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Inhibitory effect of acetyl-11-keto-β-boswellic acid on androgen receptor by interference of Sp1 binding activity in prostate cancer cells

Hui-Qing Yuan [a,1], *Feng Kong* [a,1], *Xiao-Ling Wang* [a], *Charles Y.F. Young* [b], *Xiao-Yan Hu* [a], *Hong-Xiang Lou* [c,*]

[a] *Department of Biochemistry and Molecular Biology, School of Medicine, Shandong University, Jinan, Shandong 250012, China*
[b] *Departments of Urology and Biochemistry and Molecular Biology, Mayo Graduate School, Mayo Clinic/Foundation, Rochester, MN 55905, USA*
[c] *Department of Natural Product Chemistry, School of Pharmaceutical Sciences, Shandong University, 44 Wenhua WestRoad, Jinan, Shandong 250012, China*

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ABSTRACT

Androgen receptor (AR)-mediated signaling is crucial for the development and progression of prostate cancer (PCa). Naturally occurring phytochemicals that target the AR signaling offer significant protection against this disease. Acetyl-11-keto-β-boswellic acid (AKBA), a compound isolated from the gum-resin of *Boswellia carterii*, caused G1-phase cell cycle arrest with an induction of $p21^{WAF1/CIP1}$, and a reduction of cyclin D1 as well in prostate cancer cells. AKBA-mediated cellular proliferation inhibition was associated with a decrease of AR expression at mRNA and protein levels. Furthermore, the functional biomarkers used in evaluation of AR transactivity showed suppressions of prostate-specific antigen promoter-dependent and androgen responsive element-dependent luciferase activities. Additionally, down-regulation of an AR short promoter mainly containing a Sp1 binding site suggested the essential role of Sp1 for the reduction of AR expression in cells exposed to AKBA. Interruption effect of AKBA on Sp1 binding activity but not Sp1 protein levels was further confirmed by EMSA and transient transfection with a luciferase reporter driven by three copies of the Sp1 binding site of the AR promoter. Therefore, anti-AR properties ascribed to AKBA suggested that AKBA-containing drugs could be used for the development of novel therapeutic chemicals.

1. Introduction

A tremendous amount of evidence indicates that androgen and androgen receptor (AR)-mediated signaling are crucial in the development and progression of PCa [1–3]. Targeting of AR signaling may represent a rational strategy for treatment of advanced PCa. Inspiration from low PCa incidence in Asian countries and evidence from the epidemiologic studies, nutrition and diet may impact PCa, and naturally occurring phytochemicals from oriental traditional medicines may offer

* *Corresponding author*. Tel.: +86 531 88382012; fax: +86 531 88382019.
E-mail address: louhongxiang@sdu.edu.cn (H.-X. Lou).

[1] They contributed equally to this work.

Abbreviations: AR, androgen receptor; ARE, androgen responsive element; hK2, human glandular kallikrein; PSA, prostate-specific antigen; Sp1, promoter specificity protein 1.

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significant protection for reducing this disease-related morbidity and mortality [4,5].

The gum resins, produced by trees of various *Boswellia* species, have been traditionally used in medicine in Asia for centuries. Extracts of gum resins exhibit anti-inflammatory effects on various inflammatory diseases [6,7]. The traditional herbal formula of gum resins which is believed to have activity against tumor and relief of pain has been included in anti-tumor prescription of ''Xihuangwan (or Xihuang Pill)'' for the treatment of cancers *in vivo* and *in vitro* in China [8–10]. Boswellic acids (BAs), a group of pentacyclic triterpenic acids, are main bioactive principles of the gum resins as indicated by their anti-inflammatory activity [11–13]. Additionally, BAs are gaining much more attention for their anti-tumor activities recently [14–18], and suggesting that BAs may be potential anticancer agents. Meanwhile, the proposed molecular mechanisms related to their functional cellular processes for tumor growth arrest include triggering apoptosis by activation of caspase-dependent signaling or suppression of IκB kinase signaling [16–20], inducing cell cycle arrest [15,16], inhibiting matrix metalloproteinase activity [20], and interfering with MAPK signaling [21]. It has been reported that acetyl-boswellic acids promote apoptosis of androgen-independent prostate cancer cells [16]. Whether the acetyl-11-keto-β-boswellic acid (AKBA), one of the ingredients of gum resins of *Boswellia carterii*, represents the active principle and exhibits inhibitory effect by targeting of AR was studied in the present study. The results showed that the AKBA inhibited cell growth and arrested cells at the G1 phase, and blocked AR expression and function in prostate cancer cell line, LNCaP.

2. Material and methods

2.1. Chemicals

The resinous exudate of *B. serrata* (2.0 kg, from Affiliated Hospital of Shandong Traditional Chinese Medical University, Jinan, China) was ground into powder and extracted with ethanol. After removing of ethanol in vacuum, the crude extract was dissolved in 1% NaOH solution, and then extracted with ethyl acetate. The ethyl acetate extract was discarded, and the aqueous solution was neutralized with diluted HCl and precipitate was produced. The precipitate was washed with distilled water, and dried to produce acid extract. The extract was subjected to silica gel column chromatography and eluted with a petroleum ether–ethyl acetate gradient of increasing amount of ethyl acetate to provide nine fractions (A–I). Fraction F (50 g) was further separated on silica gel column chromatography to afford AKBA and followed by purification on Phenomenex C18 reversed phase high performance liquid chromatography (RP-HPLC) and resulted in high purity of AKBA (>99.9%). The AKBA was prepared in dimethyl sulfoxide (DMSO) as a stock and stored as small aliquots at −20 °C. Dihydrotestosterone (DHT) (Sigma) was dissolved in ethanol.

2.2. Cell culture and treatments

Human prostate cancer cell lines, LNCaP (The American Type Culture Collection, Rochville, MD) and PC3 (The Cell Bank of Chinese Academy of Sciences, Shanghai) were routinely maintained in 50 ml or 75 ml flasks, 6-well or 24-well plate culture dishes in RPMI 1640 medium (HyClone) supplemented with 10% fetal bovine serum (JRH biosciences, Australia) and 5% CO_2 at 37 °C until reaching approximately 50–70% confluence. Cells were maintained in serum-free RPIM 1640 medium for further 24 h to deplete endogenous steroid hormones, and then treated with DHT (10 nM) with or without AKBA (30 μM or as indicated) in the medium containing 5% charcoal stripped serum. Ethanol and DMSO were also used as control vehicles. Medium with or without chemicals were changed daily.

2.3. Cell proliferation assays

Proliferation of the LNCaP cells in the presence of AKBA was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) colorimetric assay. The LNCaP cells were propagated in 24-well culture plates and grown under the conditions described above. The cells were then treated with vehicle, DHT alone or DHT combined with desired concentrations of AKBA for further 48 h. Cell growth response to the chemicals was detected by measuring the absorbance of formazan crystals produced by living cultured cells at 570 nm. Three replicates were used for each treatment and the assay was repeated at least three times.

2.4. Cell cycle analyses

The LNCaP cells were seeded in 75 ml flasks and incubated under the conditions described above. After treatment with chemicals for 48 h, cells were sedimented, washed with ice-cold PBS, and then fixed in 70% ethanol. Cell cycle analyses were carried out with propidium iodide staining and flow cytometry using the Becton Dickinson FACScan.

2.5. Quantitative PCR

After exposed to chemicals for 24 h, LNCaP cells in 6-well plates were collected and total RNA were extracted by using Trizol (Invitrogen Life Technologies). Changes in AR gene expression after AKBA treatment were quantified using real time RT-PCR on the Takara Thermal Cycle Dice TP800 Detection System (Takara Biotechnology, Japan). AR transcript was detected from M-MLV reverse transcriptase-amplified cDNA, with one aliquot designated to receive no enzyme. Quantitative PCR (TaqMan PCR) was performed using Premix Ex Taq™ reagent according to the manufacturer's recommended protocols (Takara Biotechnology, Dalian, China). For each 25 μl TaqMan PCR reaction, synthesized cDNA corresponding to 100 ng total RNA as template, a final concentration of 400 nM primers, 120 nM of probe, 2X TaqMan PCR Mix and PCR-grade water were mixed together. Sequence specific primers for AR were: 5′-AAGGCTATGAATGTCAGCCCA-3′ (sense) and 5′-CATTGAGGCTAGAGAG CAAGGC-3′ (antisense). Probes harbored the fluorescence reporter FAM at the 5′-end and TAMRA at the 3′ end, FAM5′-TGTGTGCTGGACACGACAA-CAACC-3′ TAMRA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as an internal control that was run in the same PCR reaction, the probe and primer combinations

were as follows: FAM 5′-AACAGCGACACCCACTCCTCCACC-3′ TAMRA, 5′-CCAGGTGGTCTCCTCTGACTT-3′ (sense), 5′-GTT-GCTGTAGCCAA ATTCGTTGT-3′ (antisense). All primers and probes were synthesized by Takara Biotech (Dalian, China). PCR assay was performed on each group in triplicate. Quality of reactions was confirmed by comparison of triplicate RT versus no enzyme control for each RNA sample. Samples with Ct (threshold cycle) values greater than 38 cycles were considered to show no amplification. The amount of target gene relative to the housekeeping gene GAPDH for each sample was analyzed with the $2_T^{-\Delta\Delta C}$ method [22]. Values were indicated as percentage of untreated control and set 100%.

2.6. Transient transfection assay

Cells were seeded in 24-well plates and grown under the conditions described above. For transfection into LNCaP cells, pGL3 basic vector (Promega) containing the AR promoter (−1380/+577) (AR 2 kb promoter, 1.0 μg/well), pGL3 basic vector driven by an AR short promoter (−87/+77) (AR short promoter, 1.0 μg/well), pGL3 basic vector with the PSA promoter (−5824/+12) (PSA 6 kb promoter, 0.8 μg/well), or pGL3-SV-40 (Promega) with three copies of Sp1 binding motif of the AR promoter (pGL3-SV40-AR-3Sp1, 1 μg/well) and pcDNA-Sp1 expressing human Sp1 (0.2 μg/well) were co-transfected by using Lipofectamine 2000 (Invitrogen Life Technologies) transfection reagent. For transfection into PC-3 cells, a human AR expression vector (pSG5-hAR, 0.5 μg/well) was co-transfected with PSA promoter (PSA 6 kb promoter, 0.8 μg/well), or pGL3-SV-40 with three tandem repeats of ARE binding sequence in the hk2 promoter (pGL3-SV40-hk2-3ARE, 0.8 μg/well). The empty vectors pGL3 basic and pGL3-SV40 were used as controls. The phRL-TK vector (0.1 μg/well, Renilla luciferase, Promega) was co-transfected to normalize transfection efficiency. After transfection, cells were treated with indicated chemicals for an additional 24 h in 5% charcoal stripped serum medium. Cell lysates were prepared for luciferase assays (Dual-luciferase Reporter Assay System, Promega). At least three independent transfections were performed.

2.7. Western blot analysis

Cells, grown in 75 ml flasks under the same conditions described above, were treated with indicated chemicals for 24 h, and then washed with ice-cold PBS. Cell lysate preparation was as described previously [23,24]. Lysates containing 50 μg of total proteins were resolved by SDS-polyacrylamide gel and electro-transferred onto nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% non-fat milk in TBST buffer (20 mM Tris–HCl, 137 mM NaCl, and 0.1% Tween 20, pH 8.0) prior to incubation with specific antibodies to $p21^{WAF1/CIP1}$ (Santa Cruz Biotechnology), cyclin D1 (Santa Cruz Biotechnology), AR (BD Biosciences, 554225), Sp1 (Santa Cruz Biotechnology) or β-tubulin (Santa Cruz Biotechnology) overnight at 4 °C. After washing and incubating with horseradish peroxidase-conjugated IgG (Dako cytomation) at room temperature for 1 h, the membranes were washed and detected by enhanced chemiluminescence substrate (ECL, Pierce Biotechnology). For detection of Sp1 or AR protein levels produced by the Sp1- or AR-expressing plasmid in transfected cells, we transfected AR expression plasmid in PC-3 cells, and cotransfected pGL3 basic reporter or AR short promoter reporter construct with Sp1 expression plasmid in LNCaP cells grown in 50 ml flasks. After 24 h transfection, the cells were treated with chemicals as indicated for an additional 24 h, and then the cell lysates were prepared for Western blot assay. The Precision Plus Protein™ Dual color Standards was purchased from Bio-Rad laboratories. The MagicMark™ Western Standard marker was from Invitrogen Life Technologies.

2.8. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from LNCaP cells with or without pretreatment with AKBA for 24 h were prepared as described previously [25] and used for EMSA. Double stranded oligonucleotides corresponding to the specific binding sequence of Sp1 in the AR promoter as described before [23,24] were labeled with digoxigenin-11-dUTP (Dig) using the reagents provided in the DIG Gel Shift Kit (Roche, Germany) according to the manufacturer's instructions. Nuclear extracts (10 μg) were incubated with 64 fmol of labeled probe at room temperature for 30 min in binding reaction solution containing of 20 mM HEPES (pH7.6), 0.2% Tween 20, 1 mM EDTA, 1 mM dithiothreitol, 30 mM KCl, 1 μg poly(dI-dC), 0.1 μg poly(L-Lys). The specificity of binding was determined by competition with addition of a 125-fold molar excess of unlabeled specific Sp1 oligonucleotides or by incubation of 2 μg specific anti-Sp1 antibody (Santa Cruz Biotechnology Inc.) with the nuclear extracts for 30 min prior to in vitro DNA binding. The protein–DNA complexes were resolved on native pre-run 6% polyacrylamide gels in 0.5× TBE buffer at 150 V for 1–1.5 h. The gels were then electro-transferred to positive charged nylon membranes (Roche, Germany). The DNA complexes were cross-linked to the membranes at 120 °C for 30 min. Subsequently, electro-blotting and chemiluminescent detection were performed according to the protocol provided in the DIG Gel Shift Kit. In addition, a serial dilutions of dot spots were generated on a nylon membrane to test the efficiency of the labeling reaction.

2.9. Statistical analysis

The data are presented as the mean ± S.D. of several independent experiments. Statistical analysis was performed using 2-tailed Student's *t*-test for paired values, and statistical significance was considered when p was <0.05.

3. Results

3.1. Effect of AKBA on cell proliferation

We first examined the changes of LNCaP cell proliferation following treatment with AKBA which was purified by HPLC as shown in Fig. 1A. Typical kinetic growth, as measured by MTT assays, was monitored in cells exposed to AKBA at various concentrations in the presence or absence of androgen as shown in Fig. 1B. AKBA treatment resulted in cell growth decreases in the presence of androgen, cell viability was significantly reduced at 20, 30, or 40 μM with 58.92%, 79.26%, and 94.33% repression, respectively. Concentration response

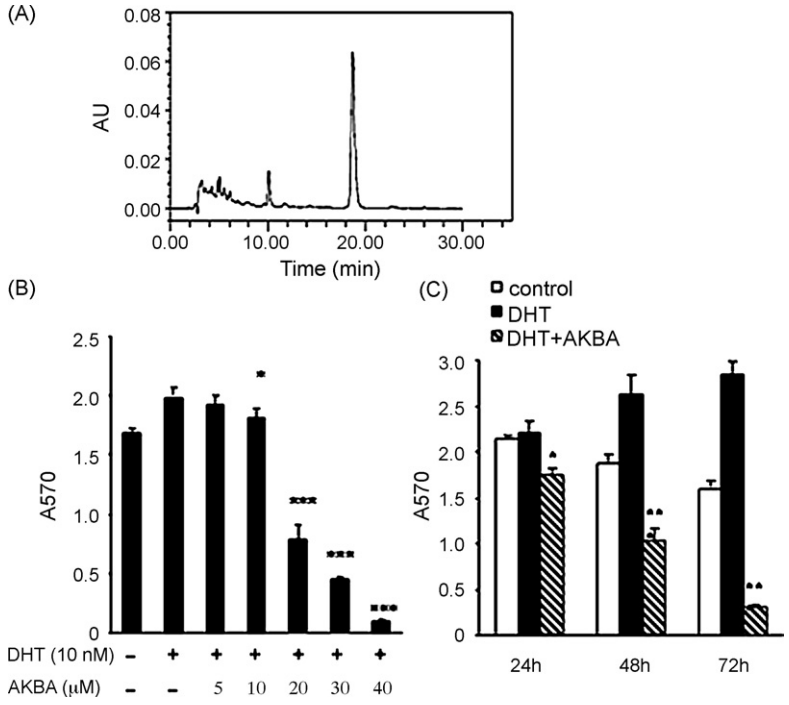

Fig. 1 – AKBA inhibits LNCaP Cell proliferation. (A) Purity of AKBA was determined by RP-HPLC. (B) Changes in cell viability after treatment with increasing concentrations of AKBA for 48 h. (C) Changes in cell viability in treatment with 30 μM AKBA for 24, 48, and 72 h. Cell viability was assayed by MTT. Control cells were treated with equal volumes of ethanol and DMSO supplemented in treated cells. Results are expressed as mean ± standard error (S.E.), and are representative of at least three separated experiments. Panel (A), $^{***}p < 0.001$ compared to the DHT treatment. Panel (B), $^{*}p < 0.05$, $^{***}p < 0.001$ compared to the DHT treatment.

data demonstrated 50% growth inhibition was estimated at 20.07 μM (IC_{50} = 20.07 μM). Therefore, AKBA at the concentration of 30 μM was used in the following experiments. Time course studies, as shown in Fig. 1C, revealed that inhibition of AKBA on cell growth in the presence of androgens became detectable (31.02% repression compared to that of DHT-treated) as early as 24 h after exposure to AKBA, and more apparently at 72 h (92.08% repression). These data indicated that cell proliferation was concentration- and time-dependently inhibited by AKBA.

3.2. *AKBA induced cell cycle arrest*

To determine the cell cycle phase after treatment with AKBA, we analyzed cell cycle by flow cytometry. Consistent with its effect on cell growth inhibition, AKBA induced significant G0/G1 arrest in LNCaP cells as shown Fig. 2A and B. AKBA treatment for 24 h resulted in accumulation of 76% of cells in G0/G1 phase compared to 61% in DHT-treated or 72% in untreated cells, respectively. The increase in G0/G1 cell population was accompanied by decrease in the number of S-phase cells. These results suggested that androgen-stimulated cell growth was dramatically quenched, and cells were arrested at the G0/G1 phase following AKBA treatment. The percentage of sub-G1 cells remained unchanged significantly in AKBA-treated cells, suggesting that AKBA did not trigger extensive apoptosis at the dose used.

Further analysis was conducted to assess the effect of AKBA on cell cycle regulatory molecules that play important roles in G1/S cell cycle progression. The results in Fig. 2C revealed that AKBA caused markedly increases in protein abundance of $p21^{WAF1/CIP1}$. In addition, the expression of cyclin D1 protein level was suppressed after AKBA treatment as shown in Fig. 2D. Taken together, the results suggested that cells were arrested at the G1 phase following AKBA treatment.

3.3. *AKBA decreased AR expression*

The importance of the AR in the proliferation and survival of PCa cells has been emphasized by many independent studies in vitro as well as in vivo [26,27]. Therefore, we wanted to determine whether growth inhibitory effect of AKBA was a result from reduction of AR expression. The first indication came from the expression changes of the AR protein level using Western blot analysis. As shown in Fig. 3A, DHT stimulation largely increased cellular AR protein level, while AKBA treatment, in the presence of 10 nM DHT, caused a significantly suppression in AR protein abundance. Meanwhile, we examined by real-time PCR whether the effect of AKBA on AR expression occurred at the transcription level. mRNA level was calculated in relation to the internal control GAPDH. The result in Fig. 3B showed that DHT-induced expression of the AR transcript was greatly reduced by a treatment with the AKBA at a 3.4-fold decrease compared with that treated with DHT alone.

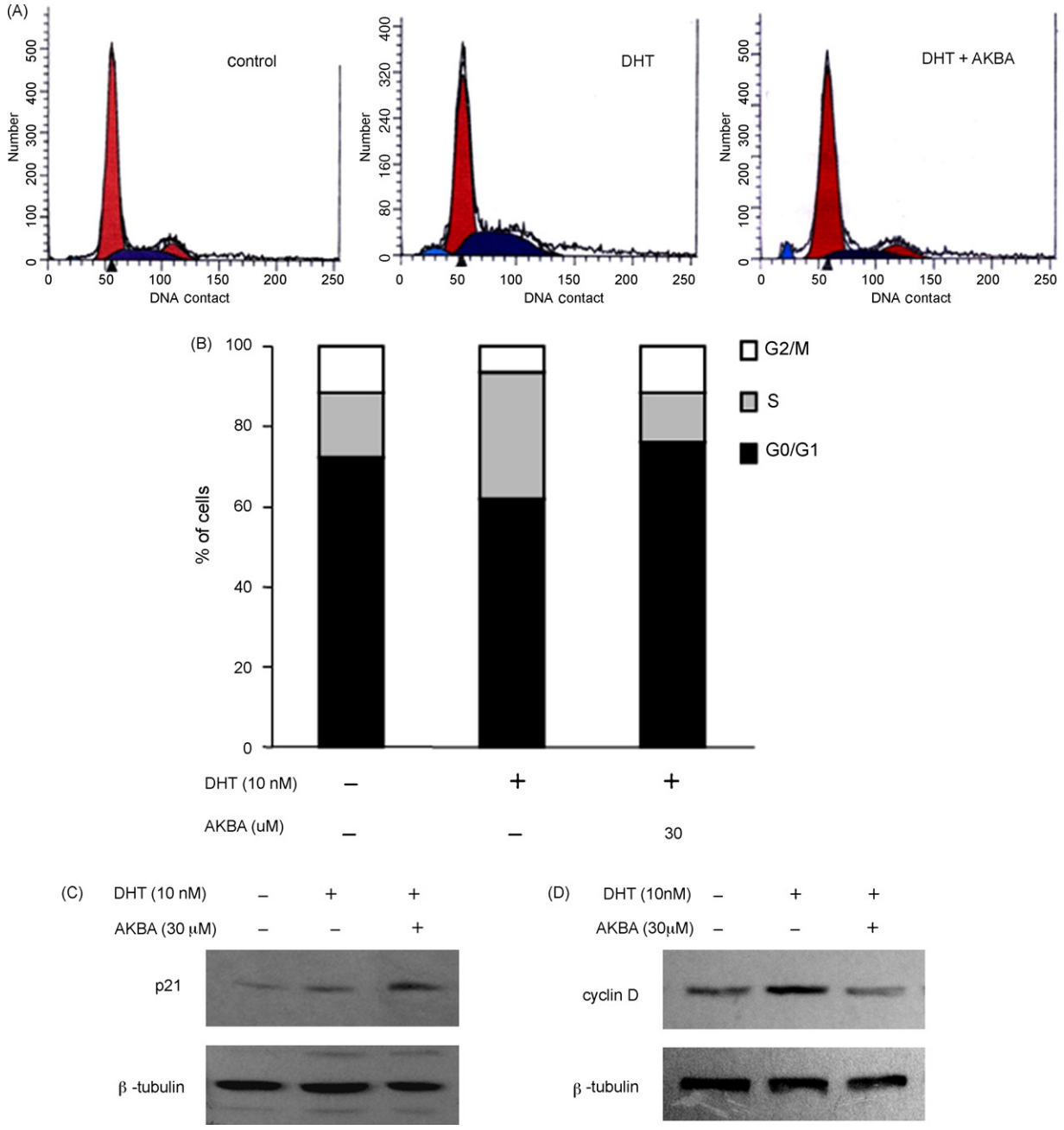

Fig. 2 – AKBA induce LNCaP cell cycle arrest. (A) Cell cycle analysis by flow cytometry. After exposure of LNCaP cells to AKBA for 48 h, cells were harvested, stained with propidium iodide and analyzed by flow cytometry. Flow cytometric histograms are representative of two separate experiments. (B) Percentage of cell populations. The data are from one of two independent experiments with similar results. (C) and (D), Western blot analysis of $p21^{WAF1/CIP1}$ and cyclin D1 protein levels in whole cell lysates from LNCaP cells treated with indicated chemicals. Beta-tubulin was served as an internal control to monitor protein loading and transferring efficiency.

To further confirm the observed effect of AKBA on AR expression, LNCaP cells were transiently transfected with a luciferase reporter driven by a 2-kb of the AR promoter. As expected, the AR promoter activity was up-regulated by DHT, and this stimulation was significantly inhibited by treatment with AKBA as seen in Fig. 3C, in an excellent agreement with the results in Fig. 3A and B. Taken together, these results indicated that the suppression effect of AKBA on AR protein level was largely mediated by reducing AR mRNA transcript abundance in the presence of androgen.

3.4. *AKBA inhibited AR transcriptional activity*

Prostate-specific antigen (PSA), a functional biomarker of androgen and AR signaling, is a gene tightly regulated by androgens through androgen response elements (AREs) to

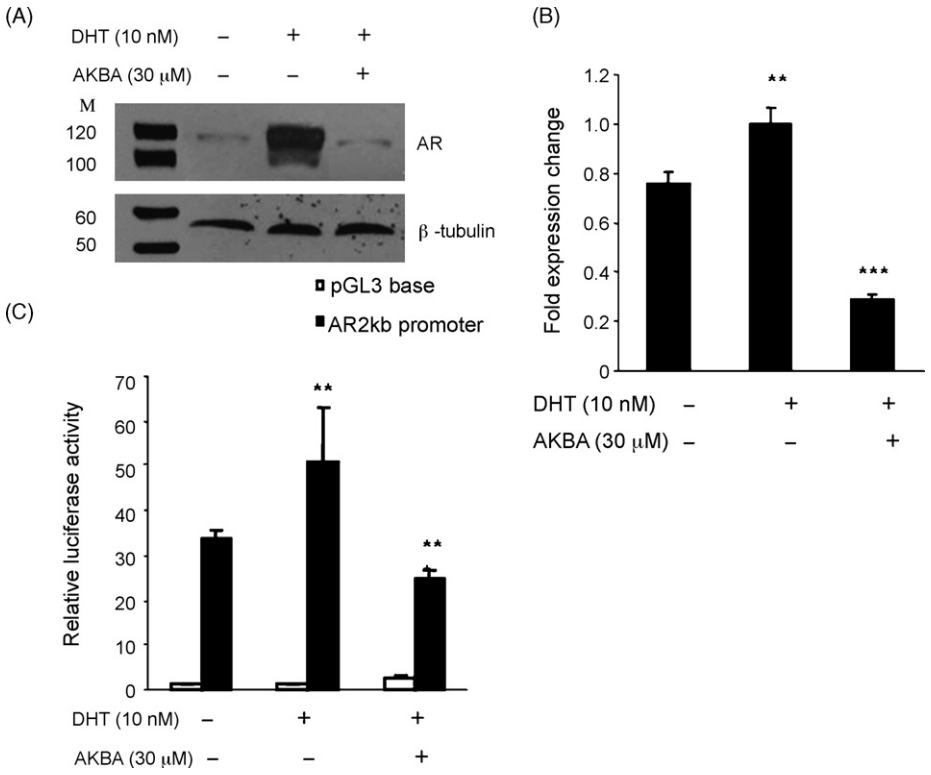

Fig. 3 – AKBA inhibit AR expressions in LNCaP cells. (A) Whole cell lysates from cells treated with control vehicles, DHT alone or DHT combined with AKBA were resolved on an 8% SDS-PAGE gel for Western blot analysis of AR protein levels. Beta-tubulin was served as an internal control. (B) Changes of AR mRNA expression were determined by quantitative PCR. RNA from AKBA-treated and control cells was subjected to real-time PCR analysis using primers specific for AR or GAPDH, respectively. Data are mean ± S.E. of three independent experiments, each performed in triplicate. $^{**}p < 0.01$ compared to the untreated. $^{***}p < 0.001$ compared to DHT treatment. (C) Effect of AKBA on AR promoter activity. LNCaP cells were cotransfected with AR 2 kb promoter-luciferase reporter (pGL3-AR2kb promoter) or the parental vector (pGL3 basic) and phRL-TK vector. After transfection, cells were exposed to AKBA or control vehicles for 24 h. Cell extracts were lysed for dual luciferase activity assays. The resulting luciferase activities were normalized to the activities of phRL-TK for the equal efficiency of the transfection. The data are expressed as mean ± S.E. from at least three separate experiments. $^{**}p < 0.01$ compared to the untreated. $^{***}p < 0.001$ compared to DHT treatment.

which the AR binds. To assess whether AKBA-mediated reduction in cellular AR protein level was accompanied by decreasing the transcriptional activity of the AR, co-transfection experiments were performed with a construct containing PSA promoter linked to a luciferase gene to examine the AR transactivity following AKBA treatment. As seen in Fig. 4A, DHT-stimulated induction of the reporter gene activity was detected, while a strong suppressive effect of AKBA was clearly observed on androgen induction of the PSA promoter. To further verify the inhibitory effect of AKBA on AR transcriptional function. We transfected the PSA promoter-luciferase reporter into PC-3 cells, lacking of AR expression. As expected in Fig. 4B, androgen dependent luciferase expression was dramatically inhibited in cells by addition of AKBA. It has been identified that the ARE sequence may play a role in determining promoter specificity, because AR binding affinity is response element-specific. We then used luciferase reporter containing three repeats of ARE in the hK2 gene to test whether DNA sequence dependent AR transcriptional activity could be affected in the presence of AKBA. Not surprisingly, the result in Fig. 4C revealed that the luciferase activity of hk2-3ARE plasmid, in the presence of DHT, was blunted with AKBA treatment. Furthermore, the result in Fig. 4D was shown that the levels of AR produced by the AR-expressing plasmid were detected in PC-3 cells, and were not affected greatly in the presence of AKBA. Therefore, AKBA treatment significantly inhibited androgen-stimulated AR transcriptional activity in addition to the suppression of AR expression.

3.5. *AKBA repressed Sp1-stimulated AR promoter activity by blocking of Sp1 DNA-binding activity*

Since Sp1 binding site is a major positive regulatory element in the AR promoter [28,29]. We were prompted to extend our investigation to determine the potential role of Sp1 in AKBA-mediated inhibitory effect on AR expression. The luciferase reporter driven by a minimal promoter region mainly containing Sp1 binding site in the AR gene was cotransfected into LNCaP cells with an expression plasmid encoding a full length Sp1 cDNA. Induction of AR short promoter-mediated luciferase activity was observed in cells cotransfected with Sp1 expression construct as shown in Fig. 5A. However, the Sp1-stimulated

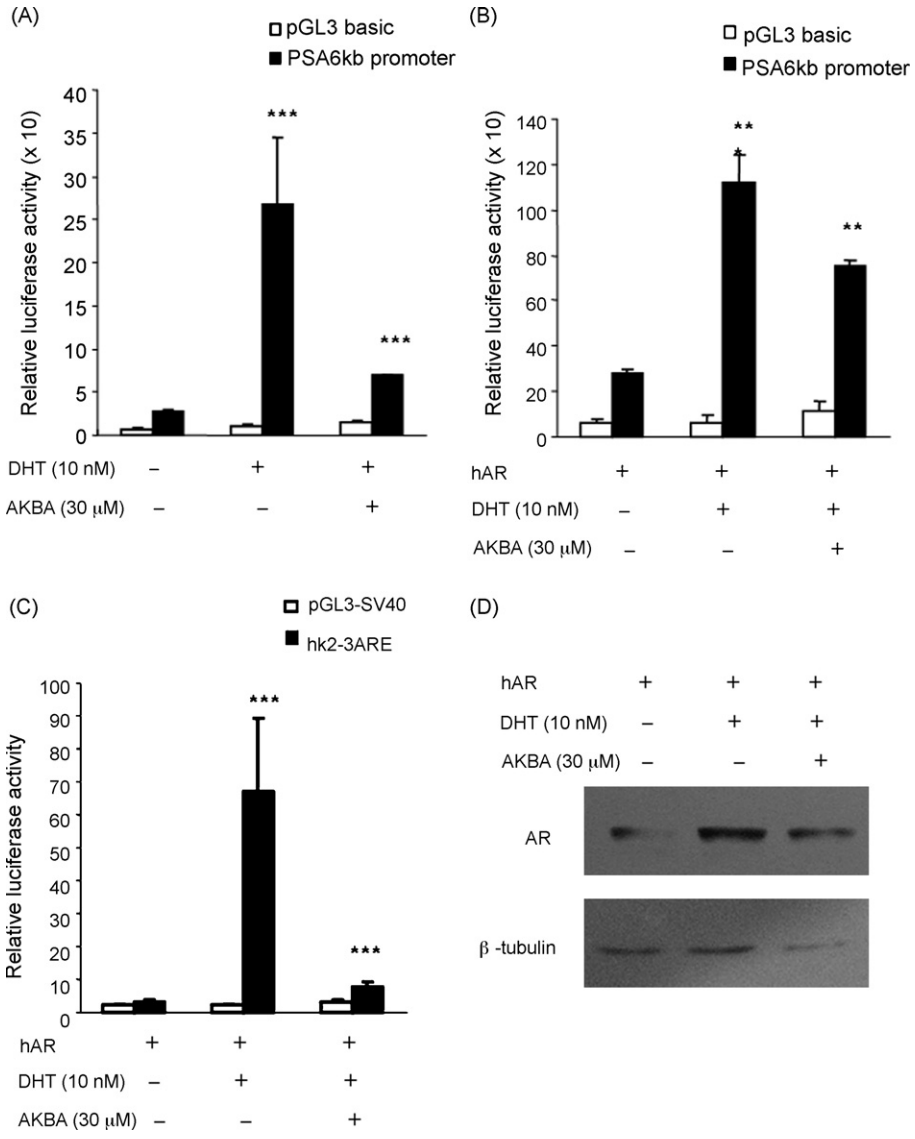

Fig. 4 – AKBA attenuate AR transcriptional activity in LNCaP cells. (A) Luciferase activities in LNCaP cells transfected with the pGL3-PSA6kb promoter luciferase reporter following treatment with chemicals for 24 h. $^{***}p < 0.001$, compared to group PSA6kb promoter without treatment, or group PSA6kb promoter + DHT and group PSA6kb promoter + DHT + AKBA. (B) Luciferase activities in PC3 cells co-transfected with the PSA6kb promoter reporter and a human AR expression construct following exposure to chemicals as indicated for 24 h. $^{***}p < 0.001$ compared to untreated control. $^{**}p < 0.01$ compared to DHT treatment. (C) Luciferase activities in PC3 cells co-transfected with the reporter containing of pGL3-SV-40 with three copies of ARE in hk2 gene (pGL3-SV40-hk2-3ARE) and a human AR expression construct following treatment with chemicals as indicated for 24 h. $^{***}p < 0.001$, compared to group hk2-3ARE without treatment, or group hk2-3ARE + DHT and hk2-3ARE + DHT + AKBA. The parental vectors pGL3 basic vector and pGL3-SV40 were included as controls. The phRL-TK, cotransfected in each transfection, was served as an internal control for normalization. The normalized, relative luciferase activities (mean ± S.E.) of at least three independent experiments were shown. (D) Western blot analysis of AR protein levels in whole cell lysates from PC-3 cells transfected with AR-expressing plasmid and then treated with indicated chemicals. Beta-tubulin was served as an internal control.

luciferase activity of the AR short promoter was markedly inhibited with AKBA treatment ($p < 0.001$). In addition, the Sp1 levels produced by Sp1 expression plasmid in transfected cells were shown in Fig. 5B, and the protein abundance was not markedly affected by AKBA. This result suggested that stimulatory function of Sp1 on AR promoter could be suppressed by AKBA in the presence of androgen.

To elucidate how the Sp1 could be affected in cells following AKBA treatment, and at least in part, responsible for downregulation of AR expression. We further determined Sp1 expression levels in cells exposed to AKBA. As shown in Fig. 5C, stimulation with DHT resulted in a slight increase in Sp1 expression compared to the vehicle control. But there was no significant change observed in the Sp1 protein level in

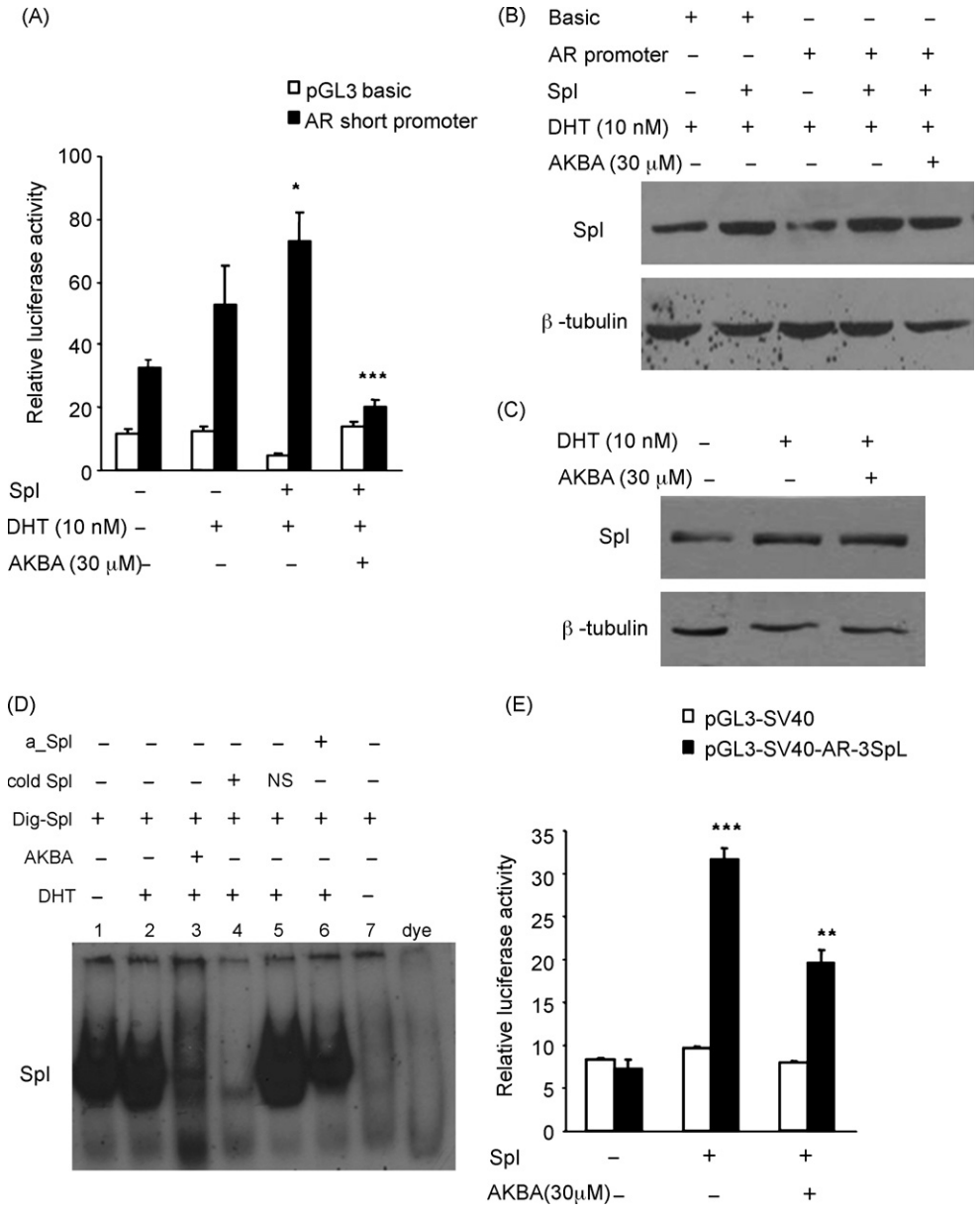

Fig. 5 – AKBA inhibits Sp1-mediated stimulation on AR promoter. (A) Inhibition of Sp1-mediated transactivation on AR short promoter by AKBA. LNCaP cells were co-transfected with the AR short promoter luciferase reporter and a human Sp1 expression construct. After transfection, cells were exposed to chemicals or remained untreated for 24 h. The parent vector pGL3 basic was included as a control. The phRL-TK was cotransfected into cells for normalization. $^{*}p < 0.05$ compared to the group without cotransfection of the Sp1 expression construct but exposure to DHT. $^{***}p < 0.001$ compared to the group cotransfection with the Sp1 expression construct, but without AKBA treatment. (B) and (C) Western blot analysis of Sp1 expression levels produced by Sp1-expressing plasmid in transfected cells or endogenous Sp1 protein abundance in LNCaP cells treated with indicated chemicals. Whole cell lysates were resolved on an 8% SDS-PAGE gel for analysis of Sp1 protein levels. Beta-tubulin was served as an internal control. (D) Effect of AKBA on Sp1 binding activity by EMSA assay. Nuclear extracts (10 μg/reaction), prepared from LNCaP cells treated with chemicals as indicated for 24 h, were incubated with Dig-labeled double-stranded Sp1 oligonucleotides for 30 min at room temperature. The Sp1-DNA complexes were resolved on a 6% non-denaturing polyacrylamide gel. Competition or Sp1 antibody treatment was carried out in the reaction with 125× unlabeled Sp1 oligonucleotides or 2 μg anti-Sp1 antibody, respectively. NS: nonspecific competitor. The experiments were repeated several times with essentially identical results. (E) LNCaP cells were co-transfected with luciferase reporter containing three copies of the Sp1 binding site of the AR promoter (pGL3-SV40-AR-3Sp1) and pcDNA-Sp1 expression plasmid. The parental vector (pGL3-SV40) served as a control. In each transfection, the phRL-TK was cotransfected in each transfection. The resulting luciferase activities were normalized to phRL-TK and expressed as relative activity ± standard error (S.E). At least three separated experiments were performed. $^{**}p < 0.01$, compared to the group transfected with Sp1 expression construct, but remained untreated. $^{***}p < 0.001$, compared to the group without transfection of Sp1 expression plasmid, and cells remained untreated.

AKBA treated cells compared to the DHT treated alone. Since Sp1 protein level was not affected by AKBA, it would be reasonable to consider if function of Sp1 may be blunted by AKBA. The specific binding sequence of Sp1 in the AR promoter was efficiently labeled with digoxigenin-11-dUTP (data not shown). The EMSA with nuclear extracts from cells exposed to AKBA, as shown in Fig. 5D, revealed that treatment with AKBA strongly inhibited binding of the extracts to the labeled Sp1 binding sequence (lane 3). The specificity of Sp1 binding was confirmed in competition experiments, a 125-fold molar excess of unlabeled Sp1, but not of non-specific oligonucleotides (AP-1 consensus sequence). The specific DNA–protein complexes were also confirmed by a significant reduction of the intensity of the band with addition of the Sp1 antibody (lane 6).

In an attempt to further demonstrate the inhibition observed above, we analyzed the effect of AKBA on the Sp1-mediated luciferase expression in cells cotransfected with a Sp1 expression construct and a luciferase reporter containing three repeats of Sp1 binding element of the AR gene. As expected in Fig. 5E, cotransfection of pGL3-SV40-AR-3Sp1-luciferase with the Sp1 expression plasmid resulted in a great increase in luciferase activity, which was suppressed by addition of AKBA. Together, these finding indicated that the AKBA inhibits specifically Sp1 activity by reducing Sp1-DNA binding ability.

4. Discussion

With the progression of PCa, the hormone refractory tumors, which usually arise after hormone ablation therapies, become resistant to conventional approaches including radiotherapy and chemotherapy [2]. Recently, taxane-related drugs have shown some efficacy for patients with hormone-refractory PCa. But significant side effects were observed in the treatment as seen commonly in most of anti-tumor chemicals [30]. AR inhibition is established for many chemicals including of naturally occurring compounds [23,24,31]. For development of effective strategies to prevent or treat PCa, discovery of novel, low toxic alternatives is still much desired.

Boswellic acid derivatives have already been known to posses potential anti-tumor properties via multiple mechanisms. In this study, we reported that AKBA were able to cause a concentration- and time-dependent inhibition of LNCaP cell growth (Fig. 1). Initiation of G1-arrest by AKBA was associated with up-regulation of $p21^{WAF1/CIP1}$ expression, and down-regulation of cellular cyclin D1 expression (Fig. 2A–D).

Interruption of the AR signaling by AKBA might result in inhibition of prostate cancer cell growth. The present studies clearly demonstrated by significant down-regulation of AR mRNA and protein expressions as well as suppression of AR-dependent PSA and hk2 expression with AKBA treatments (Figs. 3 and 4). Further analysis demonstrated that the Sp1-mediated stimulation in AR promoter activity was dramatically attenuated by AKBA treatment. However, the Sp1 protein maintained at basal expression levels and was not altered significantly in cells exposed to AKBA. We previously reported that association of AR and Sp1 was involved in quercetin-mediated inhibition on AR expression and transactivity [23]. Interestingly, quercetin treatment did not alter Sp1 DNA binding activity. In the case of AKBA, impaired Sp1 binding activity seemed to play a role in the regulation of AR expression.

Several independent studies have revealed that boswellic acid derivatives are able to induce growth arrest and apoptosis by interference with the NF-κB pathway [16,19]. From our data (not shown), AKBA treatment had no detectable inhibition in the translocation of p65 into the nucleus. In addition, the DNA-binding activity of NF-κB remained unaffected in the treatment with AKBA as well (data not shown). In contrast to androgen-independent PC-3, NF-κB activity in LNCaP cells was low [32,33]. Our data suggest that inhibition of NF-κB activity by AKBA may not be a major mechanism contributed for decrease of AR expression in LNCaP cells.

Collectively, in line with other reports, AKBA had the ability to induce cell growth arrest and reduce the AR expression and transcriptional activity in LNCaP cells. Inhibition of the Sp1 binding activity by AKBA appeared to be a mechanism for down-regulation of AR promoter activity. It suggested that AKBA might be a potential candidate to be developed for PCa.

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