regulation of prostate cancer, introduce new insights in prostate cancer management. Histone deacetylases (HDACs) are a pivotal ingredients of regulating gene expression by the epigenetic machinery; it also act as oncogenes in some cancer types (6). HDAC1, a class I family member, is the initial mammalian deacetylase to be recognized and is the most widely described HDAC to date (7).

HDAC inhibitors are in clinical trials for treatment a variety of malignancies. (8). HDACi can prompt cell-cycle arrest, differentiation and cell death in vitro and induce selective apoptosis in tumor cells at drug concentrations and leave normal cells rather unharmed (9-11). The cyclin-dependent kinase inhibitor, p21, is one of the known targets of HDAC inhibitor-mediated de-repression. Treatment of several cancer cell types with any of HDAC inhibitors induces the transcriptional up-regulation of p21 antiproliferative gene. Overexpression of HDACs is detected in several cancer types, with corresponding decreases in p21 expression. For example, prostate cancer cells overexpress HDAC1. (12)

Belinostat (PXD101, trade name Beleodaq), a is a developing drug for the treatment of hematological malignancies and solid tumors (13). Further, successful investigations in animal models (14, 15) and also in phase I clinical studies, introduce belinostat as a tumor growth inhibitor at non-toxic concentrations(13, 16, 17). 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 (18-23). 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 some gene such as p21 *in vitro* (24-27).

According to the importance of prostate cancer in human communities and also the ambiguous molecular mechanism of belinostat, the present study was conducted to determine the effect of belinostat in an advanced prostate cancer cell line (PC3).

MATERIALS AND METHODS

Cell culture and the treatments

Human prostate cancer cell line (PC3) was bought from the National Cell Bank of Iran-Pasteur Institute. This cells cultivated in tissue culture flasks75 with Dulbecco's Modified Eagle's Medium (DMEM; sigma) and allowed attaching to them. Cells incubated at 37°C with 95% humidity and 5% CO2 in medium that supplemented by 10% Fetal Bovine Serum (FBS; Invitrogen), 1 % penicillin and streptomycin. This concentration of growing cells was implemented for further investigations. Belinostat drug (Torento Research ;B131400) dissolved in DMSO/PBS and prepared as a 10 mM stock (28).

It was 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 RT-PCR analyses, respectively.

MTT assay and IC50 evaluation

Before treatment with belinostat 5×10^4 numbers of PC3 cells were seeded into 24-well plates (Becton-Dickinson) to allow attaching

and growing for 24 hours. After another one day MTT assay (Chemicon; Temecula, CA) was done for determining the IC50 value for belinostat. In MTT assay that is a spectrophotometrical method, diminished yellow color of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) induced by mitochondrial succinate dehydrogenase will be measured. The emerging MTT dye subsides into the mitochondria ,as an insoluble, colored (dark purple) formazan product, which could be detectable due to reacting with DMSO, as yields dark purple colored formazan crystals, at 570 nm of spectrophotometer. The experiment was conducted in a triplicate repeat. To confirm the data of IC50 MTT assay was either executed. For this reason, 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 used to measure the number of apoptotic cells in a triplicate repeat. Three 24-well plates of 24- hours cultured cells received a single selected dose of belinostat and allowed to further grow in 24, 48 and 72 hours (experimental groups), while three other wells did not treat with any reagent, as control group. After that, 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, eBiscience, 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. After treatment with DNaseI (Cinagen, Iran), total RNA (500 ng) 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.

Real-Time PCR

Applied Biosystems™ Real-Time PCR instruments was used to carry out Real time PCR. The Maxima SYBR Green/RoxqPCRMaster Mix kit (Fermentas) was used for real time PCR and Primer sequences are shown in Table1.

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 Ct ($^{2-\Delta\Delta ct}$) method. The relative expression ratio between the amount of *P21 and HDA1 genes* 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 expression of P21 and HDAC1 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

PC-3 cells metabolic activity was proved by the dark purple formazan crystals that were seen in cells treated with MTT solution.

The number of cells was reduced, dependent on the drug concentration as shown by the half-maximal IC50 index. According to results of this study, the belinostat IC50 on PC-3 cells was 1 μM at 24 h (Figure 1).

Flowcytometry:

To determine the apoptosis 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 24-h treatment increased apoptosis (Figure 2, $P < 0.001$), however, apoptosis at 72-h compared to control group increased significantly (Figure 2, $P < 0.001$) but the greatest effect on apoptosis at 48 hours was observed (Figure 2, $P < 0.001$).

Real-Time PCR

The effects of belinostat, on the P21 and HDAC1 mRNA expression levels in PC3 cells were studied. Real-Time PCR analysis revealed the up-regulation of P21 gene expression after treatment with 1 μM belinostat at different time durations (24, 48 and 72 h). Increased expression of P21 at 24-h treatment was not significantly (Figure 3, $P < 0.01$) while increasing its expression at 48 hours compared to the control group was statistically significant (Figure 3, $P < 0.01$). Significant increase in P21 expression at 72-h treatment was observed, that was more than 48 hour group. (Figure 3, $P < 0.01$).

Real-Time PCR analysis revealed the down-regulation of HDAC1 mRNA expression after treatment with 1 μM belinostat at 48h. HDAC1 MRNA expression was significantly reduced at 48 hours While changes in expression were very low at 24 hours. Unlike the other two groups increase HDAC1 mRNA expression was observed in 72 hours, but this increase was not significant (Figure 4, $P < 0.01$).

DISCUSSION

Prostate cancer, as a hormone sensitive tumor, its growth is triggered by the androgen receptor activation in combination with nuclear co-regulatory proteins including HDACs. The histone deacetylases regulate transcriptional activity of hormone receptors such as androgen receptor (4, 5). HDAC inhibitors, broadly used as anti-cancer agents, given their toxicity profiles and antitumor activities (29).

We studied the effects of belinostat on the P21 and HDAC1 gene expression in prostate cancer cell line (PC-3). Real-Time PCR analysis revealed that belinostat induces significant increase in P21 gene expression at 48 hours (P <

0.01). On the other hand, significant down-regulation of HDAC1 gene expression was observed after treatment with belinostat. The results of flowcytometric analysis showed that belinostat induced apoptosis in PC3 cells as greatest effect observed at 48 hours.

Bolden J, et al (2006) published that HDACi treatment can induce tumor cell death via cell cycle arrest (30). Michael T Buckley, et al (2007) revealed that belinostat inhibits cell growth and proliferation in a dose-dependent manner and affected cell cycle arrest in the cell lines of urinary bladder cancer (28). Chowdhury S, et al (2011) reported that belinostat treatment in all cancer cell lines tested showed a time-dependent increase in apoptotic cell death (31). Kalipso Halkidou, et al (2004), for the first time, showed the abrogating effect of HDACi's repressive activity in the epithelial prostate cell differentiation (12). Longgui Wang et al, (2009) indicated that the expression of HDACs and their related co-repressors are increased in prostate cancer (5). In our study significant down-regulation of HDAC1 gene expression was observed after treatment with 1 µM belinostat at 48 hours; therefore, in addition to anti-androgens, HDACi's could act as a therapeutic agent in prostate cancer.

Apoptosis and growth arrest can be regulated by metabolic sensors, especially the p21 and its regulatory pathway (32). C.-Y. Gui, et al indicated that suberoylanilide hydroxamic acid (SAHA), as a HDAC inhibitor, triggered a noticeable down regulation of HDAC1 and Myc and also increase in RNA polymerase II in proteins bound to the p21(WAF1) promoter (33). In accordance with previous studies, in our research belinostat induced significant increase in p21 gene expression ($P < 0.01$); and induces growth arrest of the cells, in consequence.

CONCLUSION

This study shows belinostat can act as HDAC inhibitor. It increased expression of p21 and decreased expression of HDAC1 gene in prostate cancer cell line (PC3), therefore can suggest the belinostat as a potential adjuvant treatment for prostate cancer.

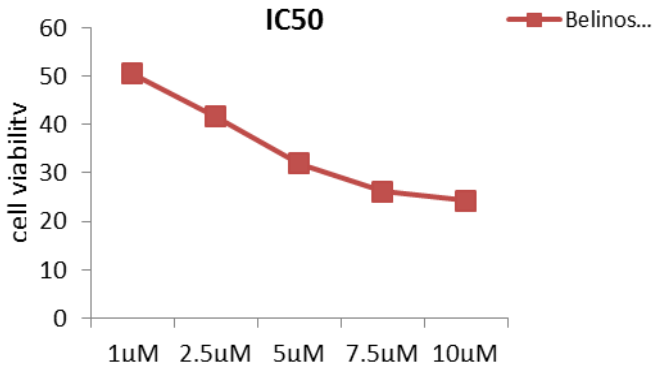
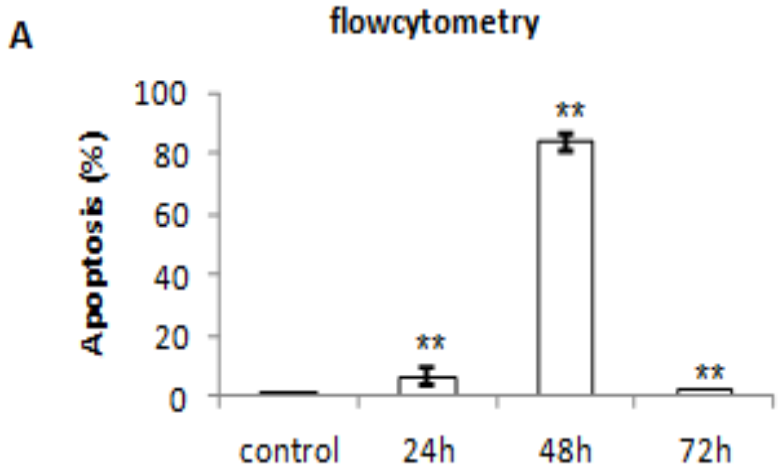


Figure 1: Inhibitory concentration (IC50) assay for half-maximal IC analysis of belinostat. In PC-3 cell line after 24 h of treatment. Cells were incubated with or without the belinostat using 1, 2.5, 5, 7.5 and 10µM doses and the relative amount of viable cells were estimated by measuring the absorbance 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 PC-3cell line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flowcytometry
Apoptosis (%)
100
80
60
40
20
0
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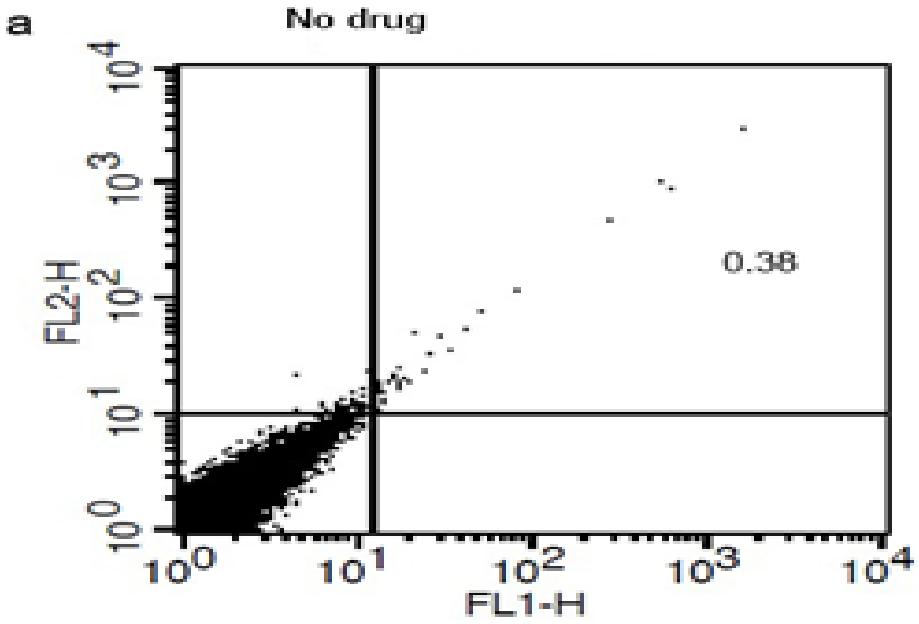
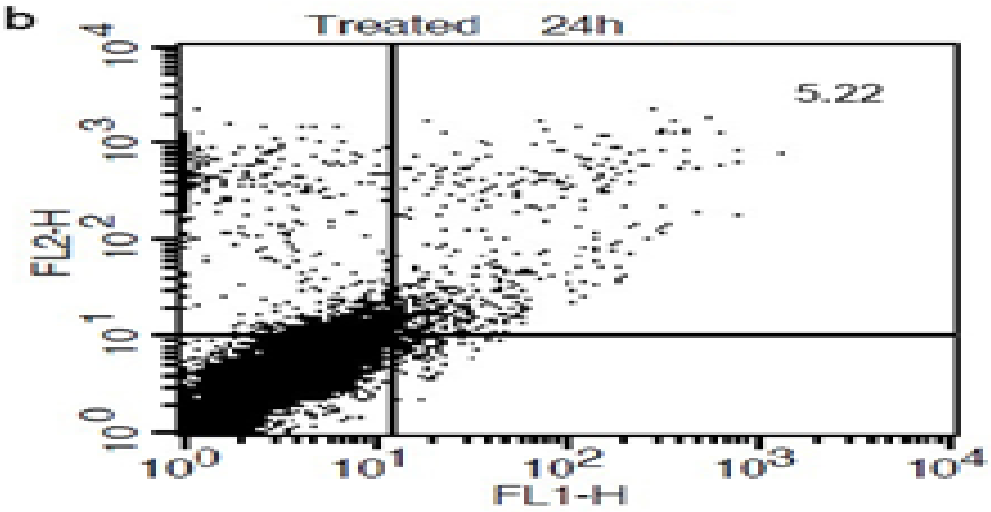
control
24h
48h
72h


a
No drug
FL2-H
FL1-H
0.38


b
Treated 24h
FL2-H
FL1-H
5.22

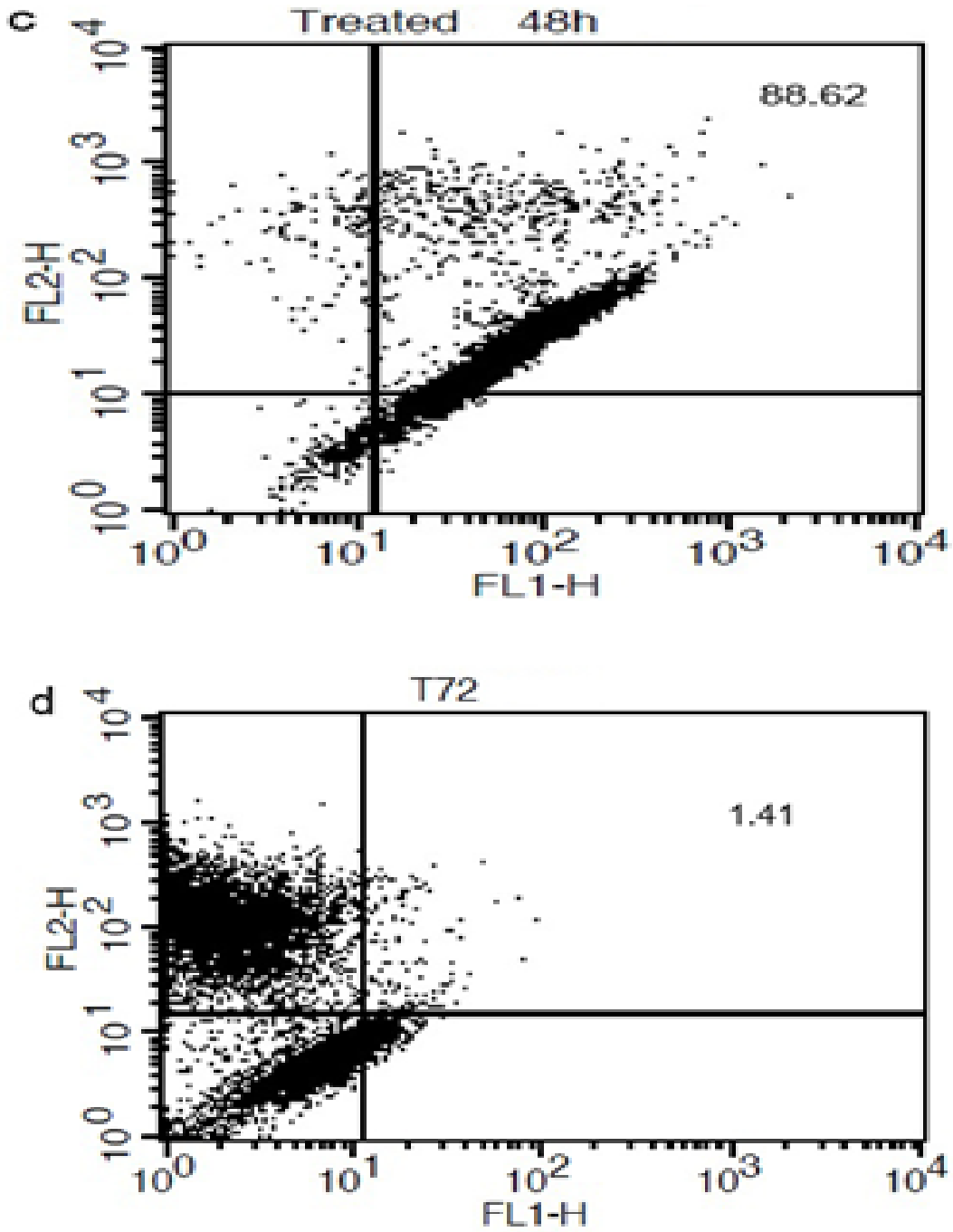


Figure 2: Relative levels of apoptotic cells in Human prostate cancer cell line (PC3) treated with belinostat drug for different times A and B. Cells incubated with the vehicle dimethyl sulfoxide (DMSO) were used as a control. (a and b) The percentage of apoptotic cells was measured using the AnnexinV 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).

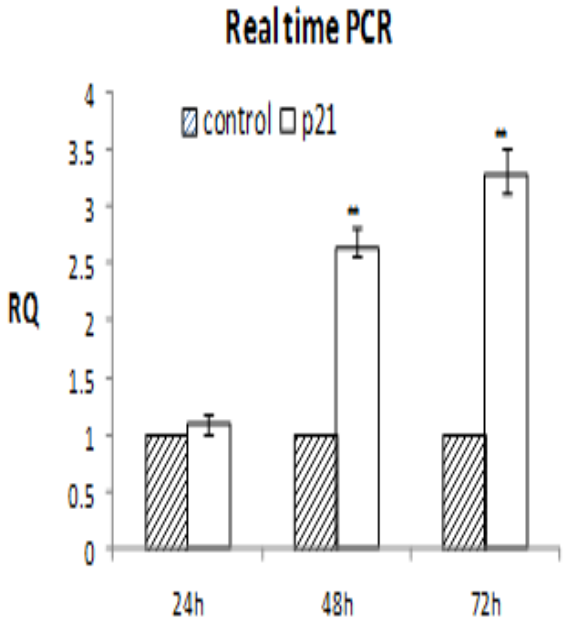


Figure 3: Changes in the P21 ratio of the mRNA levels in 1 μM belinostat at different times (24, 48 and 72 h) treated prostate cancer cell line (PC-3) in comparison with control cells. ** *p<0.01*

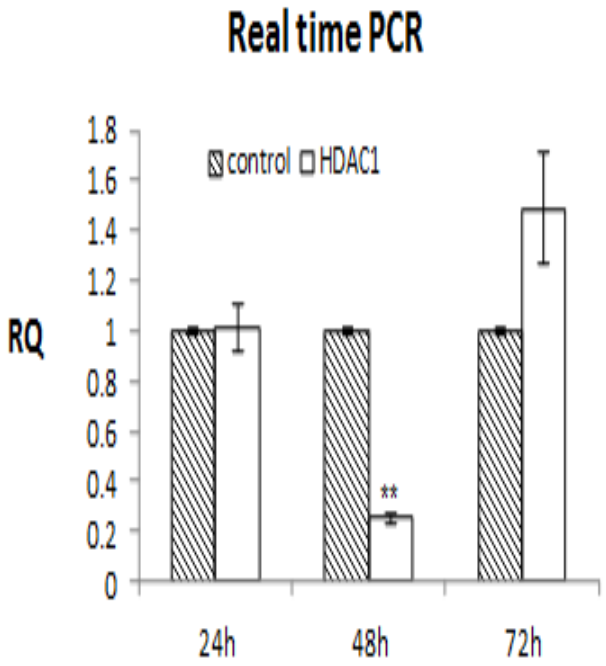


Figure 4: Changes in the HDAC1 ratio of the mRNA levels in 1 μM belinostat at different times (24, 48 and 72 h) treated prostate cancer cell line (PC-3) in comparison with control cells. ** *p<0.01*

Table 1. Primer sequences used for real-time PCR

Gene		Primer sequence (5' to 3')
GAPDH	F	AAGCTCATTTCCTGGTATG
	R	CTTCCTCTTGTGCTCTTG
P21	F	GACCAGCATGACAGATTTCTACC
	R	CAGCACTCTTAGGAACCTCTCA
HDAC1	F	CGCTCCATCCGTCCAGATAACAT
	R	ACAGCACTTGCCACAGAACCA

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