3,6-diazabicyclo[3.3.1]heptanes Induces Apoptosis and Arrests Cell Cycle in Prostate Cancer Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Prostate cancer, non-cutaneous malignant tumor, is the second common cause of cancer related mortalities in American men and is responsible for 13% of deaths related to cancer. The present study investigated the anti-cancer effects of 3,6-diazabicyclo[3.3.1]heptane on LNCaP and PC3 prostate cancer cells *in vitro* and on tumor growth *in vivo* in BALB/C nude mice.

Material/Methods: Reduction of cell viability by 3,6-diazabicyclo[3.3.1]heptane was evaluated by sulphorhodamine-B staining and apoptosis onset using annexin V and propidium iodide (PI) staining. The 2',7'-dichlorofluorescein-diacetate stain was used for assessment of reactive oxygen species (ROS) formation while as western blotting for analysis of protein expression.

Results: The viability of LNCaP and PC3 cells was reduced significantly (*P*<0.05) by 3,6-diazabicyclo[3.3.1]heptane in dose-based manner. At 30 µM of 3,6-diazabicyclo[3.3.1]heptane the viability of LNCaP and PC3 cells was reduced to 32 and 28%, respectively. The 3,6-diazabicyclo[3.3.1]heptane treatment increased apoptosis in LNCaP cells to 43.31% at 30 µM. The cell cycle in LNCaP cells was arrested in G1 phase on treatment with 3,6-diazabicyclo[3.3.1]heptane. The expression of cyclin D1 and p21 proteins was significantly increased by 3,6-diazabicyclo[3.3.1]heptane in LNCaP and PC3 cells. The growth of prostate tumor was also suppressed *in vivo* in mice by 3,6-diazabicyclo[3.3.1]heptane treatment.

Conclusions: In summary, the study demonstrated that LNCaP and PC3 prostate cancer cell viability is suppressed by 3,6-diazabicyclo[3.3.1]heptane treatment. The suppression of prostate cancer cell viability by 3,6-diazabicyclo[3.3.1]heptane involves apoptosis induction, cell cycle arrest and upregulation of p21 expression. Therefore, 3,6-diazabicyclo[3.3.1]heptane can be a potential chemotherapeutic agent for prostate cancer.

MeSH Keywords: Apoptosis • Cyclin-Dependent Kinase 4 • Lymphatic Metastasis

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Background

Prostate cancer is the non-cutaneous malignant tumor detected most commonly in American men and accounts for around 30% of all the diagnosed cancers [1]. It is the second common cause of cancer related mortalities in American men and is responsible for 13% deaths related to cancer [1]. Recent data has revealed that 2.2 lakh men have prostate cancer and more than 27 000 men died of it in the United States alone [2]. In the majority of patients, prostate cancer has glandular origin [1–3]. The early stage of prostate cancer is generally asymptomatic because the malignancy arises from peripheral region of the gland which is far from the prostatic urethra. At the advanced stage, prostate cancer involves urethra and/or bladder neck which is accompanied by obstruction of urethra [2,3]. The symptoms at this stage include slow passage or intermittent flow of urine [3]. There is also erectile dysfunction if the tumor encroaches locally on neurovascular bundles [3]. In around 95% of patients, prostate cancer metastasizes to the bones leading to osteoblastic metastases which is associated with the bone pain [4]. Some other characteristics of prostate cancer include anemia due to involvement of bone marrow, and edema in the lower body because of lymphatic system obstruction [4]. Therefore, development of treatment for prostate cancer is of immense importance not only to inhibit the tumor but also to prevent the secondary disorders.

The ionotropic acetylcholine nicotinic receptors (nAChRs), after activation, play an important role in several physiological processes and their modulation is beneficial for the treatment of different pathological disorders [5]. The potential therapeutic significance of these receptors has been subject of extensive research which led to the development of bridged piperazines by structural modification of epibatidine [6,7]. The compound, 3,8-diazabicyclo[3.2.1]octane has been found to possess high affinity for $\alpha4\beta2$ and acts as a potent central analgesic molecule [8]. Taking into account the potential activity of new pharmacophore model of the 2,5-diazabicyclo[2.2.1]heptane, extensive structural modification can lead to the synthesis of a novel series of compounds, 3,6-diazabicyclo[3.1.1]heptane (3,6-DBH) [9–11]. In the present study cytotoxicity of 3,6-diazabicyclo[3.3.1]heptane against LNCaP and PC3 cells *in vitro* and in a mice model *in vivo* were investigated. The study showed that 3,6-diazabicyclo[3.3.1]heptane exhibited a toxicity effect on prostate cancer cells both *in vitro* as well as *in vivo*.

Material and Methods

Cell lines and culture

LNCaP and PC3 cell lines were supplied by the Chinese Academy of Sciences, Shanghai, China. The cell lines were maintained in Dulbecco's Modified Eagle's medium (DMEM) mixed with fetal bovine serum (10%). The medium also contained streptomycin (100 µg/mL), penicillin (100 U/mL) and L-glutamine (2 mM). The cells were cultured in an incubator under 5% CO_2 atmosphere at 37°C.

Cell viability assay

The viability of LNCaP and PC3 cells following 48 hours of 3,6-diazabicyclo[3.3.1]heptane treatment was measured by MTT assay. Briefly, the cells were distributed at 2×10^5 cells/well density in 96-well plates and cultured for 24 hours. Then 3,6-diazabicyclo[3.3.1]heptane at 5, 10, 15, 20, 25, and 30 µM concentrations was added to the wells and plates were incubated for 48 hours. Following treatment, 0.5 mg/mL solution of MTT (20 µL) was put into each well and incubation was continued for 4 hours more. Then medium was discarded and DMSO (150 µL) was added to the plates followed by absorbance measurement at 487 nm using a microplate reader.

Apoptosis analysis by Annexin V-FITC and propidium iodide (PI) double staining

The apoptosis induction by 3,6-diazabicyclo[3.3.1]heptane in LNCaP cells was detected by flow cytometry. The cells were exposed to 20, 25, and 30 µM of 3,6-diazabicyclo[3.3.1]heptane or phosphate-buffered saline (PBS) (control) for 48 hours. The cells were harvested, washed 2 times with ice-cold PBS and then treated with 1x binding buffer at 1×10^6 cells/mL density. Then stained of the cells with Annexin VFITC and PI solution was performed for 20 minutes under darkness at room temperature. The cell apoptosis was detected using flow cytometry (Beckman Coulter, Inc., Brea, CA, USA).

Detection of reactive oxygen species (ROS) production in LNCaP cells

The 2',7'-dichlorofluorescein-diacetate (DCFHDA) staining was used for analysis of changes in reactive oxygen species (ROS) production by 3,6-diazabicyclo[3.3.1]heptane in LNCaP cells. The cells were incubated for 48 hours with 20, 25, and 30 µM of 3,6-diazabicyclo[3.3.1]heptane. After treatment, the cells were twice washed with PBS and then re-suspended in serum free culture medium mixed with 10 µM of DCFH-DA dye. The stained cells were observed for ROS production by flow cytometry.

Cell cycle analysis by flow cytometry

The LNCaP cells were incubated for 48 hours with 20, 25, and 30 µM of 3,6-diazabicyclo[3.3.1]heptane and then washed using PBS. The cells were subjected to fixing with 70% cold ethyl alcohol overnight followed by treatment with 20 µg/mL

RNase A at 37°C. Then cells were stained with a solution of PI (10 µg/mL) at 37°C for 45 minutes. The FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) was used for analysis of cell cycle distribution and DNA content in the cells.

Western blotting

LNCaP cells at 1×10^6 cell/well density were treated with 20, 25, and 30 µM of 3,6-diazabicyclo[3.3.1]heptane for 48 hours. The cells were collected, trypsinized and subsequently treated with radio immunoprecipitation assay (RIPA) lysis buffer (1000 µL; 50 mM Tris-base, 1 mM EDTA, 150 mM sodium chloride, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate). The lysate was centrifuged for 15 minutes at 12 000×g at 4°C and protein concentration in the supernatant was determine using bicinchoninic acid (BCA) method. The protein samples (20 µL) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel by the electrophoresis (80 V) and subsequently transferred to polyvinylidene difluoride membranes. The membranes were earlier blocked by incubation with 50 g/L skimmed milk for 45 minutes at room temperature. Then membrane incubation with primary antibodies against p21, cyclin D1, and GAPDH (all from Santa Cruz Biotechnology Inc., CA, USA) was carried at 4°C overnight. The membranes were washed extensively with PBS and Tween-20 for 5 minutes prior to incubation for 1 hour with goat antirabbit-labelled horseradish peroxidaseconjugated secondary antibodies (Abcam) at room temperature. The enhanced chemiluminescence detection kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used for developing the membranes for imaging.

Animals

Seventy BALB/C nude mice were obtained from the Beijing HFK Bioscience Co., Ltd., China. The mice were tagged and maintained in plastic cages individually under standard laboratory conditions with 12 hours light/dark cycles, at a constant temperature of 23°C and humidity of 60%. Food and water were provided ad libitum.

Treatment strategy

The mice were randomly assigned into 7 groups of 10 mice each: normal control, untreated, and 5 treatment groups (1, 2, 3, 4, and 5 mg/kg 3,6-diazabicyclo[3.3.1]heptane treatment groups). All the mice except mice in the normal control group were subcutaneously implanted LNCaP cells (1×10^6) on the left flank immediately above the hind limb. The mice in the treatment groups were intra-peritoneally administered 1, 2, 3, 4, and 5 mg/kg doses of 3,6-diazabicyclo[3.3.1]heptane on day 2 of tumor implantation. The normal control and untreated groups received equal volumes of normal saline. All the mice were closely monitored during the 45 days of study starting
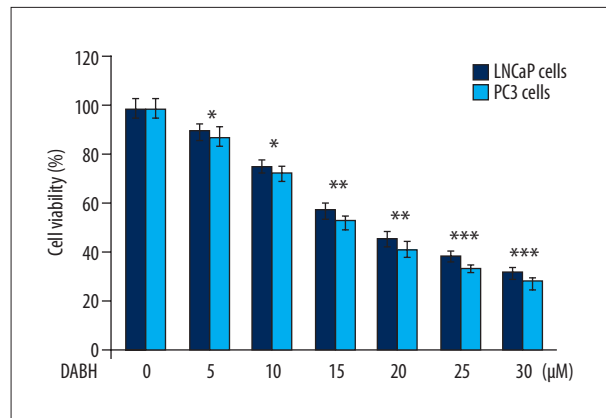
Figure 1. Effect of 3,6-diazabicyclo[3.3.1]heptanes on LNCaP and PC3 cell viability. LNCaP and PC3 cells were incubated with 5, 10, 15, 20, 25, and 30 µM concentrations of 3,6-diazabicyclo[3.3.1]heptane or phosphate-buffered saline (control) for 48 hours. The cell viability is presented as the mean±standard error of the mean (SEM) relative to control. * $P<0.05$, ** $P<0.02$, *** $P<0.01$ versus control.

from the day 1 of tumor administration to measure survival rate. On day 46 of tumor implantation, the mice were sacrificed to excise the tumor under sodium sorbitol anesthesia. The volume of excised tumors was measured using calipers.

Statistical analysis

The data presented are the mean±standard deviation of 3 experiments. The comparison among the groups was carried out using ANOVA followed by Tukey's or Dunnett's test. The Statistical analysis of the data was performed using SPSS 17.0 software (IBM Corp., Armonk, NY, USA). The values at $P<0.05$ were considered to indicate a statistically significant difference.

Results

Effect of 3,6-diazabicyclo[3.3.1]heptane on LNCaP and PC3 cell proliferation

The toxicity effect of 3,6-diazabicyclo[3.3.1]heptanes on LNCaP and PC3 cells were investigated MTT assay (Figure 1). The data showed cytotoxic effect of 3,6-diazabicyclo[3.3.1]heptane against LNCaP and PC3 cells in concentration-based manner. The viability of LNCaP cells was suppressed to 32% on treatment with 30 µM of 3,6-diazabicyclo[3.3.1]heptane at 48 hours. Treatment with 30 µM of 3,6-diazabicyclo[3.3.1]heptane reduced PC3 cell viability to 28% in comparison to 100% in the control group.
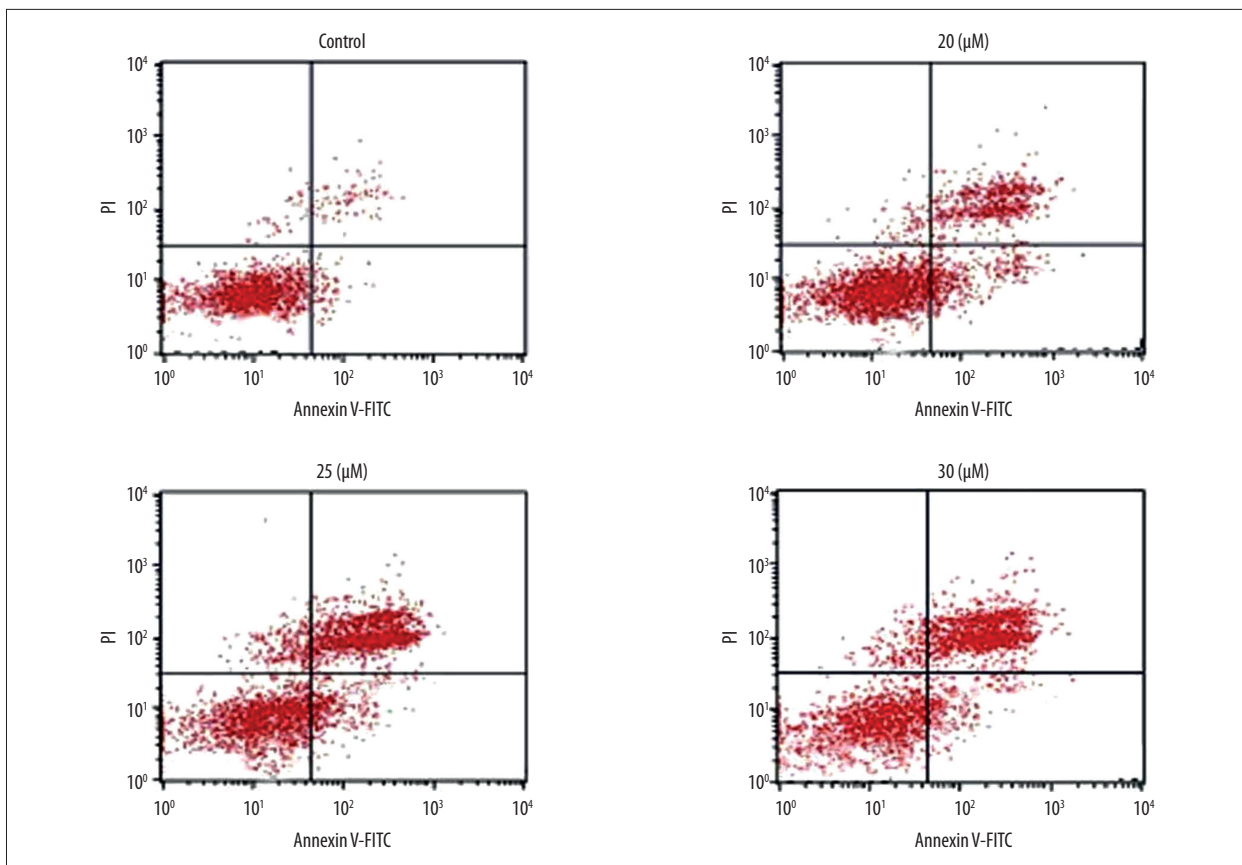
Figure 2. Effect of 3,6-diazabicyclo[3.3.1]heptane on apoptosis in LNCaP cells. The 3,6-diazabicyclo[3.3.1]heptane was added to plates at 20, 25, and 30 µM and apoptosis was analyzed using annexin V/propidium iodide (PI) binding by flow cytometry. Magnification; 4×10.

3,6-diazabicyclo[3.3.1]heptane induces LNCaP cell apoptosis

The annexin V/PI stained LNCaP cells were observed by flow cytometry following treatment with 20, 25, and 30 µM of 3,6-diazabicyclo[3.3.1]heptane (Figure 2). The number of apoptotic LNCaP cells increased significantly ($P<0.05$) with the increase in 3,6-diazabicyclo[3.3.1]heptane concentration at 48 hours. The apoptotic cell proportion increased from 1.8% in control to 19.54%, 32.76%, and 43.31%, respectively on treatment with 20, 25, and 30 µM of 3,6-diazabicyclo[3.3.1]heptane.
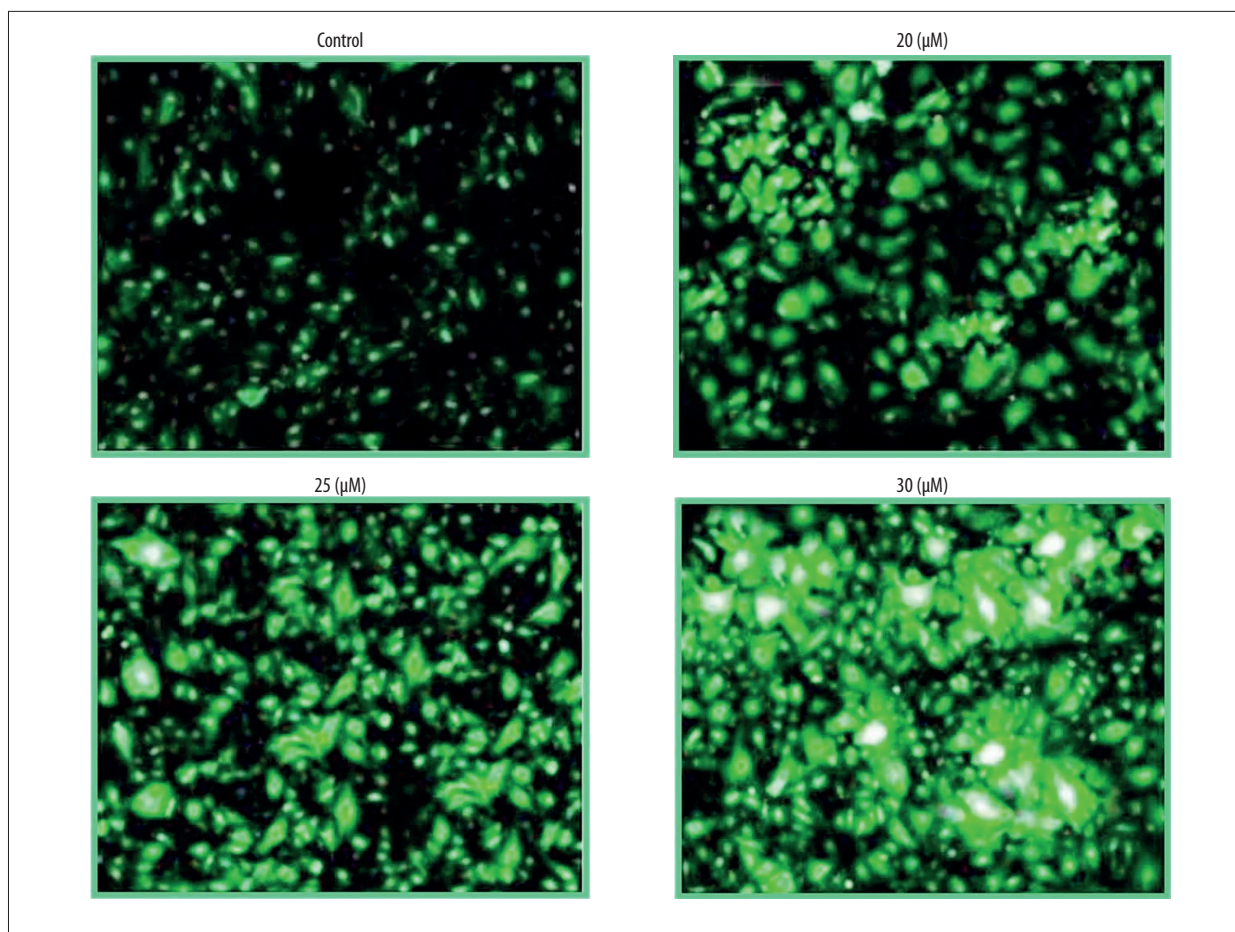
ROS production in LNCaP cells by 3,6-diazabicyclo[3.3.1] heptanes

The changes in ROS level by 3,6-diazabicyclo[3.3.1]heptane in LNCaP cells were analyzed to understand the mechanism of cytotoxicity (Figure 3). The proportion of DCFH-DA stained LNCaP cells showed a significant increase on increasing 3,6-diazabicyclo[3.3.1]heptane concentration from 20 to 30 µM. To confirm whether increase in ROS formation is associated with the cytotoxicity the changes in LNCaP cells viability by

3,6-diazabicyclo[3.3.1]heptanes were determined following glutathione pre-treatment (Figure 3). The data showed that glutathione pre-treatment prevented cytotoxic effect of 3,6-diazabicyclo[3.3.1]heptane against LNCaP cells.

3,6-diazabicyclo[3.3.1]heptane arrests cell cycle in LNCaP cells

The changes in LNCaP cell cycle progression on treatment with 20, 25, and 30 µM of 3,6-diazabicyclo[3.3.1]heptane were analyzed by flow cytometry (Figure 4). Treatment of LNCaP cells with 3,6-diazabicyclo[3.3.1]heptane caused arrest of cell cycle in sub-G1 phase. The proportion of cells in sub-G1 phase increased from 48.66% in control to 69.87% on treatment with 30 µM of 3,6-diazabicyclo[3.3.1]heptane. However, the percentage of LNCaP cells in S and G2/M phases showed a significant decrease with increase in 3,6-diazabicyclo[3.3.1]heptane from 20 to 30 µM.

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Figure 3. Effect of 3,6-diazabicyclo[3.3.1]heptane on ROS formation in LNCaP cells. The cells treated with 20, 25 and 30 μM of 3,6-diazabicyclo[3.3.1]heptanes were analysed for ROS generation by DCFH DA dye. Magnification; 4×10.

3,6-diazabicyclo[3.3.1]heptane increases cyclin D1 in LNCaP cells

Western blotting showed a marked increase in cyclin D1 protein expression in LNCaP cells on treatment with 3,6-diazabicyclo[3.3.1]heptane (Figure 5). The expression of cyclin D1 was found to increase in concentration-based manner on treatment with 3,6-diazabicyclo[3.3.1]heptane. In LNCaP cells the level of p21 was increased with increase in concentration of 3,6-diazabicyclo[3.3.1]heptane from 20 to 30 μM.

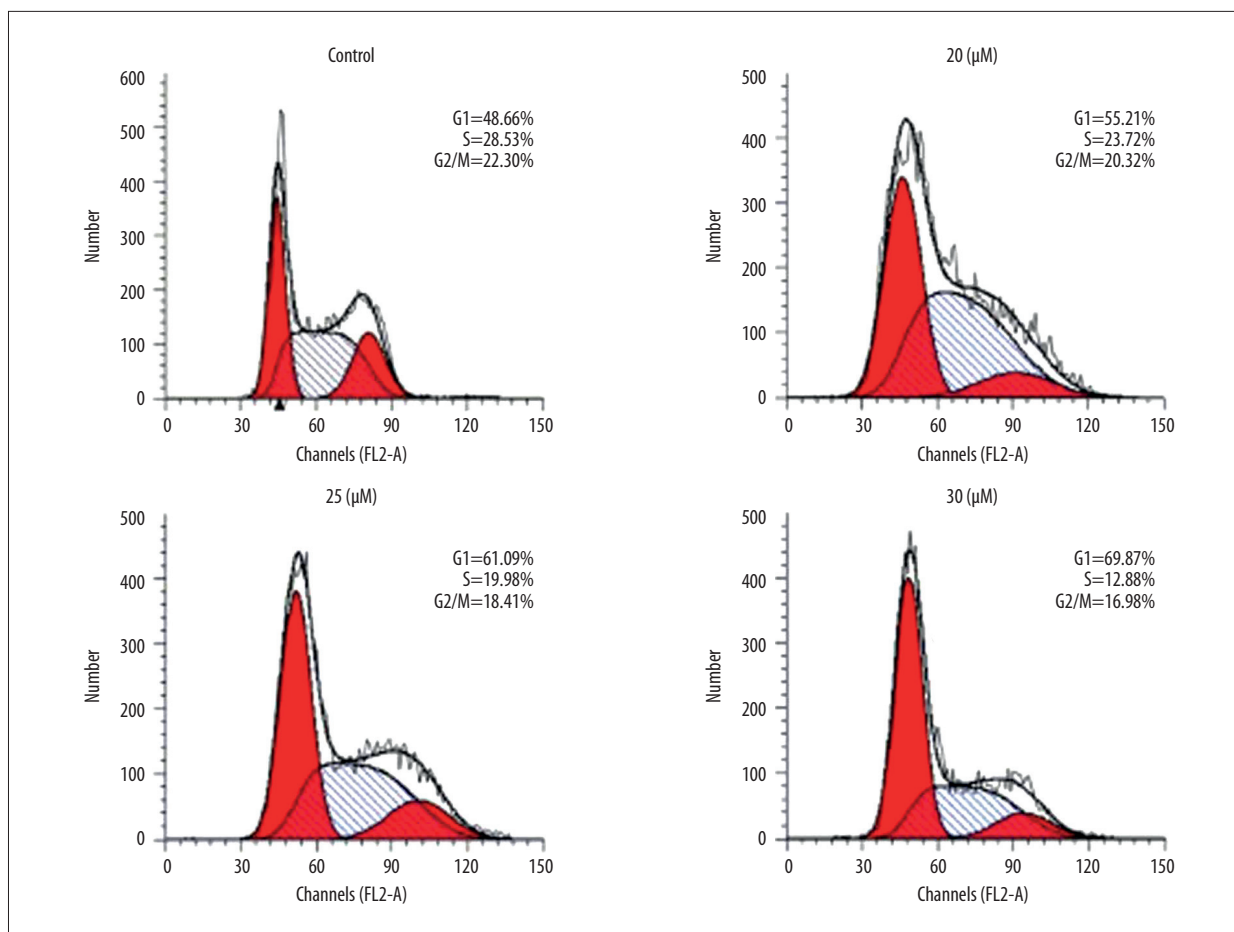### Effect of 3,6-diazabicyclo[3.3.1]heptane on *in vivo* tumor growth

The 3,6-diazabicyclo[3.3.1]heptanes treatment significantly increased survival of the mice bearing prostate cancer (Figure 6A). In the 5 mg/kg 3,6-diazabicyclo[3.3.1]heptanes treatment group all the mice were alive during the study. In the untreated group 70% mice died while as in 2 mg/kg 3,6-diazabicyclo[3.3.1] heptanes treatment group 50% mice died during the study. The tumor size was markedly higher in the untreated mice

which survived during the study compared to the normal control (Figure 6B). Treatment of the tumor implanted mice with 3,6-diazabicyclo[3.3.1]heptane significantly ($P<0.05$) reduced tumor size in comparison to the untreated group.

Discussion

The present study demonstrated the cytotoxicity of 3,6-diazabicyclo[3.3.1]heptane against LNCaP and PC3 cells *in vitro* and in mice model *in vivo*. The study showed that 3,6-diazabicyclo[3.3.1]heptane exhibits toxicity effect on prostate cancer cells both *in vitro* as well as *in vivo*.

Cell apoptosis which plays a prominent role in the regulation of carcinoma is controlled by various genes [12]. There are different types of signaling pathways which initiate apoptosis through activation of proteases, commonly known as caspases [13]. The activation of apoptotic signaling process by different agents causes death of cells instead of direct killing [14]. In the present study 3,6-diazabicyclo[3.3.1]heptane treatment lead to suppression of
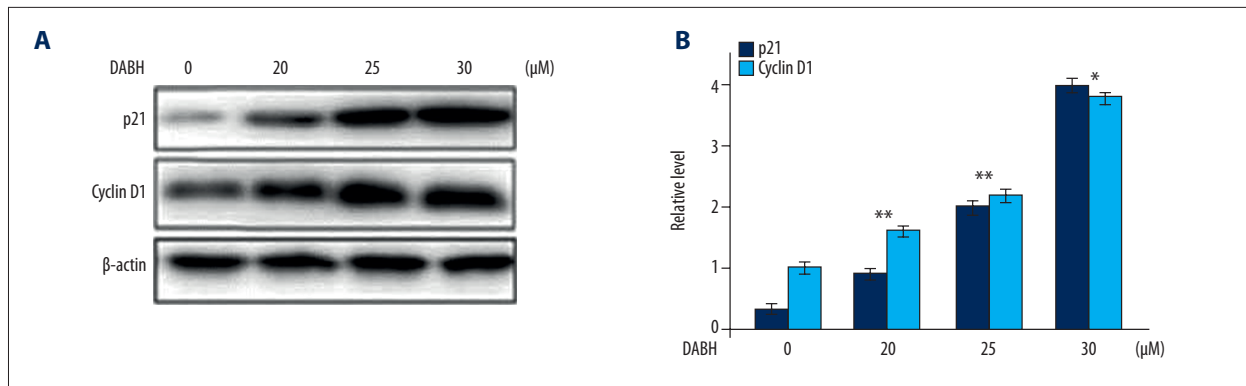
Figure 4. Effect of 3,6-diazabicyclo[3.3.1]heptane on LNCaP cell cycle progression. The cells treated with 20, 25, and 30 μM of 3,6-diazabicyclo[3.3.1]heptane, were stained with propidium iodide (PI) and analyzed by flow cytometry.

proliferation in LNCaP and PC3 prostate cancer cells. The study showed a concentration-based suppression of prostate cancer cell proliferation on exposure to 3,6-diazabicyclo[3.3.1]heptane. To understand the 3,6-diazabicyclo[3.3.1]heptane induced cytotoxicity in LNCaP and PC3 prostate cancer cells flow cytometry was used for analysis of cell apoptosis. The results from present study showed that 3,6-diazabicyclo[3.3.1]heptane treatment significantly promoted apoptosis in LNCaPs cells. These observations confirmed that 3,6-diazabicyclo[3.3.1]heptane activated apoptosis process in LNCaPs cells. It is well established that reduction of glutathione by chemotherapeutic agents increases generation of ROS leading to apoptosis of carcinoma cells [15]. The over-production of ROS in cells has been observed prior to their death [16,17]. In the present study treatment of LNCaP cells with 3,6-diazabicyclo[3.3.1]heptane increased production of ROS in dose-based manner. The ROS generation was markedly higher in LNCaP cells on treatment with 3,6-diazabicyclo[3.3.1]heptane for 48 h.

The cell cycle, a highly regulated cellular process consists of a sequence of events which control reproduction. Synthesis of DNA takes place at replication origin where licensing machinery

unwinds the double helical structure to expose individual strands to enzymes [18]. In the M phase following S phase is associated with the chromosomal segregation and division of nucleus and cells. Generally, a gap phase between G1 and S phases regulates progression of cells from one cell cycle to another and the process is under the control of intracellular and extracellular signals [19,20]. In cell cycles G1 has prime importance because it is during this phase that cells either exit or decide to undergo cell cycle [21,22]. In the present study 3,6-diazabicyclo[3.3.1]heptane treatment of LNCaP cells increased cell population in G1 phase. The cell percentage in the S and G2 phases was significantly decreased. These findings suggested that LNCaP cells on treatment with 3,6-diazabicyclo[3.3.1]heptane decided to exit the cell cycle in G1 phase. Progression of the cells through different phases is controlled strictly by various factors. The two important Ser/Thr protein kinases known as CDK4 and CDK6 play a vital role in the cell cycle progression through G1 phase. Cyclin D1 restricts progression of cells from G1 to S phase by binding either to CDK4 or CDK6 [23]. In the present study 3,6-diazabicyclo[3.3.1]heptane treatment markedly promoted the expression of cyclin D1

Figure 5. Effect of 3,6-diazabicyclo[3.3.1]heptane on cyclin D1 in LNCaP cells. (**A**) The cells treated with indicated doses of 3,6-diazabicyclo[3.3.1]heptane were analysed by western blotting. (**B**) The data was quantified. * $P<0.05$, ** $P<0.01$ vs. control.
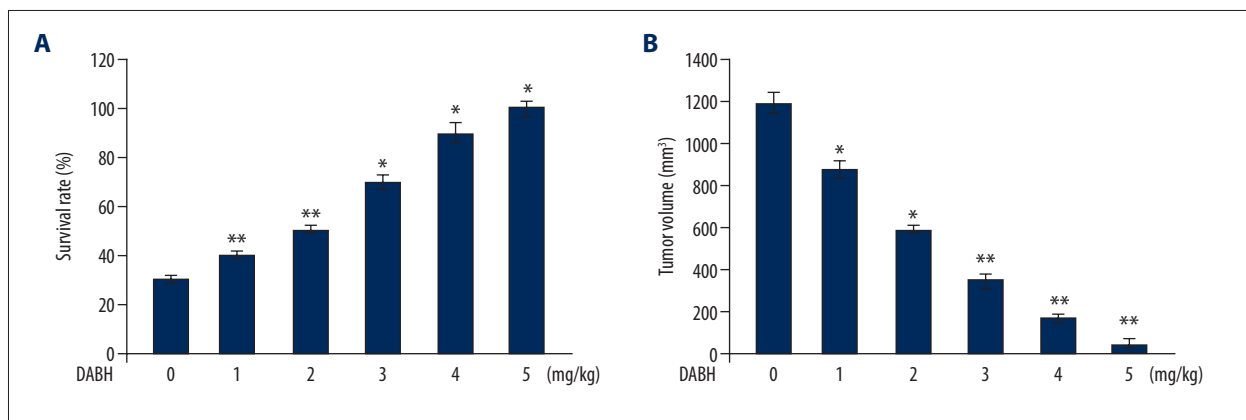
Figure 6. Effect of 3,6-diazabicyclo[3.3.1]heptane on *in vivo* tumor growth. (**A**) The mice implanted with 1×10^6 LNCaP cells were intraperitonally injected 1, 2, 3, 4, and 5 mg/kg doses of 3,6-diazabicyclo[3.3.1]heptane on day 1. The survival of mice was observed during the 45 days of tumor implantation. (**B**) The living mice were sacrificed using sodium sorbitol anesthesia on the day 45 to excise the tumor for measurement of volume. * $P<0.05$, ** $P<0.01$ versus control.

in LNCaP cells in concentration-based manner. The p21 plays a vital role in the regulation of tumor growth by activation of tumor suppressor activities [24]. In the present study 3,6-diazabicyclo[3.3.1]heptane treatment promoted the expression of p21 in LNCaP cells. The results from *in vivo* studies showed that 3,6-diazabicyclo[3.3.1]heptane treatment of mice implanted with LNCaP tumor cells inhibited tumor development in dose-based manner. The survival rate of the prostate tumor mice was significantly increased on treatment with 3,6-diazabicyclo[3.3.1]heptane.

Conclusions

The present study demonstrated that 3,6-diazabicyclo[3.3.1] heptane suppresses prostate carcinoma cell proliferation by promoting ROS production, arrest of cell cycle and up-regulation of cyclin D1 expression. Moreover, the tumor growth in mice model was also suppressed on treatment with 3,6-diazabicyclo[3.3.1]heptane. Therefore, 3,6-diazabicyclo[3.3.1]heptane can be used for the development of treatment for prostate cancer.

Conflict of interest

None.

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