

# Berberine, a natural product, induces G<sub>1</sub>-phase cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma cells

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## Abstract

Berberine, a naturally occurring isoquinoline alkaloid, has been shown to possess anti-inflammatory and antitumor properties in some *in vitro* systems. Here, we report that *in vitro* treatment of androgen-insensitive (DU145 and PC-3) and androgen-sensitive (LNCaP) prostate cancer cells with berberine inhibited cell proliferation and induced cell death in a dose-dependent (10–100 μmol/L) and time-dependent (24–72 hours) manner. Treatment of nonneoplastic human prostate epithelial cells (PWR-1E) with berberine under identical conditions did not significantly affect their viability. The berberine-induced inhibition of proliferation of DU145, PC-3, and LNCaP cells was associated with G<sub>1</sub>-phase arrest, which in DU145 cells was associated with inhibition of expression of cyclins D1, D2, and E and cyclin-dependent kinase (Cdk) 2, Cdk4, and Cdk6 proteins, increased expression of the Cdk inhibitory proteins (Cip1/p21 and Kip1/p27), and enhanced binding of Cdk inhibitors to Cdk. Berberine also significantly ( $P < 0.05–0.001$ ) enhanced apoptosis of DU145 and LNCaP cells with induction of a higher ratio of Bax/Bcl-2 proteins, disruption of mitochondrial membrane potential, and activation of caspase-9, caspase-3, and poly(ADP-ribose) polymerase. Pretreatment with the pan-caspase inhibitor z-VAD-fmk partially, but significantly, blocked the berberine-induced apoptosis, as also confirmed by the comet assay analysis of DNA fragmentation, suggesting that berberine-induced apoptosis of human prostate cancer cells is mediated primarily through the caspase-dependent pathway. The effectiveness of berberine in checking the growth of androgen-insensitive, as well as androgen-

sensitive, prostate cancer cells without affecting the growth of normal prostate epithelial cells indicates that it may be a promising candidate for prostate cancer therapy. [Mol Cancer Ther 2006;5(2):296–308]

## Introduction

Prostate cancer is one of the leading causes of cancer-related deaths in men worldwide (1). In the United States, one of nine men over the age of 65 years is diagnosed with prostate cancer (1, 2). The major cause of the mortality associated with this disease is the metastasis of cancer cells that fail to respond to hormone ablation therapy (3, 4). As surgery and current chemotherapeutic options seem to be inadequate in curing or controlling prostate cancer, there is a pressing need for the identification of alternative chemopreventive and chemotherapeutic strategies.

Phytochemicals show promise in this area as their potential chemopreventive or chemotherapeutic actions in prostate cancer have been indicated by epidemiologic and experimental studies (5–7). Of 121 prescription drugs in use for cancer treatment, 90 are derived from plant species and ~74% of these drugs were discovered by investigating a folklore claim (8, 9). Berberine, a naturally occurring isoquinoline alkaloid (Fig. 1A), is present in the roots, rhizome, and stem bark of a number of important medicinal plants [e.g., *Berberis vulgaris* (barberry), *Berberis aquifolium* (Oregon grape), *Berberis aristata* (tree turmeric), and *Tinospora cordifolia*]. The potential effectiveness of berberine is indicated by its use in the Indian Ayurvedic (10), Unani, and Chinese systems of medicine since time immemorial. Berberine has been shown to possess anti-inflammatory activities *in vivo* (11) and preliminary studies have been conducted to determine its anticarcinogenic activity in skin (12). It has been shown to inhibit activator protein 1, a key transcription factor in inflammation and carcinogenesis, in human cell lines (13) and has been shown to possess antitumor properties and effectively inhibit cyclooxygenase-2 transcriptional activity in human colon cancer cells (14, 15). Berberine has been shown to inhibit DNA topoisomerase II (16), and in fact, several classes of compounds that inhibit eukaryotic topoisomerase I or II have antitumor activity (17). Therefore, for the first time, we attempted to examine the chemotherapeutic effect of berberine on prostate cancer cells in *in vitro* system.

Prostate cancer in human progresses from an androgen-responsive to an androgen-unresponsive state and, at the time of clinical diagnosis, most prostate cancers represent a mixture of androgen-responsive and androgen-unresponsive cancer cells (18). Androgen-responsive prostate cancer cells undergo rapid apoptosis on androgen

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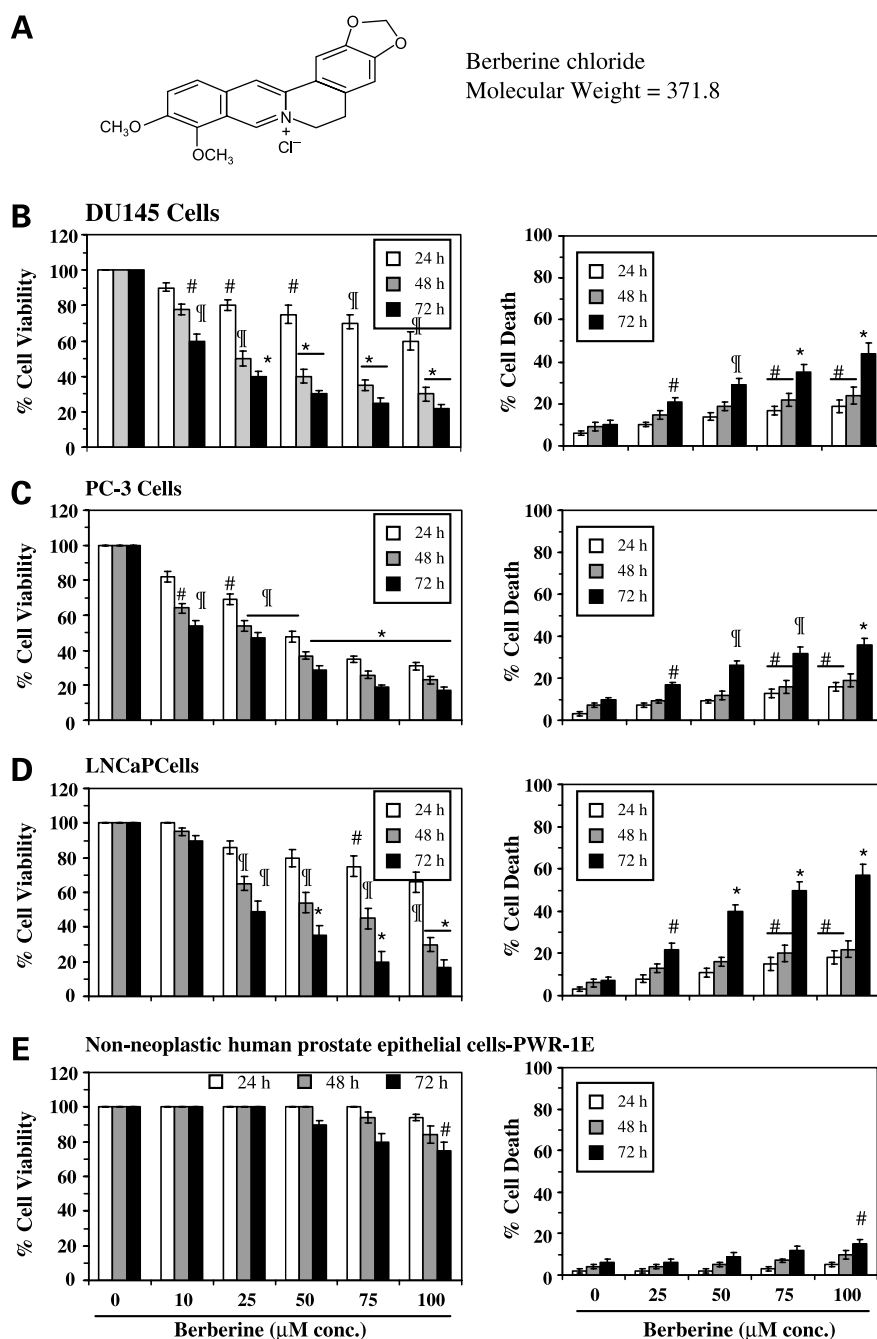
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**Figure 1.** *In vitro* treatment of human prostate carcinoma cells with berberine inhibits the proliferation potential and induces cell death in a dose- and time-dependent manner. **A**, chemical structure of berberine chloride. **B** to **E**, dose- and time-dependent effect of berberine on the viability of human prostate carcinoma cells and nonneoplastic human prostate epithelial (PWR-1E) cells. **B**, DU145 cells; **C**, PC-3 cells; **D**, LNCaP cells; and **E**, PWR-1E cells. *Left*, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods. *Columns*, mean percent of vehicle-treated (0.2% ethanol in medium) control cells from eight replicates; *bars*, SE. *Right*, the cytotoxic effect of berberine on prostate cells was determined using the trypan blue dye exclusion assay as described in Materials and Methods. *Columns*, mean percent of dead cells from three experiments; *bars*, SE. #,  $P < 0.05$ ; †,  $P < 0.01$ ; \*,  $P < 0.001$ , versus control group (non-berberine).



ablation whereas androgen-unresponsive cells generally evade apoptosis during androgen withdrawal. Mortality among prostate cancer patients generally occurs from the abnormal proliferation and invasion of these androgen-unresponsive cells (4) but it has proved difficult to identify agents that can eradicate these cells without incurring toxic responses in uninvolved cells. We therefore undertook a detailed study of the sensitivity of prostate cancer cells to berberine in which we tested the efficacy of berberine against human prostate cancer cells *in vitro*. We show that

berberine imparts antiproliferative effects against both androgen-unresponsive (DU145 and PC-3) and androgen-responsive (LNCaP) human prostate cancer cells and that this effect is mediated through interference with cell cycle progression and induction of apoptosis. Our study also provides insights into the mechanism by which berberine inhibits cell cycle progression and induces apoptosis in prostate cancer cells. Under the conditions used in this study, berberine did not affect the viability of nonneoplastic human prostate epithelial cells.

## Materials and Methods

### Reagents and Antibodies

For our experimental purpose, we selected the chloro-derivative of berberine because of its greater solubility in solvents in comparison with its parent compound. We refer to berberine chloride as berberine throughout this report. The berberine chloride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and all other chemicals employed in this study were of analytic grade and purchased from Sigma Chemical Co. (St. Louis, MO). The Annexin V-conjugated Alexa Fluor 488 Apoptosis and JC-1 Mitochondrial Membrane Potential Detection Kits were purchased from Molecular Probes, Inc. (Eugene, OR). The primary antibodies were purchased as follows: antibodies for Bax, active caspase-9, and active caspase-3 were purchased from Cell Signaling Technology (Beverly, MA); antibodies for Bcl-2, cytochrome *c*, cyclin D1, cyclin D2, cyclin E, cyclin-dependent kinase (Cdk)-2, Cdk4, Cdk6, Cip1/p21, Kip1/p27, and  $\beta$ -actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and anti-poly(ADP-ribose) polymerase was from Upstate Cell Signaling Solutions (Lake Placid, NY). The secondary antibodies, horseradish peroxidase-linked antimouse immunoglobulin G and antirabbit immunoglobulin G, were purchased from Santa Cruz Biotechnology. The pan-caspase inhibitor z-VAD-fmk was purchased from R&D Systems, Inc. (Minneapolis, MN). DMEM, penicillin, streptomycin, fetal bovine serum, and trypsin/EDTA were purchased from Cellgro (Herndon, VA). The protein assay kit was purchased from Bio-Rad (Hercules, CA) and the enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

### Cell Culture

Human prostate carcinoma cell lines LNCaP, DU145, and PC-3 and the nonneoplastic human prostate epithelial cell line PWR-1E were purchased from the American Type Culture Collection (Manassas, VA). The prostate cancer cell lines were cultured as monolayers in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100  $\mu$ g/mL penicillin, and 100  $\mu$ g/mL streptomycin (Invitrogen, Carlsbad, CA), and maintained in an incubator with a humidified atmosphere of 95% air and 5% CO<sub>2</sub> 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) and maintained in an incubator under the conditions described above. In all treatments, berberine (berberine chloride) was dissolved in a small amount of ethanol [maximum concentration, 0.2% (v/v)]. The subconfluent cells (60–70% confluent) were treated with varying concentrations of berberine in complete cell culture medium and cells treated only with vehicle (ethanol, 0.2% in media) served as control.

### Cell Proliferation/Viability Assay

The effect of berberine on the proliferative capacity of the cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide assay as previously described (19). Briefly,  $1 \times 10^4$  cells per well (DU145, PC-3, LNCaP, and PWR-1E) were plated in 96-well culture plates. After overnight incubation, the cells were treated with varying concentrations of berberine (0, 10, 25, 50, and 100  $\mu$ mol/L) for 24, 48, and 72 hours with eight replicates. In this assay, the resulting formazan crystals that had formed dissolved in DMSO (150  $\mu$ L). Absorbance was recorded at 540 nm with a reference at 650 nm serving as the blank. The effect of berberine on cell viability was assessed as percent cell viability compared with vehicle-treated control cells, which were arbitrarily assigned 100% viability.

### Cell Death Assay

The cytotoxic effects of berberine were determined using the trypan blue dye exclusion assay. Briefly,  $5 \times 10^4$  cells were seeded into each well of six-well culture plates under standard culture conditions and kept overnight in an incubator. The next day, the cells were treated with berberine (0, 25, 50, and 100  $\mu$ mol/L final concentration) for 24, 48, and 72 hours. At the stipulated time point, the cells were harvested after brief trypsinization and the cells that had taken the dye were counted using a microscope with a hemocytometer. The cytotoxic effects of berberine are expressed as the mean percentage ( $\pm$  SE) of dead cells in each treatment group from three independent experiments.

### DNA Cell Cycle Analysis

Subconfluent cells were treated with berberine (0, 25, 50, and 100  $\mu$ mol/L) in culture medium as described above for 48 hours. The cells were then harvested, washed with cold PBS, and processed for cell cycle analysis. Briefly,  $1 \times 10^5$  cells were resuspended in 50  $\mu$ L of cold PBS, to which cold methanol (450  $\mu$ L) was added, and the cells were then incubated for 1 hour at 4°C. After centrifugation, the pellet was washed with cold PBS, suspended in 500  $\mu$ L PBS, and incubated with 5  $\mu$ L RNase (20  $\mu$ g/mL final concentration) for 30 minutes. The cells were kept on ice for 10 minutes and incubated with propidium iodide (50  $\mu$ g/mL final concentration) for 1 hour in the dark. The cell cycle distribution of the cells of each sample was then determined using a FACSCalibur instrument (BD Biosciences, San Jose, CA) equipped with CellQuest 3.3 software in the Fluorescence-Activated Cell Sorting (FACS) Core Facility of the Comprehensive Cancer Center of the University of Alabama at Birmingham. ModFit LT cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

### Quantification of Apoptotic Cells

Berberine-induced apoptosis in human prostate cancer cells and normal prostate epithelial cells was determined by flow cytometry using the Annexin V-conjugated Alexa Fluor 488 (Alexa488) Apoptosis Detection Kit following the instructions of the manufacturer and as previously described by us (19). Briefly, after overnight serum starvation, cells were treated with berberine (0, 25, 50, and 100  $\mu$ g/mL) for 48 and 72 hours. The cells were then harvested, washed in PBS, and incubated with Alexa488 and propidium iodide for cellular staining in binding

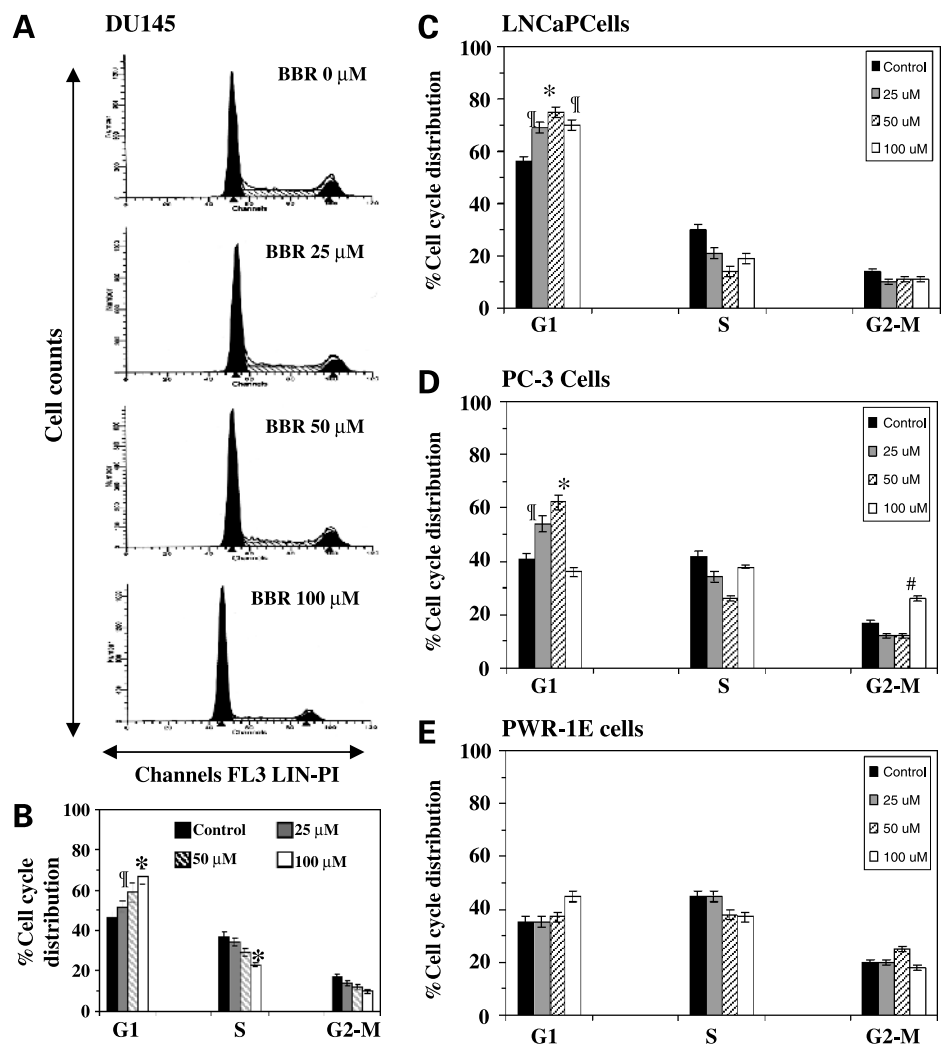
buffer at room temperature for 10 minutes in the dark. The stained cells were analyzed by FACS using a FACSCalibur instrument (BD Biosciences) equipped with CellQuest 3.3 software. The early apoptotic cells stained with Alexa488, which give green fluorescence, are represented in the lower right quadrant of the FACS histogram, and the late apoptotic cells stained with both Alexa488 and propidium iodide, which have red-green fluorescence, are represented in the upper right quadrant of the histogram. In experiments in which the pan-caspase inhibitor (z-VAD-fmk) was used, the inhibitor was added 2 hours before the addition of the berberine.

#### Immunoblotting and Immunoprecipitation

Cells were lysed as previously described (19), the lysate cleared by centrifugation at  $14,000 \times g$  for 10 minutes, and the supernatant fraction used for immunoblotting. For immunoblotting of cytochrome *c*, mitochondria-free cytosolic fraction from control and berberine-treated cells was prepared (20). Proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 5% nonfat dry milk in blocking buffer [20 mmol/L

TBS (pH 7.5) containing 0.1% Tween 20], the membrane was incubated with the desired primary antibody for 1 hour at room temperature. The membrane was then incubated with appropriate peroxidase-conjugated secondary antibody and the immunoreactive bands were visualized using the enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ) method. To ensure equal protein loading, each membrane was stripped and reprobed with antiactin antibody to normalize for differences in protein loading.

For Cdk inhibitor-Cdk binding assay, DU145 cells were treated with vehicle or 100  $\mu\text{mol/L}$  berberine for 48 hours, washed with ice-cold PBS, and whole-cell lysates prepared as previously described (19). Aliquots containing 200  $\mu\text{g}$  of lysate protein were cleared with protein A/G-plus agarose beads (Santa Cruz Biotechnology) for 45 minutes at  $4^\circ\text{C}$ . Cip1/p21 and Kip1/p27 proteins were immunoprecipitated from whole-cell lysates using specific antibodies (4  $\mu\text{g}$ ) after incubation for 8 hours, followed by the addition of protein A/G-plus agarose beads (50  $\mu\text{L}$ ; Santa Cruz Biotechnology), and incubation was continued overnight



**Figure 2.** Effect of berberine on cell cycle progression of human prostate carcinoma cells. Cells were cultured in complete medium and treated either with vehicle (0.2% ethanol in medium) or 25, 50, or 100  $\mu\text{mol/L}$  berberine. After 48 h of treatment, cells were harvested, washed with cold PBS buffer, and digested with RNase. Cellular DNA was stained with propidium iodide and flow cytometric analysis was done to determine the cell cycle distribution as described in the Materials and Methods. **A**, cell cycle distribution in DU145 cells after treatment with different doses of berberine. **B**, a summary of cell cycle distribution data in **A**. **C** to **E**, summaries of cell cycle distribution data for similarly treated LNCaP, PC-3, and PWR-1E cells. *Columns*, mean of three independent experiments; *bars*, SE. #,  $P < 0.05$ ; †,  $P < 0.01$ ; \*,  $P < 0.001$ , versus non-berberine-treated control group.

at 4°C. Immunoprecipitates were washed with lysis buffer and subsequently subjected to SDS-PAGE on 12% gels followed by immunoblotting using Cdk2, Cdk4, and Cdk6 antibodies.

#### Measurement of Mitochondrial Membrane Potential

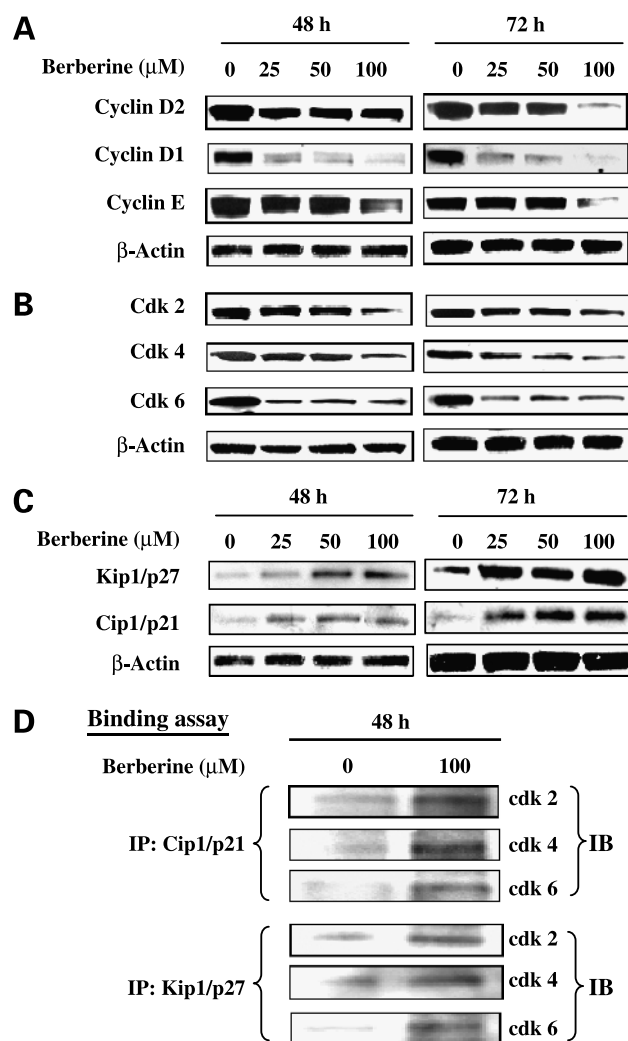
The mitochondrial membrane potential was determined quantitatively by flow cytometry using the fluorescent lipophilic cationic probe JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) Detection Kit following the instructions of the manufacturer (Molecular Probes). JC-1 is selectively concentrated or accumulates within intact mitochondria to form multimer J-aggregates emitting fluorescence light at 590 nm. The monomeric form emits light at 527 nm after excitation at 490 nm. Thus, the color of the dye changes from orange to green, depending on the mitochondrial membrane potential, and can be analyzed by FACS with green fluorescence in channel 1 (FL1) and orange emission in channel 2 (FL2). Briefly, semiconfluent DU145 cells were treated with berberine (0, 25, 50, and 100 µmol/L) for 48 hours, harvested, and washed with PBS;  $1 \times 10^6$  cells were incubated in 1 mL PBS containing 10 µg JC-1 for 15 minutes at 37°C in the dark. Stained cells were washed, resuspended in 500 µL PBS, and used for immediate FACS analysis.

#### Caspase-3 Activity Assay

The activity of caspase-3 was measured using the colorimetric protease assay ApoTarget Kit (BioSource International, Inc., CA) following the protocol of the manufacturer. Briefly, the cells were treated with berberine (25 or 50 µmol/L) with or without pan-caspase inhibitor (z-VAD-fmk). The z-VAD-fmk (60 µmol/L) was added 2 hours before the addition of the berberine. After 48 hours, the cells were harvested using trypsinization and cell lysates prepared as described (19). Samples of the cell lysates (100 µg protein per sample) were mixed with reaction buffer and 200 µmol/L substrate (DEVD-pNA for caspase-3) and incubated for 3 hours at 37°C in the dark. The assay conditions were standardized such that the products of the reaction remained in the linear range of detection. The absorbance was then measured at 405 nm and the sample readings calculated by subtracting the absorbance of blank samples.

#### Measurement of DNA Damage by the Comet Assay

Berberine-induced DNA damage was determined using the comet assay. Cells were treated with berberine (0, 25, and 50 µg/mL) for 48 hours in complete medium, and the comet assay was done as described earlier (21). Briefly, after treatment with berberine and pan-caspase inhibitor, the cells were harvested and resuspended in ice-cold PBS. Approximately  $1 \times 10^4$  cells in a volume of 75 µL of 0.5% (w/v) low-melting-point agarose were pipetted onto a frosted glass slide coated with a thin layer of 1.0% (w/v) agarose, covered with a coverslip, and allowed to set on ice for 10 minutes. Following removal of the coverslip, the slides were immersed in ice-cold lysis solution containing 2.5 mol/L NaCl, 10 mmol/L Tris, 100 mmol/L Na<sub>2</sub>-EDTA, and 1% (w/v) *N*-lauroyl-



**Figure 3.** Effect of berberine on G<sub>1</sub> cell cycle regulatory proteins in DU145 cells. The DU145 cells were cultured in complete medium and treated either with vehicle or 25, 50, and 100 µmol/L berberine for 48 or 72 h, then subjected to SDS-PAGE followed by Western blot analysis, as described in Materials and Methods, with antibodies for cyclin D1, cyclin D2, and cyclin E (A); Cdk2, Cdk4, and Cdk6 (B); or Kip1/p27 and Cip1/p21 (C). β-Actin was used to verify equal gel loading. Representative blots from three independent experiments with identical observations. D, Cip1/p21 and Kip1/p27 were immunoprecipitated followed by Western blot analysis for Cdk2, Cdk4, and Cdk6 as detailed in Materials and Methods. IB, immunoblotting; IP, immunoprecipitation.

sarcosine, adjusted to pH 10.0, and 1.0% Triton X-100 was added immediately before use. After 2 hours at 4°C, the slides were placed into a horizontal electrophoresis tank filled with buffer [0.3 mol/L NaOH, 1 mmol/L EDTA (pH 13)] and subjected to electrophoresis for 30 minutes at 300 mA. Slides were transferred to neutralization solution (0.4 mol/L Tris-HCl) for 3 × 5-minute washes and stained with ethidium bromide for 5 minutes. After a final wash in double-distilled water, the gels were covered with glass coverslips. Slides were viewed using the 20× objective of a Zeiss Axioskop

microscope equipped with epifluorescence optics. For each sample, the tail lengths (in micrometers) of a minimum of 50 comets were analyzed. The length of the comet was quantified as the distance from the centrum of the cell nucleus to the tip of the tail in pixel units and the tail length was expressed as a mean  $\pm$  SE from 50 comets.

#### Statistical Analysis

The statistical significance of difference in between control and treated groups was determined by paired *t* test or one-way ANOVA followed by Bonferroni's multiple comparison tests.  $P < 0.05$  was considered statistically significant.

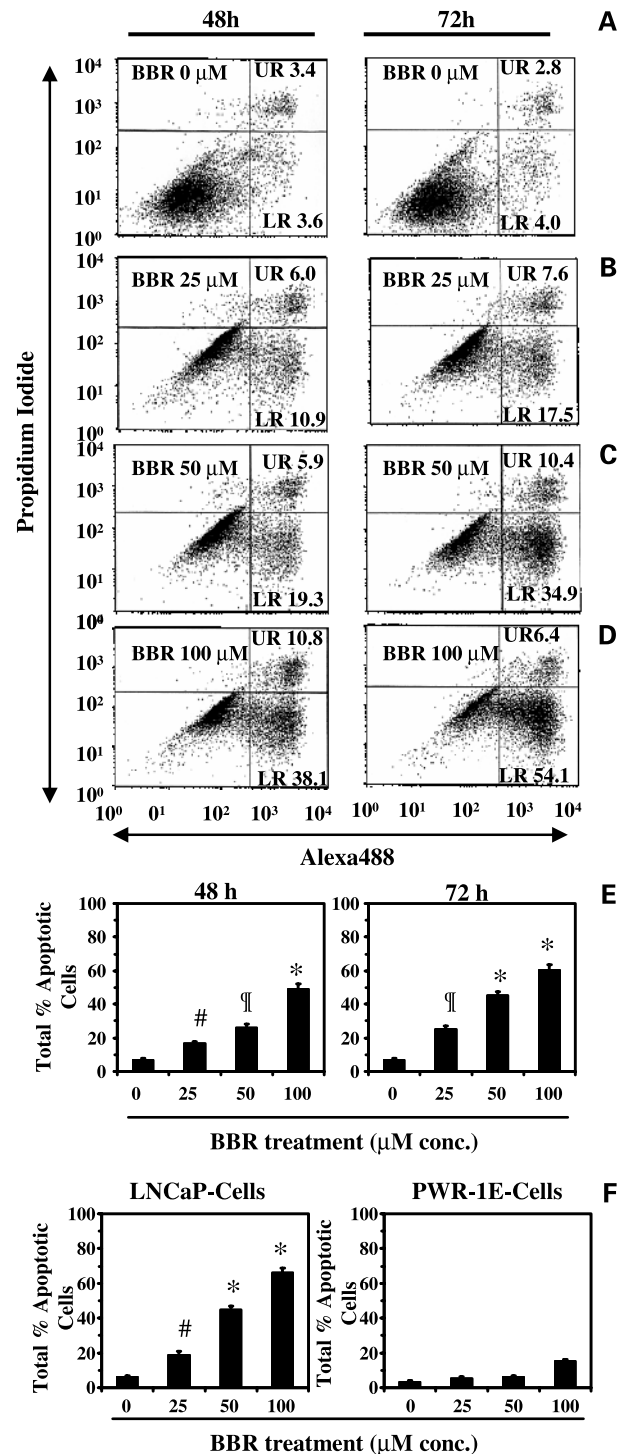
### Results

#### Berberine Inhibits Proliferation and Viability and Induces the Death of Prostate Cancer Cells but not of Normal Prostate Epithelial Cells

We first determined the antiproliferative effects of berberine on human prostate carcinoma cells, including androgen-sensitive (LNCaP) and androgen-insensitive (DU145 and PC-3) cells, as well as normal human prostate epithelial cells (PWR-1E). The cells were treated with 0, 10, 25, 50, 75, and 100  $\mu\text{mol/L}$  berberine for 24, 48, and 72 hours. The treatment of DU145 cells with berberine (10–100  $\mu\text{mol/L}$ ) resulted in a significant reduction in cell proliferation/viability as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, ranging from 10.0% to 40% ( $P < 0.05$ –0.01) after 24 hours, 22% to 75% ( $P < 0.05$ –0.001) after 48 hours, and 41% to 80% ( $P < 0.01$ –0.001) after 72 hours of treatment (Fig. 1B, left). Similar effects were obtained on treatment of PC-3 and LNCaP cells (Fig. 1C and D, left) except that berberine affected the PC-3 cells more rapidly than the DU145 and LNCaP cells, with the reduction in viability that was achieved in these cells by 24 hours approaching the reduction, which was approximately achieved in the other cell lines after 72 hours, especially at the higher doses. In contrast, the sensitivity of the PWR-1E cells to the cytotoxic effects of berberine was much lower, with berberine only having a significant effect on the viability (25% reduction,  $P < 0.05$ ) of the PWR-1E cells at the higher doses (100  $\mu\text{mol/L}$ ) and longer treatment times (72 hours). Thus, these data suggest

that berberine does have a cytotoxic effect on prostate tumor cells, having an equal effect on androgen-sensitive and androgen-insensitive cells, but is not cytotoxic to normal prostate epithelial cells.

In additional separate experiments, we determined the effect of berberine on the viability of the human prostate



**Figure 4.** Berberine induces apoptosis in human prostate carcinoma DU145 and LNCaP cells but not in normal human prostate epithelial PWR-1E cells. **A to D**, cells were treated with varying concentrations of berberine, 0  $\mu\text{g/mL}$  (**A**), 25  $\mu\text{g/mL}$  (**B**), 50  $\mu\text{g/mL}$  (**C**), and 100  $\mu\text{g/mL}$  (**D**), for 48 h (left) and 72 h (right), then harvested for analysis of apoptosis using the Annexin V-Alexa Fluor 488 (Alexa488) Apoptosis Vybrant Assay Kit as detailed in Materials and Methods. Lower right (LR) quadrant, percentage of early apoptotic cells (Alexa488-stained cells); upper right (UR) quadrant, percentage of late apoptotic cells (Alexa488 + propidium iodide-stained cells). **E** and **F**, total percent of apoptotic cells summarized after 48 and 72 h of berberine treatment for DU145 cells (**E**) and after 48 h of berberine treatment for LNCaP (**F**, left) and PWR-1E (**F**, right) cells. Columns, mean of three experiments; bars, SE. #,  $P < 0.05$ ; ¶,  $P < 0.01$ ; \*,  $P < 0.001$ , versus control.

cancer cell lines using the trypan blue dye exclusion assay. Treatment of DU145, PC-3, and LNCaP cells with berberine at concentrations of 25, 50, 75, and 100  $\mu\text{mol/L}$  for 24, 48, and 72 hours resulted in significant cell death (Fig. 1, *right*). As shown in Fig. 1B (*right*), when compared with control-treated cells, treatment of DU145 cells with berberine (25, 50, 75, and 100  $\mu\text{mol/L}$ ) resulted in a 6% to 19% ( $P < 0.05$ ) increase in cell death at 24 hours, a 9% to 24% ( $P < 0.05$ ) increase at 48 hours, and a 10% to 44% ( $P < 0.05$ – $0.001$ ) increase in cell death at 72 hours. The PC-3 and LNCaP cells exhibited an almost identical reduction in viability as the DU145 cells under these conditions (Fig. 1C and D, *right*). Further, we tested whether berberine has any toxic effect on nonneoplastic human prostate epithelial PWR-1E cells. Interestingly, we did not find significant cytotoxicity or cell death by berberine on PWR-1E cells; however, a marked increase in cell death (15%,  $P < 0.05$ ) in PWR-1E cells was observed only at the maximum concentration of berberine (100  $\mu\text{mol/L}$ ) used in this study and after the maximum duration of treatment (72 hours; Fig. 1E, *right*). Moreover, the berberine-induced death of PWR-1E cells at this dose and time point was significantly less ( $P < 0.001$ ) than the effects of the same dose of berberine on prostate cancer cells at the same time point (Fig. 1B–D, *right*). Thus, berberine seems to be capable of exerting a cytotoxic effect on prostate cancer cells without incurring cytotoxicity to normal prostate epithelial cells under the present experimental conditions.

#### Berberine Induces G<sub>1</sub>-Phase Cell Cycle Arrest in Prostate Cancer Cells

Based on the preliminary assays in which we determined the effects of berberine on cell proliferation and viability, we selected doses of 25, 50, and 100  $\mu\text{mol/L}$  for further *in vitro* mechanistic studies. As we found a significant growth inhibitory effect of berberine on prostate cancer cells, we determined the possible inhibitory effect of berberine on cell cycle progression. As summarized in Fig. 2A, treatment of DU145 cells with berberine for 48 hours resulted in a significantly higher number of cells in the G<sub>1</sub> phase at the concentrations used, 25  $\mu\text{mol/L}$  (52%), 50  $\mu\text{mol/L}$  (59%,  $P < 0.01$ ), and 100  $\mu\text{mol/L}$  (67%,  $P < 0.001$ ), compared with nonberberine-treated control (46%). As, in each case, there was a concomitant reduction in the number of cells in the S and G<sub>2</sub>-M phases, this experiment suggested that berberine induces G<sub>1</sub>-phase cell cycle arrest in DU145 cells. Similar but slightly more pronounced results were obtained when the effects of berberine on DU145 cells were determined at 72 hours (data not shown). Similar results were obtained on analysis of the effects of berberine treatment on cell cycle progression of LNCaP (Fig. 2C) and PC-3 (Fig. 2D) cells although, in the case of PC-3 cells, the highest dose of berberine used (100  $\mu\text{mol/L}$ ) did not induce G<sub>1</sub> arrest but rather a significant accumulation of cells in the G<sub>2</sub>-M phase ( $P < 0.05$ ). Treatment of the PWR-1E cells with berberine at doses of 25 and 50  $\mu\text{mol/L}$  for 24 hours did not result in induction of cell cycle arrest as compared with cells that were not treated with berberine, although a

nonsignificant higher level of G<sub>1</sub> arrest was observed in PWR-1E cells treated with the highest dose of berberine (100  $\mu\text{mol/L}$ ). These data suggest that inhibition of cell proliferation or induction of cell death in both androgen-sensitive and androgen-insensitive prostate cancer cells by berberine may be associated with the induction of G<sub>1</sub> arrest, and that this effect of berberine occurs in cancer cells but not in normal cells.

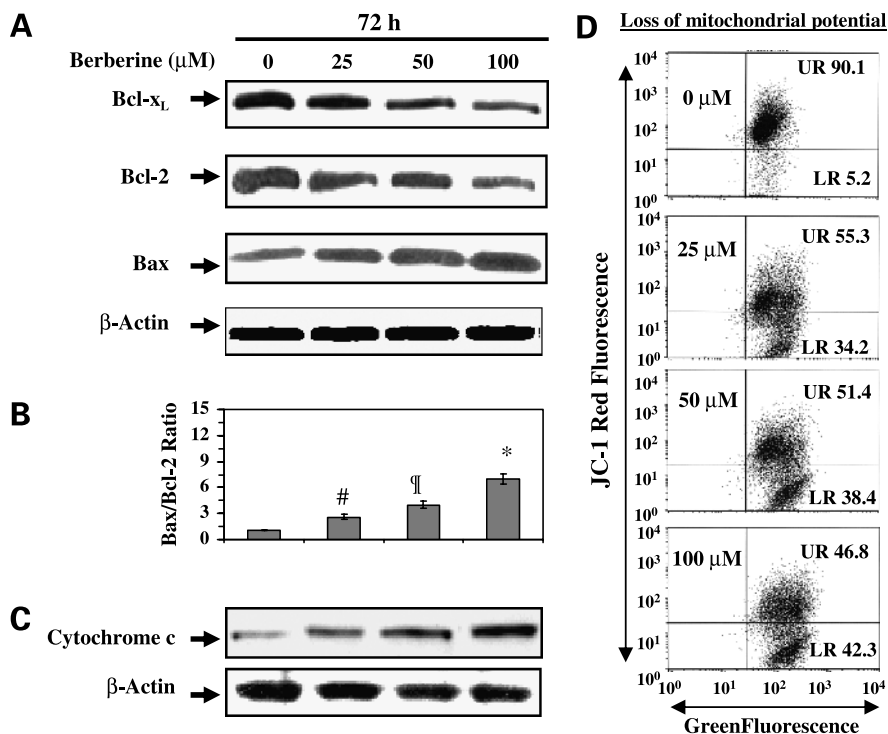
#### Berberine Down-Regulates Cyclins and Cdks and Up-Regulates Cip1/p21 and Kip1/p27 in DU145 Cells

As it has been shown that Cdks, Cdk inhibitors, and cyclins play essential roles in the regulation of cell cycle progression (22), we examined the effect of berberine on the expression of these cell cycle regulatory proteins. We selected DU145 cells for these mechanistic studies as berberine seemed to be almost equally effective in suppressing the growth and inducing G<sub>1</sub>-phase arrest of cell cycle progression in DU145, PC-3, and LNCaP cells. As shown in Fig. 3, treatment with berberine resulted in a marked reduction in the expression of cyclins D1, D2, and E (Fig. 3A) in a dose-dependent manner after 48 and 72 hours of treatment. Importantly, cyclin D1 was the most potently inhibited cyclin by berberine in tumor cells. Similarly, a pronounced reduction in the expression of Cdk2, Cdk4, and Cdk6 was observed at 48 and 72 hours (Fig. 3B), with the reduction in the expression of Cdk6 being more pronounced than the reduction in expression of either Cdk2 or Cdk4. The levels of these regulatory proteins in the berberine-treated cells did not differ from the levels in the control cells when the treatment was limited to 24 hours (data not shown).

The Cdk inhibitors Cip1/p21 and Kip1/p27 regulate the progression of cells in the G<sub>0</sub>-G<sub>1</sub> phase of the cell cycle and induction of these proteins causes a blockade of the G<sub>1</sub> to S transition, thereby resulting in a G<sub>0</sub>-G<sub>1</sub> phase arrest of the cell cycle (23). It has been shown that a loss of functional Cdk inhibitors in human cancers can lead to uncontrolled cell proliferation due to an increase in the levels of the Cdk-cyclin complex (24). Analysis of the expression of Kip1/p27 and Cip1/p21 by Western blot analysis indicated that berberine treatment of DU145 cells for 48 and 72 hours resulted in a dose-dependent enhancement of their expression (Fig. 3C). These changes were not due to differences in the amounts of proteins loaded on the gels as the equivalent protein loading was confirmed by probing stripped blots for  $\beta$ -actin as shown.

The Cdk inhibitory proteins suppress cell cycle progression by binding to and inhibiting the kinase activity of the Cdk-cyclin complex (22, 25, 26). Therefore, we next assessed whether berberine promotes the interaction between Cdk inhibitor and Cdk. To assess this effect, Cip1/p21 and Kip1/p27 were immunoprecipitated from total cell lysates and their binding of Cdk2, Cdk4, and Cdk6 was assessed using Western blotting. As compared with vehicle-treated controls, treatment with berberine was found to enhance the binding of Cdk2, Cdk4, and Cdk6 with Cip1/p21 and Kip1/p27 (Fig. 3D). These observations suggest that the berberine-induced enhancement of the levels of Cdk inhibitors plays an important role in the

**Figure 5.** **A**, *in vitro* treatment of DU145 cells with berberine for 72 h results in a dose-dependent reduction in the expression of antiapoptotic proteins Bcl- $x_L$  and Bcl-2 while increasing the expression of the proapoptotic protein Bax as estimated by Western blot analysis. **B**, treatment of DU145 cells with berberine significantly increases the Bax/Bcl-2 protein ratio. *Columns*, mean of three independent experiments; *bars*, SE. #,  $P < 0.05$ ; †,  $P < 0.01$ ; \*,  $P < 0.001$ . **C**, berberine also induces the release of cytochrome *c* from mitochondria. At the end of the treatment period, cell lysates were prepared and Western blot analysis was done to determine the expression of different proteins as detailed in Materials and Methods. A representative blot is shown from three independent experiments with identical observations, and equivalent protein loading was confirmed by probing stripped blots for  $\beta$ -actin as shown. **D**, berberine induces dose-dependent loss of mitochondrial membrane potential in DU145 cells. Cells were treated with berberine (0, 25, 50, and 100  $\mu\text{mol/L}$ ) for 72 h, then harvested and stained with JC-1 probe and analyzed by flow cytometry as described in Materials and Methods. *Lower right quadrant*, the percentage of cells that emit only green fluorescence can be attributed to depolarized mitochondrial membrane.



berberine-induced inhibition of  $G_1$  arrest of cell cycle progression in androgen-insensitive human prostate carcinoma DU145 cells, possibly through their inhibition of Cdk kinase activity.

#### Berberine Induces Apoptosis in Both Androgen-Sensitive (LNCaP) and Androgen-Insensitive (DU145) Human Prostate Carcinoma Cells

To determine whether the berberine-induced loss of the proliferation capacity and cell viability of the prostate cancer cells was associated with the induction of apoptosis, DU145 and LNCaP cells were treated with berberine as described above and the numbers of apoptotic cells were assessed using the Annexin V-conjugated Alexa Fluor 488 (Alexa488) Apoptotic Detection Kit as previously described (19). Apoptotic cells were counted as late or early apoptotic cells, which are shown respectively in the upper right and lower right quadrants of the histograms presented in Fig. 4 (27). After 24 hours of treatment, the berberine-induced apoptosis of DU145 and LNCaP cells was not significantly greater than that of vehicle-treated controls (data not shown). Berberine treatment of DU145 cells for 48 hours resulted in a significant dose-dependent enhancement in the number of apoptotic cells at both the early and late stages of apoptosis (Fig. 4A–D): 0  $\mu\text{mol/L}$  (vehicle control, 7%), 25  $\mu\text{mol/L}$  (16.9%,  $P < 0.05$ ), 50  $\mu\text{mol/L}$  (25.2%,  $P < 0.01$ ), and 100  $\mu\text{mol/L}$  (48.9%,  $P < 0.001$ ). Similar results were obtained on berberine treatment of DU145 cells for 72 hours except that the percentages of apoptotic cells were higher (Fig. 4A–D, *right*). The total percent of apoptotic cells in DU145 cells caused by berberine treatments after 48 and 72 hours is summarized in Fig. 4E. Treatment of

LNCaP cells with berberine for 48 hours also resulted in a significant dose-dependent induction of apoptosis: 0  $\mu\text{mol/L}$  (vehicle control), 6  $\pm$  1%; 25  $\mu\text{mol/L}$ , 19  $\pm$  2% ( $P < 0.05$ ); 50  $\mu\text{mol/L}$ , 45  $\pm$  2% ( $P < 0.001$ ); and 100  $\mu\text{mol/L}$ , 66  $\pm$  3% ( $P < 0.001$ ; Fig. 4F, *left*), again indicating that berberine is equally effective in androgen-insensitive and androgen-sensitive cells. Berberine treatment of PWR-1E cells for 48 hours did not result in significant enhancement of apoptosis of PWR-1E cells (Fig. 4F, *right*). Although a slightly higher number of apoptotic cells was observed on treatment of these cells with 100  $\mu\text{mol/L}$  berberine for 48 hours, this did not reach significance and was significantly less ( $P < 0.001$ ) than the levels of apoptosis of DU145 and LNCaP cells induced by treatment with the same concentration of berberine for the same time period. This suggests that at least under the experimental conditions used in this study, berberine is not toxic to normal prostate epithelial cells.

#### Berberine Treatment Differentially Affects the Levels of Bcl-2 Family Proteins in DU145 Cells

The proteins of the Bcl-2 family play critical roles in the regulation of apoptosis by functioning as promoters (e.g., Bax) or inhibitors (Bcl-2 or Bcl- $x_L$ ) of cell death process (28–31). As the levels of berberine-induced apoptosis in DU145 and LNCaP cells were almost identical, we selected DU145 cells for further analysis of the mechanisms underlying berberine-induced apoptosis. Because berberine-induced maximum cell death, apoptosis, and changes in cell cycle regulatory proteins were found at 72 hours after its treatment, we selected this time point for further mechanistic studies. We first used Western blotting to



detect Bcl-x<sub>L</sub>, Bcl-2, and Bax in the cells treated with berberine (0, 25, 50, and 100 μmol/L) for 72 hours (Fig. 5A). This revealed that treatment of DU145 cells with berberine resulted in a dose-dependent reduction in the levels of the antiapoptotic proteins Bcl-x<sub>L</sub> and Bcl-2 with a concomitant increase in the levels of proapoptotic protein Bax compared with the cells that were not treated with berberine (Fig. 5A). Stripping and reprobing of the blots for actin expression confirmed equal sample loading in all the blots. Thus, berberine treatment can alter the protein levels of key members of the Bcl-2 family in a manner that favors an increase in the ratio of Bax/Bcl-2, which may contribute to the susceptibility of cancer cells to berberine-induced apoptosis (Fig. 5B; refs. 3, 29).

#### **Berberine Induces the Disruption of Mitochondrial Membrane Potential and Increases the Release of Cytochrome *c***

An early event in apoptosis is the disruption of the mitochondrial membrane potential. This event, which can be induced by a variety of stimuli (32, 33) including translocation of Bax from the cytosol to the mitochondria, triggers release of cytochrome *c* and other apoptogenic molecules from the mitochondria to the cytosol (34, 35). In turn, these contribute to the activation of caspases and subsequent cell death. To evaluate the effects of berberine on the release of mitochondrial cytochrome *c* into the cytosol, cellular subfractions were prepared from DU145 cells that had been treated with berberine (25, 50, and 100 μmol/L) for 72 hours. Western blot analysis revealed that berberine resulted in a dose-dependent release of cytochrome *c* into the cytosol (Fig. 5C), which suggests disruption of the mitochondrial membrane potential. To verify that berberine induces disruption of the mitochondrial membrane potential, we labeled the cells with the cationic lipophilic dye JC-1, which accumulates within mitochondria in a potential-dependent manner. On disruption of the mitochondrial membrane potential, the fluorescence emission of JC-1 changes from orange to green. As shown in Fig. 5D, treatment of DU145 cells with berberine for 72 hours resulted in a dose-dependent increase in the number of green fluorescence-positive cells from 5.2% in non-berberine-treated cells to 34.2%, 38.4%, and 42.3% on treatment with 25, 50, and 100 μmol/L berberine, respectively, thus confirming the disruption of the mitochondrial membrane potential on berberine treatment.

#### **Berberine Induces the Activation of Caspase-3 and Poly(ADP-ribose) Polymerase: Relationship with the Induction of Apoptosis and DNA Damage**

Once in the cytosol, cytochrome *c* binds to Apaf-1 and recruits and activates procaspase-9 in the apoptosome (32, 33, 36, 37), active caspase-9 cleaves and activates executioner caspases, including caspase-3 (36, 37), which cleave a broad spectrum of cellular target proteins, including poly(ADP-ribose) polymerase, thus leading to cell death (38, 39). Therefore, we determined the effect of berberine on the activation of caspase-9, caspase-3, and poly(ADP-ribose) polymerase. Treatment of DU145 cells

with berberine (0, 25, 50, and 100 μmol/L) for 72 hours resulted in a dose-dependent increase in the cleavage of caspase-9, caspase-3, and poly(ADP-ribose) polymerase when compared with the cells which were not treated with berberine (Fig. 6A). The membranes were also checked for the level of β-actin as a loading control. The role of caspase-3 activation in berberine-induced apoptosis was further confirmed using a colorimetric caspase-3 activity assay. Treatment of DU145 cells with berberine for 72 hours resulted in a dose-dependent increase in caspase-3 activity (data not shown). Further, we observed that caspase-3 activity was significantly higher in berberine-treated than in vehicle-treated DU145 cells (Fig. 6B). Treatment of these cells with berberine plus the pan-caspase inhibitor (z-VAD-fmk) for the same period of time resulted in a significant decrease in caspase-3 activity ( $P < 0.05$ ). Thus, these data suggest that caspase-3 activation is involved in the berberine-induced apoptosis of DU145 cells.

#### **Pan-caspase Inhibitor Blocks Berberine-Induced Apoptosis in Prostate Cancer Cells**

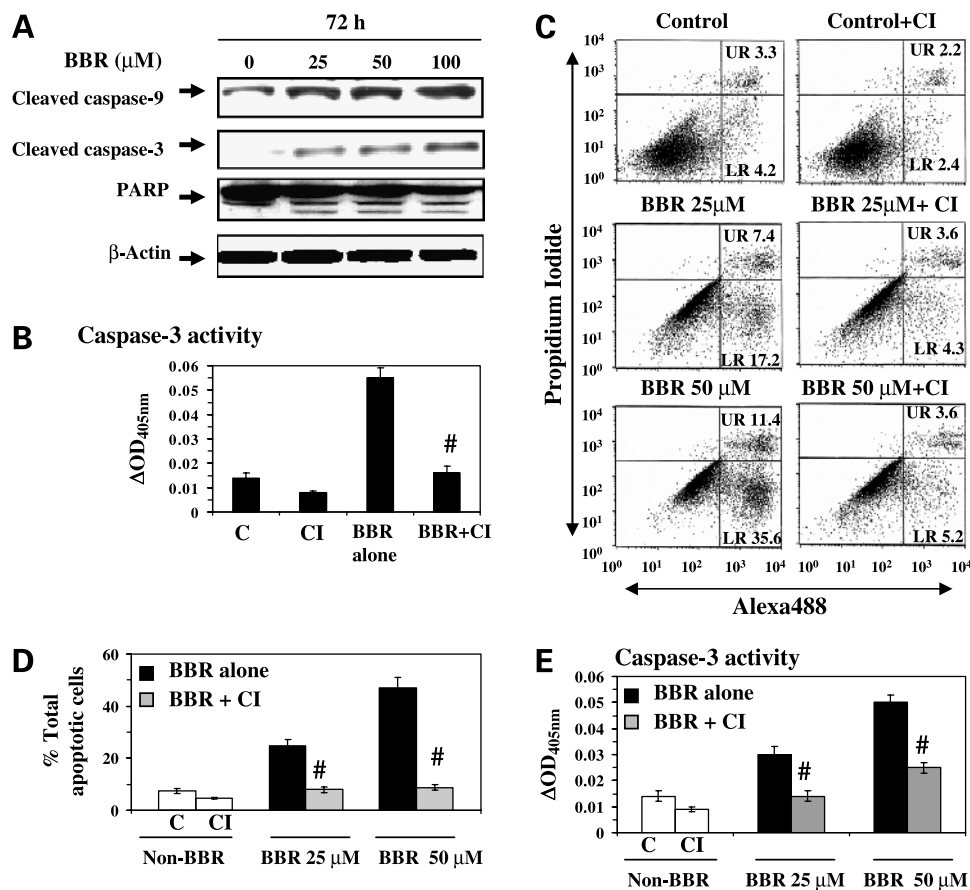
As the addition of the pan-caspase inhibitor (z-VAD-fmk) inhibited berberine-induced caspase-3 activation, we sought to determine whether the induction of apoptosis by berberine also is reduced or blocked by this inhibitor. DU145 cells that had been treated with berberine, with or without z-VAD-fmk (60 μmol/L) for 72 hours, were stained using the Alexa488 Apoptosis Detection Kit as detailed previously (19). In the absence of the inhibitor, berberine treatment resulted in a dose-dependent increase in apoptosis of DU145 cells: 0 μmol/L (vehicle control), 7.5%; 25 μmol/L, 24.6%; and 50 μmol/L, 47.0% (Fig. 6C). In the presence of the pan-caspase inhibitor (z-VAD-fmk), the berberine-induced apoptosis was reduced significantly: 25 μmol/L, 80% ( $P < 0.001$ ); 50 μmol/L, 89% ( $P < 0.001$ ; Fig. 6C and D). Taken together, the results indicate that berberine-induced apoptosis in DU145 cells is mediated primarily through the activation of caspases. These results were further confirmed by caspase-3 activation assay. Preincubation of cells with z-VAD-fmk resulted in a significant reduction of berberine-induced increase in caspase-3 activity, as shown in Fig. 6E. Finally, we determined the effect of berberine on cellular DNA damage using the comet assay, which was used as a biomarker of apoptosis. As shown in Fig. 7A and B, treatment of DU145 cells with berberine (25 and 50 μmol/L) for 72 hours resulted in significant DNA damage ( $P < 0.001$ ) compared with cells that were not treated with berberine, as estimated by measuring the length of the comet. Treatment of DU145 cells with the pan-caspase inhibitor (z-VAD-fmk) together with berberine for 72 hours blocked the DNA damage in cells compared with the cells which were not treated with pan-caspase inhibitor but treated only with berberine. DNA damaging effect in terms of DNA fragmentation was determined by measuring the tail length of the comet under a microscope. The data of tail lengths in micrometers were represented as mean ± SE from at least 50 DNA-damaged cells in each treatment group (Fig. 7B).

## Discussion

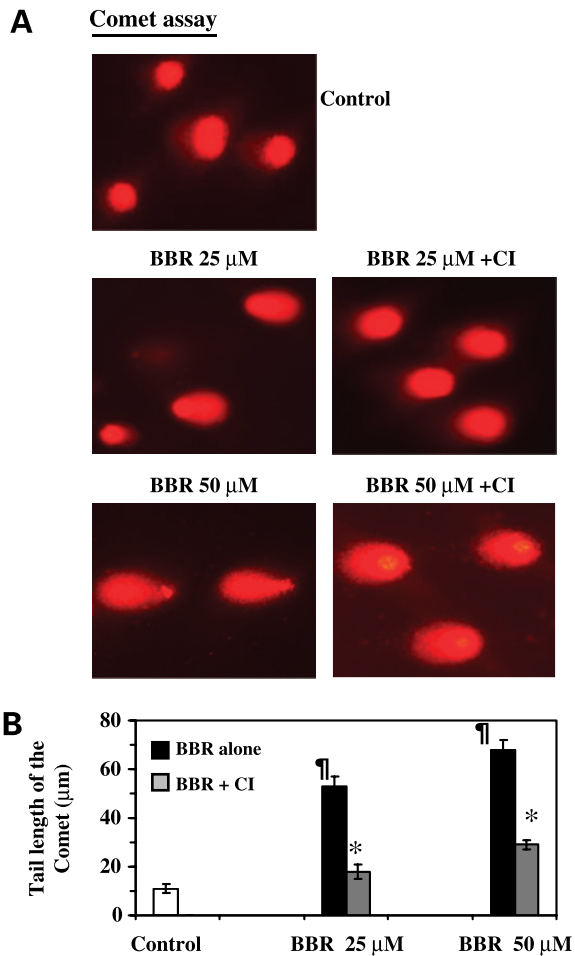
The evaluation of ancient herbal medicines may indicate novel strategies for the treatment of prostate cancer, which remains the leading cause of cancer-related deaths in American men (1). In our present investigation, we show that a naturally occurring isoquinoline alkaloid, berberine, significantly inhibits the proliferation and reduces the viability of DU145 and PC-3 as well as LNCaP cells (Fig. 1), which suggests that berberine may be an effective chemotherapeutic agent against both androgen-sensitive and androgen-insensitive prostate cancer cells. Importantly, we found that berberine did not exhibit toxicity to nonneoplastic human prostate

epithelial cells under the conditions used, except for a moderate reduction in cell viability at higher concentrations when cells were treated *in vitro* for an extended period of time.

Control of cell cycle progression in cancer cells is considered to be a potentially effective strategy for the control of tumor growth (22, 23) as the molecular analyses of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies (40, 41). Our *in vitro* data indicated that treatment of both androgen-sensitive (LNCaP) and androgen-insensitive (DU145, PC-3) cells with berberine resulted in significant G<sub>1</sub>-phase arrest of cell cycle progression, which indicates



**Figure 6.** *In vitro* treatment of DU145 cells with berberine increases the cleavage of caspase-9, caspase-3, and poly(ADP-ribose) polymerase (PARP). **A**, DU145 cells were treated with varying concentrations of berberine (0, 25, 50, and 100 μg/mL) for 72 h, then cells were harvested and samples were prepared for analysis of the cleavage of caspase-9, caspase-3, and poly(ADP-ribose) polymerase using Western blot analysis as detailed in Materials and Methods. Equal protein loading was confirmed by probing stripped blots for β-actin as shown. Representative blot from three independent experiments with very similar results. **B**, berberine (50 μmol/L)–induced activity of caspase-3 in DU145 cells was determined after 72 h of treatment in the absence or presence of 60 μmol/L of pan-caspase inhibitor (z-VAD-fmk). The inhibitor was added to the cells 2 h before treatment with berberine. The activity of caspase-3 in cell lysates from different treatment groups was determined using a colorimetric protease assay (ApoTarget Kit). Columns, mean absorbance from three independent experiments as a measure of caspase-3 activity; bars, SE. C, control (non-berberine); BBR, berberine; CI, pan-caspase inhibitor. **C**, the effect of berberine (25 and 50 μmol/L) on apoptosis of DU145 cells was determined after 72 h of treatment in the absence or presence of 60 μmol/L of pan-caspase inhibitor (z-VAD-fmk). The selected dose of pan-caspase inhibitor was standardized in trial experiments to achieve >90% inhibition in apoptosis. Percent of apoptotic cells in different treatment groups (as shown in figure) was analyzed by flow cytometry using Annexin V-Alexa Fluor 488 Apoptotic Vybrant Assay Kit as detailed in Materials and Methods. **D**, summary of the total number of apoptotic cells in each treatment group in **C**. Columns, mean from three independent experiments; bars, SE. **E**, the caspase-3 activity in cell lysates from the experiment in **C** was measured using a colorimetric protease assay (ApoTarget Kit), which reflects that treatment of berberine with pan-caspase inhibitor blocked berberine-induced caspase-3 activity in DU145 cells. #,  $P < 0.001$ , versus berberine alone.



**Figure 7.** **A**, treatment of DU145 cells with berberine (25 and 50  $\mu\text{mol/L}$ ) for 72 h induced DNA damage, which was measured using the comet assay. The treatment of DU145 cells with berberine plus pan-caspase inhibitor (z-VAD-fmk) blocked berberine-induced DNA damage in these cells, which may result in inhibition of berberine-induced apoptosis. The comet assay was used to determine the DNA damage by berberine in DU145 cells in the form of DNA fragmentation as detailed in Materials and Methods. **B**, the tail of the comet was measured in each cell under a microscope and expressed in micrometers. *Columns*, mean from at least 50 cells in each treatment group; *bars*, SE. <sup>†</sup>,  $P < 0.001$ , significant increase in tail length versus control. \*,  $P < 0.001$ , significant decrease in tail length versus berberine treatment alone.

that one of the mechanisms by which berberine may act to inhibit the proliferation of cancer cells is inhibition of cell cycle progression. Notably, this effect was not seen in a normal prostate epithelial cell line (Fig. 2). Our finding of a significant decrease in cyclins D1, D2, and E and Cdk2, Cdk4, and Cdk6 in DU145 cells on berberine suggests the disruption of the uncontrolled cell cycle progression of these cells (Fig. 3) and that the berberine-induced  $G_1$  arrest is mediated through the up-regulation of Cip1/p21 and Kip1/p27 proteins, which enhances the formation of heterotrimeric complexes with the  $G_1$ -S Cdk and cyclins thereby inhibiting their activity (Fig. 3D). Based on the data (Figs. 2 and 3), it seems that cyclin D1 and Cdk6

are responsible for most of the cell cycle arrest observed in response to berberine because these regulators are effectively inhibited at the lowest dose of berberine (25  $\mu\text{mol/L}$ ). Kip1/p27 is up-regulated in response to antiproliferative signals (42). The increased expression of  $G_1$  cyclins in cancer cells provides an uncontrolled growth advantage because most of these cells either lack Cdk inhibitors or the expression of Cdk inhibitors is not at a sufficient level to control Cdk-cyclin activity (26). Similar to DU145, the treatment of PC-3 and LNCaP cells with berberine resulted in identical effects of  $G_1$ -phase arrest of cell cycle progression, but berberine did not affect the cell cycle progression machinery in PWR-1E cells (Fig. 2C–E). Further studies are needed to examine the possibility that p53 status influences cell cycle arrest by berberine as well as the chemotherapeutic effect of berberine on cell cycle regulatory checkpoints in PC-3 and LNCaP cells.

$G_1$ -phase arrest of cell cycle progression provides an opportunity for cells to either undergo repair mechanisms or follow the apoptotic pathway. In the case of advanced prostate cancer, cancer cells become resistant to apoptosis and do not respond to the cytotoxic effects of most of the available chemotherapeutic agents (43). Therefore, identification of agents that can induce apoptosis in hormone-refractory prostate cancer cells is of high priority. We therefore determined the effect of berberine on the induction of apoptosis in both DU145 and LNCaP cells. Our flow cytometry data indicate that treatment of DU145 and LNCaP cells with berberine resulted in significant induction of apoptosis and that this effect was not seen in normal prostate epithelial PWR-1E cells (Fig. 4). Apoptosis plays a crucial role in eliminating the mutated neoplastic and hyperproliferating neoplastic cells from the system and therefore is considered as a protective mechanism against cancer progression (44). Acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer. Therefore, berberine seems to be a potent chemotherapeutic agent for prostate cancer inhibition.

Apoptosis is tightly regulated by antiapoptotic and proapoptotic effector molecules, including proteins of the Bcl-2 family, and can be mediated by several different pathways. As the activity of the regulatory molecules can be lost in cancer cells or be affected by other chemotherapeutic drugs, it is important to elucidate the mechanisms by which antiapoptotic drugs exert their effects, especially in androgen-unresponsive DU145 cells. Therefore, we investigated the contribution of Bcl-2 family proteins to berberine-induced apoptosis of prostate cancer cells and, in particular, androgen-unresponsive DU145 cells. The proteins of the Bcl-2 family either promote cell survival (e.g., Bcl-2 and Bcl-x<sub>1</sub>) or induce programmed cell death (e.g., Bax; refs. 45, 46). The ratio of Bax/Bcl-2 is critical for the induction of apoptosis and this ratio determines whether cells will undergo apoptosis (3, 29). An increase in the ratio of Bax/Bcl-2 stimulates the release of cytochrome *c* from mitochondria into the cytosol. The cytosolic cytochrome *c* then binds to Apaf-1, leading to the activation of caspase-3 and poly(ADP-ribose) polymerase (38, 39). We found that

treatment of DU145 cells with berberine resulted in an increase in the expression of Bax protein and a decrease in the expression of Bcl-2 and Bcl-x<sub>L</sub> (Fig. 5A) and increased the ratio of Bax/Bcl-2 (Fig. 5B). This may be responsible for the concomitant execution phase of apoptosis that we observed, which included disruption of mitochondrial membrane potential and increased release of cytochrome *c* from mitochondria to cytosol (Fig. 5C). As the level of cytochrome *c* increases in the cytosol, it interacts with Apaf-1 and ATP forms a complex with procaspase-9 (apoptosome), leading to activation of procaspase-9 and caspase-3 (36). Activated caspase-3 is the key executioner of apoptosis and cleaved caspase-3 leads to cleavage and inactivation of key cellular proteins, such as poly(ADP-ribose) polymerase (36, 37). We found that berberine treatment of DU145 cells resulted in a dose-dependent activation of caspase-9 and caspase-3 and cleavage of poly(ADP-ribose) polymerase (Fig. 6A). The involvement of a berberine-induced increase in caspase-3 and its effect on apoptosis were further confirmed by measuring its activity (Fig. 6B) and induction of apoptosis by flow cytometry. The blockade of berberine-induced apoptosis in DU145 cells by the pan-caspase inhibitor z-VAD-fmk, together with the concomitant decrease in caspase-3 activity, confirmed the role of caspase-3 in the berberine-induced apoptosis. As DNA damage is a feature of apoptotic cell death, we further confirmed DNA damage using the comet assay and that treatment with berberine plus pan-caspase inhibitor resulted in a significant inhibition of DNA damage (Fig. 7).

In conclusion, the results of the present study indicate that berberine inhibits proliferation and induces G<sub>1</sub>-phase arrest and apoptosis in human prostate cancer cells but not in normal human prostate epithelial cells. In addition, we provide mechanistic evidence that berberine-induced apoptosis in prostate carcinoma cells, particularly hormone-refractory prostate carcinoma cells, is mediated through enhanced expression of Bax, disruption of the mitochondrial membrane potential, and activation of caspase-3.

## References

- Jemal A, Tiwari RC, Murray T, et al. Cancer statistics. *CA Cancer J Clin* 2004;54:8–29.
- Bosland MC, McCormick DL, Melamed J, Walden PD, Jacquotte AZ, Lumey LH. Chemoprevention strategy for prostate cancer. *Eur J Cancer Chemoprev* 2002;11:18–27.
- Tang DG, Porter AT. Target to apoptosis: A hopeful weapon for prostate cancer. *Prostate* 1997;32:284–93.
- Denmeade SR, Lin XS, Isaacs JT. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. *Prostate* 1996;28:251–65.
- Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. *Science* 1997;278:1073–7.
- Kucuk O. Chemoprevention of prostate cancer. *Cancer Metastasis Rev* 2002;21:111–24.
- Barnes S. Role phytochemicals in prevention and treatment of prostate cancer. *Epidemiol Rev* 2001;23:102–5.
- Craig WJ. Phytochemicals: guardians of our health. *J Am Diet Assoc* 1997;97:S199–204.
- Craig WJ. Health-promoting properties of common herbs. *Am J Clin Nutr* 1999;70:S491–9.
- Sathyavathi GV, Gupta AK, Tandon N, et al. Medicinal plants of India. Vol. 2. New Delhi (India): Indian Council of Medical Research; 1987. p. 230–9.
- Iizuka N, Miyamoto K, Okita K, et al. Inhibitory effect of *Coptidis rhizome* and berberine on the proliferation of human esophageal cancer cell lines. *Cancer Lett* 2000;148:19–25.
- Nishino H, Kitagawa K, Fujiki H, Iwashima A. Berberine sulfate inhibits tumor-promoting activity of teleocidin in two-stage carcinogenesis on mouse skin. *Oncology* 1986;43:131–4.
- Fukuda K, Hibiya Y, Mutoh M, et al. Inhibition of activation protein 1 activity by berberine in human hepatoma cells. *Planta Med* 1999;65:381–3.
- Lin JG, Chung JG, Wu LT. Effects of berberine on arylamine *N*-acetyltransferase activity in human colon tumor cells. *Am J Chin Med* 1999;27:265–75.
- Fukuda K, Hibiya Y, Mutoh M, Koshiji M, Akao S, Fujiwara H. Inhibition by berberine of cyclooxygenase-2 transcriptional activity in human colon cancer cells. *J Ethnopharmacol* 1999;66:227–33.
- Kim SA, Kwon Y, Kim JH, Muller MT, Chung IK. Induction of topoisomerase II-mediated DNA cleavage by a protoberberine alkaloid, berberrubine. *Biochemistry* 1998;37:16316–24.
- Liu LF. DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* 1989;58:351–75.
- Gleave M, Bruchoovsky N, Goldenberg SL, Rennie P. Intermittent androgen suppression for prostate cancer: rationale and clinical experience. *Eur Urol* 1998;34:37–41.
- Roy AM, Baliga MS, Elmets CA, Katiyar SK. Grape seed proanthocyanidins induce apoptosis through p53, Bax, and caspase-3 pathways. *Neoplasia* 2005;7:24–36.
- Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell* 1996;86:147–57.
- Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988;175:184–91.
- Grana X, Reddy P. Cell cycle control in mammalian cells: role of cyclins, cyclin-dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CDKIs). *Oncogene* 1995;11:211–9.
- Pavletich NP. Mechanisms of cyclin-dependent kinase regulation: structures of cdk, their cyclin activators, and CIP and INK4 inhibitors. *J Mol Biol* 1999;287:821–28.
- Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim Biophys Acta* 2002;1602:73–87.
- Morgan DO. Principles of CDK regulation. *Nature (Lond)* 1995;374:131–4.
- Hunter T, Pine J. Cyclins and cancer II: cyclin D and CDK inhibitors come of age. *Cell* 1994;79:573–82.
- Katiyar SK, Roy AM, Baliga MS. Silymarin induces apoptosis primarily through a p53-dependent pathway involving Bcl-2/Bax, cytochrome *c* release, and caspase activation. *Mol Cancer Ther* 2005;4:207–16.
- Adams JM, Cory S. Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem Sci* 2001;26:61–6.
- Reed JC. Regulation of apoptosis by bcl-2 family proteins and its role in cancer and chemoresistance. *Curr Opin Oncol* 1995;7:541–6.
- Hockenbery D, Nunez G, Millman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990;348:334–6.
- Chao DT, Korsmeyer SJ. Bcl-2 family: regulators of cell death. *Annu Rev Immunol* 1998;16:395–419.
- Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998;281:1309–12.
- Liu X, Kim C, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell* 1996;86:147–57.
- Susin SA, Lorenzo HK, Zamzami N, et al. Molecular characterization of mitochondrial apoptosis-inducing factors. *Nature* 1999;397:441–6.

35. Hengartner MO. The biochemistry of apoptosis. *Nature* 2000;407:770–6.
36. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998;281:1312–6.
37. Wolf BB, Green DR. Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J Biol Chem* 1999;274:20049–52.
38. Yang J, Liu X, Bhalla K, et al. Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science* 1997;275:1129–32.
39. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997;275:1132–6.
40. Kastan MB, Canman CE, Leonard CJ. P53, cell cycle control and apoptosis: implications for cancer. *Cancer Metastasis Rev* 1995;14:3–15.
41. Molinari M. Cell cycle checkpoints and their inactivation in human cancer. *Cell Prolif* 2000;33:261–74.
42. Toyoshima H, Hunter T. P27, a novel inhibitor of G<sub>1</sub> cyclin-CDK protein kinase activity, is related to p21. *Cell* 1994;78:67–74.
43. Pilat MJ, Kamradt JM, Pienta KJ. Hormone resistance in prostate cancer. *Cancer Metastasis* 1998–99;17:373–81.
44. Hickman JA. Apoptosis induced by anticancer drugs. *Cancer Metastasis Rev* 1992;11:121–39.
45. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999;13:1899–911.
46. Crompton, M. Bax, Bid and the permeabilization of the mitochondrial outer membrane in apoptosis. *Curr Opin Cell Biol* 2000;12:414–9.