Berberine inhibits p53-dependent cell growth through induction of apoptosis of prostate cancer cells

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Abstract. Berberine has anti-tumor properties in some cancer cells including prostate cancer, but the exact mechanisms and *in vivo* effects are unclear. We investigated anti-cancer activity of berberine *in vitro* and *in vivo*, and possible mechanisms in prostate cancer cells. Berberine treatment inhibited cell cancer growth in a concentration (0-50 μM) and time- (0-48 h) dependent manner without any growth inhibition in normal human prostate epithelial PWR-1E cells. However, the p53 expressing LNCaP cells were more susceptible against berberine than the p53 lacking PC-3 cells. The cell arrest in G_0/G_1 phase, apoptotic cell death and the expression of apoptotic cell death proteins Bax and caspase-3 was much higher in berberine-treated LNCaP cells than those in PC-3 cells. Exploration of p53 siRNA or pifithrin-α, a p53 inhibitor to the LNCaP cells, suppressed berberine-induced cell death and expression of apoptosis-related proteins. In xenograft *in vivo* studies, berberine reduced tumor weights and volumes accompanied with apoptotic cell death and increased expression of apoptotic cell death proteins, however, the extent of inhibitory effect was more significant in LNCaP cell-bearing mice. Therefore, these results indicated that berberine inhibited p53-dependent prostate cancer cell death.

Introduction

Chemoprevention, which refers to the administration of synthetic or natural agents to prevent the initiation and/or promotional events associated with carcinogenesis, is being increasingly appreciated as an attractive approach for the management of cancer development (1). In recent years, apoptosis has come to be a good way to eliminate pre-cancerous and/or cancer cells (2,3). Thus, chemopreventive agents that can modulate apoptosis may be able to affect the steady-state cell population, which may be useful in the management and therapy of cancer development. Many natural compounds inducing apoptosis have been developed as preventive and/or treatment agents of cancer development (4,5).

Berberine is an isoquinoline alkaloid that has been isolated from *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (Coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric). Berberine possesses a wide range of biochemical and pharmacological activities such as, anti-diarrheal (6), anti-arrhythmic (7), and anti-inflammatory (8) activity. Berberine has been examined for anti-cancer activity following evidence of anti-neoplastic properties (9,10). Berberine has been shown to inhibit effectively human colon cancer cell growth by inhibiting cyclooxygenase-2 transcriptional activity (11), arylamine N-acetyltransferase activity (12), and DNA topoisomerase I and II (13), and by acting intercalating agent (14,15). Therefore, it is possible that berberine may have anti-cancer activities through multiple anti-cancer mechanisms depending on the cell type and status (16).

p53, a tumor suppressor protein, serves as the major obstruction for carcinogenesis. Indeed, approximately 50% of human cancers bear p53 gene mutations (17). p53 is activated as a transcription factor in response to e.g., oncogene activation, hypoxia and DNA damage, resulting in growth arrest and/or apoptosis by stimulating the expression of various p53 target genes such as $p21^{WAF1/CIP1}$, Bax, Puma, Noxa, Apaf-1, Fas, and DR5 (18,19). p53 can also transcriptionally repress the expression of anti-apoptotic proteins, e.g., Bcl-2, Bcl-XL or survivin (20). *Bax* gene product dimerizes with Bcl-2 and prevents the activity of Bcl-2 from blocking apoptosis (21). The Bax protein controls cell death through activation of caspases-9 and -3 (22). Several natural compounds such as curcumin (23), resveratrol (24), (-)-Epigallocatechin-3-gallate (EGCG) (25), capsaicin (26) and glycolic acid (27) have been reported to induce p53-dependent apoptosis in human ovarian, liver, prostate, leukemic and keratinocyte cancer cells. We recently found that berberine induced p53-dependent apoptosis of neuroblastoma cells (unpublished data).

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In the present study, we investigated the inhibitory effects of berberine on human prostate cancer cell growth via apoptotic cell death, and whether berberine-induced apoptosis is p53-dependent in prostate cancer LNCaP (p53 expressing) and PC-3 (p53 lacking) cells in *in vitro* and *in vivo*.

Materials and methods

Cell culture and treatment. Human prostate cancer LNCaP cells, PC-3 cells, and the non-neoplastic human prostate epithelial cell line PWR-1E were purchased from the American Type Culture Collection (Manassas, VA, USA). The prostate cancer cell lines were cultured in RPMI-1640 supplemented with 10% FBS, 100 mg/ml penicillin-streptomycin and maintained in an incubator with a humidified atmosphere 5% CO_2 at 37˚C. The PWR-1E cells were cultured in keratinocyte growth medium supplemented with 5 ng/ml human recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract (Gibco/Invitrogen, Carlsbad, CA, USA). Berberine was purchased from Sigma Aldrich (St. Louis, MO, USA). In all treatment, berberine was dissolved in dimethyl sulfoxide (DMSO) and made up to the maximum final concentration of 0.05% (v/v) in the complete cell culture medium.

p53 siRNA transfection. Sequences of oligonucleotides of p53 siRNA were purchased from Samchully Pharm. Co. (Seoul, Korea). The sequence of the p53 oligonucleotide is: sense 5'(CUACUUCCUGAAAACAACG)d(TT)3' and anti-sense 5'(CGUUGUUUUCAGGAAGUAG)d(TT)3'. siRNA oligonucleotides were transfected using the siPORT™ NeoFX™ (Ambion, Cambridgeshire, UK). In preliminary studies, cells were treated with 10-200 nM of p53 siRNA according to the manufacturer's instructions in order to determine the optimum concentration to down-regulate the target genes. In later studies, cells were transfected with 10, 50, and 100 nM siRNA for 4 h, and then the cell were grown for a further 48 h in normal medium. The effect of siRNA treatment on sensitivity to apoptosis was assessed by flow cytometry 48 h after berberine treatment. The effect on cell viability of p53 siRNA treatment was determined by cell viability test 24 and 48 h after berberine treatment. Treatment of cells with the selective p53 inhibitor, pifithrin-α (Calbiochem, CA), was performed by pretreatment the cells with the inhibitor 24 h prior to addition of berberine.

Cell viability assay. Cell viability assay was performed as described previously (28). Cells were plated in 96-well plates, and subconfluent cells were subsequently treated with berberine (0, 5, 10, 20, and 50 μM) for 24, 48, and 72 h. After treatment, cell viability was measured by Cell Counting Kit-8 (CCK-8) system according to the manufacturer's instructions (Dojindo, Kumamoto, Japan).

Cell cycle analysis by flow cytometry. Subconfluent cells were treated with berberine (0, 5, 10, 20, and 50 μM) in culture medium for 48 h and 50 μM concentration of berberine in complete medium for 0, 6, 12, 24, 48, and 72 h. Flow cytometric analysis was done as previously described

(28). The cell cycle distribution was determined using a FACSCalibur instrument (BD Biosciences, San Jose, CA).

Apoptosis evaluation. Apoptosis assays were carried out in the berberine treated cells by using flow cytometric analysis and by observing fluorescence microscopy after the terminal nucleotidyl transferase-mediated nick end-labeling (TUNEL) method using the *In Situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The cells were then observed through a fluorescence microscope (magnification x400, Leica Microsystems AG, Wetzlar, Germany). TUNEL staining assay as previously described (28).

Immunofluorescence staining. Immunofluorescence staining was done as described previously (28). The cells were cultured with medium containing berberine (10 and 50 μM) or vehicle for 48 h. Primary polyclonal antibodies for p53 (1:400 dilution) and anti-rabbit-biotinylated secondary antibodies Alexa Fluor 568 (Molecular Probes Inc., Eugene, OR) (1:200 dilution) were used. Upon nuclear stain and mount in antifade medium with DAPI (Vector Laboratories, Inc.), immunofluorescence images were acquired using a confocal laser scanning microscope (TCS SP2; Leica Microsystems AG) equipped with a 630x oil immersion objective.

Western blot analysis. Western blot analysis was done as described previously (28). The cells were harvested and homogenized in lysis buffer Pro-Prep (iNtRON Biotechnology Co., GyeongGi-do, Korea). The membrane was immunoblotted with primary specific antibody: mouse monoclonal antibodies against CDK2, CDK4, cyclin D1, cyclin E and p21 (1:1000 dilution, Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), rabbit polyclonal for p53, Bax (1:500 dilution, Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA), Bcl-2, PARP and cleaved caspase-3 (1:1000 dilution, Cell Signaling Technolology, Inc. Beverly, MA, USA). The blot was then incubated with the corresponding conjugated anti-mouse and anti-rabbit immunoglobulin G-horseradish peroxidase (1:4000 dilution, Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with the ECL Western blotting detection system. The relative density of the protein bands was scanned by densitometry using MyImage (SLB, Seoul, Korea) and quantified by Labworks 4.0 software (UVP Inc., Upland, CA). The tumor fragments were homogenized in lysis buffer Pro-Prep, and then, equal amounts of proteins (80 μg) were studied under the same conditions and course.

Anti-tumor activity of berberine in vivo xenograft. Six-week-old male BALB/c athymic nude mice were purchased from Japan SLC (Hamamatsu, Japan). Prostate cancer PC-3 and LNCaP cells were injected subcutaneously ($1x10^7$ tumor cells/0.1 ml PBS/animals) with a 27-gauge needle into the right lower flanks of the mice. Tumors were removed from mice, and same size tumor fragments (3x3x3 mm) were then implanted s.c. into the right flank of other BALB/c athymic nude mice with a 13-gauge trocar needle. When the tumors had reached an average volume of ~100-300 mm³, the tumor-bearing nude mice were intraperitoneally injected with
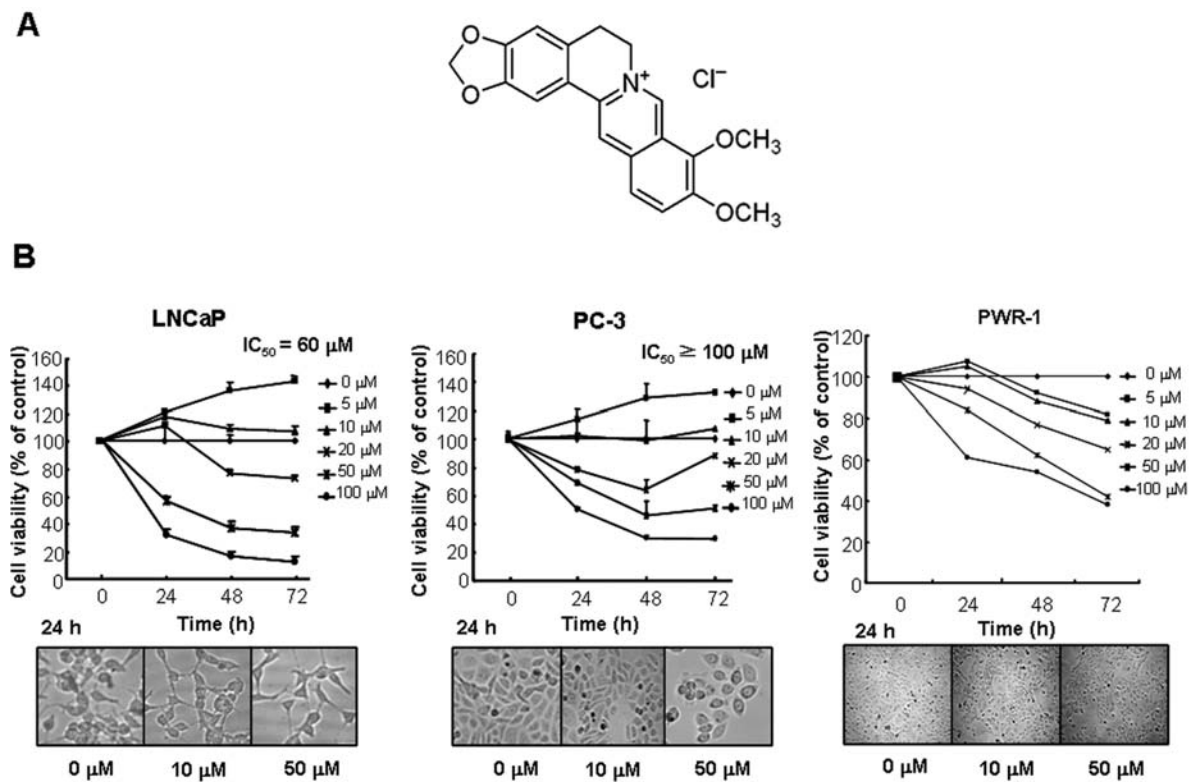
Figure 1. Effect of berberine on cell viability in human prostate cancer cells. (A) Chemical structure of berberine. (B) Concentration- and time-dependent effect of berberine on the cell viability. As detailed in Materials and methods, the cells were treated with berberine (5-100 μM) for 24, 48, and 72 h, and their viability was determined by CCK-8 kit. The data are expressed by the percent of control cells (without the berberine treatment) as the mean ± SD of 3 experiments with replicates. After 24 h of berberine treatment, the cells morphologic change was observed under phase contrast microscope (magnification x200).

berberine (5 and 10 mg/kg, twice per week for 4 weeks). The group treated with saline was used as the control. The weight and tumor volume of the animals were monitored once per week. The tumor volumes were measured with vernier calipers and calculated by the following formula: (A x B²)/2, where A is the larger and B is the smaller of the 2 dimensions of the tumor. At the end of the experiment, the animals were sacrificed with cervical dislocation. The tumors were separated from the surrounding muscles and dermis, excised and weighed.

Immunohistochemistry. All specimens were fixed in formalin and paraffin-enclosed for examination. Sections 5-μm thick were stained with hematoxylin and eosin (H&E) and immuno-histochemistry for proliferating cell nuclear antigen (PCNA) and cleaved caspase-3. Paraffin-embedded sections were deparaffinized in xylene, rehydrated through graded alcohols to water, and then incubated for 10 min in PBS. The endo-genous peroxidase was inactivated by incubation for 10 min in 3% peroxide in methanol and rehydrated for 10 min in PBS. The sections were blocked for 30 min with 3% normal horse serum diluted in PBS; the sections were then blotted and incubated with primary mouse anti-human PCNA monoclonal antibody (Santa Cruz Biotech) for 4 h at room themperature and primary rabbit anti-cleaved caspase-3 antibodies (Cell Signaling Tech, Danvers, MA) for overnight at 4˚C. The slides were incubated with biotinylated horse antibody for 2 h and washed in PBS, followed by the avidin-

biotin-peroxidase complex (ABC, Vector Laboratories, Inc., Burlingame, CA). The slides were washed and the peroxidase reaction developed with diaminobenzidine and peroxide, then counterstained with hematoxylin, mounted in aqua-mount, and evaluated by light microscopy. A negative control was performed in all cases by omitting the primary antibody.

Results

Effect of berberine on cell growth in prostate cancer cells. Berberine chloride shown in Fig. 1A, was studied to assess the inhibitory effect of berberine on cell growth of prostate cancer cells LNCaP and PC-3 cells, as well as normal human prostate epithelial cells (PWR-1E). The cells were treated with varying concentrations of berberine (0, 5, 10, 20, 50, and 100 μM) for 24, 48, and 72 h. Berberine inhibits cell proliferation in prostate cancer cells at the concentration between 30 to 100 μM in a concentration- and time-dependent manner. However, the wild-type p53 expressed LNCaP cells (IC$_{50}$ = 60 μM) were more susceptible against berberine than that of the cells PC-3 (IC$_{50}$ ≥100 μM) lacking p53. In contrast to the effect of berberine in cancer cell lines, non-neoplastic human prostate epithelial cells (PWR-1E) were not affected by these concentrations of berberine.

Berberine induces G_0/G_1-phase cell cycle arrest and decreases the levels of G_0/G_1 regulatory proteins. We determined the possible inhibitory effect of berberine on cell cycle progression

A

BBR (µM)	LNCaP			PC-3		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
0	58.6	11.8	29.6	55.8	19.1	25.1
5	62.1	14.1	23.8	61.8	13.3	24.9
10	62.5	16.1	21.4	62.3	13.6	24.1
20	72.6	9.8	17.6	66.8	11.8	21.4
50	78.9	7.0	13.1	61.8	6.4	31.8

B

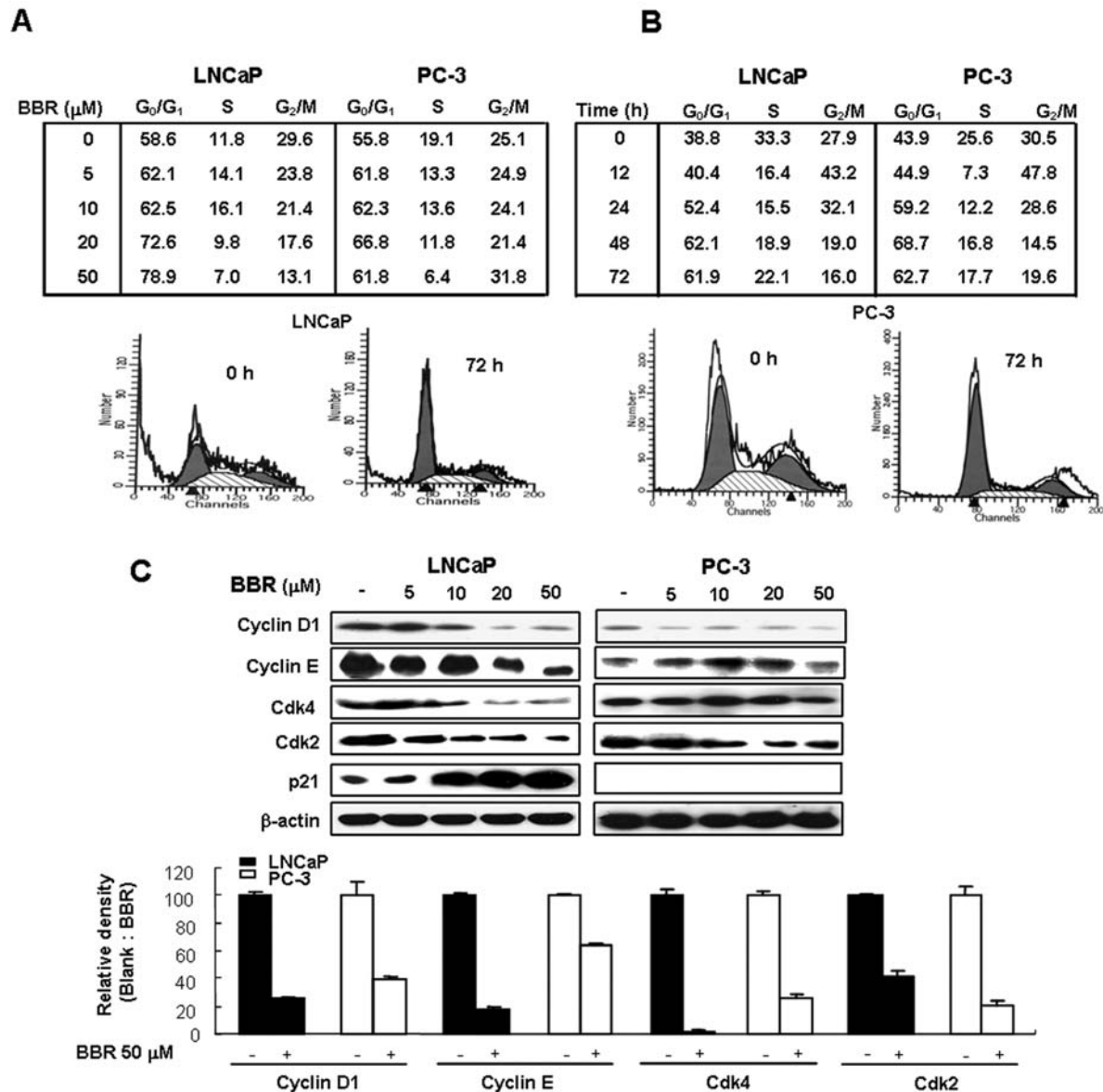| Time (h)	LNCaP			PC-3		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
0	38.8	33.3	27.9	43.9	25.6	30.5
12	40.4	16.4	43.2	44.9	7.3	47.8
24	52.4	15.5	32.1	59.2	12.2	28.6
48	62.1	18.9	19.0	68.7	16.8	14.5
72	61.9	22.1	16.0	62.7	17.7	19.6

C

Figure 2. Effect of berberine on cell cycle progression and cell cycle regulatory proteins expression in human prostate cancer cells. (A) Concentration-dependent cell cycle progression. The cells were treated with 0, 5, 10, 20, and 50 µM of berberine in complete medium. After 48 h of treatment, cells were harvested and digested with RNase A. Cellular DNA was stained with propidium iodide (PI) and flow cytometric analysis was performed to analyze the cell cycle distribution as detailed in Materials and methods. (B) Time-dependent cell cycle progression. The cells were treated with 50 µM concentration of berberine in complete medium for 0, 6, 12, 24, 48, and 72 h. After various times of treatment, cells were harvested and analyzed for DNA content by PI staining. Similar results were obtained from three independent experiments. (C) Effect of berberine on the expression of cell cycle-related proteins (cyclins, Cdks, and p21) was determined using Western blot analysis. The cells were treated with or without berberine for 24 h. Total cell lysates were processed for Western blot analysis as described in Materials and methods. ß-actin was used as an internal control. The relative density was analyzed by densitometry. The data are expressed as the mean ± SD of 3 experiments with replicates.

to investigate cell growth property of berberine. As summarized in Fig. 2, compared with vehicle treatment, berberine treatment resulted in a significantly higher number of cells in the G_0/G_1 phase in a concentration- and time-dependent manner (Fig. 2A and B). The number of cells at G_2/M and S phase was also decreased in the two cell lines treated with increasing concentrations of berberine. After 6 h of treatment, the G_0/G_1 phase of cells gradually increased by the treatment of berberine up to 72 h. It is noteworthy that treatment of berberine (50 µM) for 48 h resulted in a significantly higher number of cells in the G_1 phase in LNCaP (78%), compared with those of PC-3 (66%) cells.

These data suggest that inhibition of cell proliferation in prostate cancer cells by berberine may be associated with the induction of G_0/G_1 arrest.

The effect of berberine on cell cycle-regulatory molecules operative in G_0/G_1 phase of the cell cycle were then determined. In order to investigate the role of cyclin/Cdk complexes in the anti-proliferative activity of berberine, the expression of G_0/G_1 regulators cyclin D1, cyclin E, Cdk2, and Cdk4 were analyzed in prostate cancer cells. Protein extracts were prepared from cells treated with varying concentrations of berberine for 24 h. The treatment with berberine resulted in a significant reduction in cyclin D1, cyclin E, Cdk2, and Cdk4

protein expression in both cell lines. The inhibition on the expression of G_0/G_1 regulatory protein in p53 expressed cells were much greater than that of p53 lacking cells (Fig. 2C). CDKIs are well known to interfere with cell cycle progression to cause phase-specific cycle arrest. Thus, the protein level of certain CDKI family member, such as p21, a crucial protein in the regulation of G_1-phase progression, was determined by Western blot analysis. Berberine treatment upregulated the expression levels of p21 in LNCaP cells (Fig. 2C). However, p21 protein was not detected in PC-3 cells. These results imply that the down-regulation of cyclin D1, cyclin E, Cdk2, and Cdk4 protein expressions may be responsible for the G_0/G_1 cell cycle arrest induced by berberine, and regulation of p21 proteins may also be involved in berberine-triggered p53-dependent arrest of cells in G_0/G_1 phase of cell cycle.

Berberine induces apoptosis through mediation of apoptotic protein expression. To assess whether cell cycle arrest resulted in the induction of apoptosis flow cytometric analysis was used to quantify the prostate cancer cells. The cells were treated with varying concentrations of berberine (0, 5, 10, 20, and 50 μM) for 48 h. The treatment of LNCaP and PC-3 cells with berberine resulted in increasing apoptosis in a concentration-dependent manner (Fig. 3A). Consistent with results of cell viability test and cell cycle arrest, berberine induced more apoptosis in wild-type p53 expressing cells LNCaP (88.5%) than in cells PC-3 (40.2%) lacking p53 after treatment of 50 μM of berberine. The increase in the percentage of apoptotic cells by berberine was further confirmed by using confocal microscopy after DAPI and TUNEL staining. As shown in Fig. 3B, DAPI-positive/TUNEL staining cells were observed over a period of 48-h exposure to berberine.

Since Bax and Bcl-2 plays a crucial role in apoptosis controlled by p53, we next determined the effect of berberine treatment of two cell lines on the levels of Bax and Bcl-2 expression. The Western blot analysis exhibited a significant increase in the protein expression of pro-apoptotic Bax (Fig. 3C). In contrast, the expression of anti-apoptotic protein Bcl-2 was significantly decreased by berberine treatment in a concentration-dependent manner. Alteration in Bax/Bcl-2 is known to initiate caspase signaling, therefore, the involvement of cleaved caspase-3 during berberine-mediated apoptotic death was also investigated. As shown by the immunoblot analysis, berberine treatment resulted in a significant increase of the expression of cleaved caspase-3 and PARP cleavage in a concentration-dependent manner. However, the extent of expression changes in the expression of Bax/Bcl-2 and cleaved caspase-3 was more significant in LNCaP cells than PC-3 cells (Fig. 3C).

Berberine induces p53 translocation to nucleus in LNCaP cells. p53 is a cellular gatekeeper for the cell growth and division (29). It has been shown that p53 can regulate cell cycle arrest, apoptosis, and DNA repair in a variety of cells. Since berberine induced more susceptibility to cells following apoptotic cell death in wild-type p53 expressing LNCaP cells, the effect of berberine on the p53 translocation into nuclear was investigated to see whether translocation of p53 into nuclear is implicated in regulation of gene expression. Cells were treated with berberine for 24 h and subjected to immunoblot analysis (Fig. 4A) and confocal microscopy (Fig. 4B). As shown in Fig. 4A, nuclear translocation of p53 proteins was highly increased in LNCaP cells in a concentration-dependent manner, but not in PC-3 cells. To further study the localization of p53 protein, the cancer cells were subjected to confocal microscopy by immunofluorescent staining of p53. As shown in Fig. 4B, the intensity of red fluorescence (Alexa 568) exhibiting p53 expression was significantly increased when exposed to 50 μM berberine for 24 h in LNCaP cells. The increased p53 proteins were concentrated on the nuclei of these cells.

Knockdown of p53 decreases berberine-induced cytotoxicity and expression of apoptosis-related proteins in LNCaP cells. To further investigate whether berberine-induced apoptosis of cancer cells is p53-dependent or not, two sets of experiments were done. First, LNCaP cells were knocked down of p53 protein expression by p53 siRNA treatment. Second, LNCaP cells were treated with berberine in the presence and absence of PFT-α, a compound that inhibits the accumulation and/or the transcriptional activity of p53 (30). The levels of cell viability, apoptotic cell death, and apoptotic protein expression in berberine-treated LNCaP cells were determined. As shown in Fig. 5Aa, transfection of p53 siRNA significantly reversed berberine-induced cell growth inhibition. Similarly, 24 h prior pretreatment of PFT-α (10 and 30 μM) also reversed the berberine-induced cell growth inhibition in LNCaP cells in a concentration-dependent manner (Fig. 5Ba). Furthermore, increase of the number of apoptotic cells by berberine was also reduced when cultures were treated with a combination of berberine and p53 siRNA/or PFT-α (Fig. 5Ab and 5Bb). In agreement with these findings, the expression of p53 and cleaved caspase-3 protein in cells treated with a combination of berberine and p53 siRNA/or PFT-α reversed berberine induced expression (Fig. 5Ac and 5Bc). These results demonstrate that berberine-induced apoptosis and inhibition of cell growth could be related with the activation of p53 signaling pathway.

Berberine inhibits growth of prostate cancer cells in vivo xenograft. To elucidate the anti-cancer effect of berberine *in vivo*, the tumor growth on prostate xenograft-bearing nude mice following berberine treatments was investigated. In xenograft studies, berberine was administrated intraperitoneally twice per week for 4 weeks to mice with tumors ranging from 100 to 300 mm^3. The body weight increase and hair coats as well as other overall behavioral activities were similar in the all group at the completion of the experiments, suggesting that berberine did not have major side effects on these mice (data not shown). Tumor volume was measured weekly, and all mice were sacrificed at the end of 4 week when tumors were dissected and weighted. The effect of berberine on growth of prostate cancer cells was significant in both xenograft model mice. Fig. 6A represents the relative tumor growth measured after treatment of berberine (5 and 10 mg/kg, n=10) and cisplatin (5 mg/kg, 5 times, n=5) in comparison with the sham control group (saline, n=10).
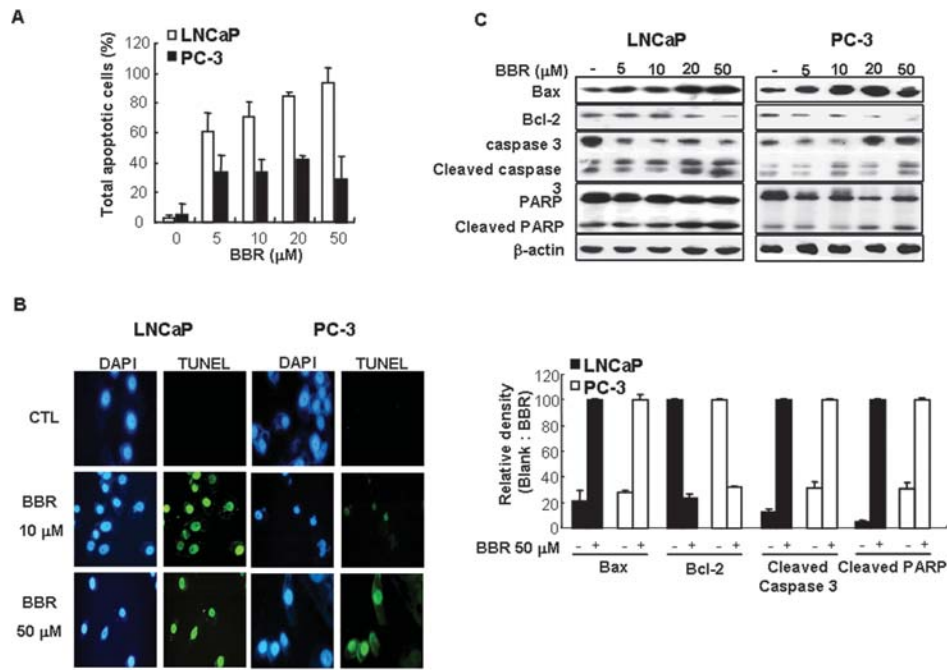
Figure 3. Effect of berberine on apoptosis and expression of apoptotic proteins in human prostate cancer cells. (A) Quantification of apoptosis by flow cytometric analysis. The cells were treated with berberine 0, 5, 10, 20, and 50 μM for 48 h, and then labeled TUNEL solution. (B) The green color in the fixed cells marks TUNEL-labeled cells. Total number of apoptotic cells in a given area was determined with DAPI nuclear staining TUNEL-labeled cells (magnification x400). The data are expressed as the mean ± SD of 3 experiments. (C) Effect of berberine on the expression of apoptosis regulatory molecules (Bax, Bcl-2, caspase-3, and PARP) was determined using Western blot analysis. The cells were treated with various concentration of berberine for 24 h. As detailed in Materials and methods, the total cell lysates were prepared for Western blot analysis. The relative density was analyzed by densitometry. The data are expressed as the mean ± SD of 3 experiments.
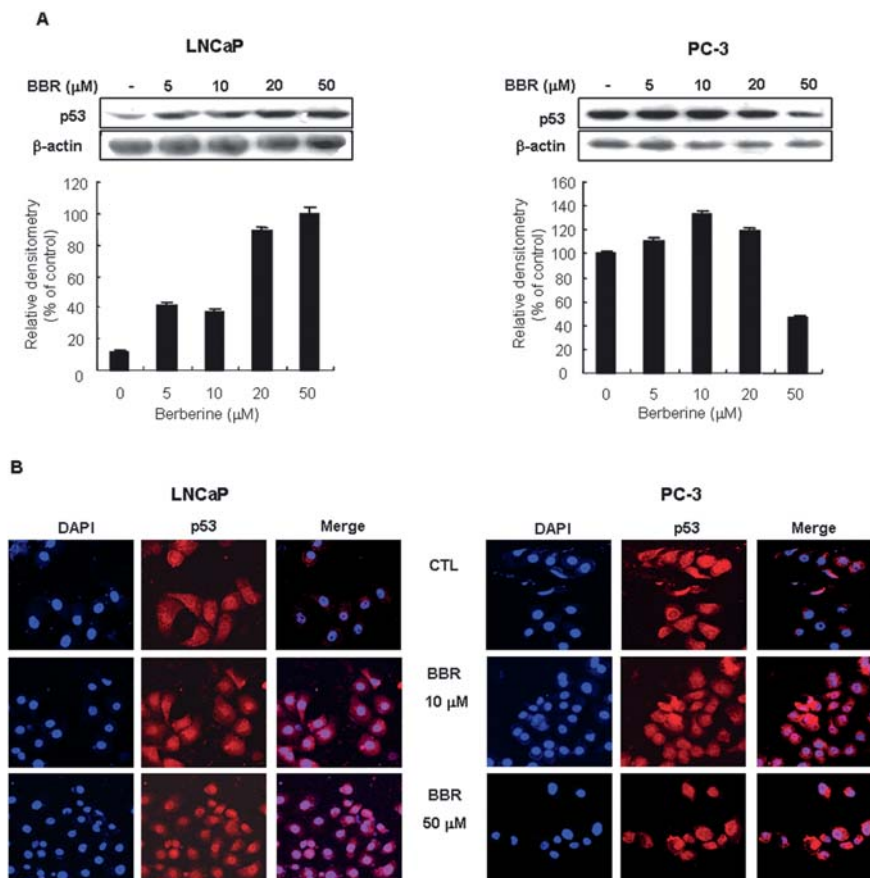

Figure 4. Effect of berberine on p53 expression and translocation to nuclear in LNCaP cells. (A) Effect of berberine p53 protein expression in nucleus fraction. The cells were treated with berberine (0, 5, 10, 20, and 50 μM) for 24 h and then harvest and lysis as detailed in Materials and methods. The data are expressed as the mean ± SD of 3 experiments. (B) The translocation of p53 was determined by immunofluorescence confocal laser scanning microscopy. After treatment with berberine for 24 h, p53 was translocation into the nucleus. The color of the cells [fluorescence for p53 (red)] was developed by Alexa-Fluor 568 (1:100) for 1 h. Similar results were obtained in three independent experiments (magnification x630).
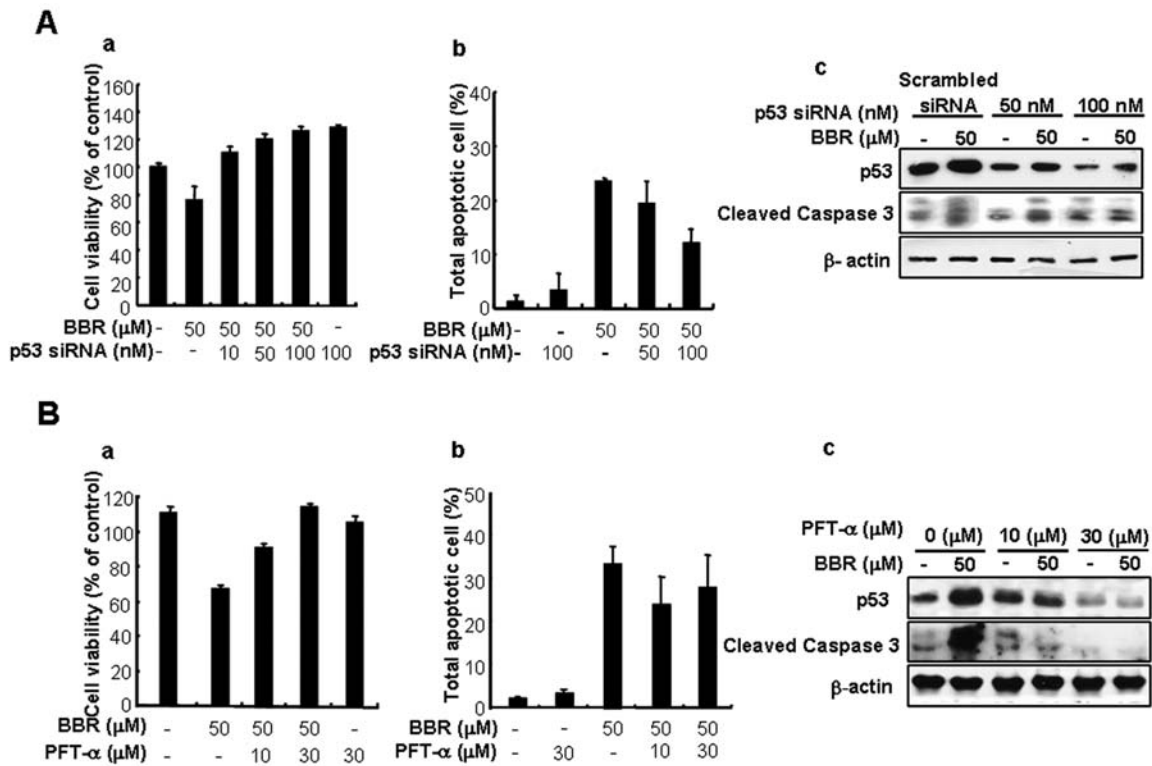
Figure 5. Effect of a p53 inhibitor or a p53 siRNA on the cell viability and apoptotic cell death in berberine-treated LNCaP cells. Suppressive effect of p53 siRNA and pifithrin-α treatment on the berberine-induced cancer cell growth inhibition, apoptosis, and expression of apoptotic regulatory proteins. (A) Cell viability (a) and apoptosis (b) in LNCaP cells transfected with p53 siRNA. The cells transfected with p53 siRNA for 4 h, and the cell were grown for a further 48 h in normal medium, and then the cells were treated with berberine for 24 h. (B) Cell viability (a) and apoptosis (b) in the LNCaP cells treated with p53 inhibitor (PFT-α). The data are expressed in terms of percent of control cells (scrambled siRNA transfected cells) as the mean ± SD of three experiments. Cell viability was determined by the CCK-8 kit. Quantification of apoptosis was determined by DAPI and TUNEL double staining assay. The data are expressed by the percent of control cells (without the berberine treatment) as the mean ± SD of 3 replicates. Expression of apoptotic proteins was determined by Western blotting in LNCaP cells transfected with p53 siRNA (Ac) and PFT-α (Bc). Using the same protocol as for (A) and (B). Western blot analysis to detect the changes in p53 and cleaved caspase-3 protein expression levels. Similar results were obtained in three independent experiments.

Tumor growth was gradually and time-dependently retarded by the treatment of berberine. Tumor volumes in 10 mg/kg berberine-treated mice compared with those saline-treated were statistically significantly smaller (1899.34±55.15 versus 1255.62±50.18 mm³ and 1537.66±36.38 versus 984.38±50.68) in PC-3 and LNCaP bearing mice, respectively (Fig. 6A). Prostate tumor weights were significantly less in 10 mg/kg berberine-treated mice compared with that of saline-treated mice (1.24±0.03 versus 0.87±0.09 in PC-3, and 1.19±0.04 versus 0.55±0.05 in LNCaP bearing mice), respectively (Fig. 6B).

The immunohistochemistry analysis of tumor section for hematoxylin and eosin revealed that berberine concentration-dependently inhibited tumor size and proliferation (Fig. 6C). We next determined the effect of berberine on the levels of cell proliferation marker PCNA, as well as apoptosis regulatory proteins, cleaved caspase-3. Using the expression of cleaved caspase-3, we confirmed that apoptosis-regulatory protein expression was significantly increased in berberine-treatment concentration-dependently in LNCaP and PC-3-bearing mice. However, the extent of expression was more significant in LNCaP-bearing mouse tumors. Consistent with the immunohistochemistry data, the expression of cleaved caspase-3, and cleaved PARP detected with Western blotting in mice treated with 10 mg/kg of berberine were significantly

increased compared to the control group, and the extent of change of these gene expressions were greater in LNCaP bearing tumor (Fig. 6D).

Discussion

In this study, we demonstrated that berberine (5-50 μM) inhibited cancer cell growth in prostate carcinoma LNCaP and PC-3 cells through induction of apoptosis *in vitro* as well as *in vivo* xenograft model. Moreover, we also demonstrated that LNCaP, p53 positive cells, are more vulnerable to berberine, suggesting that activation of p53-dependent apoptosis pathway is involved in berberine-induced prostate cancer cell growth inhibition. Similar to our finding, previous other reports have demonstrated that berberine (25-200 μM) inhibited human gastric carcinoma SNU-5 cells (31) and colon cancer cell growth (11) *in vitro*. Moreover, several studies have reported that berberine (15-75 and 10-100 μM respectively) also induced apoptosis of human epidermoid carcinoma A431 cells and prostate cancer cells (DU145, PC-3, and LNCaP) (32,33). Letasiova *et al* also reported that berberine inhibited murine melanoma B16 cell growth *in vitro* and in B16-bearing nude mice (34). These data suggest that berberine could be an agent for treatment of many cancer cell types with micromole range concentration.
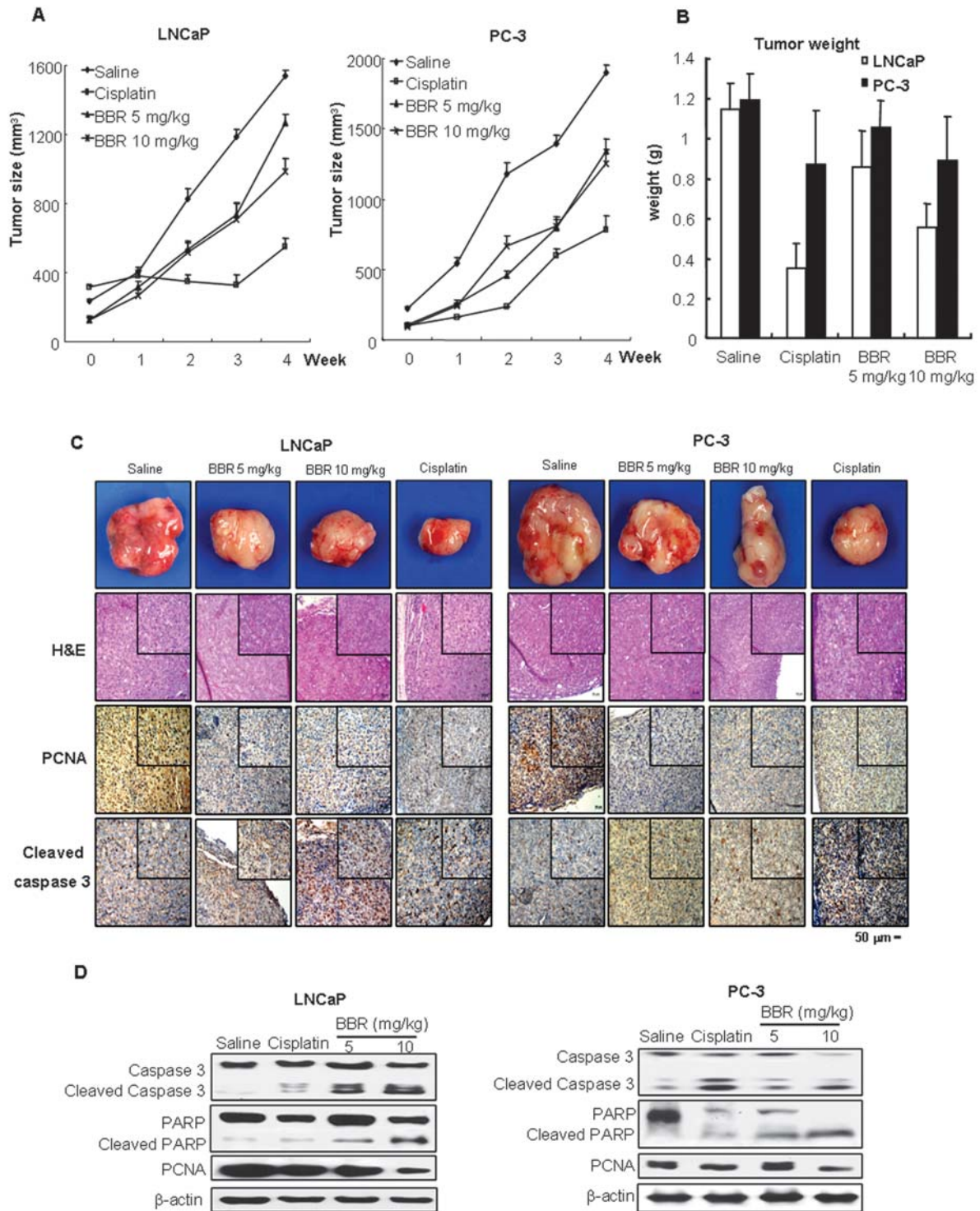
Figure 6. Anti-tumor activity of berberine in prostate cancer xenograft. Cells (1x10^7) were injected into the right lower flanks of nude mouse (n=10). Treatments were started on day 14 after tumor cell injection. Berberine was administered i.p. at 5 or 10 mg/kg twice per week for 4 weeks. At the end of the experiment, the animals were sacrificed and separated tumors. (A) Tumor burden was measured once per week using a caliper, and calculated volume [(width)^2 x (length)/2]. Tumor volume is presented as means ± SD. (B) The tumor tissues were harvested at day 30 from the mice treated with or without berberine. The tumors were separated from the surrounding muscles and dermis, excised and weighed. Tumor weight is presented as means ± SD. (C) Representative photographs of each group are shown at day 30 from the mice (upper panel). Lower panel represented microscopic images of tumor sections stained with H&E, anti-proliferating cell nuclear antigen (PCNA), and anti-cleaved caspase-3 in xenograft tumors (magnification x100). (D) Combination of the three randomly selected tumor tissues each from ten individual mouse in control and berberine-treated groups were used for total cell lysate preparation and analyzed by Western immunoblotting for the expression of cleaved caspase-3, PCNA, and cleaved PARP as described in Materials and methods. Similar results were obtained in three independent experiments. Scale bar, 50 µm.

The most common anti-apoptotic lesion in cancers is the inactivation of p53 tumor-suppressor gene (17). It has been well established that p53 plays multiple tumor suppressive roles in cells upon introduction of stresses to regulate cell cycle G_1 and G_2 arrest or apoptosis, in part depending on the cell type (35). In most cases, induction of p53 leads to an

irreversible inhibition of cancer cell growth, most decisively by activating the apoptosis pathway. Several naturally-occurring anti-tumor agents have been shown to induce cell cycle arrest followed by apoptosis of cancer cells in a p53-dependent manner such as human gastric cancer cell death by arsenic trioxide (36), bladder cancer T24 cell death by ellagic acid (37), lung cancer A549 cell death by plumbagin (38), and leukemia cell death by capsaicin (26). Moreover, p53-dependent apoptosis of cancer cells by berberine was also found in gastric carcinoma SNU-5 cell line (31). Therefore, it is possible that p53 pathway critically contributes to berberine-induced apoptotic cell death of prostate cancer cells. To delineate whether berberine-induced apoptosis in prostate cancer cells is p53-dependent or not, we compared cancer cell vulnerability against berberine in p53 expressing and lacking cells. Our present data showed that berberine inhibited cell growth in prostate cancer LNCaP (p53 wt) and PC-3 (p53 mutant) cells, however, the growth inhibitory effect of berberine is more effective in LNCaP cells. Subsequently, we also found that berberine increased the expression and translocation of p53 proteins into nucleus in p53 positive LNCaP cells in a concentration-dependent manner, but there was no change in mutant p53 expressing cell line PC-3. In addition, the inhibition of p53 by treatment with a p53 siRNA or a specific p53 inhibitor, PFT-α, attenuated berberine-induced cell growth inhibition and induction of apoptosis in LNCaP cells. These data suggest that berberine-induced cancer cell growth and apoptotic cell death could be involved with the activation of p53 signaling pathway.

Molecular analyses of human cancers have revealed that cell cycle regulators are frequently involved in most of the common malignancies (39). Among them, p21^{WAF1}, a downstream target of p53, is critical in the regulation of cancer cell cycle. p21^{WAF1} is an inhibitor of cyclin-dependent kinases 2, 4 and 6 and its upregulation causes the cells to undergo G_1 arrest. The present data showed that consistent with the greater effect on cell growth inhibition, berberine induced cell arrest in the G_0/G_1 phase of LNCaP cells that was much greater than that of PC-3. We also found that berberine increased p21 expression in LNCaP cells but it was not detected in PC-3 cells. In addition, remarkably greater changes of the expression of cell cycle-related proteins, cyclin D1, cyclin E, Cdk2 and Cdk4 in LNCaP cells than that of PC-3 cells were also observed. These effects correlated well with the induction of apoptosis by berberine. That is, berberine more effective and significantly (2-fold higher) induced apoptosis in LNCaP cells compared to the effect in PC-3 cells. Activation of caspase-3 (determined by the increase of the expression of cleaved caspase-3 and PARP) was more increased in LNCaP cells. Furthermore, p53 siRNA and inhibitor of p53 reversed berberine-induced apoptosis of p53-positive cells as well as the p53-mediated apoptosis regulatory protein (caspase-3) expression. These data together suggest that p21-dependent cell cycle arrest followed by the activation of caspase-3 may be critical in the p53-dependent berberine-induced apoptosis of prostate cancer cells. Caspase activation mechanism is involved in berberine-induced human epidermoid carcinoma A431 cells, prostate cancer cells, promonocytic U937 cancer cells,

leukemia HL-60 cells, and human hepatoma cell death (32,33,40-42). More recently, when we were preparing our study caspase-3 activation was suggested as a critical cell death signal in prostate cancer cell death by berberine (33).

Our *in vivo* study with prostate xenograft-bearing nude mice following berberine treatment we found that intra-peritoneal administration of berberine at a 10 mg/kg caused a substantial decline in tumor volume and weight of prostate cancer PC-3 and LNCaP xenograft, and that the extent of tumor growth inhibition by berberine was greater in LNCaP cells, suggesting that berberine more effectively inhibited p53 positive cancer cell growth. There is increasing evidence that p53 may directly regulate androgen signaling (25). The p53 protein has been shown to interact with several steroid receptors including the androgen receptor (AR) (43). LNCaP cells express AR, but PC-3 cells do not express AR. It is known that AR expression prostate cancer can be more susceptible for anti-cancer agents (44,45). For example, lycopene differentially induced apoptosis at concentration 10 nM in LNCaP cells and at concentration 1 μM in PC-3 cells. Thus, it is possible that berberine may be more effective in cancer cell growth in LNCaP cells expressing p53 as well as AR. To investigate whether berberine could be developed into an effective drug, we examined the absorption, distribution, metabolism, excretion and toxicity (ADME/Toxicity) using a prediction program (preADME version 1.0.2). Berberine has good oral and intestinal absorption as determined by the Caco-2 and MDCK cell permeability assay, and was found to easily pass through the brain-blood barrier. Berberine was also evaluated not to be rodent carcinogenic. In conclusion, the present study demonstrated that berberine inhibited prostate cancer cell growth by affecting G_0/G_1 phase cell cycle arrest followed by apoptosis in a p53 signal-dependent manner, suggesting that berberine can be useful as an anti-cancer agent.

Acknowledgements

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