

Bufalin induces apoptosis through activation of both the intrinsic and extrinsic pathways in human bladder cancer cells

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Abstract. Bufalin, a major digoxin-like immunoreactive component of the Chinese medicine Chansu, is prepared from toad venom. This compound has been shown to exert a potential for anticancer activity against various human cancer cell lines *in vitro*. However, the detailed molecular mechanisms of its induction of apoptosis are still unclear. In this study, we investigated the apoptosis-inducing effect of bufalin in T24 human bladder cancer cells. Our data revealed that bufalin treatment resulted in a concentration-response growth inhibition of T24 cells by inducing cell cycle arrest at the G2/M phase and apoptosis, as evidenced by formation of apoptotic bodies, chromatin condensation and accumulation of cells in the sub-G1 phase. Apoptosis induction of T24 cells by bufalin showed correlation with proteolytic activation of caspase-3, -8 and -9, and concomitant degradation of poly (ADP-ribose) polymerases, and collapse of the mitochondria membrane potential. In addition, bufalin treatment resulted in an increase of the Bax/Bcl-2 (or Bcl-xL) ratio and caused down-regulation of inhibitor of apoptosis protein (IAP) family members. The increase in apoptosis by bufalin treatment was also associated with up-regulation of death receptor-related factors. Our data indicate that the growth inhibitory effects of bufalin occur through blockade of the G2/M phase, and that these cancer cells do not enter cell cycle progression and die through apoptosis via both intrinsic and extrinsic pathways.

Introduction

Bladder cancer has become a global health matter. Incidence of bladder cancer is higher in males than in females and varies considerably among countries, with the highest rates in developed communities. However, the burden of bladder cancer will

increase in less developed areas of the world (1). The most common type of bladder cancer is transitional cell carcinoma, and other types include squamous cell carcinoma and adenocarcinoma (2,3). Transitional cell carcinomas are more aggressive than other types of bladder cancer; therefore, in order to improve clinical management of this serious disease, novel treatment strategies are urgently needed.

Apoptosis is one of the known processes of genetically programmed cell death. It is indispensable for development, maintenance of tissue homeostasis, and elimination of unwanted or injured cells from multicellular organisms (4). In addition, abnormal regulation of apoptosis has been involved in many human diseases, such as neoplasms, including cancer, autoimmune disease, ischemia, and viral infection (5). Several gene products have been demonstrated to be critical in the regulation of apoptosis. For instance, caspases, a group of cysteine proteases, play key roles in apoptosis (6,7). Caspases are synthesized as proenzymes, which are activated by cleavage of the prodomain at a specific aspartic acid cleavage site (7,8). Caspase activation is often regulated by various cellular factors, including members of the Bcl-2 and IAP family, and death receptor-related gene products (9,10). However, most cancer cells can block apoptosis, which allows them to survive despite genetic and morphologic transformations. Thus, understanding the mechanisms of apoptosis is important for prevention and treatment of many disorders, including cancer.

Bufalin is a cardiotonic steroid of the components of a Chinese medicine, Chansu, which is obtained from the skin and parotid venom gland of toads. Due to the similarity in the chemical structure between bufalin and digoxin, it is expected that bufalin has a digoxin-like function (11,12). Bufalin also functions as either an inhibitor of Na⁺/K⁺-ATPase or DNA topoisomerase II, which is the target of several anticancer therapy drugs, including etoposide, adriamycin and genistein (13). In addition, bufalin has been known to exert anticancer activities through induction of cell cycle arrest and apoptosis in numerous other solid cancer cells, including lung adenocarcinomas (11,14), gastric cancer (15), prostate cancer (13), ovarian cancer (16), and cervical cancer cells (17), but not in normal cells. Therefore, bufalin is thought to be a valuable anticancer medicine; however, cellular and molecular mechanisms underlying the anticancer effects of bufalin are not yet completely understood.

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Table I. Sequence of primers used for RT-PCR.

Gene name	Sequence
GAPDH	Sense: 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' Antisense: 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'
Bax	Sense: 5'-ATG GAC GGG TCC GGG GAG-3' Antisense: 5'-TCA GCC CAT CTT CTT CCA-3'
Bcl-2	Sense: 5'-CAG CTG CAC CTG ACG-3' Antisense: 5'-GCT GGG TAG GTG CAT-3'
Bcl-xL	Sense: 5'-CGG GCA TTC AGT GAC CTG AC-3' Antisense: 5'-CA GGA ACC AGC GGT TGA AG-3'
XIAP	Sense: 5'-GAA GAC CCT TGG GAA CAA CA-3' Antisense: 5'-CGC CTT AGC TGC TCT CTT CAG T-3'
cIAP-1	Sense: 5'-TGA GCA TGC AGA CAC ATG C-3' Antisense: 5'-TGA CGG ATG AAC TCC TGT CC-3'
cIAP-2	Sense: 5'-CAG AAT TGG CAA GAG CTG G-3' Antisense: 5'-CAC TTG CAA GCT GCT CAG G-3'
Fas	Sense: 5'-TCT AAC TTG GGG TGG CTT TGT CTT C-3' Antisense: 5'-GTG TCA TAC GCT TTC TTT CCA T-3'
FasL	Sense: 5'-GGA TTG GGC CTG GGG ATG TTT CA-3' Antisense: 5'-AGC CCA GTT TCA TTG ATC ACA AGG-3'
DR4	Sense: 5'-CAG AAC GTC CTG GAG CCT GTA AC-3' Antisense: 5'-ATG TCC ATT GCC TGA TTC TTT GTG-3'
DR5	Sense: 5'-GGG AAG AAG ATT CTC CTG AGA TGT G-3' Antisense: 5'-ACA TTG TCC TCA GCC CCA GGT CG-3'
TRAIL	Sense: 5'-ATG GCT ATG ATG GAG TCC AG-3' Antisense: 5'-TTG TCC TGC ATC TGC TTC AGC-3'

In the present study, we conducted an investigation of the biochemical mechanisms of apoptosis by bufalin in human bladder carcinoma cells for the first time. Bufalin was found to inhibit cell growth at G2/M phase of the cell cycle and to induce apoptosis in a dose-dependent manner, which was associated with both mitochondria-regulated and death receptor-initiated pathways.

Materials and methods

Reagents and antibodies. The reagents 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO). Caspase activity assay kits and carbocyanine iodide (JC-1) were obtained from R&D Systems (Minneapolis, MN) and Calbiochem (San Diego, CA), respectively. An enhanced chemiluminescence (ECL) kit and an RNase kit were acquired from Amersham Corp. (Arlington Heights, IL) and Qiagen (La Jolla, CA), respectively. Bufalin was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO, vehicle). Antibodies against caspase-3, -8, -9, poly (ADP-ribose) polymerase (PARP), Bcl-2, Bcl-xL, Bax, Bid, XIAP, cIAP-1, -2, Fas, FasL, death receptor (DR)4, DR5, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) were

purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against actin was obtained from Sigma-Aldrich. Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulins were acquired from Amersham Crop. All other chemicals not specifically mentioned here were purchased from Sigma-Aldrich.

Cell culture and cell viability assay. Human bladder carcinoma T24 and EJ cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified environment with 5% CO₂ at 37°C. Cell viability assay was performed using the MTT assay. For this assay, cells grown to 70% confluence were treated with various concentrations of bufalin for 24 h. After treatment, 0.5-mg/ml MTT solution was added, followed by incubation for 2 h at 37°C in the dark. Absorbance of each well was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Sunnyvale, CA).

DNA flow cytometric analysis. After treatment with bufalin, cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and fixed with 75% ethanol at 4°C for 30 min, and the DNA content of the cells was stained

using a DNA staining kit (CycleTEST PLUS Kit, Becton-Dickinson, San Jose, CA) with PI. DNA contents at each phase of the cell cycle were then determined by FACSCalibur (Becton-Dickinson) and analyzed by the CellQuest software (Becton-Dickinson). The sub-G1 population was calculated in order to estimate the apoptotic cell population (18).

Nuclear staining with DAPI. For the assessment of apoptosis, morphological changes of nuclei were visualized following DNA staining by the fluorescent dye, DAPI. Cells were seeded at 1×10^5 cells/ml in 6-well plates and incubated with bufalin. Following incubation for 24 h, cells were fixed with 3.7% para-formaldehyde for 20 min at room temperature, and washed with PBS. Cells were then stained with 2.5 $\mu\text{g/ml}$ DAPI solution for 10 min at room temperature. Cells were then washed twice with PBS and stained nuclei were observed using a fluorescence microscope (Carl Zeiss, Germany).

Mitochondrial membrane potential (MMP, $\Delta\psi_m$) assay. MMP of intact cells was measured by a DNA flow cytometer with the lipophilic cation JC-1. JC-1 is a ratiometric, dual-emission fluorescent dye, which is internalized and concentrated by respiring mitochondria and can therefore reflect changes in MMP in live cells. It has two excitation wavelengths: at low values of MMP, it remains a monomer (FL-1, green fluorescence; 527 nm) while it forms aggregates at high MMP (FL-2, red fluorescence; 590 nm) according to the recommended procedure. For this study, cells were trypsinized and cell pellets were re-suspended in PBS and incubated with 10 μM JC-1 for 20 min at 37°C. Cells were subsequently washed once with cold PBS, suspended, and analyzed using a flow cytometer (19).

Protein extraction and Western blot analysis. Cells were harvested and lysed with lysis buffer (20 mM sucrose, 1 mM EDTA, 20 μM Tris-Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM MgCl_2 and 5 $\mu\text{g/ml}$ aprotinin) for 30 min. Protein concentration was measured using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. For Western blot analysis, an equal amounts of protein were subjected to electrophoresis on SDS-polyacrylamide gels and transferred by electroblotting to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibody, and visualized by ECL solution according to the recommended procedure (20).

RNA extraction and reverse transcription-PCR. Total RNA was prepared using an RNase kit and primed with random hexamers for synthesis of complementary DNA using AMV reverse transcriptase (Amersham Corp.) following the manufacturer's protocols. Polymerase chain reaction (PCR) was performed in a Mastercycler (Eppendorf, Hamburg, Germany) using the primers indicated in Table I. Conditions for PCR reactions were 1 cycle (94°C for 3 min), 35 cycles (94°C for 45 sec; 58°C for 45 sec; and 72°C for 1 min) and 1 cycle 72°C for 10 min). Amplification products obtained by PCR were electrophoretically separated on 2% agarose gels and visualized by ethidium bromide (EtBr) staining.

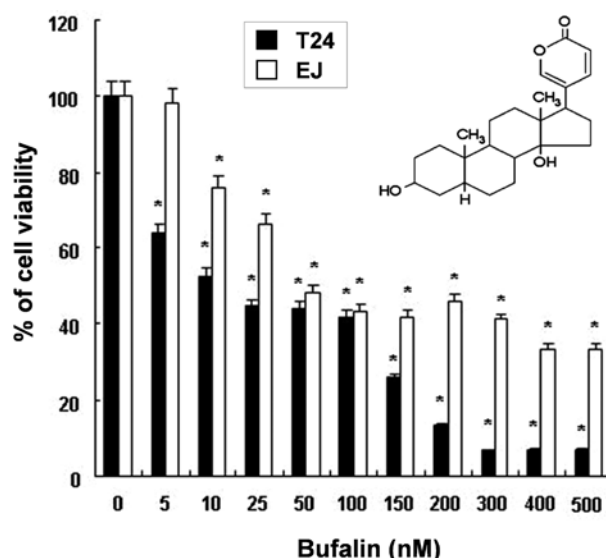


Figure 1. Inhibition of cell viability by bufalin treatment in human bladder carcinoma cells. T24 and EJ cells were seeded at 2×10^5 cells/ml, and incubated for 24 h. Cells were treated with varying concentrations of bufalin for 24 h, and cell viability was measured by the metabolic-dye-based MTT assay. Data are expressed as mean \pm SD of three independent experiments. *Significant differences from other treatments, $p < 0.05$.

Determination of caspase activity. Enzymatic activities of caspases were measured using colorimetric assay kits according to the manufacturer's procedures. Briefly, cells treated with bufalin at the indicated times were harvested and lysed in the supplied lysis buffer for 30 min on an ice bath. After centrifuging at 14,000 rpm for 20 min, supernatants were collected and incubated with the supplied reaction buffer containing dithiothreitol (DTT) and the colorimetric tetrapeptides, Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (pNA) substrate for caspase-3, Ile-Glu-Thr-Asp (IETD)-pNA substrate for caspase-8, and Leu-Glu-His-Asp (LEHD)-pNA substrate for caspase-9, respectively, at 37°C for 2 h in the dark. Caspase activities were calculated by the change in the absorbance at 405 nm using an ELISA reader (21).

Statistical analysis. All data are presented as the mean \pm SD. Significant differences among the groups were determined through the unpaired Student's t-test. A value of $p < 0.05$ was accepted as an indication of statistical significance. All results presented were obtained from at least three independent experiments.

Results

Inhibition of cell growth and induction of apoptosis by bufalin in bladder cancer cells. To examine the effects of bufalin on cell growth, T24 and EJ cells were stimulated with various concentrations of bufalin for 24 h and cell viability was measured by the MTT assay. As shown in Fig. 1, treatment with bufalin resulted in significant inhibition of cell viability, and this effect occurred in a concentration-dependent manner. In particular, T24 cells revealed much stronger growth inhibitory effects, as compared with EJ cells. Thus, the next experiments were carried out in T24 cells. Direct observation

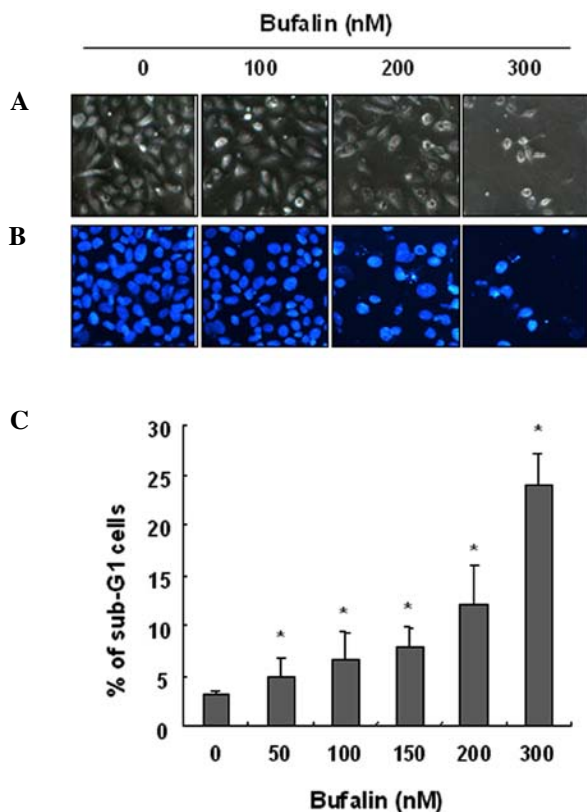


Figure 2. Induction of apoptosis of T24 cells by bufalin treatment. (A) Following treatment with the indicated concentrations of bufalin, cells were observed using an inverted microscope (magnification, x200). (B) Cells grown under the same conditions as (A) were stained with DAPI for 10 min, washed with PBS, and then photographed with a fluorescence microscope using a blue filter (magnification, x400). (C) To quantify the degree of apoptosis induced by bufalin, cells grown under the same conditions were evaluated for sub-G1 DNA content, which represents fractions undergoing apoptotic DNA degradation, using a flow cytometer. Each point represents the mean \pm SD of three independent experiments. *Significant differences from other treatments, $p < 0.05$.

using an inverted microscope showed that numerous morphological changes had occurred in cells treated with bufalin. In particular, cell shrinkage and cytoplasm condensation appeared in a concentration-dependent manner after bufalin treatment (Fig. 2A). To elucidate whether growth inhibition by bufalin was associated with apoptosis, we examined apoptotic features by measurement of chromatin condensation of the nuclei and the amount of cells in the sub-G1 phase. Treatment with bufalin resulted in a significant number of cells with chromatin condensation and formation of apoptotic bodies in a concentration-dependent manner, whereas these features were not observed in the control cells (Fig. 2B). In addition, treatment with bufalin resulted in increased accumulation of cells with sub-G1 DNA content. These results suggest that bufalin inhibits proliferation of T24 cells through induction of apoptotic cell death.

Induction of G2/M cell cycle arrest by bufalin in T24 cells.

Using a flow cytometer, cell cycle patterns of T24 cells were examined in order to determine whether bufalin treatment of cells influenced cell cycle progression. Analysis of the cell cycle distribution of cells showed marked accumulation in the G2/M phase of the cell cycle, which was associated with a decrease

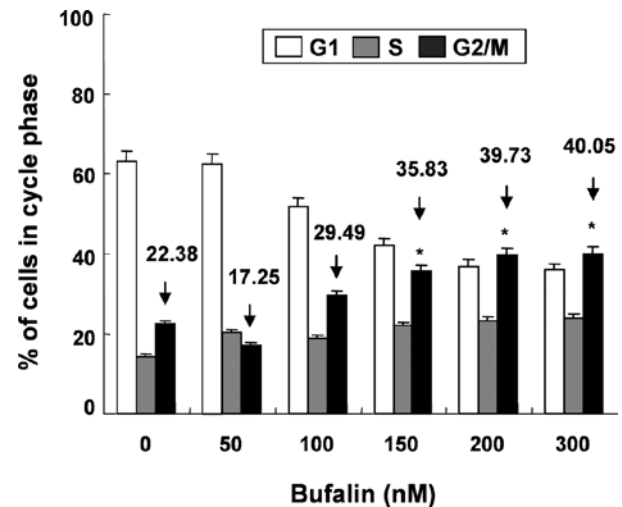


Figure 3. Cell cycle arrest at the G2/M phase by bufalin treatment in T24 cells. Cells were exposed to various concentrations of bufalin for 24 h, and flow cytometric analysis was performed for analysis of DNA contents. Percentages of cells in the G2/M phase are presented. Data represent the mean \pm SD of three independent experiments. The significance was determined using a Student's t-test ($p < 0.05$ vs. untreated control).

in the G1 phase when compared with untreated control cells (Fig. 3). Therefore, these results indicate that the anti-proliferative effect of bufalin was associated with cell cycle arrest at the G2/M phase, and that cells treated with bufalin do not enter cell cycle progression and die through apoptosis.

Activation of caspases by bufalin treatment in T24 cells.

Caspases are very important regulators of the apoptotic pathway. Therefore, we examined expression levels and activities of caspase-3, -8, and -9 during bufalin-induced apoptosis. Immunoblotting results showed that bufalin treatment resulted in concentration-dependent down-regulation of pro-caspase-3, -8, and -9 proteins (Fig. 4A). To further quantify the proteolytic activation of caspases, cell lysates containing equal amounts of total proteins from cells treated with bufalin were estimated for *in vitro* caspase activities using DEVD-pNA, IETD-pNA, and LEHD-pNA as substrates for caspase-3, -8, and -9, respectively. As shown in Fig. 4B, treatment with bufalin resulted in a marked increase in the activity of caspase-3, -8, and -9. In addition, bufalin treatment led to progressive proteolytic cleavage of PARP proteins, an endogenous caspase-3 substrate (Fig. 4A).

Loss of MMP by bufalin treatment in T24 cells.

To investigate the role of mitochondria in bufalin-induced apoptosis of T24 cells, alteration of the MMP values was measured using a flow cytometer. Exposure of T24 cells to various concentrations of bufalin for 24 h led to a distinct reduction in MMP levels, which occurred in a dose-dependent manner (Fig 5; ~2.0- and 3.6-fold by 200 and 300 nM of bufalin, respectively). These results suggest that dysfunction of mitochondria plays an important part in the process of bufalin-induced apoptotic cell death.

Modulation of Bcl-2 and IAP family members by bufalin treatment in T24 cells. Next, we examined the effects of bufalin

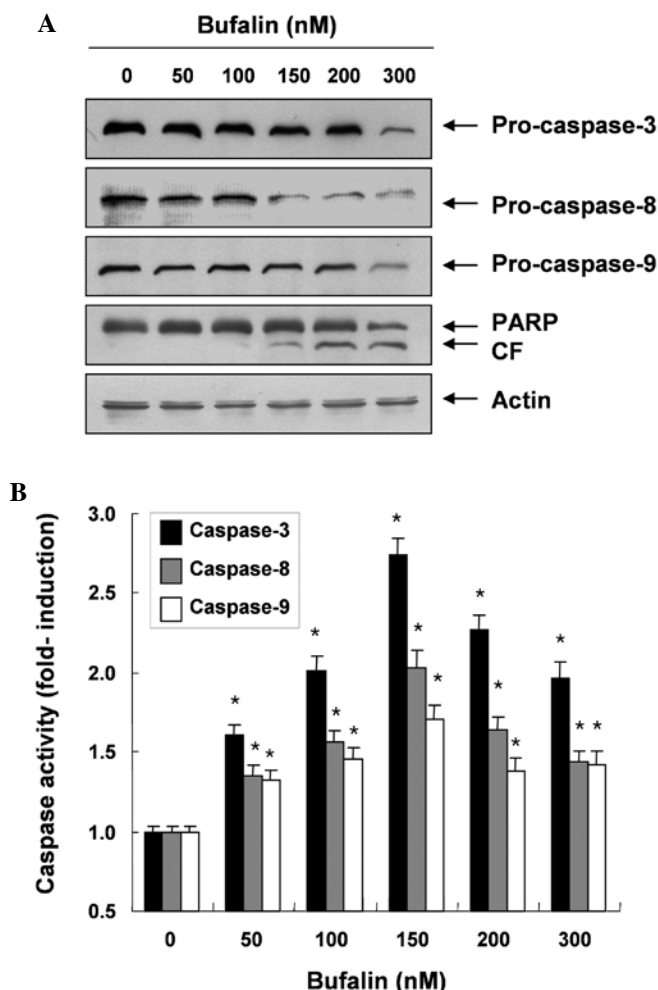


Figure 4. Activation of caspases by bufalin treatment in T24 cells. (A) Cells were incubated with the indicated concentration of bufalin for 24 h and then lysed. Equal amounts of cell lysates (30 μ g) were subjected to electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were then probed with the indicated antibodies and proteins were visualized using an ECL detection system. Actin was used as an internal control. (B) Cells grown under the same conditions as (A) were collected and lysed. Aliquots were incubated individually with DEVD-pNA, IETD-pNA, and LEHD-pNA for caspase-3, -8, and -9 at 37°C for 1 h. Released fluorescence products were measured. Data represent the mean of three independent experiments. A Student's t-test (* p <0.05) was used for analysis of the statistical significance of the results. CF, cleavage form.

on the levels of Bcl-2 family members. As shown in Fig. 6, mRNA and protein levels of anti-apoptotic Bcl-2 and Bcl-xL expression were partially inhibited in response to bufalin treatment and the pro-apoptotic protein Bid, a BH3-only pro-apoptotic member of the Bcl-2 family, was truncated in a concentration-dependent manner, whereas levels of pro-apoptotic Bax were increased in cells treated with bufalin. Under the same conditions, expression levels of IAP family members were also examined. Bufalin treatment resulted in a concentration-dependent decrease in the expression of IAP family members, including XIAP, cIAP-1, and cIAP-2 at both the transcriptional and translational levels (Fig. 6).

Induction of death receptor-related gene products by bufalin treatment in T24 cells. In order to further determine which apoptosis pathway contributes to bufalin-induced apoptosis,

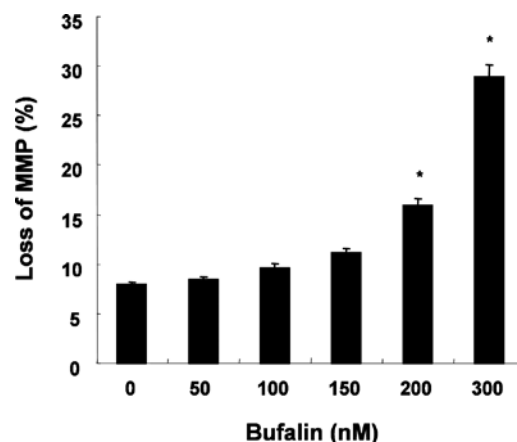


Figure 5. Loss of MMP by bufalin treatment in T24 cells. Cells grown under the same conditions as Fig. 4 were stained with carbocyanine iodide (JC-1) and then incubated at 37°C for 20 min, after which the mean JC-1 fluorescence intensity was detected using a flow cytometer. Data represent the mean \pm the SD of representative experiments performed at least three times. The significance was determined by a Student's t-test (* p <0.05 vs. untreated control).

the death receptor and corresponding pro-apoptotic ligands were examined by Western blot analysis and RT-PCR. Results showed that bufalin treatment induced a concentration-dependent increase in the mRNA and protein levels of Fas, DR4, DR5, and TRAIL, but did not alter FasL expression (Fig. 7). These results indicate that the extrinsic pathway may be involved in the bufalin-induced apoptosis in T24 cells.

Discussion

In recent years, various studies have demonstrated that bufalin, a major immunoreactive component of the skin and parotid venom gland of toads, may impinge on cellular signaling pathways and may often recruit them to induce apoptosis in cancer cells (11,13-17,22). However, the molecular mechanisms responsible for the pro-apoptotic effects of bufalin in cancer cells are not fully understood. In the present study, bufalin was found to significantly inhibit cell proliferation at the G2/M phase of the cell cycle, and to induce apoptosis through an intrinsic as well as an extrinsic pathway in human bladder carcinoma T24 cells.

Apoptosis is the end point of a cascade of molecular events that are initiated by several stimuli and ultimately lead to activation of proteolytic enzymes (caspases) responsible for cell death (6,7). In general, apoptosis is mediated through two major pathways, the extrinsic (death receptor-mediated) and intrinsic (mitochondrial-mediated) pathways (23,24). In the case of bufalin-induced apoptosis, some reports have indicated involvement of a variety of caspases in several human cancer cell lines (11,13-15); however, the pathway involved in bufalin-induced apoptosis has not yet been determined. The death receptor pathway is initiated by binding of ligands, such as FasL and TRAIL, to cell surface transmembrane receptors, such as Fas, DR, and DR5, which then activates caspase-8 and cleavage of Bid (tBid), further activating a series of caspase cascades, resulting in apoptotic cell death (25). However, changes in mitochondrial integrity in response to a broad range of physical and chemical stimuli can trigger the intrinsic pathway of apoptosis (5,24). Once in the cytosol, cytochrome c

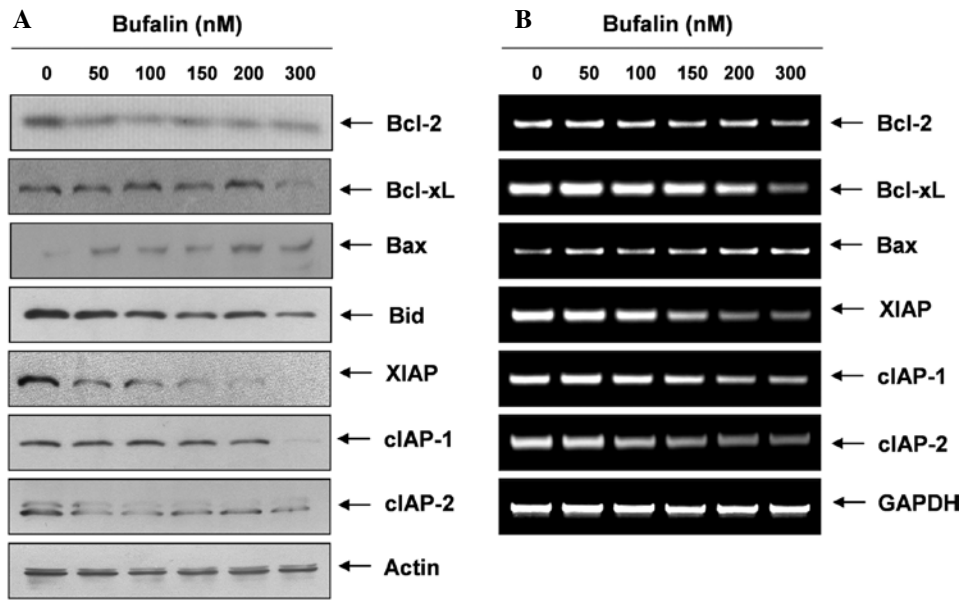


Figure 6. Modulation of expression of Bcl-2 and IAP family members by bufalin in T24 cells. (A) Cells were stimulated with the appropriate doses of bufalin for 24 h, lysed, and cellular proteins (30 μ g) were separated by SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. Membranes were probed with the indicated antibodies and proteins were visualized using an ECL detection system. Actin was used as an internal control. (B) Total RNAs were isolated from cells grown under the same conditions as (A) and reverse-transcribed. Resulting cDNAs were then subjected to PCR with the indicated primers and the reaction products were separated on 2% agarose gels and visualized by ethidium bromide staining. GAPDH was used as an internal control. Data are representative of two experiments.

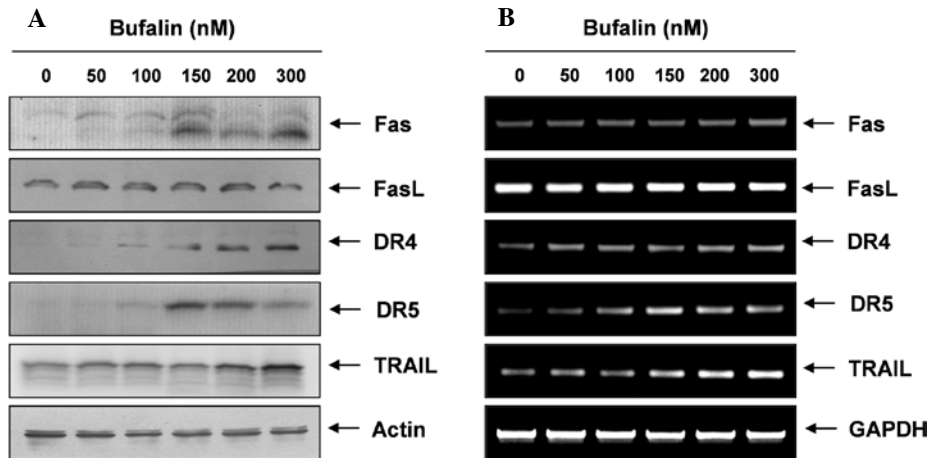


Figure 7. Effects of bufalin on the expression of death receptor-related factors in T24 cells. Cells were treated with the indicated concentration of bufalin for 24 h. Cells were lysed and then equal amounts of cell lysates (30 μ g) were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with the indicated antibodies and proteins were visualized using an ECL detection system. Actin was used as an internal control. (B) Total RNAs were isolated from cells grown under the same conditions as (A) and reverse-transcribed. Resulting cDNAs were then subjected to PCR with the indicated primers and the reaction products were separated on 2% agarose gel and visualized by ethidium bromide staining. GAPDH was used as an internal control. Data are representative of two experiments.

can activate caspase-9, which in turn cleaves and activates the key executioner, caspase-3, which is either partially or totally responsible for proteolytic cleavage of many key proteins, including PARP, which is important for cell viability, but also serves as a marker of apoptosis when cleaved (26). Our data have demonstrated that bufalin induced an increase in the levels of Fas, DR4, DR5, and TRAIL expression (Fig. 7), key regulators of the extrinsic apoptosis pathway, and the enzymatic activity of both extrinsic and intrinsic caspase cascades, such as caspase-8 and -9 (Fig. 4). In addition, down-regulation of whole Bid expression and loss of MMP values were

observed in bufalin-treated T24 cells (Figs. 5 and 6). These results indicate that bufalin may activate the extrinsic apoptosis pathway and increase mitochondrial dysfunction. This, in turn, results in activation of caspase-9, leading to activation of caspase-3, which is associated with degradation of PARP, a caspase-3 target protein (Fig. 4). Overall these results suggest that bufalin induces apoptosis in T24 cells through activation of the intrinsic and the extrinsic pathway.

The intrinsic apoptotic pathway is also controlled by several factors, including Bcl-2 and IAP family proteins. Members of the Bcl-2 family regulate apoptosis either as an activator (e.g.,

Bax and Bad) or as an inhibitor (e.g., Bcl-2 and Bcl-xL); therefore, it has been suggested that the Bax/Bcl-2 ratio is a key factor in the regulation of products of the apoptotic process (9,10). On the other hand, members of the IAP family of inhibitors of apoptosis proteins function negatively through binding to and inhibiting several caspases (26,27). Our data revealed that bufalin-induced apoptosis was related to down-regulation of the anti-apoptotic Bcl-2 and Bcl-xL as well as to up-regulation of the pro-apoptotic Bax (Fig. 6). Further studies have demonstrated that exposure of T24 cells to bufalin resulted in down-regulation of IAP family members, including XIAP, cIAP-1, and cIAP-2, in a concentration-dependent manner (Fig. 6). The data indicated that bufalin induced an increase in the Bax/Bcl-2 ratio, leading to mitochondrial dysfunction, and activation of caspases by bufalin was associated with inhibition of IAP family expression.

In summary, our results indicate that bufalin induces strong suppression of proliferation of T24 cells at the G2/M phase of the cell cycle and induces apoptosis through activation of the mitochondrial mediated-intrinsic caspase pathway along with the death receptor-mediated extrinsic pathway. Although conduct of further studies is warranted, we believe that bufalin has important antineoplastic properties and may be part of an effective strategy for the treatment of human bladder cancer.

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