The Effects of Sodium Butyrate as a Histone Deacetylase Inhibitor on the Activation of Toll-Like Receptor 4 in Prostate Cancer Cells

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Research Article

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Abstract

I. Background: Prostate cancer is the most diagnosed cancer in men and the covalent acetylation and deacetylation of histone proteins by the histone deacetylase (HDAC) enzymes can be considered a novel therapeutic target in PCa cells. Sodium butyrate (NaBu) is one of the most studied HDAC inhibitor (HDACi) which is a promising potential anticancer drug. Toll-like receptors (TLRs) are expressed on prostate cells and TLR4 expression is increased in prostate cancer cells and HDACi alter TLR-inducible gene expressions. Therefore, we aimed to evaluate the effects of NaBu on TLR4 mediating signaling pathways in two different PCa cells (DU-145 and LNCaP) for the first time.

II. Methods and Results: The cytotoxicity of NaBu in PCa cells was determined by WST-1 assay. Apoptotic effects of NaBu were analyzed by Annexin V and AO/PI assays. Subcellular localization of TLR4, IRF3 and NF-κB proteins was evaluated by IF assay. Our results showed that NaBu significantly inhibited the viability of PCa cells and increased the percentage of apoptotic cells ($p<0.01$). However, DU-145 cells were more sensitive to NaBu than LNCaP cells. Furthermore, NaBu can induce the cytoplasmic TLR4 and IRF3 expression in particularly DU-145 cells without affecting nuclear translocation of NF-kB in PCa cells.

III. Conclusions: NaBu induces apoptotic cell death and regulated the TLR4/IRF3 signaling pathways in DU-145 cells but not in LNCaP cells. Therefore, PCa cells differentially responded to NaBu treatment due to probably AR status. Therefore, further investigations should be performed to assess the molecular mechanisms underlying cell response to NaBu-induced TLR-mediated signaling pathways *in vitro* and *in vivo.*

Introduction

Prostate cancer (PCa) is the most diagnosed malignant tumor in men [1, 2]. Castration-resistant prostate cancer (CRPC), the advanced form of prostatic cancer, is a heterogenous disease with poor outcome and characterized by disease progression after surgical castration or androgen deprivation. The continuing presence of androgen receptors in CRPC cells and re-activation of androgen axis cause the growth and survival of PCa cells [3, 4]. 10–20% of patient with prostate cancer develop CRPC within 5 years. > 84% of patients with CRPC have metastasis at the time of diagnosis. In those without metastases, 33% of patients develop metastasis within 2 years of diagnosis [5]. Therefore, the underlying molecular mechanisms of PCa progression and the identification of new targets are urgently needed to improve life quality and treatment response of PCa patients.

The covalent acetylation and deacetylation of histone proteins can be considered a novel therapeutic target in PCa cells, and these modifications are mediated by the histone deacetylase (HDAC) family of enzymes [6, 7]. Sodium butyrate (NaBu), the sodium salt of butyric acid, is one of the most studied HDACi. NaBu has a wide range of effects on different cancer cells as a promising potential anticancer drug by altering gene expression through chromatin modification due to inhibiting cell proliferation and inducing apoptosis at lower concentrations [8–10]. In some studies, NaBu inhibits the cell growth and

induces apoptosis in DU-145, PC-3 and LNCaP cells in a dose-dependent manner by increased of p21 and decreased of Bcl-xL, CDK4, CDK6 and procaspase-3 protein levels [11]. Thus, the induction of apoptosis by HDAC inhibitors could be a potential candidate for the treatment of certain types of cancer [12–15].

Toll-like receptors are a family of transmembrane receptors that have important roles in the innate immune response [16]. TLRs contribute to the enhancement of the immune response by recognizing pathogen-related molecular patterns (PAMPs) in pathogen invasion and endogenous damage-related molecular patterns (DAMPs) in different diseases such as prostate cancer (PCa) [17]. Higher expression of TLRs is not related only with innate immune cells, but also observed in cancer cells [18].

TLRs are expressed on prostate cells and increased expression of TLR4 in prostate cancer cells is identified by Kundu et al. [19]. TLR4 activation initiates two different TLR4 signaling pathways which inducer of tumor growth (myeloid differentiation primary response 88 (MyD88)-dependent signaling) or inhibitor of PCa development (MyD88-independent TRIF-dependent signaling) [20]. Firstly, activated TLR4 can phosphorylate MYD88-dependent expression of pro-inflammatory cytokines via NF-κB signaling pathway and the activation of NF-κB (TLR4/NF-κB signaling) contributes the pathogenesis of inflammatory response as a pro-inflammatory role in PCa [21]. Secondly, in TRIF-dependent pathway, activated TLR4 can phosphorylate interferon regulatory factor-3 (IRF3) and IRF3 translocate to the nucleus to initiate the transcription of Type I interferon genes, especially IFN-β. Thus, the activation of IRF3 (TLR4/IRF3 signaling) results in an anti-inflammatory response [22, 23].

In the literature, HDACi alter TLR-inducible gene expressions and suppress TLR-inducible inflammatory responses in macrophages and dendritic cells [24–27]. On the other hand, the study of Xiao et al. [28] shows that NaBu can alter the expression level of TLR4, MAPK and NF-κB signaling activations and pro-inflammatory response in colon cancer cells and suggests that NaBu mediates innate immunity in colon cancer cells through two distinct TLR4 signaling pathways. In this context, further studies are required to identify the role of NaBu on the activation of TLR4. Therefore, we aimed to evaluate the effects of NaBu on TLR4 mediating signaling pathways (TLR4/NF-κB or TLR4/IRF3) in two different PCa cells [DU-145 (hormone independent) and LNCaP (hormone dependent)], for the first time.

Material And Methods

Cell culture

In this study, DU-145 and LNCaP cells were used as PCa cells and obtained from the American Type Culture Collection (ATCC, USA). Both cell lines were grown in RPMI-1640 (Gibco, USA) medium (Gibco, USA) with 10% FBS (Gibco, Scotland) supplemented with 100 IU/mL penicillin (Gibco, USA) and 100 mg/ml streptomycin (Gibco, USA). Cells were incubated at 37°C in a humidified 5% CO_2 for 24 h before experimental use.

Cell Viability (Wst-1) Assay

The cytotoxic effect of NaBu was determined with WST-1 assay. The PCa cells ($2x10^5$ cell/well) were grown in the presence of different concentrations of NaBu (1, 2.5, 5, and 10 mM) in 96-well plates and incubate for 24h (Mu et al., 2013). The WST-1 dye was added to the wells and incubated for 30 min at 37°C. The optical density (OD) value was measured by a spectrophotometer (Allsheng, China). All experiments were performed at least three times.

Annexin V Analysis

To evaluate the rate of apoptotic cell death induced by NaBu, Annexin V analysis was performed. PCa cells were cultured in 6-well plates ($5x10^5$ cells/well) and treated with 1 and 5 mM NaBu according to the WST-1 results. The cells were washed with cold phosphate buffer solution (PBS) and stained with Annexin V Assay kit (Millipore) according to the kit instructions. Afterwards staining cells were examined with Muse Cell Analyzer (Millipore).

Acrydine Orange/ Propidium Iodide Staining

To confirm the apoptotic effect of NaBu on the morphological changes of PCa cells, AO/PI dual staining was performed. The PCa cells were cultured in 6-well plates ($5x10^5$ cells/well) and treated with 1 and 5 mM NaBu according to the WST-1 results. Afterwards treated cells were fixed with 4% paraformaldehyde (PFA) then stained by AO and PI dye at room temperature. The stained cells were washed twice with PBS and captured with EVOS FL Cell Imaging System (Thermo Fisher Scientific).

Immune Florescence Assay (If)

Subcellular localization of TLR4, IRF3 and NF-κB protein was analyzed in PCa cells using immune florescence (IF) method. For this purpose, PCa cells ($5x10^5$ cells/well) were cultured in 6-well plates and then exposed to different concentrations (1 and 5 mM) of NaBu for 24 h. After fixation, permeabilization and incubation with primary antibody of TLR4 [mouse monoclonal TLR4 (sc-293072), IRF-3 (sc-33641) and NF-κB, Santa Cruz Biotechnology, USA], and mouse IgG H&L Alexa FluorVR 488 seconder antibody (Abcam, UK) were used to detect subcellular localization of selected proteins. Then, the nucleus of cells was stained with DAPI. The images were photographed with an EVOS FL Cell Imaging System (Thermo Fisher Scientific, MA, USA).

Statistical Analysis

All data was statistically analyzed by GraphPad Prism software (V60.1, CA). Obtained data were expressed in a mean ± SD of the three repeated experiments. The differences between control and NaBu treated cells were compared by One way-ANOVA. $P < 0.05$ value was considered statistically significant.

Results

The cytotoxic effects of NaBu on PCa cells

To determine the cell proliferation rate of PCa cells following treatment with NaBu for 24 h, WST-1 analysis was conducted (Fig. 1). A dose-dependent significant decrease was detected in both PCa cells for 24-hour treatment period ($p < 0.001$). We determined that NaBu treatment more inhibited DU-145 cell viability at 1, 2.5, 5, and 10 mM (70.5 ± 0.9%, 52.3 ± 0.7%, 32.5 ± 0.8%, and 26.4 ± 0.4%, respectively) than LNCaP cells (67.4 ± 0.8%, 64.4 ± 0.7%, 41.4 ± 0.8%, and 29.7 ± 0.9%, respectively) ($p < 0.001$). Additionally, the inhibition of PCa cell viability was more profound at 1 and 5mM concentrations for 24 treatment period. Thus, NaBu exhibited greater anti-proliferative activity in both PCa cells at 1 and 5 mM concentrations for 24 h and we selected these concentrations of NaBu for further analysis.

The effects of NaBu on apoptotic cell death in PCa cells

To investigate the apoptotic effects of NaBu, we performed the Annexin-V assay (Fig. 2). According to the Annexin V assay results, 1 and 5 mM NaBu treatment more increased the percentage of late apoptotic cells than the percentage of early apoptotic cells in both DU-145 and LNCaP cells (Fig. 2A). Additionally, 1 and 5 mM NaBu treatment increased the percentage of total apoptotic cells (66.5 ± 0.7% and 62.3 ± 0.7%, respectively) in DU-145 cells compared to the control (6.9 ± 0.3%) (Fig. 2B, $p < 0.01$). On the other hand, 1 and 5 mM NaBu treatment significantly increased the percentage of total apoptotic cells (37.3 ± 0.4% and 49.6 ± 0.8%, respectively) in a dose-dependent manner in LNCaP cells compared to the control (1.3 ± 0.5%) (Fig. 2A, $p < 0.01$). Thus, NaBu treatment more induced apoptotic cell death in DU-145 cells than LNCaP cells.

The effects of NaBu on the cell morphology of PCa cells

To further explore the apoptotic effects of NaBu, AO/PI dual staining was conducted (Fig. 3). AO/PI dual staining results showed that NaBu treatment exhibited apoptotic morphological changes in both PCa cells. After treatment of 1 and 5 mM NaBu for 24 h, chromatin condensation, nuclear blebbing, rounded cells, and cytoplasmic shrinkage were observed. However, the apoptotic effect of NaBu was more obvious in DU-145 cells than LNCaP cells. Our results were consistent with the WST-1 and Annexin-V assay results.

The effects of NaBu on subcellular localization of TLR4 signaling pathway proteins in PCa cells

We next determined the subcellular localizations of TLR4, IRF3 and NF-κB as downstream proteins of TLR4 signaling pathways in PCa cells (Fig. 4). For this purpose, we performed immune fluorescence (IF) assay following administration of 1 and 5 mM of NaBu for 24 h in DU-145 and LNCaP cells. The obtained results demonstrated that treatment with 1 mM NaBu induced cytoplasmic expression of TLR4, IRF3 and NF-κB compared to control cells and higher expression of nuclear IRF3 was observed in DU-145 cells. However, both cytoplasmic and nuclear NF-κB expression was decreased in DU-145 cells following 5 mM

NaBu treatment. On the other hand, treatment with 1 and 5 mM NaBu induced cytoplasmic expression of TLR4 compared with control in LNCaP cells. Additionally, we observed cytoplasmic IRF3 and NF-κB at 1 mM NaBu treatment. However, nuclear translocation of NF-κB and IRF3 was not observed after 5 mM NaBu treatment in LNCaP cells. Thus, NaBu treatment induced the cytoplasmic TLR4 and IRF3 expression in particularly DU-145 cells without affecting nuclear translocation of NF-κB in PCa cells.

Discussion

Herein, we evaluated the effects of NaBu as a histone deacetylase inhibitor on TLR4-mediating signaling pathways in PCa cells, along with its mechanism of action. In our study, we demonstrated that NaBu at 1 and 5 mM significantly decreased cell proliferation and induced apoptosis in PCa cells. We also found that NaBu increased the cytoplasmic TLR4 expression in both PCa cells. However, our results showed that NaBu administration could differently regulate the IRF3, and NF-κB protein expressions as downstream proteins of TLR4-dependent signaling pathways.

The effects of HDAC inhibitors on suppressing cell proliferation, blocking the G1/M or G2 phase of the cell cycle and inducing apoptotic cell death on various cancer cell lines have been identified in several studies [29, 30]. In a study of Mu et al. [31], a significant inhibition of growth and induction of apoptosis are observed in DU145 and PC3 cells after NaBu treatment in a dose-dependent manner through the downregulation of Bcl-xl and Bcl-2 and the overexpression of Bax and Bak. Qui et al. [32] demonstrate that NaBu inhibits cell growth and induces apoptosis in LNCaP, DU-145 and PC-3 cell lines via over-expression of p21 expression resulting CDK2- CDK4 and CDK6 down-regulation. Condensed and fragmented nuclei are also detected in the cell morphology after NaBu treatment [32].

Studies evaluating the contribution of HDACs into gene expression regulated by TLRs have identified that HDACs inhibit or activate TLRs expression and thus HDACi mediate the activation of TLRs and their signaling pathways and inflammatory response [26, 33, 34]. It has also been demonstrated that different classes of HDACs differently affect the inflammatory response and therefore HDAC act as a pro- or anti-inflammatory function [35]. Furthermore, NaBu inhibits class I HDAC activity and exhibits anti-inflammatory properties through inducing the expression of anti-inflammatory mediators and inhibited the expression of TNF-α and IL-6 [36, 37]. According to our results, the nuclear translocation of NF-κB was not observed while IRF3 translocated to the nucleus with an increasing level of apoptotic cell death in DU-145 cells after NaBu treatment. Furthermore, NF-κB and IRF3 translocation into the nucleus was not observed after NaBu treatment despite of higher apoptotic cell death in LNCaP cells. Our preliminary results showed that NaBu-induced apoptotic effect could be regulated by TLR4/IRF3 signaling pathway in DU-145 cells but not in LNCaP cells.

The androgen receptor (AR) signaling pathway plays a critical role in the development of PCa through the induction of cell proliferation [38, 39]. DU-145 cells are castration resistant cells PCa cells and DU145 cells have a methylated AR gene and very low AR gene expression. HDACs regulate AR expression in prostate cells, and thus HDACi are a potential molecular target for CRPC [40]. In prostate cancer, NaBu

increases the expression levels of acetylated histone H3 and H4 and thereby AR expression in androgen dependent human prostate cancer LNCaP cells. Additionally, NaBu leads to a decrease in the expression of the cell cycle regulatory proteins via G0/G1 phase arrest [41]. Paskova et al. [42] have reported that NaBu effects AR coregulators expression (SMRT and p300), transcription activity and histone acetylation in prostate cancer cells (LNCaP, C4-2) without affecting normal prostate cells (RWPE-1). Furthermore, the activation of TLR4 plays a contradictory role in the progression of prostate cancer. In the study of Gatti et al. [43], TLR4 expression is reduced when pathologic grade increases in human prostate tumor tissues. Additionally, DU-145 cells exhibit TLR4 expression and activated TLR4 increases the levels of pro-inflammatory cytokines through NF-κB signaling pathway. On the other hand, Rezania et al. [43] state that TLR4 protein expression is not observed in DU-145 cells. However, the proliferation of Du-145 cells is increased in response to TLR4 ligand treatment. In our study, we demonstrated that TLR4 protein expression was different in LNCaP and DU-145 cells. Interestingly, we observed lower expression of TLR4 in PCa cells. However, NaBu induced TLR4 expression and IRF3 translocation into the nucleus in castration resistant and AR-independent DU-145 cells not in AR-dependent LNCaP cells. Therefore, this feature could relate to different response of PCa cells to NaBu treatment in terms of TLR4-mediating signaling pathways or other TLRs associated IRF3 activation.

In conclusion, our findings suggest that NaBu can induce apoptotic cell death and regulate the TLR4/IRF3 signaling pathways in hormone independent DU-145 cells. However, NaBu-induced apoptotic effect was not mediated by TLR4-mediated signaling pathways in hormone dependent LNCaP cells. Thus, NaBu-induced apoptotic cell death could be regulated by the other apoptotic cell death pathways in LNCaP cells. Furthermore, NaBu treatment could lead to different responses in PCa cells due to probably AR status. Therefore, further investigations should be performed to assess the pro-inflammatory and anti-inflammatory cytokine levels, molecular mechanisms underlying cell response to NaBu-induced TLR-mediated signaling pathways *in vitro* and *in vivo.*

Declarations

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Conflicts and financial of interest The authors have no conflicts and financial of interest to declare that are relevant to the content of this article.

Ethics approval No ethics approval was received for conducting this study.

Authors' contributions ADO, GGE, NK and OT conceptualized, design and coordinated the investigation. ADO, GGE and NK conducted the analyses and coordinated the investigation. ADO and OT analyzed the data and carried out the statistical analysis. All authors participated in writing, reading and approval of the final manuscript.

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Figures

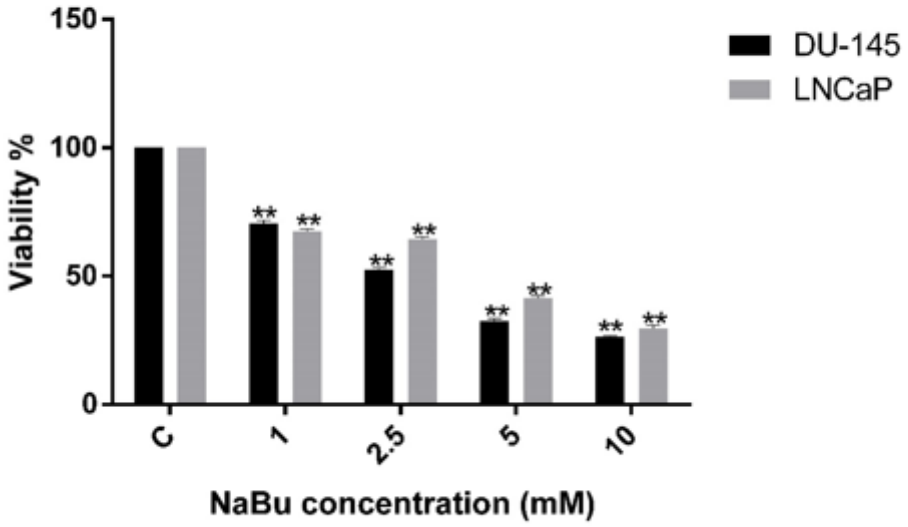

Figure 1

The cytotoxic effects of NaBu in DU-145 and LNCaP cells determined by WST-1 assay. Different concentrations of NaBu (1, 2.5, 5 and 10 mM) were treated to the cells for 24h ($p<0.01$**).

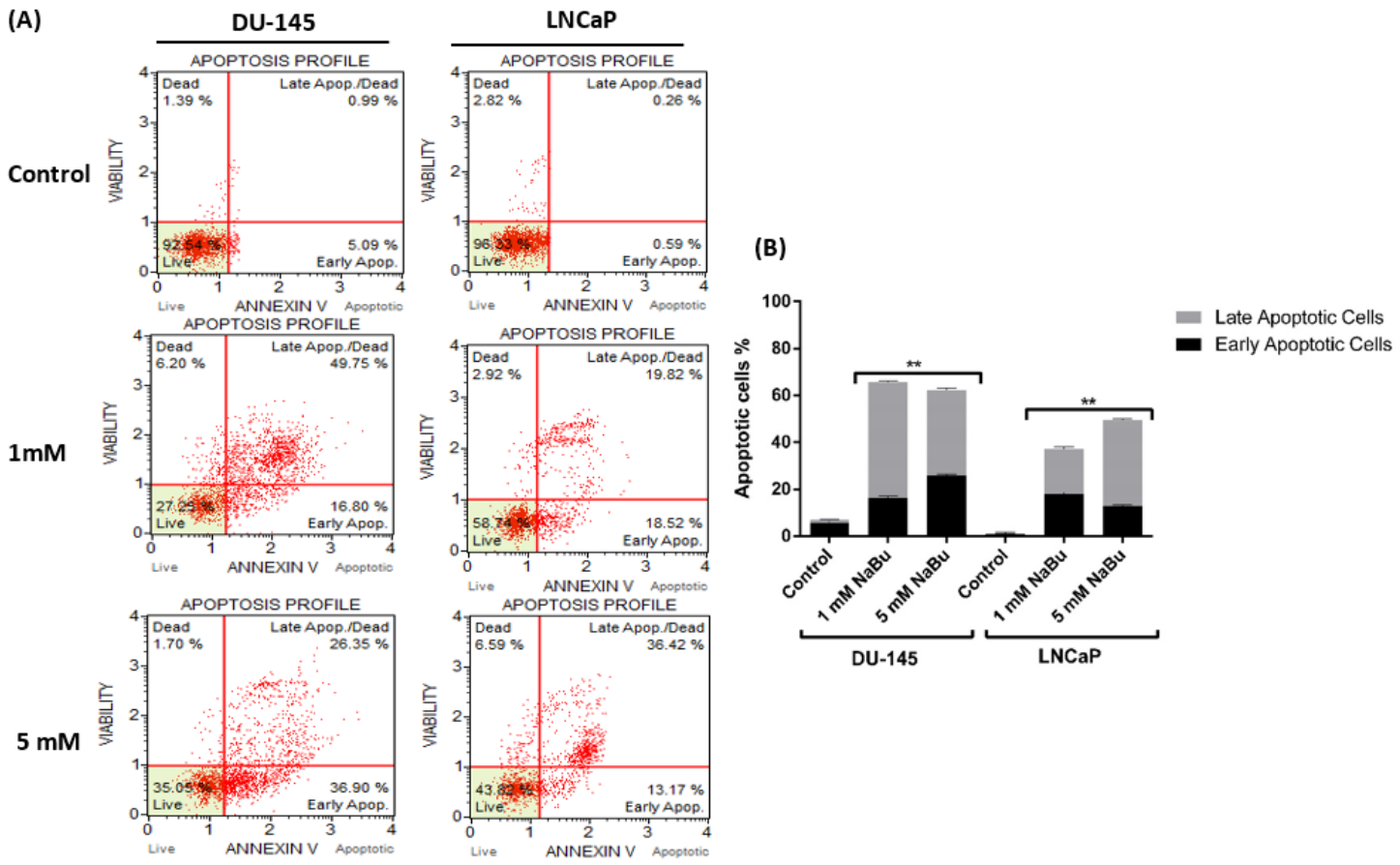

Figure 2

NaBu induced apoptotic cell death in PCa cells were determined by Annexin V analysis. (A) Annexin V diagrams of DU-145 and LNCaP cells after treated with 1 and 5 mM of NaBu compared with control. (B) Statistical comparison of the mean of percentage of early and late apoptotic cell death in PCa cells ($p<0.05$*, $p<0.01$**).

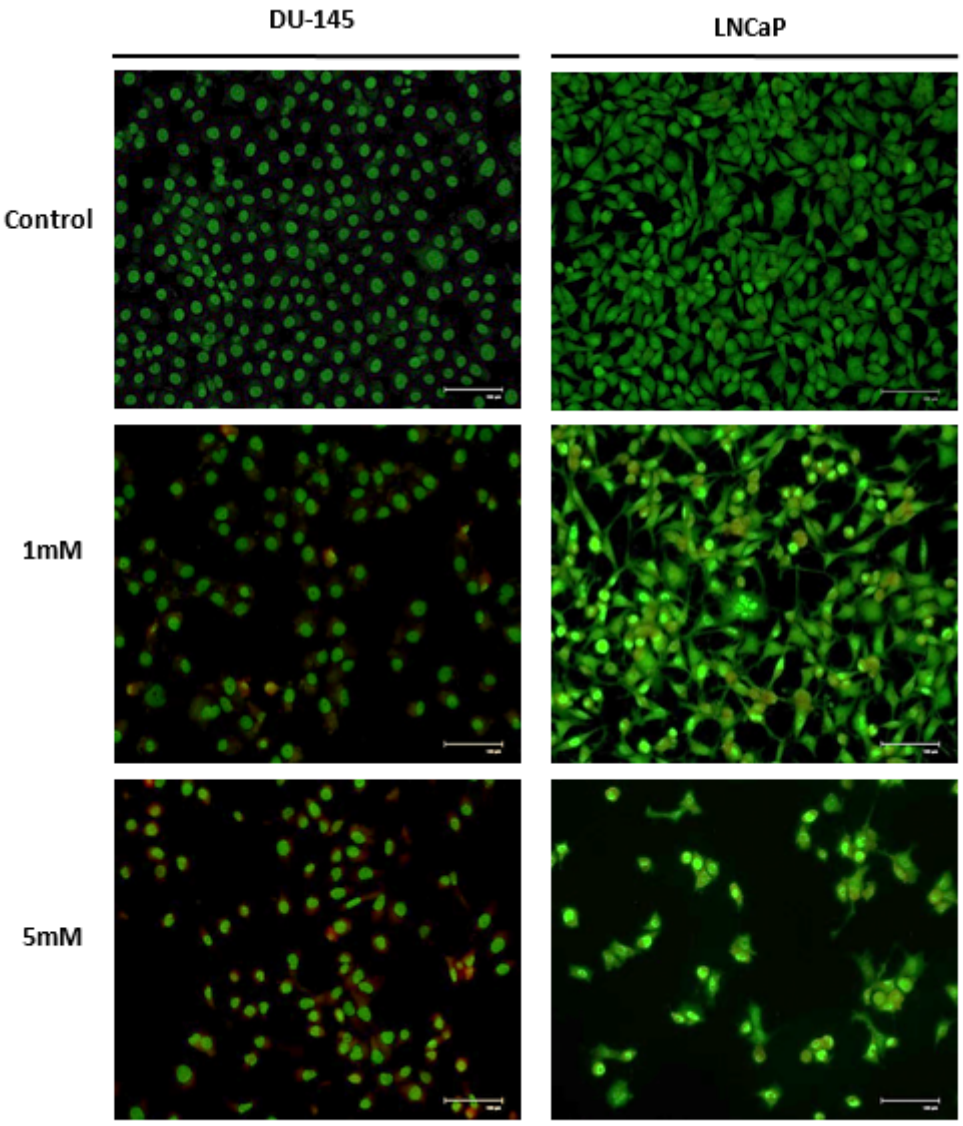

Figure 3

The effects of NaBu on cell morphology analyzed by AO/PI dual staining in PCa cells. The cells were treated with 1 and 5mM of NaBu for 24 h.

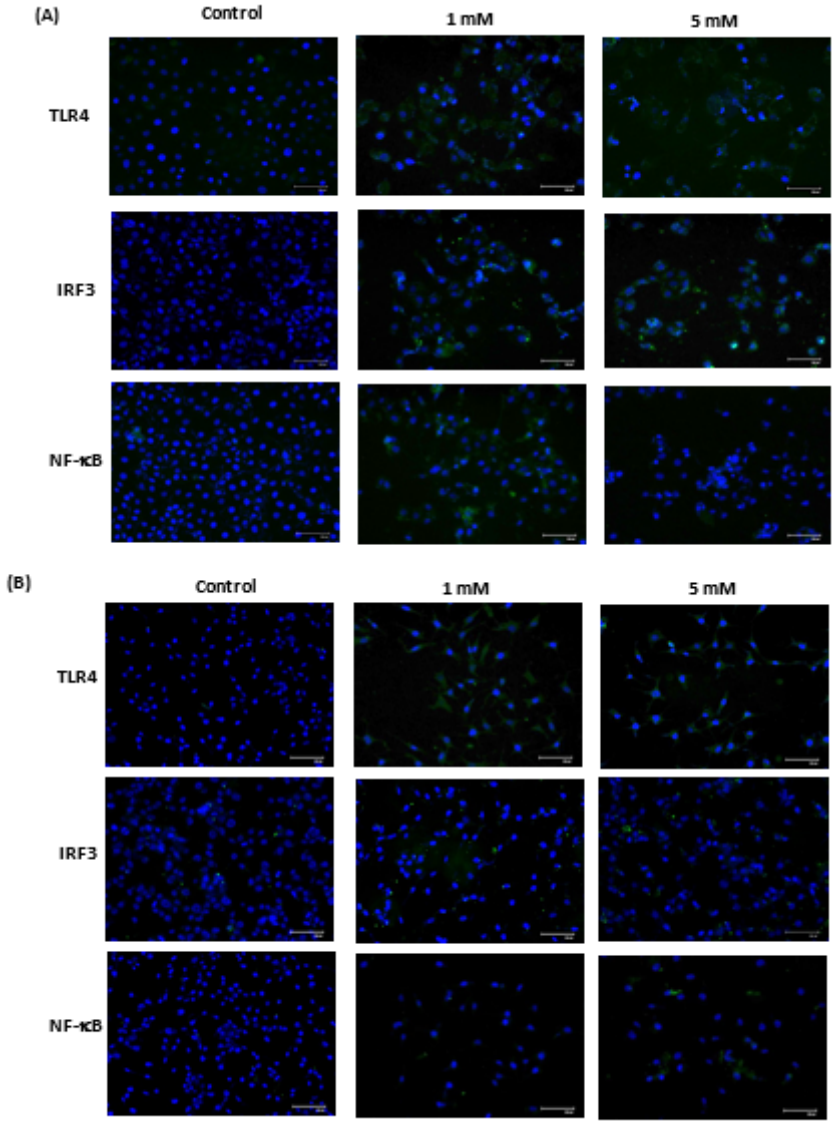

Figure 4

Subcellular localization of TLR4, IRF3 and NF-□B in (A) DU-145 and (B) LNCaP cells following treatment with 1 and 5 mM NaBu compared with control.