

Incaspitolide A isolated from *Carpesium cernuum* L. inhibits the growth of prostate cancer cells and induces apoptosis via regulation of the PI3K/Akt/xIAP pathway

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Abstract. *Carpesium cernuum* L. is a traditional medicine primarily used in Southwestern China, and it has been shown to exhibit a range of biological properties, including anti-inflammatory and antitumor activities. Incaspitolide A (IA) is a sesquiterpene isolated from *C. cernuum* L. The aim of the present study was to investigate the antiproliferative effects of IA on PC-3 prostate cancer cells and determine the underlying mechanism. Results from a Cell Counting Kit-8 assay demonstrated that IA significantly reduced the numbers of viable PC-3 cells in a time and dose-dependent manner. Phase-contrast microscopy revealed that the number and morphology of cells were markedly altered. Hoechst and EdU staining assays showed that IA reduced the proliferation of PC-3 cells. Flow cytometry analysis revealed that IA arrested cell cycle progression at the S phase and promoted cell apoptosis in a dose-dependent manner. Western blot analysis demonstrated that treatment with IA resulted in downregulation of phosphorylated (p-) PI3K, p-Akt, X-linked inhibitor of apoptosis (xIAP), CKD2, cyclin A2 and pro-Caspase-3 protein expression, and upregulation of cleaved poly(ADP-ribose) polymerase and P53 expression. The present results suggested that IA inhibited the growth of PC-3 cells and induced apoptosis. The underlying mechanism appeared to involve the inhibition of the PI3K/Akt/xIAP pathway. The present study indicated that IA may serve as a therapeutic for

the management of prostate cancer and provided a theoretical basis for the pathogenesis of prostate cancer.

Introduction

According to the 2019 Cancer Statistics Report from the United States, prostate cancer ranks highest with regard to new cases, and second highest in terms of cancer-associated deaths amongst men (1). Prostate cancer is a very common type of cancer, which threatens the health and life of men. Additionally, the metastatic potential of prostate cancer, and the risk of development of hormone-resistant recurrence is high (2,3). The prevalence of prostate cancer increases with age, and differs significantly with region, being higher in Europe and America than in Asia. The mortality and morbidity rates of patients with prostate cancer in Asian countries, including China, have been increasing in recent years, but remain lower than that of Western countries (4-6). The risk factors for prostate cancer have not been fully clarified, but may include age, ethnicity, family history, genetics, obesity and levels of sex hormones (7). Currently, the first-line chemotherapeutic drugs used to treat prostate cancer are associated with serious side effects and, the cancer may develop resistance when used long-term, particularly when they are administered during the early stages of the disease. The use of these drugs is thus restricted to specific cases (8). Therefore, there is an urgent need to identify novel agents to prevent and treat prostate cancer. Candidate drugs should ideally be able to reduce proliferation, migration and invasion of these highly metastatic tumors.

Natural compounds often exhibit inhibitory effects on a variety of cellular signaling pathways and are thus investigated to identify their bioactive molecules for use as potential anti-cancer drugs (9). Chinese herbal medicines are an important resource for discovery of novel compounds which may assist in treating a range of diseases and have formed an important part of the Chinese health care system for several millennia.

A medicinal herb known as 'Yan guan tou cao' belongs to the *Carpesium cernuum* L. family and is widely distributed in the southern regions of China (10). It consists of an abundance of chemical constituents and has been shown to possess several biological effects, including

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antitumor (11-13), anti-inflammatory (14,15), analgesic and detoxifying effects (16). It primarily consists of sesquiterpenoid lactones (17,18), sterols and aromatic compounds (19), and glycosides (20). It has been reported that the sesquiterpenoid lactones derived from *C. cernuum* L. are effective cytotoxic (16) and antitumor agents (21) IA (Fig. 1A), a known sesquiterpenoid (22), isolated from *C. cernuum* L., can inhibit the growth of A549, BGC-823, MCF-7 and other cancer cell lines (23). However, to the best of our knowledge, the mechanisms by which IA exerts its effects on cancer cells have not been determined. Therefore, the aim of the present study was to determine the antitumor effects of IA in prostate cancer cells, and explore the potential underlying molecular mechanism, by assessing the expression of key signaling proteins associated with the major signal transduction pathways.

Material and methods

Materials. The entire plant of *C. cernuum* L. was collected from Zhenning, Guizhou Province, China, and identified by Professor Qing-wen Sun (Guiyang College of Traditional Chinese Medicine). Primary extraction, isolation and purification of IA was performed as described previously (24). IA was obtained (32.6 mg) from fraction-5 (82.0 g) (Fr. 5). Based on the physical and spectral data, it was identified as IA (25,26). The purity of IA was analyzed by high performance liquid chromatography (HPLC; Fig. S1 and Table SI). IA was dissolved in chromatographic methanol to obtain a concentration of 1.00 mg/ml. Chromatographic methanol alone was used as the control. HPLC (LC-20AD; Shimadzu Corporation) was performed on a Waters SunFire C18 column (150x4.6 mm, 5 μ m) with mobile phase consisted of methanol:water (65:35, V/V) at the flow rate of 1.0 ml/min at 210 nm and 23°C, and the injection volume was 10 μ l.

Reagents. The primary reagents and kits used were the same as those described previously (27). Antibodies against phosphorylated (p-) PI3K (rabbit; cat. no. 3242), PI3K (rabbit; cat. no. 13666), p-Akt (rabbit; cat. no. 4060), Akt (rabbit; cat. no. 4691), cleaved-poly(ADP-ribose) polymerase (PARP; rabbit; cat. no. 5625), PARP (rabbit; cat. no. 9532), X-linked inhibitor of apoptosis (xIAP; rabbit; cat. no. 14334), CDK2 (rabbit; cat. no. 10122), P53 (rabbit; cat. no. 10442), cyclin A2 (CCNA2; rabbit; cat. no. 18202), Caspase-3 (rabbit; cat. no. 9662) and GAPDH (rabbit; cat. no. 5174) were purchased from Cell Signaling Technology, Inc. IA was dissolved in DMSO and stored at a constant temperature of 4°C.

Cell culture. PC-3 cells were purchased from the American Type Culture Collection (CRL-1435) and were immediately warmed to 37°C and cultured in RPMI-1640 (Beijing Solarbio Science & Technology Co., Ltd.) medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific Inc.), 100 μ g/ml penicillin and 100 μ g/ml streptomycin in a humidified incubator at 37°C with 5% CO₂.

Cell counting kit-8 (CCK-8) assays. CCK-8 assays were used to analyze the proliferation of PC-3 cells. PC-3 cells were cultured in 96-well plates with 150 μ l RPMI-1640 for 24 h at 37°C. Control group cells were untreated (0 μ M IA), whereas

the experimental group cells were treated with 5, 10, 20, 40 and 80 μ M IA for 24, 48 and 72 h, at 37°C. The final concentration of DMSO in the cells was <1% in all groups. Subsequently, 10 μ l CCK-8 solution was added, and cells were cultured for a further 3 h, at which point 150 μ l DMSO was added. A microplate reader was used to measure the absorbance at 450 nm and the IC₅₀ values were calculated using GraphPad Prism version 5.0 (GraphPad Software, Inc.). Experiments were repeated three times.

EdU assay. EdU analysis was performed using the BeyoClick™ Edu-488 cell proliferation test kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions to observe individual proliferating cells. PC-3 cells (5x10⁵/well-5x10⁶/well) were cultured in 6-well plates with 1 ml RPMI-1640 for 12 h at 37°C and then the cells were treated with 0, 10, 20 and 40 μ M IA for 24 h at 37°C. Fluorescence microscopy (magnification, x100) was used to visualize the EdU positive cells. EdU analysis was performed using ImageJ software v.1.50 (National Institutes of Health).

Phase-contrast microscopy. The morphological changes associated with apoptosis of PC-3 cells were observed in cells following the aforementioned treatments using phase-contrast light microscopy.

Apoptosis assay. Apoptosis was evaluated by fluorescence microscopy and by flow cytometry. The methods of culture, administration and treatment were the same as that described for the cell cycle distribution analysis. For the microscopy analysis, treated cells were washed twice with cold PBS, after which the cells were stained with Hoechst 33342 at 4°C for 20 min, and a fluorescence microscope was used to observe the changes in nuclear morphology. For the flow cytometry analysis, the cells were centrifuged at 1,000 x g/min at 4°C for 5 min, resuspended in 100 μ l buffer, stained with an Annexin V-FITC/PI double staining apoptosis kit (Dojindo Molecular Technologies, Inc.) at room temperature for 10 min in the dark and finally resuspended in 400 μ l buffer. Cell apoptosis was analyzed using a flow cytometer and the data obtained were analyzed using CytExpert version X (Beckman Coulter, Inc.).

Cell cycle assay. PC-3 cells were cultured in 6-well plates and treated with different concentrations of IA (0, 10, 20 and 40 μ M) for 24 h. The cells were digested and collected, washed with cold PBS, fixed using 70% cold ethanol at 4°C for 24 h. The cells were then washed twice with cold PBS and treated with RNase. After staining with PI, flow cytometry analysis was used to determine the cell cycle phase distribution, and the data obtained were analyzed using ModFit software (BD Biosciences).

Western blotting. Treated cells were lysed using lysis buffer (0.01% (v/v) Triton X-100, 20 mM Tris and 150 mM NaCl). Cells were scraped from the plates, and the lysates were centrifuged at 1.2x10⁴ x g at 4°C for 5 min, to obtain the total protein. A BCA protein assay was used to determine the protein concentration, and 10% SDS-PAGE was used to resolve protein samples (50 μ g). The proteins were transferred to PVDF membranes, after which blocking, and incubation

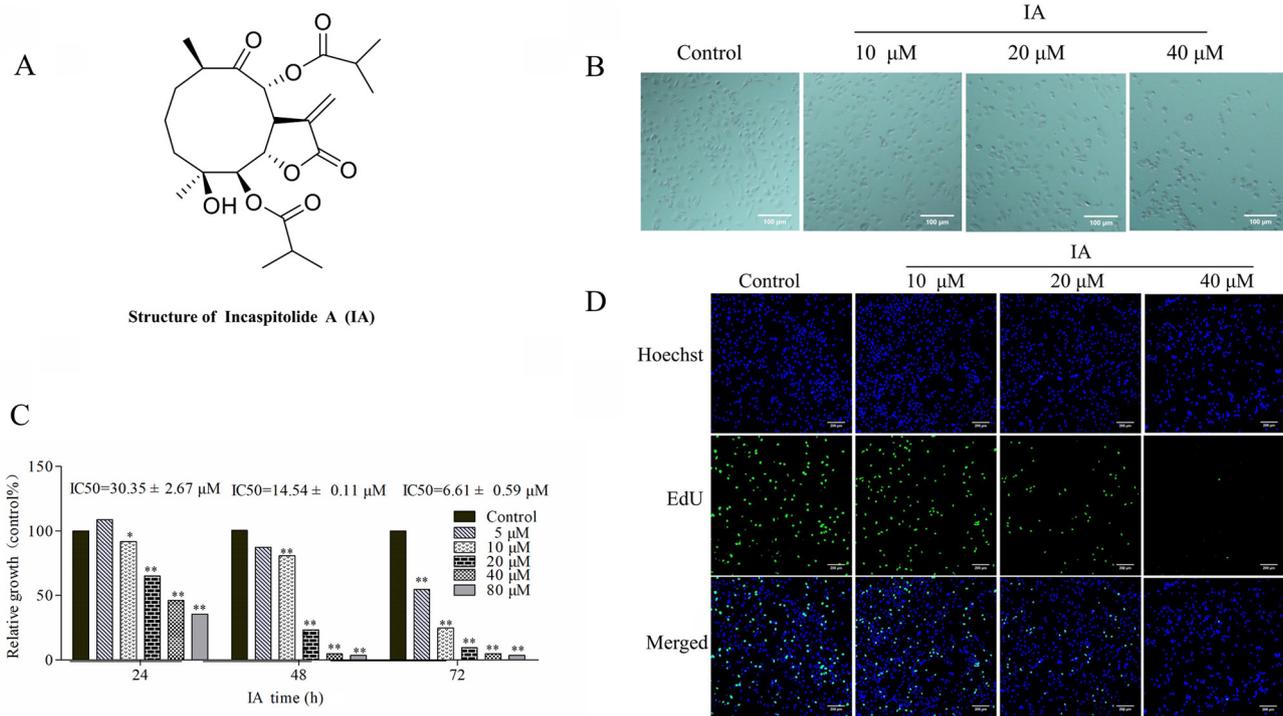


Figure 1. Effect of IA on the morphology and proliferation of PC-3 cells. (A) Structure of IA isolated from *Carpesium cernuum* L.. (B) Morphology of PC-3 cells following treatment with IA for 24 h. Phase-contrast images were captured using an inverted light microscope, from three independent experiments. Scale bar, 100 μm; magnification, x200. (C) Cytotoxic effects of IA on PC-3 cells. PC-3 cells were treated with 5-80 μM IA for 24, 48 or 72 h. A Cell Counting Kit-8 assay was used to measure the numbers of viable cells, and data are presented as a percentage of treated cells to untreated cells. (D) PC-3 cells were treated with 0, 10, 20 or 40 μM IA for 24 h, and then proliferation was evaluated by an EdU staining assay. Scale bar, 200 μm; magnification, x100. Data are presented as the mean ± standard deviation of three repeats. *P<0.05, **P<0.01 vs. control group. IA, incasitolide A.

with the primary and secondary antibodies was performed as described by Mao *et al* (28). The following primary and secondary antibodies were used: p-PI3K (1:1,000), PI3K (1:1,000), p-Akt (1:1,000), Akt (1:1,000), cleaved-PARP (1:1,000), PARP (1:1,000), xIAP (1:1,000), CDK2 (1:1,000), P53 (1:1,000), CCNA2 (1:1,000), Caspase-3 (1:1,000), GAPDH (1:1,000) and goat anti-rabbit antibody (1:1,000). Densitometry analysis was performed using ImageJ version 1.50 (National Institutes of Health).

Statistical analysis. Data are presented as the mean ± standard deviation. Multiple comparisons were performed using a one-way ANOVA with the Dunnett's post hoc test. A Student's t-test was used to analyze differences between two groups. P<0.05 was considered to indicate a statistically significant difference. Data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, Inc.).

Results

Effect of IA on the morphology and proliferation of PC-3 cells.

As evident from its chemical structure, IA is a sesquiterpenes lactone (Fig. 1A). In the present study, IA was isolated from *C. cernuum* L. with a purity >98% (Fig. S1 and Table S1). After being treated with IA for 24 h, the morphological changes of PC-3 cells were observed using an inverted light microscope, and images were captured from the three independent experiments. The majority of the cells exhibited an irregular shape, and were smaller in size when treated with 10, 20 or 40 μM IA for 24 h

compared with the control untreated PC-3 cells (Fig. 1B). Based on the images (Fig. 1B), it was hypothesized that survival was decreased, partly as a result of detachment and loss of adhesion to the surface, thus losing the effects of adhesion. As treatment concentration increased, the cells eventually lost adhesion to the surface and floated freely (Fig. 1B).

PC-3 cells were treated with IA (5-80 μM) for 24, 48 or 72 h, and proliferation was examined using a CCK-8 assay. The IC₅₀ values of IA were 30.35±2.67, 14.54±0.11 and 6.61±0.59 μM for 24, 48 and 72 h in PC-3 cells (Fig. 1C). Together, these results suggest that the effects of IA on PC-3 cells were time and dose-dependent. In subsequent experiments, cells were treated with IA for 24 h. To confirm the inhibitory effect of IA on cell proliferation, EdU staining and Hoechst staining were used. The cells were treated with 0, 10, 20 or 40 μM. The results indicated that IA treatment for 24 h markedly inhibited the number of actively proliferating PC-3 cells compared with the untreated control; the numbers of green fluorescent cells labeled with EdU were markedly decreased following IA administration (Fig. 1D).

Effects of IA on apoptosis of PC-3 cells. Hoechst-stained cells were analyzed using fluorescence microscopy to assess the nuclear changes and the formation of apoptotic bodies in the cells treated with IA for 24 h. Compared with the control group, the different concentrations of 10, 20 or 40 μM IA treatment group exhibited a decreasing nuclei trend (Fig. 2A).

Apoptosis was further evaluated by annexin V/PI double staining and flow cytometry. PC-3 cells were either untreated

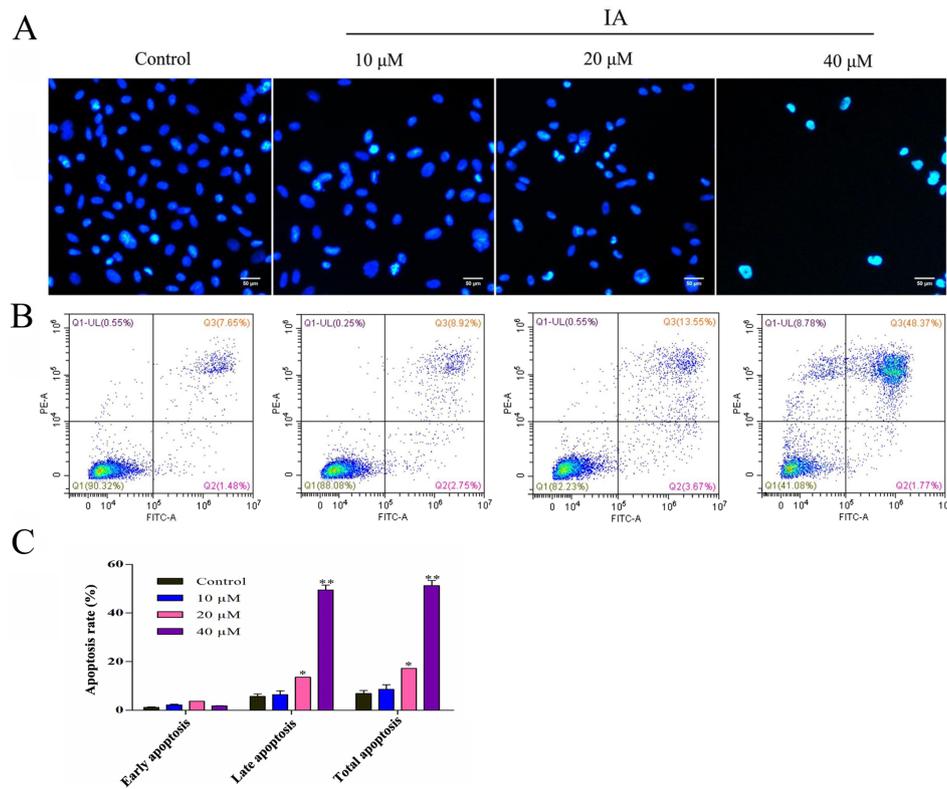


Figure 2. Effects of IA on apoptosis of PC-3 cells. (A) PC-3 cells were treated with IA for 24 h, and then fixed and stained with Hoechst. Changes in the morphology of the nuclei as well as the formation of apoptotic bodies were observed by fluorescence microscopy. Scale bar, 50 μ m; magnification, x400. (B and C) PC-3 cells were treated with 0, 10, 20 or 40 μ M IA for 24 h, and then double stained with Annexin V/PI. Flow cytometry analysis was used to detect and analyze cell apoptosis in each group. Data are presented as the mean \pm standard deviation of three repeats. * P <0.05, ** P <0.01 vs. control group. IA, incaspitolide A.

(control) or treated with 10, 20 or 40 μ M IA for 24 h. The apoptotic rate of PC-3 cells increased significantly following IA treatment in a dose-dependent manner (Fig. 2B and C). The apoptotic rates of PC-3 cells treated with 0, 10, 20 or 40 μ M IA were 9.13, 11.67, 17.22 and 50.14%, respectively. These results indicated that IA promoted cell apoptosis in a dose-dependent manner.

Effects of IA on cell cycle progression in PC-3 cells. The effect of IA on PC-3 cell cycle phase distribution was detected by flow cytometry. Compared with the control group, the cells treated with different concentrations of IA exhibited a decrease in the percentage of G₁/G₀ phase cells and an increase in the proportion of cells in the S phase (Fig. 3A and B). Following treatment with 0, 10, 20 or 40 μ M IA for 24 h, the percentage of PC-3 cells in the S phase was 9.55 \pm 0.58, 9.12 \pm 1.06, 18.92 \pm 0.16, 21.18 \pm 1.05%, respectively. In addition, the protein expression levels of cell cycle-related proteins were examined by western blotting. As the concentration of IA was increased, the protein expression levels of CDK2 and CCNA2 were significantly decreased, whereas the protein expression levels of P53 were significantly increased (Fig. 4A and H-J). These results suggested that the cell cycle was arrested at the S phase, and the effects of IA were dose-dependent.

Effects of IA on the PI3K/Akt/xIAP signaling pathway in PC-3 cells. PC-3 cells were treated with 0, 10, 20 or 40 μ M IA for 24 h. Western blot analysis revealed that IA treatment resulted

in significant changes in the expression levels of key proteins associated with the PI3K/Akt/xIAP signaling pathway. IA treatment decreased the protein expression levels of p-PI3K, p-Akt, pro-caspase-3 and xIAP significantly compared with the control cells, whereas the levels of cleaved-PARP significantly increased, all in a dose-dependent manner (Fig. 4A-G). Thus, it was hypothesized that IA may induce apoptosis of PC-3 cells by inhibiting the activity of the PI3K/Akt/xIAP signaling pathway.

Discussion

Prostate cancer is one of the primary types of cancer threatening the health and life of men. There is an urgent need to identify novel therapeutic agents to treat prostate cancer, and a well-established source of potential compounds are natural products. Recently, there has been increased interest in the anticancer activity of various sesquiterpenoid lactones.

The results of the present study demonstrated that the survival rates of PC-3 cells decreased following IA treatment, in a dose and time-dependent manner. The proliferation of PC-3 cells was also reduced by IA treatment in a time-dependent manner. Thus, for subsequent experiments, the PC-3 cells were treated with 0, 10, 20 or 40 μ M IA for 24 h. After 24-h treatment, the total number of PC-3 cells was decreased, as well as active proliferation, both in a dose-dependent manner.

The pathogenesis of cancer has several aspects, which are closely associated with cell cycle arrest and inhibition

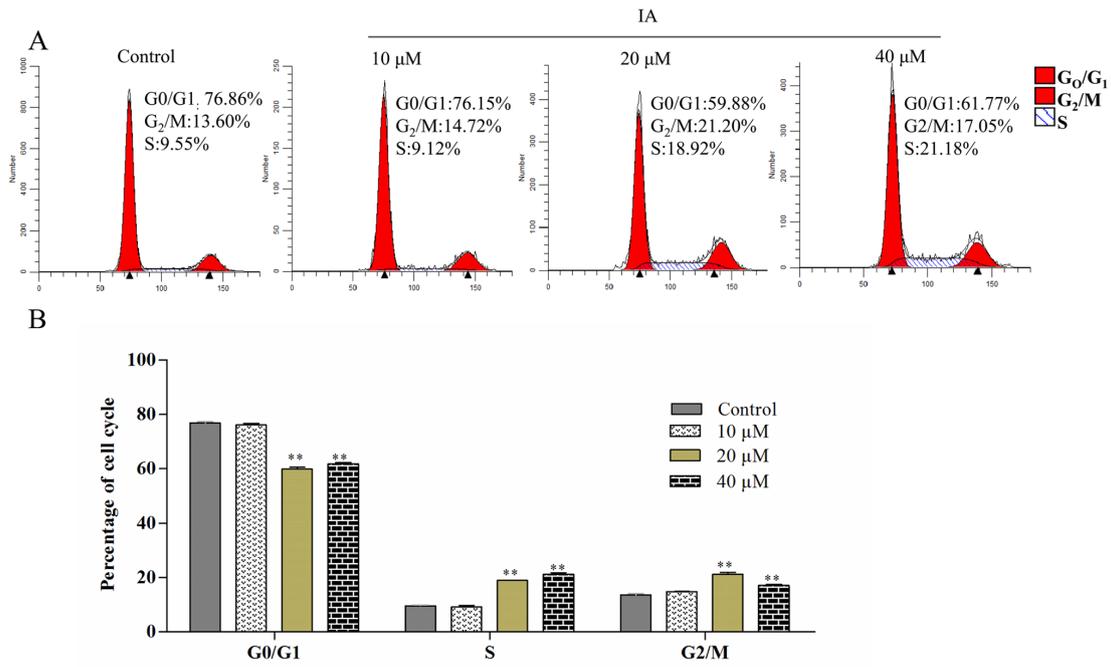


Figure 3. Effect of IA on cell cycle phase distribution of PC-3 cells. Cells were treated with 0, 10, 20 or 40 μM IA for 24 h, and then stained with PI. DNA content was measured using flow cytometry. (A) Representative plots. (B) The percentage of PC-3 cells at different stages of the cell cycle is shown in the bar graph. Data are presented as the mean ± standard deviation of three repeats. **P<0.01 vs. control group. IA, incaspitolide A.

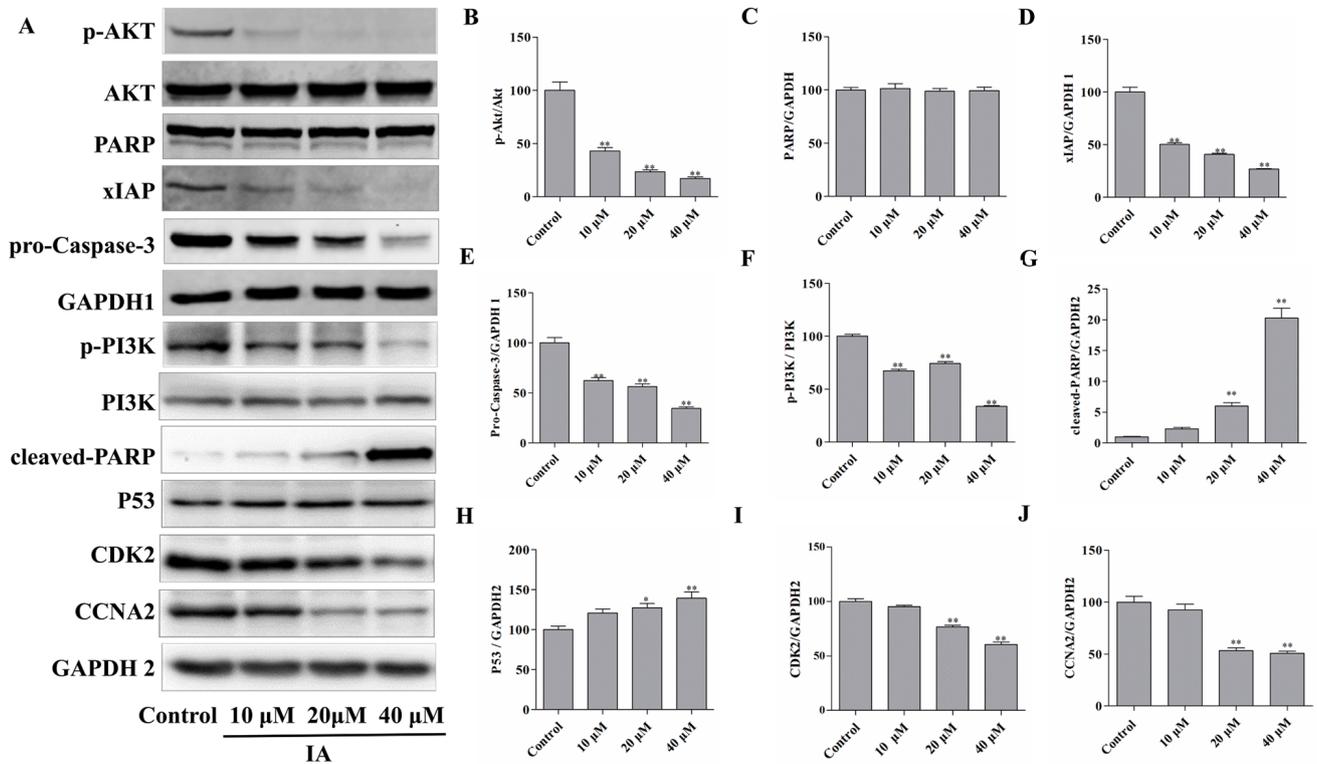


Figure 4. Effects of IA on the PI3K/Akt/xIAP signaling pathway in PC-3 cells. PC-3 cells were treated with 0, 10, 20 or 40 μM IA for 24 h, and total protein was extracted and analyzed by western blotting. (A) Representative images from the western blot analysis. (B-J) Quantitative analysis of the protein expression was performed using ImageJ. GAPDH was used as a loading control. Data are presented as the mean ± standard deviation of three repeats. *P<0.05, **P<0.01 vs. control group. IA, incaspitolide A; p-, phosphorylated; PARP, poly (ADP-ribose) polymerase; xIAP, X-linked inhibitor of apoptosis; CCNA2, cyclin A2.

of apoptosis. The results of the present study revealed that, in cells treated with IA, the cell cycle was arrested in the S phase and apoptosis was induced based on the appearance

of apoptotic bodies in the nuclei. The apoptotic rate increased in a dose-dependent manner. Apoptosis is a crucial physiological process and is required for normal development

and maintenance of tissue homeostasis. Additionally, downregulation of apoptosis has been extensively shown to be crucially involved in the development of cancer (29). The results of the Hoechst staining analyses demonstrated that IA induced apoptosis in PC-3 cells, in a dose-dependent manner. The sensitivity of prostate cancer cells to IA, and the extent of apoptosis was confirmed by flow cytometry. It was shown that exposure to 40 μ M IA resulted in 48.37% of cells being in a late apoptotic or necrotic state, higher than the 1.77% of early apoptotic cells, suggesting that IA treatment accelerated cell death.

Several signal transduction pathways can regulate the same physiological process, and thus a high degree of coordination in cell growth and programmed cell death pathways is required (30,31). Apoptosis signaling pathways have been shown to be promising targets for the development of novel anticancer drugs (32). It is well-established that the PI3K/Akt pathway is a fundamental intracellular signaling pathway involved in cell cycle regulation (33), and there is a direct relationship between cellular quiescence, proliferation and cancer cell viability (34). Activation of PI3K promotes the conversion of diphosphoinositide into triphosphoinositide, resulting in activation of Akt and xIAP (35). The inhibitory effects of IA on the PI3K/Akt/xIAP pathway were examined in PC-3 cells, and the expression levels of proteins associated with this pathway were measured in cells treated with different concentrations of IA. Firstly, IA treatment was demonstrated to inhibit the PI3K/Akt/xIAP signaling pathway. In addition, P53 expression was significantly upregulated, while CDK2 and CCNA2 were downregulated following IA treatment, consistent with the cell cycle arrest and cell proliferation inhibition phenotype obtained from the functional assays. Furthermore, the downregulation of xIAP and the upregulation of cleaved-PARP were consistent with the increased apoptosis observed by flow cytometry. AKT is a key regulator of cell growth and survival, which is essential for cancer growth (36). xIAP, a known caspase inhibitor, can inhibit apoptosis induced by different stimuli (37). Apoptosis of cancer cells can be induced by downregulation of p-Akt and xIAP (38,39).

The results of the present study demonstrated that IA could inhibit the antiapoptotic mechanism of the cells through the regulation of expression of various proteins, and the results were consistent with previous studies (35,40,41) of the PI3K/Akt/xIAP pathway. The present results highlight a potential candidate compound for the management of prostate cancer and provide a theoretical basis for the pathogenesis of prostate cancer.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MC conceived and designed the study. YH performed the majority of the experiments and wrote the manuscript. CY and HG performed experiments. JM and LZ analyzed the data. JM and CY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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