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Shikonin inhibits prostate cancer cells metastasis by reducing matrix metalloproteinase-2/-9 expression via AKT/mTOR and ROS/ERK1/2 pathways

Yongqiang Chen, Lu Zheng, Junquan Liu, Zhonghai Zhou, Xiliang Cao, Xiaoting Lv, Fuxing Chen *

Department of Central Laboratory, 97th Hospital of PLA, 226 Tongshang Road, Xuzhou 221004, China

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ABSTRACT

Metastasis is one of the most important factors related to prostate cancer therapeutic efficacy. In previous studies, shikonin, an active naphthoquinone isolated from the Chinese medicine Zi Cao, has various anticancer activities both in vivo and in vitro. However, the mechanisms underlying shikonin's anticancer activity are not fully elucidated on prostate cancer cells. In the present study, we aimed to investigate the potential effects of shikonin on prostate cancer cells and the underlying mechanisms by which shikonin exerted its actions. With cell proliferation, flow cytometric cell cycle, migration and invasion assays, we found that shikonin potently suppressed PC-3 and DU145 cell growth by cell cycle arrest at the G2 phase and metastasis in a dose-dependent manner. Mechanically, we presented that shikonin could suppress the metastasis of PC-3 and DU145 cells via inhibiting the matrix metalloproteinase-2 (MMP-2) and MMP-9 expression and activation. In addition, shikonin significantly decreased the phosphorylation of AKT and mTOR in a dose-dependent manner while it induced extracellular signal-regulated kinase (ERK), p38 mitogen activated protein kinase (MAPK) and c-Jun N terminal kinase (JNK) phosphorylation. Further investigation of the underlying mechanism revealed that shikonin also induced the production of reactive oxygen species (ROS) that was reversed by the ROS scavenger dithiothreitol (DTT). Additionally, DTT reversed the shikonin induced activation of ERK1/2, thereby maintaining MMP-2 and MMP-9 expression and restoring cell metastasis. Together, shikonin inhibits aggressive prostate cancer cell migration and invasion by reducing MMP-2/-9 expression via AKT/mTOR and ROS/ERK1/2 pathways and presents a potential novel alternative agent for the treatment of human prostate cancer.

1. Introduction

Prostate cancer is the most commonly occurring cancer in men in the developed world, and the second most common worldwide, with over 900,000 new cases and 250,000 deaths estimated to occur annually [1]. Surgical and hormonal therapies have shown beneficial effects for early-stage, hormone-responsive disease. However, as the disease progresses, tumors become metastasis, castration resistant and no longer respond to hormonal deprivation therapies [2] and few treatment options are available for more aggressive prostate cancer [3]. Key components of the metastatic process in biologically aggressive prostate tumors include proliferation, migration, invasion, and angiogenesis. The steps of metastasis require degradation of extracellular matrix (ECM) constituents via proteolytic enzymes [4]. Among these proteolytic enzymes, matrix metalloproteinases (MMPs), including MMP-2 and -9, are the chief ECM-degrading enzymes [5]. Therefore, inhibition of MMP expression and/or inhibition of the activities of MMP enzyme can be used as favorable targets for preventing cancer metastasis [6–8].

Natural products from plants continue to attract attention for the discovery of novel cancer chemopreventive agents in recent years [9,10]. Shikonin, an active naphthoquinone, is abundant in the Chinese medicine Zi Cao (gromwell), the dried root of *Lithospermum erythrorhizon* (Sieb. et Zucc), *Arnebia euchroma* (Royle, Johnst) or *Arnebia guttata* (Bunge) [11]. It has been reported to have anti-inflammatory [12,13], anti-bacterial [14], and antitumor functions in many human cancer cell lines [15–19]. These studies suggest that shikonin could be a promising agent for cancer treatment. However, the mechanisms underlying shikonin's anticancer activity are not fully elucidated on prostate cancer PC-3 and DU145 cells, although it has been studied in some cancer cell lines.

Therefore, in the present study, we focused on the in vitro anti-migration and anti-invasion of shikonin on human prostate cancer PC-3 and DU145 cells and investigated the possible molecular mechanisms involved in the process. Our results suggest that shikonin inhibits MMP-2/-9 expression and activation via ROS/ERK1/2 and AKT/mTOR signaling pathways, thereby suppressing PC-3 and DU145 cell proliferation, migration and invasion. The findings provide a better understanding of

* Corresponding author at: Department of Central Laboratory, 97th Hospital of PLA, 226 Tongshang Road, Xuzhou 221004, China. Tel./fax: +86 516 83349771.
E-mail address: chenfuxingtitc@163.com (F. Chen).

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the related mechanisms and an experimental foundation for the clinical use of shikonin for the treatment of prostate cancer.

2. Materials and methods

2.1. Reagents and antibodies

Shikonin (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) with a concentration of 8 mM and stored at −80 °C. The concentrations used in this study were 0.5, 1, 2, 4, and 8 μM. The controls were treated with the same amount of DMSO as used in the corresponding experiments. The primary antibodies of MMP-2, MMP-9, GAPDH, Bcl-2, Bax, Akt, p-Akt (Ser473), mTOR and p-mTOR (Ser2448), and p-ERK (T202/Y204) were obtained from Epitomics Inc. (Burlingame, CA); p-JNK (Thr183/Tyr185), and p-p38MAP kinase (Thr180/Tyr182) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); and dithiothreitol (DTT) was purchased from Sigma-Aldrich. Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

PC-3 and DU145 cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere.

2.3. Cell viability assay

PC-3 and DU145 cells were seeded (5000 cells/well) in a 96-well flat bottomed titer plate and incubated for 24 h at 37 °C in 5% CO_2 atmosphere. Different dilutions of shikonin were added and incubated further for 48 h. Before 4 h of completion of incubation, 10 μL MTT (5 mg/mL) was added and then the supernatants were discarded carefully, and 200 μL DMSO was added to each well. The 96-well culture plates were shaken for 10 min in the dark. The OD was assessed by an SEAC automatic enzyme immunoassay analyzer (Beijing XiYake Technology Co., Ltd.) at a wavelength of 540 nm. The number of viable cells was presented relative to untreated controls.

2.4. Cell morphology

The cells were plated in 12 well plates at a density of 1×10^5 cells/well and grown for 24 h. Different concentrations of shikonin were added and they were grown at 37 °C in a humidified 5% CO_2 for 48 h. For the cell morphology experiment, the culture plates were examined under a phase contrast microscope and photographed.

2.5. Cell cycle analysis

About 5×10^5 cells/well in 6-well plates with (0–2 μM) shikonin were incubated for different time periods, and then the cells were harvested by centrifugation. The cells were fixed gently (drop by drop) with 70% ethanol (in PBS) in ice overnight and were then resuspended in PBS containing 40 μg/mL PI and 0.1 mg/mL RNase (Sigma). After 30 min at 37 °C, the cells were analyzed with a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at a 488 nm wave-length and the cell cycle was determined.

2.6. Measurement of ROS generation

ROS levels were detected using a flow cytometer (Becton-Dickinson, San Jose, CA, USA). After treatment, cells were harvested and washed with PBS and suspended in 1640 medium containing 10 μM 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA; Beyotime Inst. Biotech, Haimen, PR China) at 37 °C for 20 min. The cells were washed again, and flow cytometry was performed.

2.7. Scratch migration assay

The scratch migration assay is a well-developed method to investigate drug effects on cell migration in vitro. PC-3 and DU145 cells were seeded at a density of 3.0×10^5 cells in 6-well plates and grown for 24 h to allow them to reach about 90% confluence. The cell monolayer was carefully scraped with a sterile 10 μL plastic pipet tip to create a scratch. Debris was removed from the culture and cells were then cultured with fresh medium. Subsequently, cell migration into the denuded areas was taken using a phase contrast microscope assessed after 24 h treatment with shikonin. The migration rate was calculated by the following formula: migration rate = (average wound distance − average no migration distance) / average wound distance × 100%.

2.8. Migration assay

For cell invasion assays, cells were treated with shikonin (0.5, 1, and 2 μM) for 24 h, and then cells were harvested and their in vitro invasiveness was determined using a Transwell chamber (Corning, NY, USA). Matrigel (Sigma, St. Louis, MO, USA) was diluted by serum-free medium to a final concentration of 2 mg/mL, and 8 μm pore polycarbonate membrane filters were coated with 50 μL of matrigel. Treated cells were then seeded into the upper chamber with 5×10^5 cells/well in 100 μL of serum-free medium, and 1 mL medium containing 20% FBS was added to the lower chamber as a chemoattractant. After incubation for 24 h at 37 °C in 5% CO_2, the matrigel coating on the upper surface of the filter was wiped with a cotton swab. Cells that invaded to the lower surface of the filter were fixed by 4% paraformaldehyde and stained with Giemsa. Cell numbers were counted in three random fields (×100) per filter. The cell motility assay was conducted with 2.5×10^3 cells/well in a similar fashion in a Transwell chamber without matrigel coating.

2.9. Analysis of MMP-2 and MMP-9 activities by gelatin zymography

After treatment, the activities of MMP-2/-9 in the conditioned medium were assessed by gelatin zymography. The protein concentration was assayed using the BCA assay reagent (Beyotime Institute of Biotechnology, Haimen, China), and samples were mixed with loading buffer and subjected to 10% SDS-polyacrylamide gel containing 0.1% gelatin as a substrate at 100 V for 3 h at 4 °C. After electrophoresis, the gels were rinsed in renaturation buffer (2.5% Triton X-100) on a shaker for 30 min to remove SDS at 4 °C and then incubated overnight at 37 °C in activation buffer (40 mM Tris–HCl, pH 8.0, 10 mM $CaCl_2$, 0.02% NaN_3). Gels were stained using 0.5% Coomassie blue R-250 for 1 h, and destained with destaining solution (20% methanol, 10% acetic acid, 70% ddH_2O) until the clear bands were visualized.

2.10. Western blot

For Western blot analysis, PC-3 and DU145 cells of different treatment groups were collected. Proteins were extracted and separated by SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, San Francisco, CA). The membranes were blocked with blocking solution (1% BSA in TBST) and then incubated overnight with primary antibodies for MMP-2, MMP-9, GAPDH, Bcl-2, Bax, cyclin D1, Akt, p-Akt, mTOR and p-mTOR, p-ERK1/2, p-JNK, and p-p38. After being washed in TBST, membranes were incubated with an alkaline phosphatase peroxidase-conjugated secondary antibody (Beyotime Institute of Biotechnology, Haimen, China). Detection was performed by the BCIP/NBT alkaline phosphatase color development kit (Beyotime Inst. Biotech, Haimen, PR China) according to the manufacturer's instructions. Bands were then recorded by a digital camera. The molecular weights of the detected proteins were deduced by