RESEARCH ARTICLE

Vitexicarpin Induces Apoptosis in Human Prostate Carcinoma PC-3 Cells through G2/M Phase Arrest

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Abstract

Vitexicarpin (3’, 5-dihydroxy-3’, 4’, 6, 7-tetramethoxy flavone), a polymethoxy flavone isolated from Vitis Fructus (Vitis rotundifolia Linne fil.), has long been used as an anti-inflammatory herb in traditional Chinese medicine. It has also been reported that vitexicarpin can inhibit the growth of various cancer cells. However, there is no report elucidating its effect on human prostate carcinoma cells. The aim of the present study was to examine the apoptotic induction activity of vitexicarpin on PC-3 cells and molecular mechanisms involved. MTT studies showed that vitexicarpin dose-dependently inhibited growth of PC-3 cells with an IC₅₀ ~ 28.8 μM. Hoechst 33258 staining further revealed that vitexicarpin induced apoptotic cell death. The effect of vitexicarpin on PC-3 cells apoptosis was tested using propidium iodide (PI)/Annexin V-FITC double staining and flow cytometry. The results indicated that vitexicarpin induction of apoptotic cell death in PC-3 cells was accompanied by cell cycle arrest in the G2/M phase. Furthermore, our study demonstrated that vitexicarpin induction of PC-3 cell apoptosis was associated with upregulation of the proapoptotic protein Bax, and downregulation of antiapoptotic protein Bcl-2, release of Cytochrome c from mitochondria and decrease in mitochondrial membrane potential. Our findings suggested that vitexicarpin may become a potential leading drug in the therapy of prostate carcinoma.

Keywords: Vitexicarpin - PC-3 cells - anticancer - apoptosis - G2/M phase arrest

Introduction

Prostate cancer is the second most common diagnosed cancer after lung cancer worldwide, and it is the third most common cause of cancer deaths in developed countries. In 2011, 240,890 men were diagnosed with prostate cancer and 33,720 men died of it as estimated by The American Cancer Society 2012. Prostate cancer incidence and mortality is comparatively higher in developed countries. Treatment of prostate cancer depends on age of patients, overall health of individual, and the stage of disease. Current available therapies include active surveillance, surgery, radiation therapy, hormone therapy, chemotherapy, and immunotherapy. However, because prostate cancer can recur in an androgen-insensitive or hormone-refractory form that is not responsive to current therapies, the mortality rate associated with recurrent cases is high (American Cancer Society, 2012). There is great demand for effective novel therapeutic agents.

Vitexicarpin (3’, 5-dihydroxy-3’, 4’, 6, 7-tetramethoxy flavone), a polymethoxy flavone isolated from Vitis Fructus (Vitis rotundifolia Linne fil.) has long been used as an anti-inflammatory herb in traditional Chinese medicine (Choudhary et al., 2009; Masaik et al., 2009). A number of recent pharmacological reports indicated that vitexicarpin induces growth inhibition, cell cycle arrest, and apoptosis in various cancer cells including the human cervical cancer cell line Hela (Chen et al., 2011; Zeng et al., 2012), hepatocellular carcinoma cell line HepG2 (Yang et al., 2011; He et al., 2012), lung epithelial cell line A549 (Koh et al., 2011), and leukemic cell line K562 (Shen et al., 2009). Molecular mechanism studies showed that vitexicarpin induces apoptosis via activation of c-Jun N-terminal kinase (JNK) in Hela cells (Zeng et al., 2012), inactivation NF-κB in A549 cells (Koh et al., 2011), and leukemic cell line K562 (Shen et al., 2009). So far, there is no information available on its effect on human prostate carcinoma cells. In the present study, we investigated effect of vitexicarpin on apoptosis in human prostate cancer cell line PC-3 using cytotoxicity assay, cell cycle analysis, and western blotting method.

Materials and Methods

Chemicals and reagents

Vitexicarpin was purchased from the National Institutes
for Food and Drug Control (Beijing, China). Characterized grade fetal bovine serum (FBS) was purchased from HyClone (USA). Dimethy sulfoxide (DMSO) was purchased from Sangon Biontech (Shanghai, China) Co., Ltd. Dulbecco’s Modified Eagle’s Medium (DMEM), [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (MTT), trypsin, Hoechst 33258, Rhodamine 123, penicillin and streptomycin were purchased from Sigma (Beijing, China). Annexin V-FITC Apoptosis Detection Kit, Cell Cycle and Apoptosis Analysis Kit, Reactive Oxygen Species Assay Kit and BCA Protein Assay kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit antihuman Caspase-3, Bax, Cyclin B1, Cytochrome c, mouse antihuman Bcl-2, β-actin, cyclin-dependent kinase (CDK1) primary antibodies were purchased from Cell Signaling (China). Horseradish peroxidase-conjugated secondary antibodies (Anti-mouse and anti-rabbit) were purchased from Santa Cruz (Beijing, China).

**Cell culture**

Human prostate cancer PC-3 cell line was purchased from American Type Culture Collection (ATCC, China) and was maintained in our lab. The cells were cultured in DMEM (supplemented with 10% FBS, 100 U/ ml penicillin, and 100 μg/ml streptomycin) at 37 °C, in a CO₂ incubator (5% CO₂, and 95% air, 95% humidity). Newly thawed PC-3 cells were past at least 3 passages before tests.

Freshly isolated mouse splenocytes were prepared from Kunming mice of SPF grade (Central Research Laboratory, Jilin University Bethune Second Hospital, China). The cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS and maintained in a CO₂ incubator (5% CO₂ and 95% air, 95% humidity).

**Cell proliferation assay**

Effect of vitexicarpin on viability of PC-3 cells was evaluated by MTT assay as reference (Khan et al., 2012). Briefly, PC-3 cells were subcultured in 96-well plates at a density of 10³/well and incubated in CO₂ incubator. After ~12h incubation, the cells were exposed to various concentrations of vitexicarpin (3.125, 6.25, 12.5, 25, 50, 100, and 200 μM) and continuously incubated for 24 h. Following incubation, 20 μl MTT (5 mg/ml) was added in each well and plate was again incubated at 37 °C. After 4 h. medium was removed carefully and 150 μl DMSO was added to each well. The plate was shaken gently for 15 min to dissolve formazen crystals. The absorbance nm was recorded at 570 by the microplate reader (Thermo Scientific, Vario Skan Flash, USA). Percentage cell viability was calculated as follow:

Cell viability (%) = (A570 treated / A570 control) × 100

The IC₅₀ values were calculated using GraphPad Prism 5.

**Cell morphological changes**

Cells were treated with vitexicarpin for 24 h, and then morphological changes of the cells were observed under a fluorescence microscope (Olympus 1×71, Japan), recorded with a CCD camera (Olympus DP72, Japan) and analyzed using DP2-BSW software.

**Flow cytometry analysis of apoptosis**

Apoptosis of PC-3 cells were determined using Annexin V-FITC/PI double staining Apoptosis Detection Kit. PC-3 cells were cultured in 6-well plates, incubated overnight, and then exposed to different concentrations of vitexicarpin for 24 h. The cells were trypsinized, washed twice with ice-chilled PBS and collected through centrifugation (1000 rpm for 5 min, 4°C). The cell pellets were resuspended in binding buffer and stained sequentially with Annexin V-FITC and PI as manufacturer’s instructions. Flow cytometry was performed on Beckman Coulter, Epics XL (USA) and data were recorded using Cell Quest software.

**DNA fragmentation analysis by Hoechst 33258 staining**

The apoptosis in PC-3 cells was detected by using Hoechst 33258 nuclei staining. Cells were treated with vitexicarpin for 24 h and collected by centrifugation at 1000 rpm for 5 min. After centrifugation, the cells were washed with PBS, stained with Hoechst 33258 (50 μg/ml) and incubated at 37°C for 30 min in the dark. At the end of incubation, cells were washed and resuspended in PBS for the observation of nuclear morphology under fluorescence microscope (Olympus 1×71, Japan), recorded with a CCD camera (Olympus DP72, Japan) and analyzed using DP2-BSW software. Apoptotic cells were defined as cells showing nuclear shrinkage and chromatin condensation.

**Flow cytometric analysis of cell cycle**

PC-3 cell cycle phase distribution was determined by using PI and flow cytometry as described previously (Khan et al., 2012). After the PC-3 cells were incubated with vitexicarpin for 24 h, the cells were collected and fixed in 70% ice-cold ethanol overnight, rinsed twice with PBS, and then stained with 50 μg/ml PI (containing 100μg/ml RNase A) at 4°C for 30 min in the dark. The DNA contents were assayed on Beckman Coulter, Epics XL (USA) using Cell Quest software.

**Determination of intracellular Reactive Oxygen Species (ROS) generation**

Intracellular ROS generations were determined using 2',7'-dichlorofluorescein-diacetate (DCFH-DA) staining method. PC-3 cells cultured in 6-well plates were treated with vitexicarpin for 24 h, and then stained with 10 μmol/L DCFH-DA at 37°C for 30 min as the manufacture’s instructions. The cells were then collected, rinsed 3 times with PBS, resuspended in 200 μl PBS. 2',7'-dichlorofluorescein (DCF) fluorescence data were acquired on Beckman Coulter, Epics XL (USA).

**Determination of mitochondrial transmembrane potential (ΔΨm)**

Rhodamine 123 staining method was used to evaluate ΔΨm in PC-3 cells. PC-3 cells cultured in 6-well plates were exposed to different concentrations of vitexicarpin for 24 h. The cells were collected in centrifuge tube, resuspended in 500 μl PBS, and then stained with 10μg/ml rhodamine 123 at 37°C for 30 min in darkroom. After
peroxidase (HRP) conjugated secondary antibodies and the PVDF membranes were incubated with horseradish dependent kinase 1 (CDK1) (1:300), respectively. Finally, Bax (1:1000), Cytochrome c (1:1000), Cyclin (1:1000), Bcl-2 (1:500), β-actin (1:500), or cyclin-dependent kinase 1 (CDK1) (1:300), respectively. Finally, the PVDF membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies and signals were detected using ECL Plus Chemiluminescence Kit on X-ray film (Millipore Corporation, Billerica, USA).

Statistical analysis of data
All data are expressed as Mean ± SEM. One way ANOVA test was used to compare control group and test values. Student’s t-test was used to determine significance when only two groups were compared, p values less than 0.05 were considered statistically significant.

Results

Vitexicarpin Reduces Cell Viability and Induces Cell Death in human prostate carcinoma PC-3 cells
Effect of vitexicarpin (chemical structure shown in Figure 1A) on human prostate carcinoma PC-3 cell viabilities were determined using MTT assay. PC-3 cells were incubated with series concentrations of vitexicarpin for 24h. As shown in Figure 1B, vitexicarpin dose-dependently inhibited proliferation of the PC-3 cells with IC50 ~ 28.78 μM. Maximum inhibition activity was achieved by 200 μM vitexicarpin. Cell morphological changes were observed under a light microscope. Cells were treated with 30 and 50 μM vitexicarpin for 24 h. As compared to DMSO control group, vitexicarpin treated group showed a significant decrease in adherent cells which accompanied an increase in floating cells in culture medium. This study also revealed significant changes in cell morphology: in contrast to normal polyclonal structure of normal cells, the vitexicarpin treated PC-3 cells acquired a round and shrunken shape (Figure 1C). We also examined the effect of vitexicarpin on mouse splenocytes using trypan blue method; it showed that vitexicarpin had little toxic effect on the cells (Figure 1D).

Vitexicarpin Induces Apoptotic Cell Death in human prostate carcinoma PC-3 cells
Cell death can be induced by apoptosis and/or necrosis. Apoptotic cell death is characterized by major features like DNA fragmentation and loss of plasma membrane integrity (Schafer, 1998). Hoechst 33258 staining analysis showed that 10, 30 and 50 μM vitexicarpin significantly increased the percentage of cleaved nuclei from 3.96 % to 12.44%, 27.83%, and 54.65 % (Figure 2A). To further investigate vitexicarpin-induced apoptotic effect, PC-3 cells were treated with different concentrations of vitexicarpin for 24 h. The cells were double stained with Annexin V-FITC/PI and analyzed on flow cytometry as described in the Materials and Methods Section. As shown in Figure 2B, treatment of cells with 10, 30, and 50 μM vitexicarpin significantly increased apoptosis rate from 2.07% ± 0.1925 to 3.87% ± 0.3031 5.62% ± 0.3964 and 25.27% ± 1.1993 respectively.

Vitexicarpin Induces Mitotic Arrest in human prostate carcinoma PC-3 cells
Cell cycle arrest is also a major cause of cell proliferation inhibition. In order to elucidate whether vitexicarpin-induced PC-3 cell growth inhibition is associated with mitotic arrest, cell cycle was profiled by PI staining and flow cytometry method. PC-3 cells were

Figure 1. Effects of Vitexicarpin on Viabilities and Morphological Characteristics of PC-3 Cells. (A) Chemical structure of vitexicarpin. (B) PC-3 cells were treated with indicated concentrations of vitexicarpin for 24 h. Cell survival rates were measured using MTT assay. Data are expressed as Mean ± SE of three independent experiments from similar results. Morphological changes of PC-3 cells (C) and mouse splenocytes (D) observed under the light microscopy after treated with indicated concentrations of vitexicarpin or DMSO (negative control) for 24 h. Scale bar=50μm

Western blotting analysis
To elucidate the mechanism of the apoptotic effect of vitexicarpin, we analyzed the apoptosis-related proteins in PC-3 cells. After incubated with (0, 30, and 50 μM) vitexicarpin for 24 h, PC-3 cells were collected, washed twice with PBS, and lyzed with lysis buffer. The lysate was centrifuged at 12000 rpm for 15min to get rid of insoluble components. For cytochrome c expression in PC-3 cells. After incubated with (0, 30, and 50 μM) vitexicarpin, we analyzed the apoptosis-related proteins and

Statistical analysis of data
All data are expressed as Mean ± SEM. One way ANOVA test was used to compare control group and test values. Student’s t-test was used to determine significance when only two groups were compared, p values less than 0.05 were considered statistically significant.
treated with different concentrations of vitexicarpin for 24 h. As shown in Figure 3, vitexicarpin dose-dependently increased the percentage of cells in G2/M phase to 26.97±0.9909%, 35.87±1.0117%, and 58.23±1.0826% versus 18.67±0.6417% in DMSO control group after treated with 10, 30, and 50 μM vitexicarpin, with a corresponding decrease in G0/G1 phase.

**Vitexicarpin Induces Increased Generation of ROS in Human Prostate Carcinoma PC-3 cells**

Cell apoptosis can be initiated via two distinct but convergent pathways: the death receptors and mitochondrial pathways (Schultz and Harrington, 2003). Mitochondria-mediated intracellular signals pathway is characterized by increased ROS generation, mitochondrial membrane potential (MMP) disruption, and glutathione (GSH) depletion (Schultz and Harrington, 2003; Antico et al., 2012). Recently, several studies showed that vitexicarpin induced apoptosis in cervical cancer cells through ROS-mediated mitochondrial signaling pathways (Gottlieb, 2001; Antico et al., 2012). It is reasonable to ask whether or not vitexicarpin could increase ROS generation in PC-3 cells. So, we measured ROS generation in PC-3 cells by using a Reactive Oxygen Species Assay Kit.

As shown in Figure 4, ROS levels in PC-3 cells were significantly increased from 5.49±0.6296% (DMSO control group) to 12.89±1.4849%, 17.19±1.0523%, and 24.06±1.616% respectively after treated with 10, 30, or 50 μM vitexicarpin (p<0.01).

**Vitexicarpin Disrupts Mitochondrial Membrane Potential (MMP) in Human Prostate Carcinoma PC-3 cells**

Because excess ROS production can induce apoptosis by depolarization of MMP, we determined MMP in PC-3 cells by using Rh-123 staining and flow cytometry assay. PC-3 cells were incubated with different concentrations

**Vitexicarpin Induces Apoptosis in Human Prostate Carcinoma PC-3 Cells through G2/M Phase Arrest**

Two classes of regulatory molecules, the cyclins and the cyclin-dependent kinases (CDKs), play key roles in cell cycle control. Different cyclin and CDK combinations determine their downstream targeted proteins. Cyclin B1/CDK1 complex is pivotal in regulating cells from G2 to M phase transition (Giono and Manfredi, 2006; Enomoto et al., 2009; Gavet and Pines, 2010). To elucidate the molecular mechanism underlying this G2/M phase blockage, we measured the expressions of Cyclin B1 and CDK1 using Western blotting analysis. The results demonstrated that the expression of these proteins decreased significantly indicating that vitexicarpin arrested the cell cycle at G2 phase in PC-3 cells. Similar results were also found in vitexicarpin treated human hepatocellular carcinoma (HCC) cells (He et al., 2012).

Apoptosis is a main reason for cell growth inhibition, and many cytotoxic agents arrest the cell cycle at specific phase and then induce apoptosis (Muschel and McKenna, 1996; Komata et al., 2003; Maddika et al., 2007; Batsi et al., 2009). So, we performed flow cytometric analysis by using propidium iodide (PI) / Annexin V-FITC double staining methods. It showed that vitexicarpin dose-dependently induced apoptosis in PC-3 cells. Previous studies have indicated that vitexicarpin induced apoptosis in hepatocellular carcinoma HepG2 (Yang et al., 2011; He et al., 2012) and human cervical cancer cells (Chen et al., 2011; Zeng et al., 2012). Also, we found vitexicarpin induced apoptotic cell death in PC-3 cells in a dose-dependent manner.

Over production of ROS induces oxidative damage including; lipid peroxidation, protein oxidation and DNA damage which results in modulation of Bcl-2 family proteins and dissipation of mitochondrial membrane potential, which are characteristic features of mitochondrial apoptosis (Khan et al., 2012). We here examined whether vitexicarpin increases intracellular ROS generation in PC-3 cells. Significant increased ROS generations were found in the vitexicarpin-treated PC-3 cells. Because excessive ROS production can induce depolarization of MMP, next we performed flow cytometric analysis to determine MMP in control and vitexicarpin-treated cells. The data showed that MMP in vitexicarpin-treated PC-3 cells was significantly decreased which is in line well with previous result found in Hela cells (Chen et al., 2011; Zeng et al., 2012).

Apoptosis can be initiated through two distinct but convergent pathways: the death receptors and mitochondrial pathways (Schultz and Harrington, 2003). The process of apoptosis is triggered by a diverse range of cellular signals. A balance between pro-apoptotic (BAX, BID, BIM, PUMA, BAK, or BAD) and anti-apoptotic (Bcl-XL, Bcl-2, BCLW, MCL1, A1, or BOO/DIVA) proteins of the Bcl-2 family controls the mitochondrial apoptosis pathway (Zinkel et al., 2006). To investigate whether vitexicarpin can trigger intrinsic apoptosis in PC-3 cells, we examined effect of the compound on expression of Bcl-2 and Bax proteins using Western blot analysis. The results demonstrated that expression of Bcl-2 gradually decreased while expression of Bax increased in cells treated with vitexicarpin, suggesting that the compound induces apoptosis through intrinsic pathway.
Release of Cytochrome c into cytosol in vitaxicarpin-treated cells further support the above findings (Gottlieb, 2001; Antico et al., 2012).

In conclusion, our data demonstrated for the first time that vitaxicarpin induced apoptosis in PC-3 cells which is associated with increased ROS generation, modulation of Bcl-2 family proteins, and disruption of MMP and activation of Caspase-3. Therefore, vitaxicarpin may become a leading drug in the development of chemotherapeutic or chemopreventive of human prostate cancer.

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