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The Effects of (11R)-13-(6-Nitroindazole)-11,13-Dihydroludartin on Human Prostate Carcinoma Cells and Mouse Tumor Xenografts

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BCD 1 Longning Wang
BCD 1 Lei Wang
BC 1 Sen Wei
CD 1 Xiaodong Wang
ACDEF 2,3 Daqing Shen

1 Department of Urology, Bin Zhou People's Hospital, Binzhou, Shandong, P.R. China
2 Medical College, Jining Medical University, Jining, Shandong, P.R. China
3 Department of Urology, The Affiliated Hospital of Jining Medical University, Jining, Shandong, P.R. China

Corresponding Author: Daqing Shen, e-mail: shendaqing2019@sina.com
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Background: This study aimed to investigate the effects of the 6-nitroindazole compound and amino analog of ludartin, (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL), on human prostate carcinoma cells *in vitro* and in mouse tumor xenografts *in vivo*.

Material/Methods: DU-145 and LNCaP human prostate carcinoma cells were cultured with increasing concentrations of NDHL. Cell viability was measured using the MTT assay, and cell apoptosis was measured by fluorescence flow cytometry. Mouse tumor xenografts were created by implanting 2×10^6 of DU-145 cells subcutaneously in the left flank. On the second day following DU-145 cell implantation, the mice in the treatment groups were injected intraperitoneally with 2, 5, and 10 mg/kg of NDHL.

Results: Treatment of DU-145 and LNCaP cells with NDHL (range, 2.5–20.0 μM) significantly reduced cell proliferation *in vitro* ($P<0.05$). The proliferation rate of DU-145 and LNCaP cells was reduced to 27% and 24%, respectively, following treatment with 20.0 μM of NDHL. Treatment with NDHL significantly increased cell apoptosis and the formation of reactive oxygen species (ROS) formation in DU-145 cells at 48 h ($P<0.05$). NDHL significantly increased the proportion of DU-145 cells in the G1 phase of the cell cycle and significantly increased the expression of cyclin D1 and p21 ($P<0.05$). Treatment of the mice in the xenograft tumor model with NDHL significantly increased survival and suppressed tumor growth ($P<0.02$).

Conclusions: NDHL inhibited cell proliferation, increased apoptosis, and caused cell cycle arrest in human prostate carcinoma cells *in vitro* and inhibited mouse tumor xenograft growth *in vivo*.

MeSH Keywords: Apoptosis • Cyclin D1 • Reactive Oxygen Species

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Background

Worldwide, prostate carcinoma is a common solid tumor and is the second most common cause of cancer-related death in men [1]. In 2010, in the United States, prostate carcinoma resulted in approximately 30,000 annual deaths [1]. There have been several studies on the effects of dietary supplements that contain selenium as treatments for prostate, lung, and colon carcinoma [2–4]. In 1996, Clark et al. reported that the incidence of prostate carcinoma was reduced by up to 63% following the introduction of dietary yeast supplemented with selenium [5]. In 1998, Yoshizawa et al. reported a 50% reduction in the risk of metastatic prostate carcinoma in patients with increased levels of selenium measured in the toenail [6].

Currently, the treatment of early-stage prostate carcinoma consists of surgery, radiation therapy, and androgen ablation [7]. However, the use of hormonal therapy can result in the development of hormone-unresponsive prostate carcinoma [7]. Therefore, the identification of novel and effective therapeutic molecules continues to be investigated. Natural products are the basis of alternative medicine and consist of plant extracts and their chemical constituents [8]. The polysaccharide class of natural products have shown a broad spectrum of activity in the treatment of human disease and possess the advantage of minimal toxicity [9–11]. The plant-based polysaccharides have been used in the pharmaceutical industry for the treatment of inflammation, tumors, and as antioxidants [12–14]. The sesquiterpene lactones are one of the largest groups of natural products that can be isolated from different plant sources and are considered to have potential as novel chemotherapeutic agents [15–17]. The 6-nitroindazole compound and amino analog of ludartin, (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL) has cytotoxic effects, but its effects in prostate carcinoma remain unknown. Therefore, this study aimed to investigate the effects of NDHL on human prostate carcinoma cells *in vitro* and in mouse tumor xenografts *in vivo*.

Material and Methods

Cell culture

DU-145 and LNCaP human prostate carcinoma cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) containing L-glutamine (2 mM) and antibiotics in a humidified atmosphere with 5% CO_2.

MTT cell viability assay following treatment with (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL)

Cytotoxicity induced by NDHL in DU-145 and LNCaP cells was assessed by the MTT assay. Briefly, DU-145 and LNCaP cells were distributed at a density of 1×10^6 cells per well in 96-well culture plates. The cells were maintained in DMEM at 37˚C for 12 h before the addition of 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 µM of NDHL. After 48 h incubation with NDHL, a 10 µl solution of MTT (5 mg/mL) was added to the cell culture wells and incubated for a further 2 h at 37°C. Dimethyl sulfoxide (DMSO) (120 µl) was added to each well to dissolve any crystals that formed. Measurement of the optical density (OD) for each well was performed at 568 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Apoptosis assay

DU-145 cells were distributed in six-well plates at 2×10^6 cells per well and cultured for 24 h. The cells were then treated with 2.5, 5, 10, and 20 µM of NDHL at 37°C for 48 h. The control cells were treated with dimethyl sulfoxide alone, without the addition of NDHL. Cell incubation was followed by trypsinization, the cells were washed twice in PBS and then stained with Annexin-V and propidium iodide (PI). The stained cells were washed twice with PBS, fixed in 10% formalin, and then washed with PBS. Cell apoptosis was observed using a fluorescence microscope (Nikon Co., Tokyo, Japan).

Cell cycle analysis

DU-145 cells plated in 60-mm culture plates at 2×10^5 cells per well and treated with 2.5, 5, 10, and 20 µM of NDHL at 37°C for 48 h. Washing in PBS was followed by trypsinization. Then cells were then fixed in 70% ethyl alcohol overnight, treated with RNase A (20 µg/ml), and stained at 37°C with 10 µg/ml of PI. The flow cytometry was performed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) to analyze the cell cycle.

Western blot

DU-145 cells were plated at 3×10^5 cells per well in RPMI 1640 medium mixed with 10% FBS in 60 mm diameter Falcon™ culture dishes (Thermo Fisher Scientific, Waltham, MA, USA). The cells were treated for 48 h with 2.5, 5, 10, and 20 µM of NDHL at 37°C. Following incubation, the harvested cells were transferred to PBS and then lysed in HEPES buffer (50 mM), pH 7.5, sodium chloride (150 mM), EDTA (1 mM), vanadate (1 mM), sodium pyrophosphate (10 mM), NaF (10 mM), NP-40 (1%), and protease inhibitors. The lysate was cleared of debris by centrifugation for 25 min at 1200×g to obtain the supernatant. A commercially available bicinchoninic acid (BCA) protein assay kit was

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used to measure the protein concentration. Protein separation was performed using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. The membrane was blocked by incubation with 5% solution of dried skimmed milk powder. Incubation of the membranes was performed overnight at 4°C with primary antibodies against GAPDH, p21, and cyclin D1 (Cell Signaling Technology, Danvers, MA, USA). After washing in PBS, the membranes were incubated with a peroxidase-labeled secondary antibody. The protein bands were visualized using an enhanced chemiluminescence (ECL) detection method (Amersham Biosciences, Little Chalfont, UK).

Assessment of reactive oxygen species (ROS) production

ROS levels in DU-145 cells following exposure to 2.5, 5, 10, and 20 μM of NDHL were assessed using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay (Sigma-Aldrich, St. Louis MO, USA). The cells were treated at 48 h to increasing concentrations of NDHL and with a 5 μM solution of DCFH-DA in DMSO for 20 min. The cells were then lysed on by treatment with 400 mM of NaOH, and the fluorescence intensity of the wells was determined using a Synergy 2 multiwell fluorescence plate reader (BioTek Instruments, Inc., Winooski, VT, USA). The wavelengths used for excitation and emission were 483 and 526 nm, respectively. ROS quantification was performed using fluorescence microscopy to measure the changes in DCFH-DA fluorescence.

Animals

Six-week-old male C57BL/6J mice (N=50) were obtained from the Experimental Animal Center, Soochow University, Suzhou, China. The mice were individually housed in plastic cages at a temperature of 30˚C temperature and 45–75% humidity under 12 h light and dark cycles. All the mice had free access to food and water.

Establishment of the mouse tumor xenograft model and treatment with NDHL

The mice were randomly divided into five groups that included the sham control group, the untreated group, and three treatment groups that were treated with 2, 5, and 10 mg/kg of NDHL. The mice in the untreated and three treatment groups were injected with 2×10^6 DU-145 cells subcutaneously in the left flank immediately above the hindlimb under sodium sorbitol anesthesia. On the second day following cancer cell implantation, the mice in the treatment groups were injected with 2, 5, and 10 mg/kg doses of NDHL by the intraperitoneal route. The mice in the control and untreated groups were given 200 μl of PBS with 1% DMSO. The mice were carefully monitored during the study period of 45 days, and the bodyweight for each mouse was recorded on alternate days.

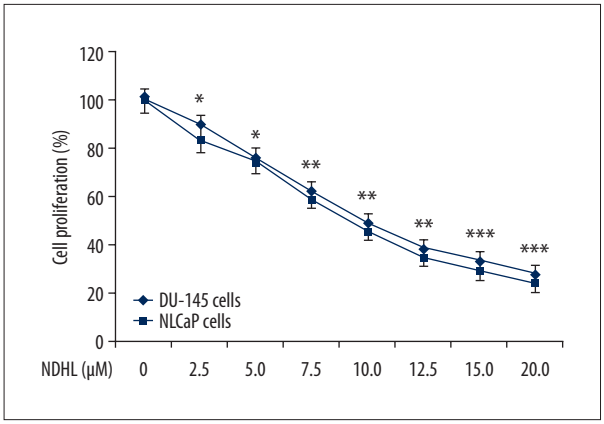

Figure 1. The effects of (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL) on DU-145 and LNCaP human prostate carcinoma cell proliferation. NDHL was added to the culture medium at increasing concentrations. Cell proliferation was assessed at 48 h by the MTT assay. * P<0.05, ** P<0.02, and *** P<0.01 *vs.* the control.

Statistical analysis

Data were presented as the mean±standard deviation (SD) of three experiments performed independently. The differences between the groups were determined using two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Data were analyzed using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA). A P-value <0.05 was considered to be statistically significant.

Results

Treatment with (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL) reduced DU-145 and LNCaP cell proliferation

Following NDHL exposure for 48 h, the DU-145 and LNCaP human prostate carcinoma cells underwent optical density assessment of proliferation (Figure 1). NDHL treatment significantly reduced the proliferation rate of DU-145 and LNCaP cells in the concentration range of 2.5–20 μM (P<0.05). Treatment with NDHL at doses >20 μM did not result in a further reduction in the proliferation of DU-145 and LNCaP cells. There was no significant reduction in DU-145 and LNCaP cell proliferation by NDHL at concentrations <2.5 μM. The proliferation of DU-145 and LNCaP cells was reduced to 27% and 24%, respectively, on treatment with 20 μM of NDHL for 48 h. NDHL treatment at 2.5 μM reduced DU-145 and LNCaP cell proliferation to 89% and 83%, respectively.

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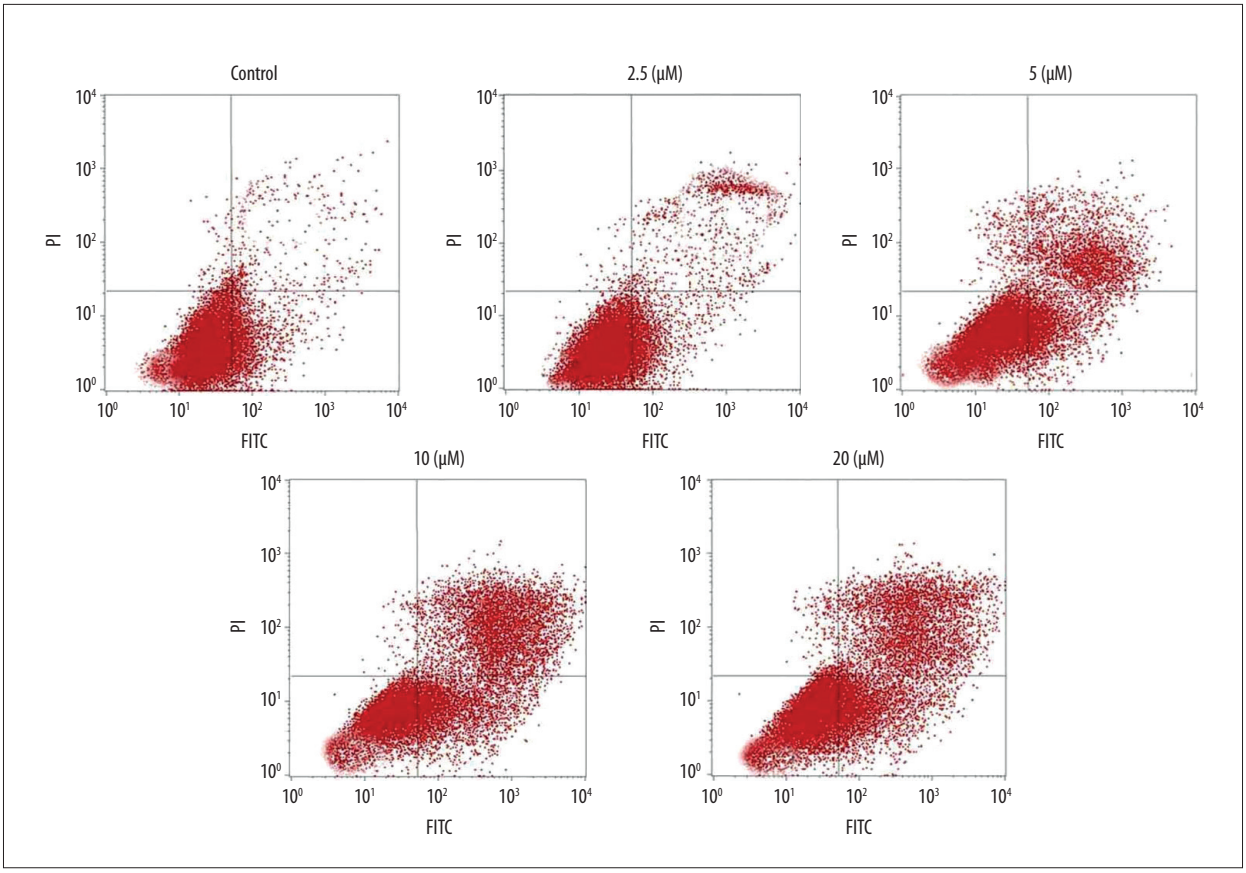

Figure 2. The effects of (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL) on DU-145 human prostate carcinoma cell apoptosis. NDHL was added to the six-well culture plates at increasing concentrations. DU-145 cells were incubated for 48 h. Cell apoptosis was detected by flow cytometry following Annexin-V and propidium iodide (PI) staining.

NDHL treatment increased DU-145 cell apoptosis

Exposure of DU-145 cells to NDHL for 48 h significantly increased the number of cells stained by annexin V ($P<0.05$) (Figure 2). The apoptotic DU-145 cells increased to 8.43%, 16.76%, 39.45%, and 57.89%, respectively, on treatment with 2.5, 5, 10, and 20 μM of NDHL. In the control DU-145 cell cultures, apoptosis was induced only in 1.98% cells at 48 h.

NDHL increased the levels of reactive oxygen species (ROS) in DU-145 cells

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) staining was used to analyze ROS generation by 2.5, 5, 10, and 20 μM of NDHL in DU-145 cells (Figure 3). The ROS generation was increased significantly ($P<0.02$) in DU-145 cells by NDHL treatment in a concentration-dependent manner. Treatment of DU-145 cells with NDHL at 20 μM increased the production of ROS by 6.5-fold.

NDHL resulted in DU-145 cell cycle arrest

NDHL treatment for 48 h significantly ($P<0.05$) increased DU-145 cells in the G1 phase of the cell cycle (Figure 4). The number of DU-145 cells in the S phase and G2/M phase on treatment with 2.5, 5, 10, and 20 μM of NDHL were reduced. Treatment with 20 μM of NDHL increased the proportion of DU-145 cells in the G1 phase to 64.23% compared with 45.68% in untreated cells. These findings showed that NDHL treatment caused DU-145 cell cycle arrest in the G1 phase.

NDHL increased cyclin D1 and p21 expression by DU-145 cells

Expression of cyclin D1 and p21 in NDHL treated DU-145 cells was assessed by Western blot (Figure 5). Treatment of DU-145 cells with 2.5, 5, 10, and 20 μM of NDHL significantly increased the expression of cyclin D1 and p21 in a concentration-dependent manner. No significant enhancement in cyclin D1 and p21 expression resulted from treatment with 2.5 μM NDHL.

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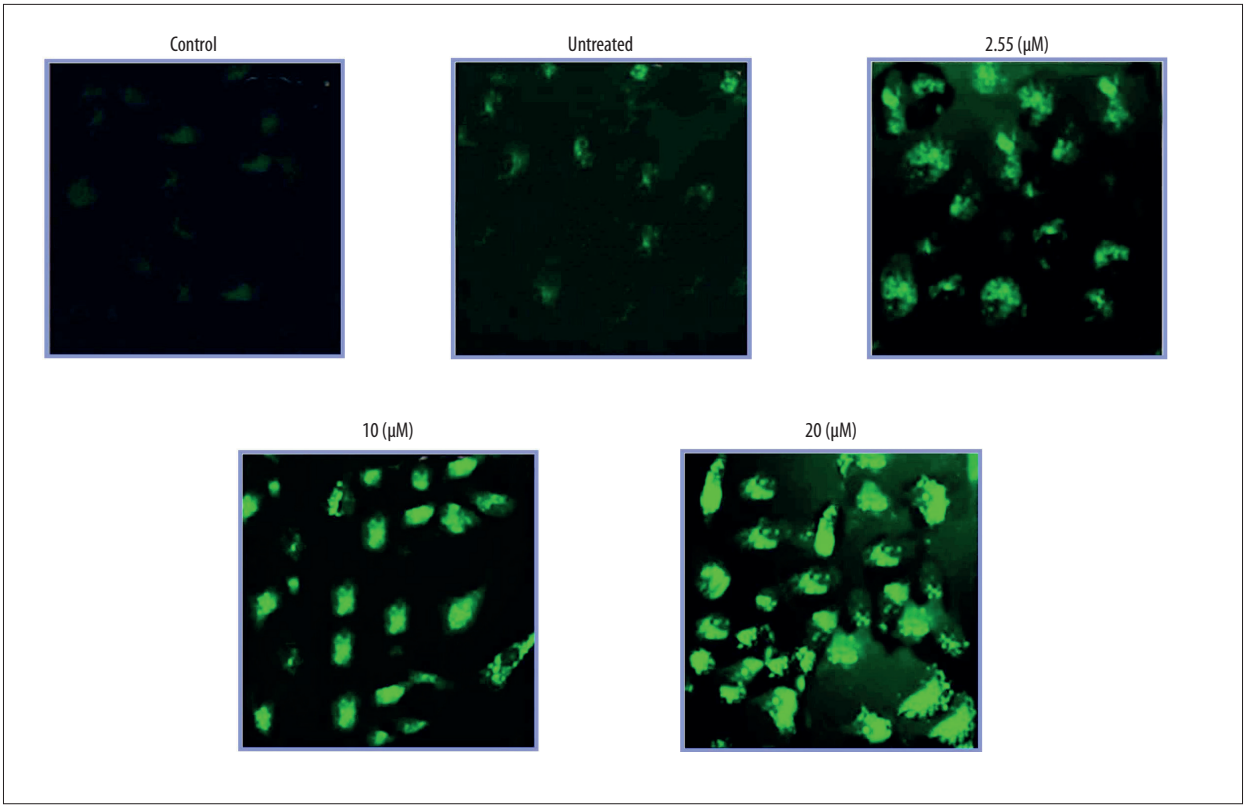

Figure 3. The effects of (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL) on the generation of reactive oxygen species (ROS) by DU-145 human prostate carcinoma cells. After 48 hours following treatment with increasing concentrations of NDHL, DU-145 cells were analyzed using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. The levels of reactive oxygen species (ROS) were analyzed by flow cytometry.

NDHL inhibited the growth of prostate carcinoma mouse DU-145 cell tumor xenografts *in vivo*

Treatment of the prostate carcinoma mouse tumor xenografts with NDHL significantly increased the mouse survival rate ($P<0.02$) compared with the untreated group (Figure 6A). Tumor growth in the prostate carcinoma mouse tumor xenografts was also significantly suppressed in a dose-dependent manner following treatment with NDHL (Figure 6B). The tumors excised from the mice treated with 10 mg/kg NDHL were significantly reduced in size compared with the untreated group.

Discussion

This study aimed to investigate the effects of (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL) on DU-145 and LNCaP human prostate carcinoma cell viability, cell proliferation, and the cell cycle *in vitro* and the growth of mouse tumor xenografts *in vivo*. The findings showed that NDHL reduced DU-145 and LNCaP cell proliferation through apoptosis induction, increased ROS production, and arrest of the cell cycle in the G1 phase. Also, NDHL inhibited the development of prostate carcinoma and increased survival of the mice with the DU-145 tumor xenografts.

Most of the biologically active sesquiterpene lactones obtained during phytochemical investigation possess anti-tumor potential [15]. The anti-tumor potential of these sesquiterpene lactones is associated with the inhibition of DNA synthesis or transcription [16]. Structure and activity relationship studies have shown that the presence of the α, β-unsaturated lactone moiety with an exocyclic double-bond enables these lactones to undergo the addition of L-cysteine or thiol-containing enzymes [17].

In the present study, the effect of NDHL on the proliferation of DU-145 and LNCaP cells was measured. The data showed that NDHL treatment suppressed the proliferation of DU-145 and LNCaP cells in a dose-dependent manner. These findings suggested that NDHL exhibited the cytotoxic effect on DU-145 and LNCaP cells. Cell apoptosis is regulated by several genes that cause the elimination of diseased cells in a controlled way [18]. The apoptotic signaling pathways operate in a sequential manner leading to the activation of proteases known as the caspases [19]. The activated apoptosis

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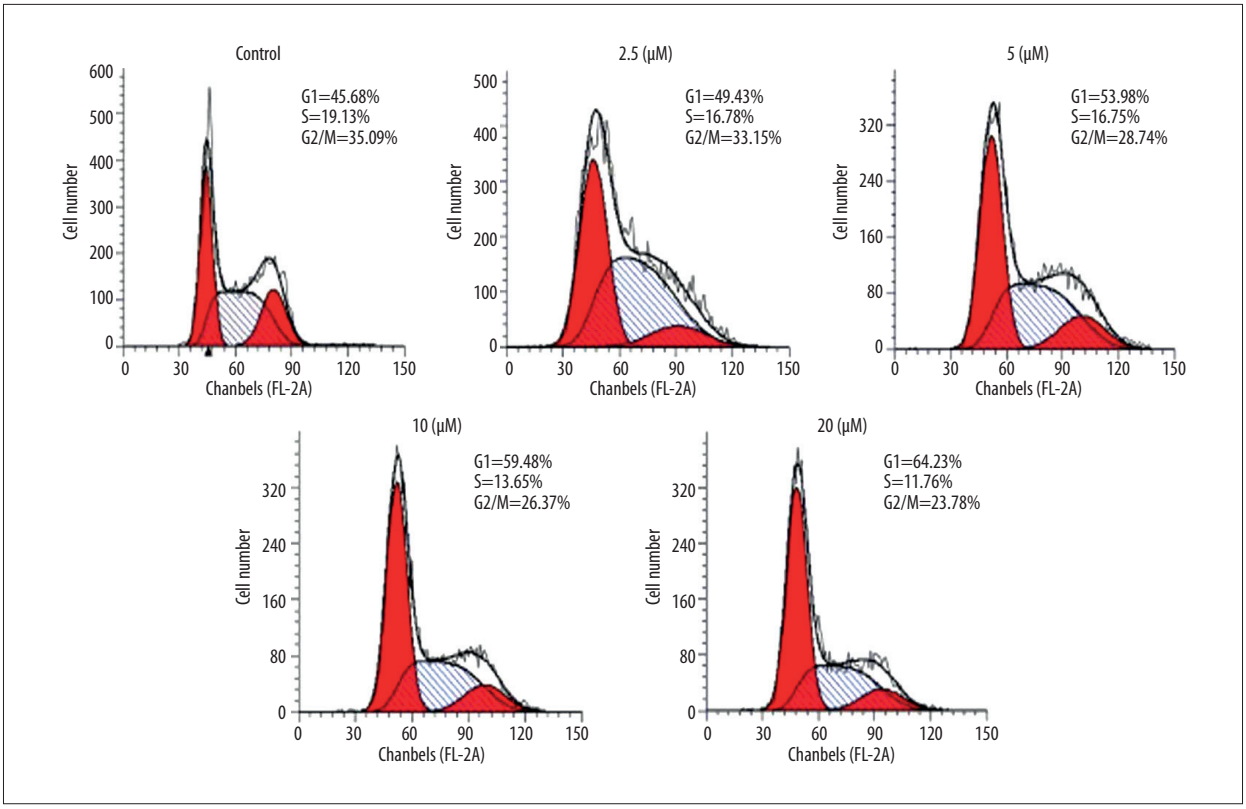

Figure 4. The effects of (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL) on the distribution of DU-145 cells in phases of the cell cycle. After 48 hours following treatment with increasing concentrations of NDHL, DU-145 cells were stained with propidium iodide (PI). The cell DNA was analyzed by flow cytometry.

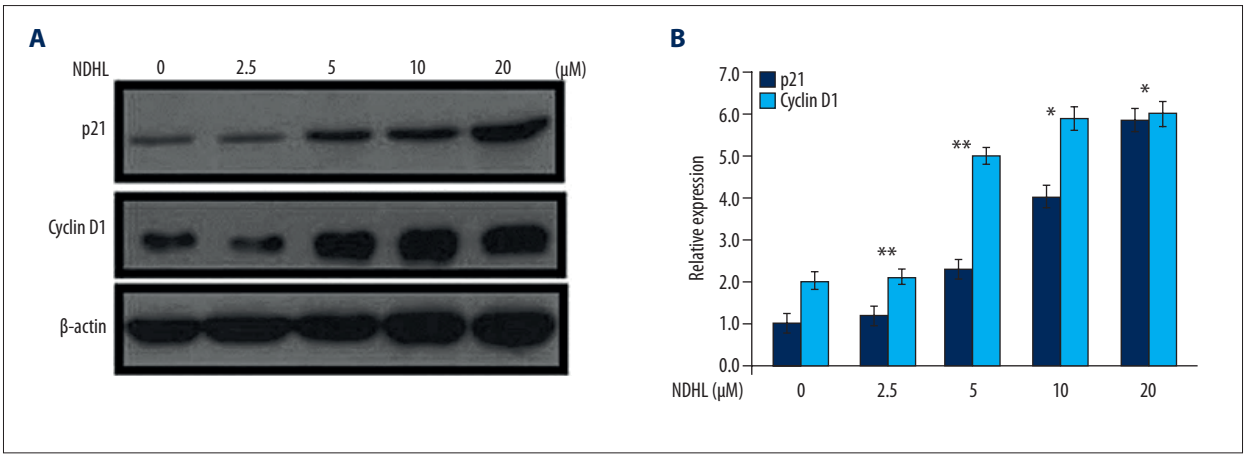

Figure 5. The effects of (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL) on cyclin D1 and p21 expression by DU-145 cells. (**A**) Treatment of DU-145 cells with increasing concentrations of NDHL after 48 h was followed by Western blot to determine the expression of cyclin D1 and p21. (**B**) Densitometric analysis of cyclin D1 and p21 expression. * P<0.05, and ** P<0.02 *vs.* the control.

signaling pathways are responsible for the removal of carcinoma cells [20]. In the present study, NDHL treatment significantly increased the percentage of apoptotic cells in the DU-145 cultured cells compared with the untreated cells. There was a significantly increased proportion of apoptotic cells in DU-145 cultures treated with NDHL compared with the untreated cells. Therefore, NDHL suppressed DU-145 cell proliferation through activation of the apoptotic signaling pathway.

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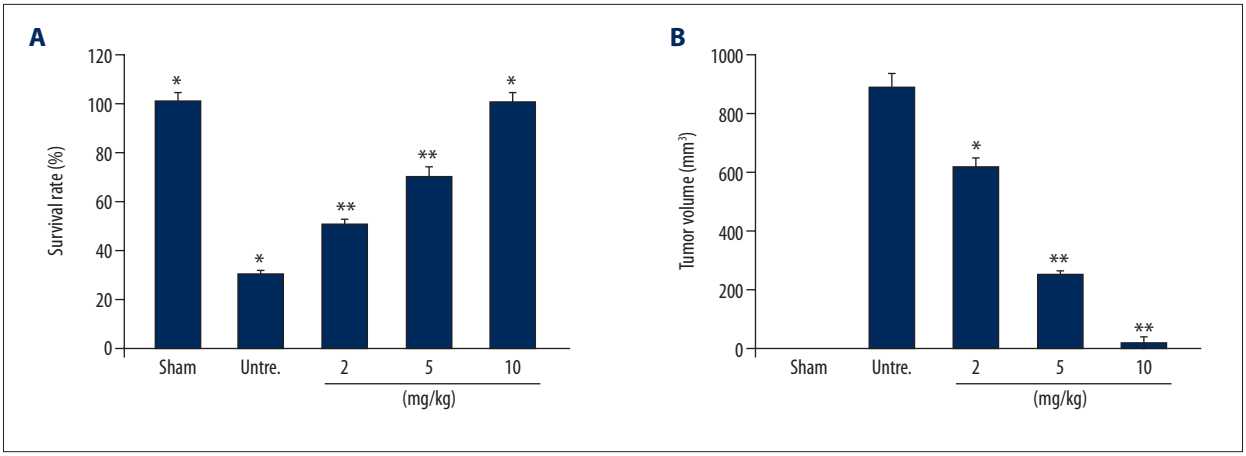

Figure 6. The effects of (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL) on the growth of mouse tumor xenografts. The mice were subcutaneously injected with 2×10^5 DU-145 cells and treated with 2, 5, and 10 mg/kg doses of NDHL. The mice were euthanized on day 45 following tumor implantation. (**A**) Survival analysis of the mice was monitored during the study period, and the data were analyzed. (**B**) The mice were euthanized on day 45, the tumors were excised, and the tumor volume was measured. * P<0.05, and ** P<0.02 *vs.* the control.

Reactive oxygen species (ROS) are metabolites with oxidative ability and are initiators of apoptosis in carcinoma cells [21]. Imbalance in the ROS generation and anti-oxidants present in the body lead to the development of oxidative stress [21]. The generation of excess ROS acts as a stimulant for the induction of DNA damage and ultimately leads to apoptosis [22]. It has been suggested the ROS production may be used as a therapeutic strategy to induce apoptosis in cancer cells [23]. The excessive generation of ROS is considered to be the beginning of apoptotic cell death in carcinoma cells [24]. Cell death by necrosis has also been found to promote the generation of ROS [24]. Necrotic cell death can be associated with the oxidative stress induced by higher levels of ROS [25]. In the present study, NDHL treatment of DU-145 cells enhanced ROS levels in a dose-dependent manner. A significant increase in ROS generation was found in DU-145 cells treated with 20 μM of NDHL.

The outcome of cells in the cell cycle is controlled by factors that include the expression of CDK4 and CDK6 (Ser/Thr protein kinases), and these molecules regulate the progression of the G1 phase [26]. Cyclin D1 acts as the restriction point for controlling the progression of cells from the G1 phase to the S phase of the cell cycle by binding to CDK4 or CDK6 [9]. Data from the present study showed that NDHL significantly increased the expression of cyclin D1 and p21 in DU-145 cells.

Treatment of DU-145 cells with NDHL resulted in the G1 phase cell cycle arrest. The G1 phase population of DU-145 cells was significantly increased, and cells in the S phase and G2/M phase decreased following treatment with NDHL. These findings suggest that NDHL arrested the cell cycle in the G1 phase by increasing cyclin D1 and p21 expression in DU-145 cells. NDHL treatment of DU-145 cell tumor xenografts in mice significantly increased the survival rate and suppressed tumor development when compared with the untreated group.

Conclusions

This study aimed to investigate the effects of the 6-nitroindazole compound and amino analog of ludartin, (11R)-13-(6-nitroindazole)-11,13-dihydroludartin (NDHL), on human prostate carcinoma cells *in vitro* and in mouse tumor xenografts *in vivo*. NDHL inhibited cell proliferation, increased apoptosis, and caused cell cycle arrest in human prostate carcinoma cells *in vitro* and inhibited mouse tumor xenograft growth *in vivo*.

Conflict of interest

None declared.

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