Berberine inhibits the proliferation of prostate cancer cells and induces G_0/G_1 or G_2/M phase arrest at different concentrations

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Abstract. Prostate cancer is the second most common disease of the male reproductive system. Berberine is a quaternary ammonium salt that is extracted from plants. The aim of the current study was to explore the antitumor activity of berberine in prostate cancer cells and identify the underlying mechanism of its effects. PC3 human and RM-1 mouse prostate cancer cells were treated with increasing concentrations of berberine, followed by analysis of the cell viability with an MTT assay. The results demonstrated that berberine markedly inhibited the proliferation of PC3 and RM-1 cells, and that the inhibitory effects to PC3 and RM-1 were enhanced in a concentration- and time-dependent manner. Flow cytometry was used to analyze the cell cycle of PC3 human prostate cancer cells, and the results demonstrated that G_0/G_1 phase arrest was induced following treatment with 10 μM berberine (P<0.05). However, with an increased concentration of berberine (50 μM) the survival rate of PC3 cells at the G_2/M phase was significantly increased compared with the cells treated with 10 μM berberine, which suggests that different cell cycle signaling pathways were activated when PC3 cells were treated with low and high concentrations of berberine. Thus, clarifying the mechanism underlying these effects in prostate cancer may provide novel molecular targets for prostate cancer therapy.

Introduction

Prostate cancer is a malignant disease that originates in the prostate gland, and it is the second most commonly observed disease of the male reproductive system (1). The incidence of prostate cancer increase with age and there are clear regional differences (2-4), for example the incidence is higher in the US and Europe compared with Asia (5,6). It has been reported that prostate cancer is the second most common cause of cancer-related mortality among males, with a high mortality

rate (7). In China, the incidence is lower in younger males, however, the mortality rate is strongly associated with age, with the highest mortality rates observed in older males (8,9). With the rise in the number of older individuals in China, the number of mortalities associated with prostate cancer has increased in recent years (8-10).

Berberine is a quaternary ammonium salt and a member of the protoberberine group of isoquinoline alkaloids. It is an important ingredient with biological properties in Chinese medicine (11,12). It is predominantly found in the roots, rhizomes, stems and bark of a number of medicinal plants, including *Mahonia aquifolium* (Oregon grape), *Phellodendron amurense* (Amur cork tree), *Berberis aristata* (tree turmeric), *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (Chinese goldthread), *Tinospora cordifolia* and *Eschscholzia californica* (Californian poppy). Berberine has a strong inhibitory effect on growth and replication in a variety of microorganisms (13,14). Berberine has primarily been used in early clinical trials to treat intestinal infections or diarrhea (15,16). In addition, it has been found that berberine has an important role in lowering blood pressure in the treatment of cardiovascular disease and diabetes (17-19).

Recently, *in vivo* and *in vitro* evidence has shown that berberine has potent anti-inflammatory and antitumor effects; however, its mechanism of action has yet to be clarified (20-23). In the present study, the antitumor effects of berberine were explored and the underlying mechanisms were investigated in PC3 human and RM-1 mouse prostate cancer cell lines.

Material and methods

Cells and reagents. PC3 human and RM-1 mouse prostate cancer cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Hyclone Corporation, South Logan, UT, USA). Berberine and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of berberine was ≥98% and it was dissolved in dimethyl sulfoxide (Sigma-Aldrich).

MTT assay. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in an atmosphere of 5% CO_2. When the cell density reached ~70%, different concentrations of berberine were added to treat the cells. In one group, the prostate cancer cells were incubated

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with 5, 10, 20 or 50 μM berberine for 24 h, while in the other group, the cells were incubated with 10 μM berberine for 24, 48 or 72 h. The survival rate of the cells was then measured via an MTT assay as previously described (24,25). The 96-well plates containing the cells were read on a microplate reader (Thermo Scientific, Rockford, IL, USA) with a test wavelength of 490 nm and a reference wavelength of 570 nm.

Flow cytometric analysis. The levels of apoptosis of the PC3 cells were tested by Annexin V/propidium iodide (PI) dual staining according to the manufacturer's instructions (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Briefly, the cells were washed in phosphate-buffered saline and treated with different concentrations of berberine (10 and 50 μM) for 24 or 48 h followed by resuspension in a binding buffer (10 mM HEPES-NaOH pH 7.4; 25 mM CaCl$_2$ and 144 mM NaCl). Subsequently, Annexin V (0.1 μg/μl) and PI (0.05 μg/μl) staining dyes were added and the cells were incubated in the dark for 30 min on ice. The cells were then subjected to fluorescence-activated cell sorting (FACS) analysis; the samples were tested by the BD FACSCalibur Cell Sorting system (BD Biosciences, San Jose, CA, USA).

Statistical analysis. SPSS statistical package version 11.5 (SPSS, Inc., Chicago, IL, USA) was used to analyze the data. All experiments were performed at least three times and results are presented as the mean ± standard error of the mean. $P<0.01$ was considered to indicate a statistically significant difference.

Results

Antitumor effects of berberine on prostate cancer cells increase with concentration. In order to explore whether berberine exhibited antitumor effects on prostate cancer cells, the PC3 human and RM-1 mouse prostate cancer cell lines were used as cellular models. The structure of berberine is shown in Fig. 1. Different concentrations of berberine were used to treat PC3 and RM-1 cells for 24 h. As shown in Fig. 2, the survival rates of PC3 and RM-1 cells as detected by an MTT assay were markedly reduced when treated with increasing concentrations of berberine. The results demonstrated that berberine has an antitumor effect and that the inhibitory effects increased in a concentration-dependent manner.

Berberine kills prostate cancer cells in a time-dependent manner. The PC3 and RM-1 cells were treated for different times with berberine at a concentration of 10 μM. The MTT assay (Fig. 3) revealed that the survival rate of prostate cancer cells was decreased in a time-dependent manner. Here, untreated cells were used as negative controls and all of the samples were analyzed in duplicates.

Berberine induces the apoptosis of human prostate cancer PC3 cells. PC3 human prostate cancer cells were treated with 10 μM or 50 μM berberine for 24 h. As shown in Fig. 4, the apoptosis rates of the PC3 cells treated with berberine were significantly higher compared with those of the untreated cells, and the apoptosis rate increased in a concentration-dependent manner.
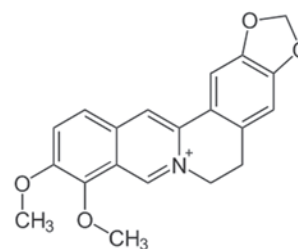

Figure 1. Chemical structure of berberine, a quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids.
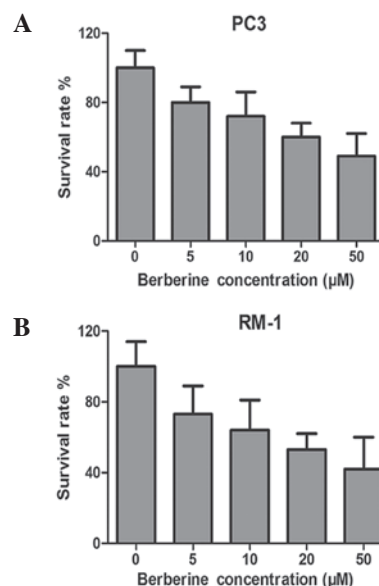

Figure 2. MTT assay. Cultured (A) PC3 and (B) RM-1 prostate cancer cells were incubated with different concentrations of berberine for 24 h, and the survival rates were measured via an MTT assay. Untreated cells served as a negative control. Data are presented as the mean ± standard error of the mean of at least three independent experiments on different cultures.
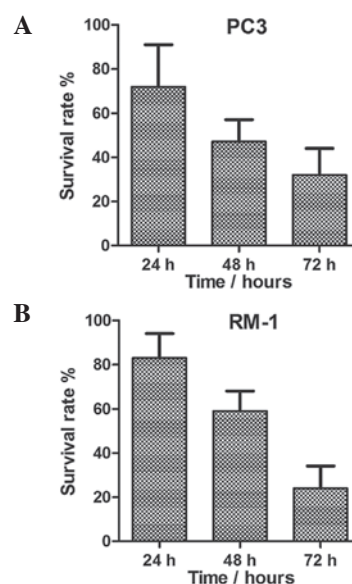

Figure 3. Survival rate of prostate cancer cells treated with berberine. The (A) PC3 and (B) RM-1 prostate cancer cell lines were incubated with berberine (10 μM) for 24, 48 and 72 h, and the survival rate of the cells was measured via an MTT assay. Untreated cells served as a negative control. Data are displayed as the mean ± standard deviation.
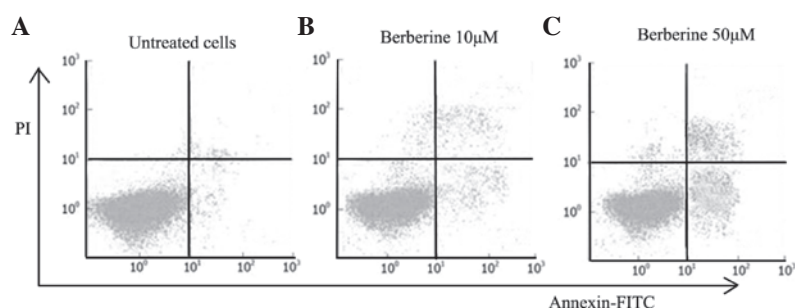
Figure 4. Cell apoptosis assay. PC3 cells (5x10^5) were plated into 6-well plates and treated with (B) 10 μM or (C) 50 μM berberine. (A) Untreated cells were used as negative controls. After 24 h, the three groups were stained with fluorescein isothiocyanate-Annexin V and propidium iodide and the percentage of apoptosis was determined. The experiments were performed at least three times.
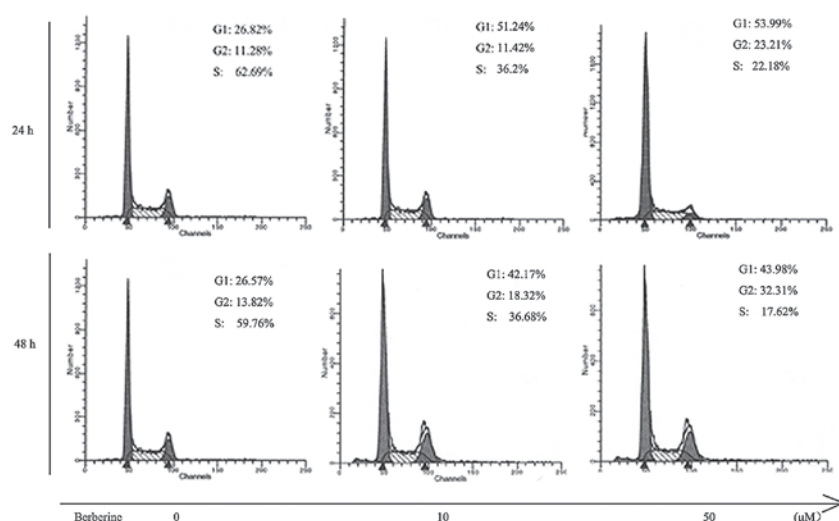

Figure 5. Cell cycle analysis of PC3 cancer cells. PC3 cells were treated with berberine (10 μM) for 24 and 48 h, and cell-cycle analysis was performed by flow cytometry using a propidium iodide staining method. The proportion of the cells in each phase was determined. The assay was repeated in three separate experiments.
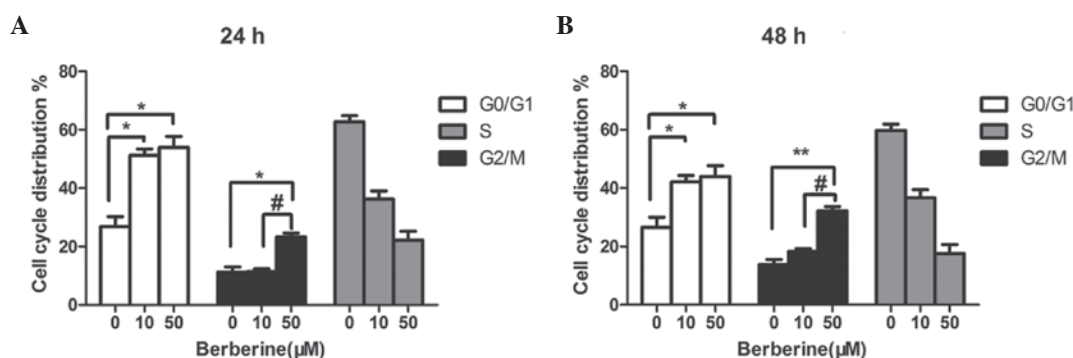

Figure 6. Distribution of PC3 cells in cell cycle phases. The cells were treated with berberine (10 μM) for (A) 24 h and (B) 48 h. Propidium iodide (PI)-positive cells were analyzed with FACS and a percent of PI-positive cells was quantified and shown in the histograms. Untreated cells were used as negative controls. *P<0.05 and **P<0.01, compared with negative controls; #P<0.01 compared with PC3 cells treated with 10 μM berberine.

Berberine induces cell-cycle arrest in the G_0/G_1 and G_2/M phases. In order to elucidate the mechanism underlying the effects of berberine, cell-cycle analysis of PC3 cells was performed using a FACS assay. PC3 cells were treated with 10 μM berberine for 24 or 48 h, and cell-cycle analysis was performed using a PI staining method (Fig. 5). As shown in Fig. 6, the distribution of PC3 cells in the cell cycle phases was

analyzed. The results demonstrated that the treatment of cells with 10 μM berberine induced G_0/G_1 phase arrest (P<0.05). However, with an increased berberine concentration of 50 μM, the survival rates of the PC3 cells in the G_2/M phase (28.4%; data not shown) were significantly increased compared with those treated with 10 μM berberine (13.2%; data not shown), which suggests that different signaling pathways associated

with the cell cycle were activated when PC3 cells were treated with low and high concentrations of berberine.

Discussion

Prostate cancer is one of the most common types of malignancies in males, which significantly affects quality of life (1,26). In the late stages of prostate cancer, treatment primarily consists of chemotherapy. However, the chemotherapeutic drugs that are currently used are expensive and have serious side-effects, hence, it is useful to identify novel natural compounds with low toxicity for the treatment of prostate cancer. Berberine is an important compound that is extracted in traditional Chinese medicine (27). It has an key role in clinical trials and in the anti-inflammatory response, and has antitumor effects (28-30). In the present study, we further explored the mechanism of anti-tumor activity of beberine on human and mouse prostate cancer cells. Berberine is primarily used in the clinical treatment of intestinal infections. Recent studies have found that berberine may have an important role in the treatment of diabetes and cardiovascular diseases in addition to antitumor activity.

In the present study, the inhibitory effects of berberine on prostate cancer and its mechanisms were investigated. PC3 human and RM-1 mouse prostate cancer cells were used as cellular models. The results of the MTT assay revealed that berberine significantly inhibited the proliferation of PC3 and RM-1 cells in a concentration- and time-dependent manner. Cell cycle distribution results showed that berberine induced G_0/G_1 phase arrest and apoptosis in PC3 prostate cancer cells, which is consistent with the results described in other studies (31-33).

Berberine inhibits the growth of a variety of tumor cells, and its antitumor mechanisms include the induction of cell cycle arrest and apoptosis (34-37). The current study determined that different signaling pathways were activated when prostate cancer cells were treated with low or high concentrations of berberine. The results demonstrated that G_0/G_1 phase arrest was induced at a lower concentrarion of berberine, and G_2/M phase arrest was observed with an increased berberine concentration of 50 μM. Different proteins were expressed at different cell cycle stages, and these proteins are associated with different signalling pathways. However, the regulatory mechanisms by which berberine induced G_2/M phase arrest remain unclear. In addition, Liu *et al* (38) found that high concentrations of berberine could induce G_2/M phase arrest in human osteosarcoma cells, and that this was not dependent on p53 function.

In conclusion, the results of the present study have provided a rationale for the development of berberine-based therapies to treat malignant tumors and have aided in the elucidation of the mechanism underlying the antitumor activity of berberine in prostate cancer cells.

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