Bufalin prevents the migration and invasion of T24 bladder carcinoma cells through the inactivation of matrix metalloproteinases and modulation of tight junctions

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Abstract. Bufalin, a cardiotonic steroid extracted from toad venom, has generally been known to possess a range of biological activities; however, only a few studies have reported the anti-metastatic activity of bufalin. In the present study, we investigated the inhibitory effects of bufalin on cell migration and invasion, two critical cellular processes that are often deregulated during metastasis, using the human bladder cancer cell line, T24. Within the concentration range that was not cytotoxic, bufalin markedly inhibited the cell motility and invasiveness of T24 cells. The inhibitory effects of bufalin on cell invasiveness were associated with the tightening of tight junctions (TJs), which was demonstrated by an increase in transepithelial electrical resistance (TER). Bufalin treatment also repressed the levels of claudin proteins (claudin-2, -3 and -4) and the major components of TJs that play key roles in the control and selectivity of paracellular transport. Furthermore, the activities of matrix metalloproteinase (MMP)-2 and -9 in T24 cells were dose-dependently inhibited by treatment with bufalin and this also correlated with a decrease in their mRNA and protein expression levels; however, the mRNA and protein levels of the tissue inhibitor of metalloproteinase (TIMP)-1 and -2 were increased. In addition, these effects were related to the increased phosphorylation of the extracellular signal-regulated protein kinase (ERK) pathway. The inhibition of ERK (PD98059) significantly prevented the bufalin-induced suppression of T24 cell migration. These findings suggest that bufalin inhibits the migration and invasion of T24 cells by modulating the activity of TJs and MMPs, possibly in association with the activation of ERK.

Introduction

Cell migration and invasion are two critical cellular processes that are often deregulated during tumorigenesis. Cancer growth is accompanied by progressive infiltration, invasion and destruction of the surrounding tissue. The metastasis and invasiveness of tumor cells are the most reliable features that differentiate malignant tumors from benign ones (1). Despite our advanced understanding of primary cancer development and progression, metastasis and the systemic spread of the cancer to secondary sites remains the leading cause of cancer-associated death (2). Therefore, the discovery of a new anti-metastatic strategy is critical in order to increase the survival and to prevent the mortality of patients with cancer.

In epithelial cells, several specialized and distinct intercellular structures, including the gap junction, tight junction (TJ), adherens junction and desmosome, are responsible for the establishment of contact between neighboring cells. Among them, TJs are the most apical component of cell/cell complexes and play a critical role in regulating the balance between differentiation, proliferation and cell death (3,4). However, during metastasis, the expression pattern of TJ proteins is frequently disrupted in epithelial tumors. Claudins, which are major integral membrane proteins that form the backbone of TJs, can form homodimers or heterodimers to produce paired strands between adjacent cells and act as a barrier to the paracellular flux of water and the solution and transmigration of other cells, thereby determining

the characteristic permeability properties of different epithelial tissues (5-7). According to the serial analysis of gene expression, the overexpression of claudins is frequently found in various cancer cells. For instance, claudin-3 and -4 have been reported to be constantly elevated in ovarian, breast, prostate and pancreatic cancers, as well as bladder cancer (8-12). Recent reports found that certain claudins, such as claudin-4 and -7 are more elevated in high-grade urothelial cancer patients as compared to low-grade patients (13,14). Therefore, the examination of the modulation of claudin family proteins is adjuvant to enhancing the accuracy of the pathological diagnosis and the addition of further information for clinical treatment (15,16).

Matrix metalloproteinases (MMPs), are a family of zinc ion-dependent endopeptidases, that consists of more than 26 endopeptidases, which are known to act on a broad spectrum of cell surface molecules (17-19). The three prominent roles of MMPs in pathological conditions may be grouped into the following types: tissue destruction, fibrosis and the weakening of the matrix. In the tumor cell invasion process, degradation of the extracellular matrix (ECM) is one of the pivotal steps. Therefore, the upregulation of MMPs has been found in the majority of malignant tumors, and has also been connected with cancer aggressiveness, stage and prognosis (20,21). Among all the members of the MMP family, MMP-2 (gelatinase-A) and -9 (gelatinase-B) have been known to be involved in the invasive metastatic possess of tumor cells, including bladder cancer cells (22-24). Since MMPs have many physiological functions in metastasis, the inhibition of the activity of MMPs is inherent for the activated MMPs, thus the tissue inhibitor of metalloproteinase (TIMP) prevents the uncontrolled action of these proteinases (21,24,25).

Bufalin is the major digoxin-like immunoreactive component, obtained from the skin and parotid venom gland of the toad, *Bufo bufo gargarizans* (26). Although bufalin has long been used as a treatment for heart failure in Oriental medicine in Asian countries, studies have shown that bufalin exhibits anticancer effects. Bufalin induces cell cycle arrest, differentiation and apoptosis in many human cancer cells such as leukemia, gastric, colon, breast, endometrial and ovarian cancer and osteosarcoma cells (27-33). Recently, Chueh *et al* (34) demonstrated that bufalin inhibited migration and invasion in human osteosarcoma cells by downregulating the levels of MMP-2, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signaling pathways. However, the anti-metastatic effects of bufalin on cancer cells have not yet been thoroughly reported and knowledge of the molecular mechanisms involved is rudimentary and remains to be delineated. Therefore, the purpose of this study was to elucidate the anti-metastatic potential of bufalin using the T24 human bladder carcinoma cell line and to investigate the underlying intracellular signal transduction pathways involved in the inhibition of metastasis. The results of this study demonstrate that bufalin inhibits two measures of metastatic potential, cell motility and invasiveness, through the modulation of the levels of TJ-associated factors and the activities of MMPs.

Materials and methods

Reagents and antibodies. RPMI-1640 medium was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Fetal bovine serum (FBS) was acquired from Gibco-BRL (Gaithersburg, MD,

USA). Bufalin was purchased from Sigma-Aldrich Chemical Corp. (St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO, vehicle) and stored in aliquots at 4˚C. The primary antibodies against MMP-2, MMP-9, TIMP-1 and TIMP-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antibodies against claudin-2, -3 and -4 were obtained from Invitrogen. The antibody against actin was obtained from Sigma-Aldrich. Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were acquired from Santa Cruz Biotechnology. All other chemicals not specifically mentioned were purchased from Sigma-Aldrich.

Cell culture and trypan blue counting. T24 human bladder carcinoma cells were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin (Gibco-BRL) at 37˚C and 5% CO_2. For the cell viability study, T24 cells were grown to 70% confluence, treated with bufalin and then cell viability was assessed by a trypan blue exclusion assay.

Colony forming assay. T24 cells were seeded into 100-mm dishes at a density of 5, 10 or $20x10^2$ cells/dish and allowed to be stabilized for 24 h at 37˚C in a culture medium. Cells were then treated with 100 nM of bufalin. After 15 days, colonies were fixed with 3.7% paraformaldehyde for 20 min and stained with hematoxylin solution for 10 min at room temperature. The dishes were washed with PBS and then counted manually and photographed.

Cell motility and wound healing assay. For the persistence migratory directionality assay, T24 cells were grown to confluence on 35 mm cell culture dishes that were coated with 20 μg/ml of rat tail collagen (Becton-Dickinson Biosciences, Bedford, MA, USA). Confluent monolayer cells were scratched using a 200 μl micropipette tip. After wounding, the cultures were washed twice with FBS-free RPMI-1640 medium to remove cell debris. The cells were supplemented with a medium containing 0.5% FBS and treated with 50 or 100 nM of bufalin. At the indicated time, the wound closure weight of cells was inspected and photographed under the microscope at x40 magnification, respectively. The assays were repeated twice and each sample was observed in triplicate (35).

In vitro invasion assay. To observe the ability of T24 cells to penetrate the ECM in the presence or absence of bufalin, Matrigel invasion assays were performed. The chambers consisted of 6.5-mm inserts with 8.0-μm pore membranes (Corning Costar Corporation, Corning, NY, USA). Each membrane was coated with 50 μl of Matrigel (Becton-Dickinson Biosciences) diluted in an ice-cold serum-free medium using a cooled pipette and incubated for 30 min at 37˚C. After the plates were ready to use, pre-warmed RPMI complete medium was added to the inside of the top chamber and rehydrated for 1 h in a 37˚C incubator. Subsequently, T24 cells were seeded in serum-free medium with or without bufalin in the upper chamber of the transwell, and medium containing 20% FBS was added to the bottom chambers. After overnight incubation, the membranes were fixed with methanol for 2 min, stained with hematoxylin for 4 min and washed with distilled water. The membranes were

Table I. Primer sequences 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'
MMP-2	Sense	5'-CTT CTT CAA GGA CCG GTT CAT-3'
	Antisense	5'-GCT GGC TGA GTA GAT CCA GTA-3'
MMP-9	Sense	5'-TGG GCT ACG TGA CCT ATG ACC AT-3'
	Antisense	5'-GCC CAG CCC ACC TCC ACT CCT C-3'
TIMP-1	Sense	5'-TGG GGA CAC CAG AAG TCA AC-3'
	Antisense	5'-TTT TCA GAG CCT TGG AGG AG-3'
TIMP-2	Sense	5'-GTC AGT GAG AAG GAA GTG GAC TCT-3'
	Antisense	5'-ATG TTC TTC TCT GTG ACC CAG TC-3'
Snail	Sense	5'-TAT GCT GCC TTC CCA GGC TTG-3'
	Antisense	5'-ATG TGC ATC TTG AGG GCA CCC-3'
E-cadherin	Sense	5'-GAA CAG CAC GTA CAC AGC CCT-3'
	Antisense	5'-GCA GAA GTG TCC CTG TTC CAG-3'
Claudin-1	Sense	5'-TCA GCA CTG CCC TGC CCC AGT-3'
	Antisense	5'-TGG TGT TGG GTA AGA GGT TGT-3'
Claudin-2	Sense	5'-ACA CAC AGC ACA GGC ATC AC-3'
	Antisense	5'-TCT CCA ATC TCA AAT TTC ATG C-3'
Claudin-3	Sense	5'-AAG GCC AAG ATC ACC ATC GTG-3'
	Antisense	5'-AGA CGT AGT CCT TGC GGT CGT-3'
Claudin-4	Sense	5'-TGG ATG AAC TGC GTG GTG CAG-3'
	Antisense	5'-GAG GCG GCC CAG CCG ACG TA-3'

removed from the insert with a small scalpel blade, mounted on a microscope slide and coverslipped. Invasive cells were observed under a microscope at x200 magnification and quantified (three fields for each membrane).

Measurement of transepithelial electrical (TER) resistance. TER was measured using an EVOM Epithelial Tissue Voltohmmeter (World Precision Instruments, Sarasota, FL, USA), equipped with a pair of STX-2 chopstick electrodes. Briefly, the T24 cells were seeded in the 8.0-μm pore size top chamber of the Transwell (Corning Costar) with serum-free medium and an assay medium containing 20% FBS was added to the bottom chamber. The cells were treated with bufalin at a concentration of 50 or 100 nM and incubated for 24 h. Two electrodes were placed at the top and bottom chambers and then resistance was estimated with the voltohmmeter.

Protein extraction and western blot analysis. The cells were treated with bufalin at the indicated time and harvested. Total cells were then gently 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 μg/ml aprotinin) for 30 min. The supernatants were collected and the protein concentrations were quantified using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). For western blot analysis, equal amounts of protein were subjected to electrophoresis on SDS-polyacrylamide gels and then electrotransferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Blots were probed with the desired antibodies, incubated with the diluted enzyme-linked secondary antibodies and visualized by enhanced chemiluminescence (ECL), according to the procedure recommended (Amersham Corp., Arlington Heights, IL, USA).

RNA extraction and reverse transcription-polymerase chain reaction (PCR). Total-RNAs were isolated using a RNeasy mini kit (Qiagen, La Jolla, CA, USA) and primed with random hexamers to synthesize complementary DNA using AMV reverse transcpriptase (Amersham) following the manufacturer's instructions. PCR was performed in a Mastercycler (Eppendorf, Hamburg, Germany) with the primers indicated in Table I. The conditions for PCR reactions were 1X (94°C for 3 min), 35X (94°C for 45 sec; 58°C for 45 sec; and 72°C for 1 min) and 1X (72°C for 10 min). The amplification products obtained by PCR were electrophoretically separated on a 1% agarose gel and visualized by ethidium bromide (EtBr) staining.

Gelatin zymographic analysis of secreted MMPs. The enzymatic activities of MMP-2 and -9 were measured by gelatin zymography. Briefly, the bufalin-treated cell culture supernatants were collected using centrifugation. The cell-free supernatant was mixed with 10X bromophenol blue (Junsei Chemical Co. Ltd., Tokyo, Japan) and zymography was conducted by precast gel (10% polyacrylamide and 0.25% gelatin). Following electrophoresis, the gel was shaken with 2.5% Triton X-100 at room temperature for 1 h and washed with distilled water two to three times. Next, the gel was incubated with a developing buffer containing 1 M Tris-HCl (pH 7.5) 50 ml, 1 M CaCl$_2$ 5 ml,
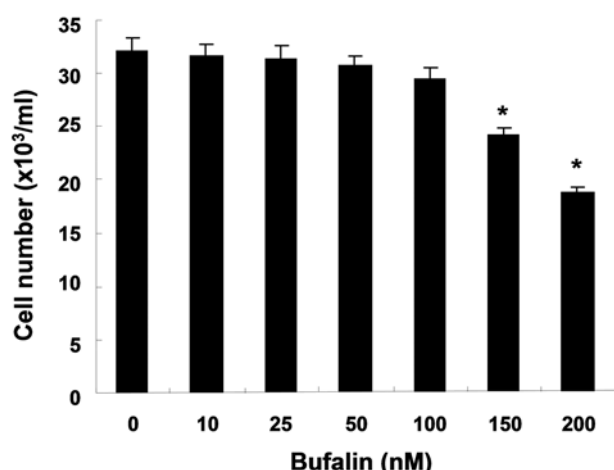
Figure 1. Effects of bufalin on the cell proliferation of T24 human bladder carcinoma cells. T24 cells were seeded at a density of $2x10^5$ cells/ml in 6 wells, incubated for 24 h and treated with the indicated concentrations of bufalin for 24 h. Cell numbers was determined using a trypan blue exclusion counting method under an inverted microscope. Data are expressed as the means ± SD of three independent experiments. *p<0.05, significantly different compared with the control group using the one-way ANOVA test.

10% NaN_3 2 ml, 20 mM $ZnCl_2$ 50 μl, H_2O at 37°C for 24 h. The following day, the gel was stained with 0.5% (w/v) Coomassie Brilliant Blue (Bio-Rad) for 30 min and then destained in methanol:acetic acid:water (1:1:8) (36).

Statistical analysis. All data are presented as the means ± SD. The significant differences among the groups were analyzed using a one-way analysis of variance followed by an ANOVA test. A value of p<0.05 was considered as an indication of statistical significance.

Results

Effects of bufalin on cell proliferation and colony formation in T24 cells. To investigate the effects of bufalin on cell proliferation, T24 cells were treated with diverse concentrations for 24 h and subjected to trypan blue exclusion. When compared with the untreated control cells, bufalin showed a dose-dependent inhibitory effect on the proliferation of T24 cells (Fig. 1). However, bufalin in the range of 10-100 nM did not have a significant cytotoxic effect on T24 cells; therefore, a concentration of bufalin within this lower range was used in the remaining experiments.
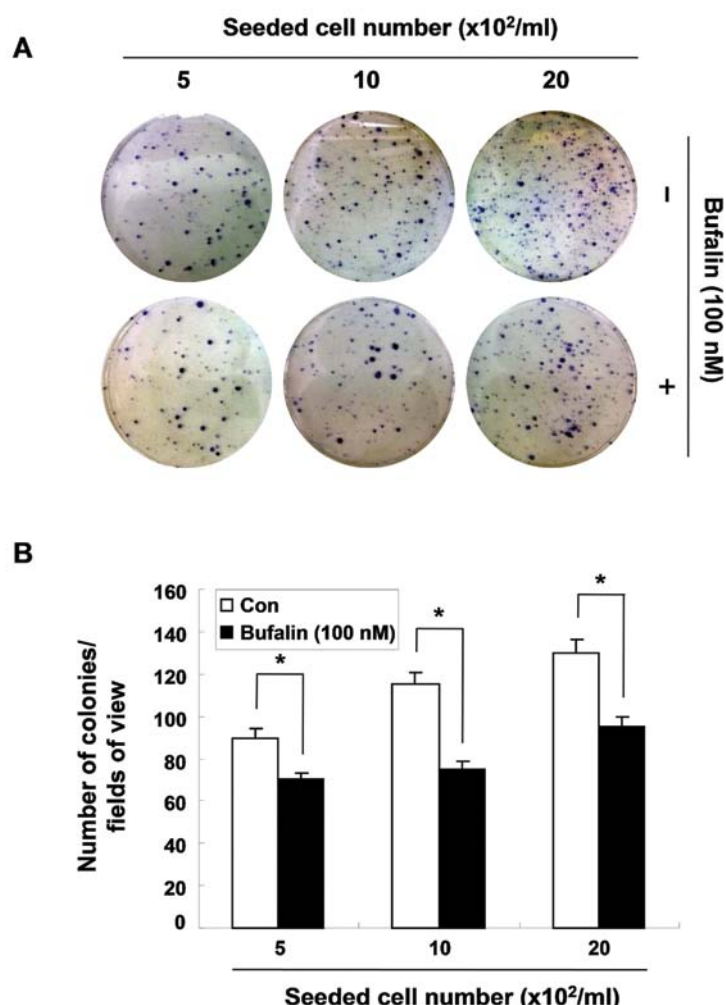

Figure 2. Effects of bufalin on colony formation in T24 cells. For clonogenic assay, T24 cells were seeded in 100-mm dishes at a density of 5, 10 and $20x10^2$ cells/dish and allowed to attach for 24 h. The cells were stimulated with 100 nM bufalin, and allowed to grow. After 15 days, colonies were fixed with 3.7% paraformaldehyde and stained with hematoxylin. (A) Stained colonies were photographed. (B) Colonies from the experiment shown in (A) were counted and plotted. Values marked as (*) indicate significant differences from other treatments (*p<0.05).
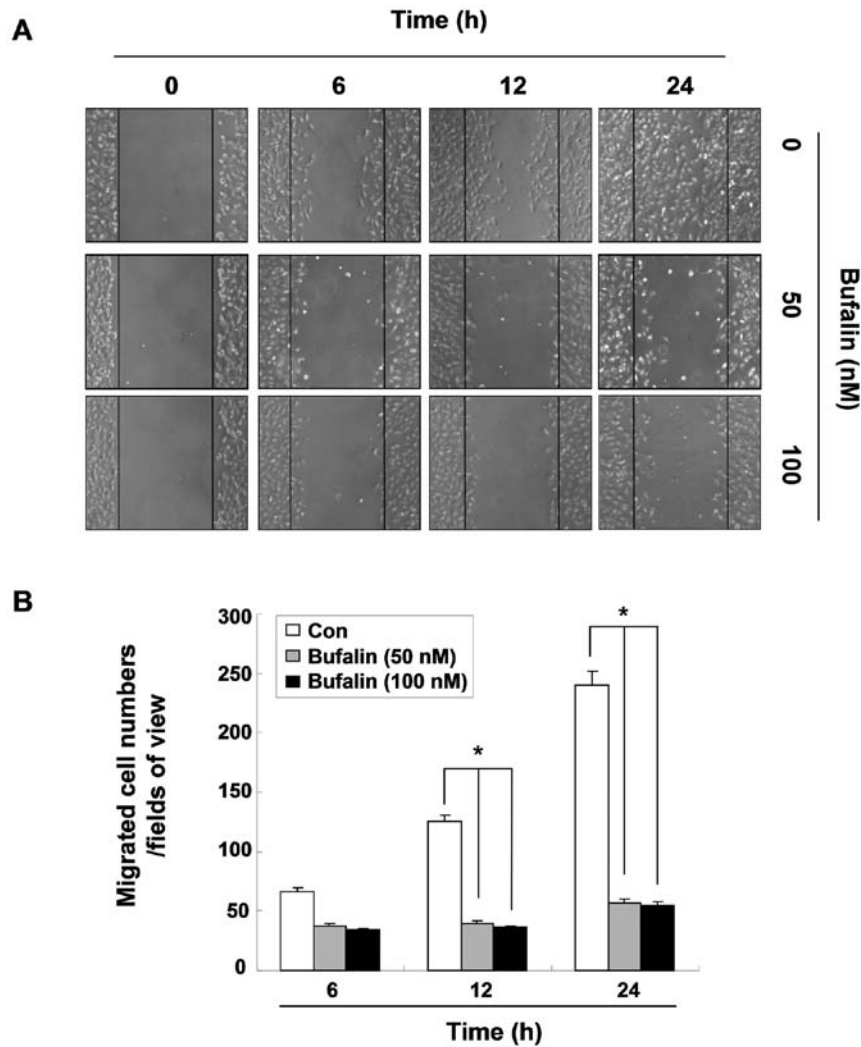
Figure 3. Inhibition of cell motility by bufalin in T24 cells. Cells were seeded at 4x10^5 cells/ml in 35-mm tissue culture dishes and scratches were made using 200 μl micropipette in confluent cultures. Subsequently the cells were washed by serum-free medium and then treated with 50 or 100 nM of bufalin in medium containing 0.5% FBS and allowed to grow. (A) At the indicated times, wound closures were photographed under a microscope (x50). (B) Migrated cell numbers within the black line were counted. The assays were repeated twice and each sample was observed in triplicate. Values marked as (*) indicate significant differences from other treatments (*p<0.05).

To confirm the cytostatic effect of bufalin, clonogenic assay was also performed. Based on trypan blue exclusion results, 100 nM concentration of bufalin were chosen for this study. Independent of the seeded number of cells, the dishes treated with bufalin exhibited less colony formation than the untreated ones, suggesting that bufalin has exerts anti-proliferative effects at a non-cytotoxic dose (Fig. 2).

Delay of migration by bufalin in T24 cells. To measure the inhibitory effect of bufalin on the motility of T24 cells, a wound healing assay, a widely used qualitative method of the study of cell migration, was carried out in the condition medium containing 0.5% FBS. After 24 h, the width of the wound was almost completely closed in the untreated dishes; however, a distinct gap still remained in the bufalin-treated dishes (Fig. 3). Quantitative data showing the migrating cells demonstrated that treatment with bufalin impeded the migration of cells compared to the control cells in a time-dependent manner. However, there was no significant difference between the two doses, 50 and

100 nM of bufalin. These results suggest that bufalin possesses the ability to reduce cell motility and migration in T24 cells.

Bufalin increases TER values and decreases cell invasion in T24 cells. To determine the interaction between the tightening of TJs and the anti-invasive activity of T24 cells stimulated with bufalin, the investigation of TER (a measure of TJ formation) values was conducted. After bufalin exposure for 24 h, TER values were considerably increased in a dose-dependent manner suggesting that bufalin increased the tightening of TJs (approximately 1.3-fold and 1.4-fold by 50 and 100 nM of bufalin, respectively, Fig. 4A). We subsequently investigated whether bufalin can reduce cell invasion using a Boyden chamber invasion assay. As shown in Fig. 4B and C, incubating cells with 50 and 100 nM of bufalin reduced cell invasion through the Matrigel chamber to 39.5 and 53.6% of the control levels at 24 h, respectively, suggesting that the upregulation of TER contributes to the inhibition of cell invasion in T24 cells.
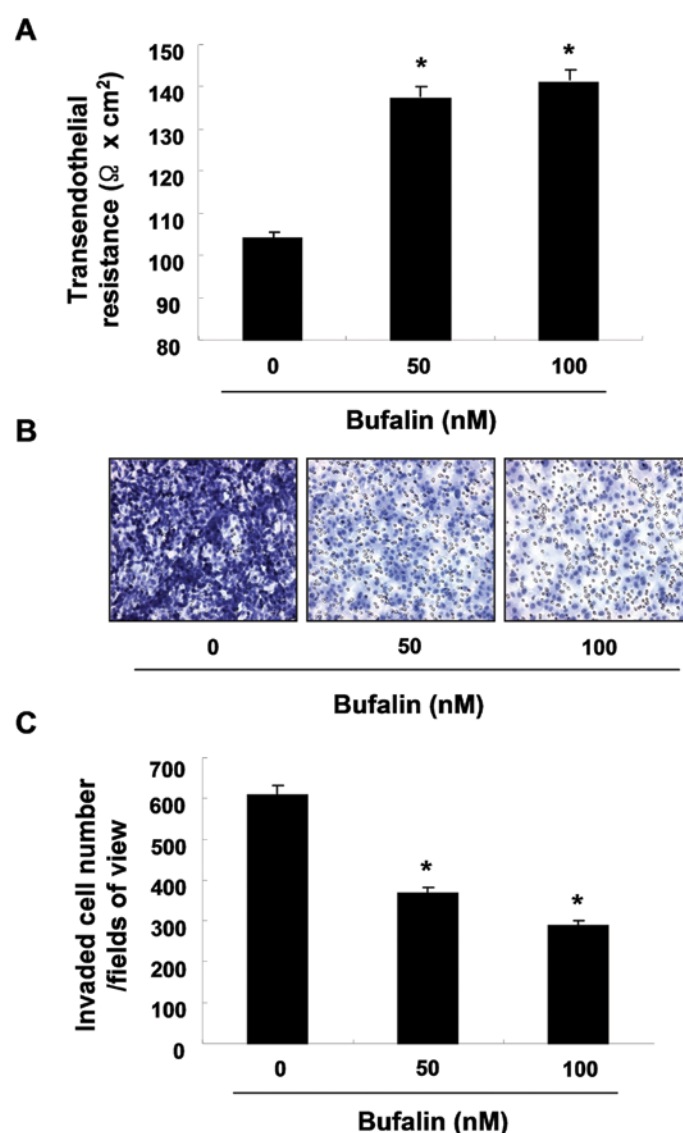
Figure 4. Effects of bufalin on cell invasion and TER values in T24 cells. (A) Cells were treated with the indicated concentrations of bufalin for 24 h and the TER value was measured using an EVOM Epithelial Tissue Voltohmmeter, as described in Materials and methods. Columns represent the mean ± SD of three independent experiments. (B and C) Cells pre-treated with 50 or 100 nM of bufalin for 6 h were plated onto the upper chamber coated with matrigel in serum-free medium with either the vehicle or bufalin. Medium containing 20% FBS was placed in the bottom chamber to act as a chemoattractant. Twenty-four hours later, the membranes were wiped from the upper chamber with a small scalper blade and stained with hematoxylin. After fixing, the membrane was observed under an inverted microscope (x50), and the numbers of invaded cells were counted. The significance was determined using the one-way ANOVA test (*p<0.05 vs. untreated control).

Bufalin modulates the expression of claudin family members. To elucidate the mechanism by which bufalin enhances TJ activity, we examined the levels of TJ components, claudins, through western blot analysis and RT-PCR analysis. As illustrated in Fig. 5, bufalin dose-dependently downregulated the levels of claudin proteins including claudin-2, -3 and -4 and considerably decreased their mRNA levels. These results indicate that this modulation of claudin family members by bufalin may be associated with the TJ tightening in T24 cells.

Bufalin inhibits enzyme activities and expression of MMPs in T24 cells. As an increase in MMP enzyme activity is important for the degradation of the ECM, which is critical to metastasis, we investigated the effect of bufalin on MMP-2 and -9 activity using western blot analysis, RT-PCR and gelatin zymography. As shown in Fig. 6, bufalin treatment led to a downregulation of

the MMP protein and mRNA levels in a dose-dependent manner, which was concomitant with decreased MMP-2 and -9 enzyme activities. However, bufalin treatment increased TIMP-1 and -2 mRNA and protein levels in a concentration-dependent manner. These results indicate that the anti-invasive effect of bufalin is associated with increased TIMP levels, as well as the inhibition of MMP expression and activity in T24 cells.

Bufalin modulates the activation of the mitogen-activated protein kinase (MAPK) pathway in T24 cells. Several lines of evidence have implicated the MAPK pathway in the regulation of MMP and TJ activity (37-39), which have been implicated in a number of cellular functions including cell survival, adhesion and metastasis. Therefore, to investigate whether the anti-invasive effect of bufalin is mediated through the modulation of the MAPK pathway, we examined the activation of the three
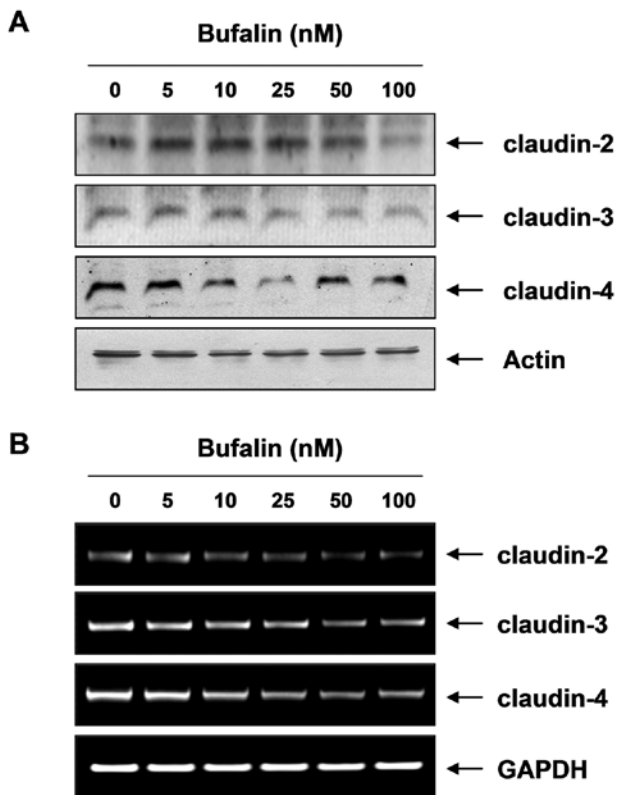
Figure 5. Effects of bufalin on the expression of claudin family members in T24 cells. (A) Cells were treated with the indicated concentration of bufalin for 24 h, lysed, and cellular proteins were separated by a SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as the internal control. (B) Total RNAs were isolated and reverse-transcribed. The resulting cDNAs were then subjected to PCR with the indicated primers and the reaction products were separated on a 1% agarose gel and visualized by EtBr staining. GAPDH was used as the internal control. Data are representative of three experiments.

MAPKs by analyzing their phosphorylated forms in western blots probed with specific anti-phosphokinase antibodies. The data demonstrated that 50 nM bufalin treatment markedly increased the phosphorylation of ERK, but not that of JNK and p38 MAPK (Fig. 7). We then evaluated the possible role of ERK signaling in bufalin-induced anti-invasive activity. As shown in Fig. 8, pre-treatment with PD98059 (an ERK inhibitor) markedly reversed the bufalin-mediated reduction of T24 cell migration, which indicates that bufalin-induced anti-invasiveness is associated with the ERK pathway.

Discussion

Bufalin is a major active component of Sum su (Chan su in Chinese), which is obtained from the skin and parotid venom gland of the toad, *Bufo bufo gargarizans* (26). Sum su has a wide range of biological applications as a cardiotonic, local anesthetic, antimicrobial, blood pressure stimulator, and has been used in respiratory and antitumor traditional medicines in Korea, China and other Asian countries (26). Moreover, a number of experimental studies on bufalin have been performed worldwide. Bufalin has revealed distinct antitumor activity, such as the inhibition of cell proliferation, induction
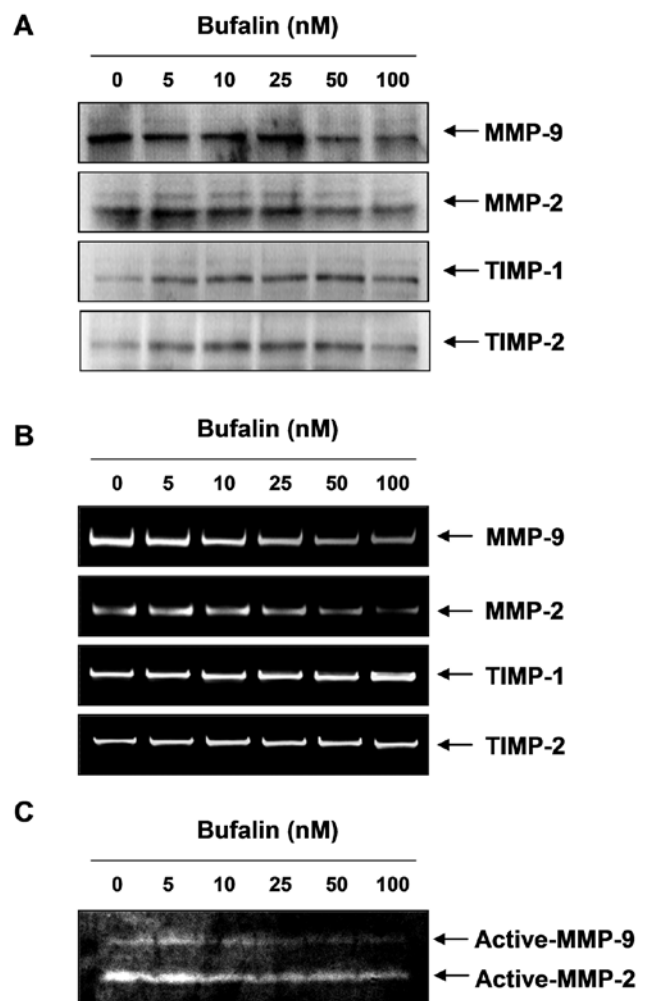

Figure 6. Inhibition of MMP expression and activity and the induction of TIMP expression by bufalin in T24 cells. (A) Cells grown under the same conditions as described in Fig. 5 were sampled, lysed, and 50 μg of proteins were separated by electrophoresis on SDS-polyacrylamide gels. Western blot analysis was then performed using the indicated antibodies and an ECL detection system. (B) After incubation with bufalin under the same conditions, total RNA was isolated and reverse-transcribed. Resulting cDNAs were then subjected to PCR. The reaction products were subjected to electrophoresis on a 1% agarose gel and visualized by EtBr staining. (C) Cells were treated with the indicated concentrations of bufalin for 24 h. The medium was collected and the activities of MMP-2 and -9 were measured by zymography as described in Materials and methods.

of cell differentiation and apoptosis, the disruption of the cell cycle, inhibition of angiogenesis, the ability to overcome anticancer drug resistance and the modulation of the immune response (27-33,40-42). Therefore, it is expected that bufalin may be a novel target to reduce cancer cell metastasis. However, the anti-metastatic activity of bufalin has not yet been extensively investigated; to our knowledge, only one study has been published so far (34). In the present study, we found that bufalin significantly inhibited the migratory and invasive activity in T24 bladder carcinoma cells by enhancing TJ activity, downregulating MMP activity and modulating TJ-related proteins *in vitro*.

TJs are completely necessary to create a tight seal of cellular sheets, which is comprised of three major integral membrane proteins, claudin, occludin and junctional adhesion
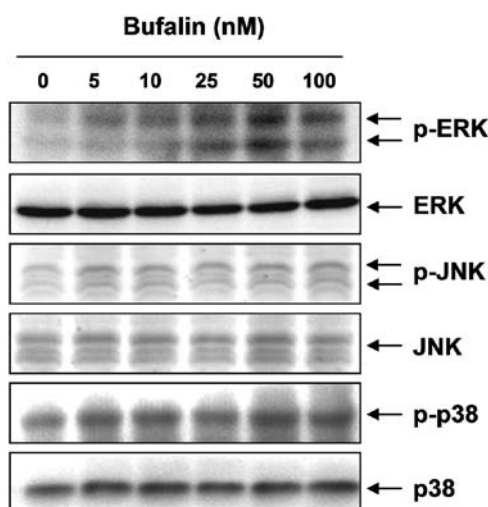
Bufalin (nM)

Figure 7. Effects of bufalin on the expression of MAPKs in T24 cells. Cells were treated with the indicated concentration of bufalin for 24 h, lysed, and cellular proteins were separated by SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system.

molecules. According to previous studies, the modulation of claudins plays an important role during oncogenic transformation exhibiting distinct tissue- and development-specific distribution patterns (8,43). Thus, the regulation of claudin proteins may be the new target for cancer treatment. Our data

showed that bufalin treatment downregulated almost all the claudin levels. In this regard, the downregulation of claudins is connected with the tightening of TJs, the inhibition of cell migration and the invasion in T24 cells. In addition, bufalin induced a significant inhibition of protein and mRNA levels and the enzyme activities of MMP-2 and -9. Simultaneously, the mRNA and protein levels of TIMP-1 and -2 were elevated in a concentration-dependent manner. MMP-2 and -9, two types of gelatinases, are key agonists in tumor invasion and angiogenesis; thus, tumor metastasis may be inhibited by blocking MMP synthesis and activation (11,44). Therefore, the data indicated that the activity of MMPs is limited to physiologically binding to one of four endogenous TIMPs; therefore, the balance of secreted MMPs and TIMPs is maintained in the connective tissue homeostasis of normal tissue. However, an imbalance of MMPs and TIMPs has usually been exhibited to lead to an excess of degradative activity (45). As shown in Fig. 6, the anti-invasive activity of bufalin in T24 cells was related with the decrease in the MMP/TIMP ratio as a key factor in the regulation of the anti-metastatic process, which subsequently blocks the degradation of the ECM and leads to the inhibition of cell invasion.

Although the MAPK pathway plays a critical role in regulating cell death and survival in many physiological and pathological settings, numerous reports have demonstrated that the function and expression of TJs and MMPs are critically mediated by the MAPK pathway, suggesting that these pathways also play an important role in tumor metastasis (37-39). Thus, we confirmed the involvement of the MAPK pathway in
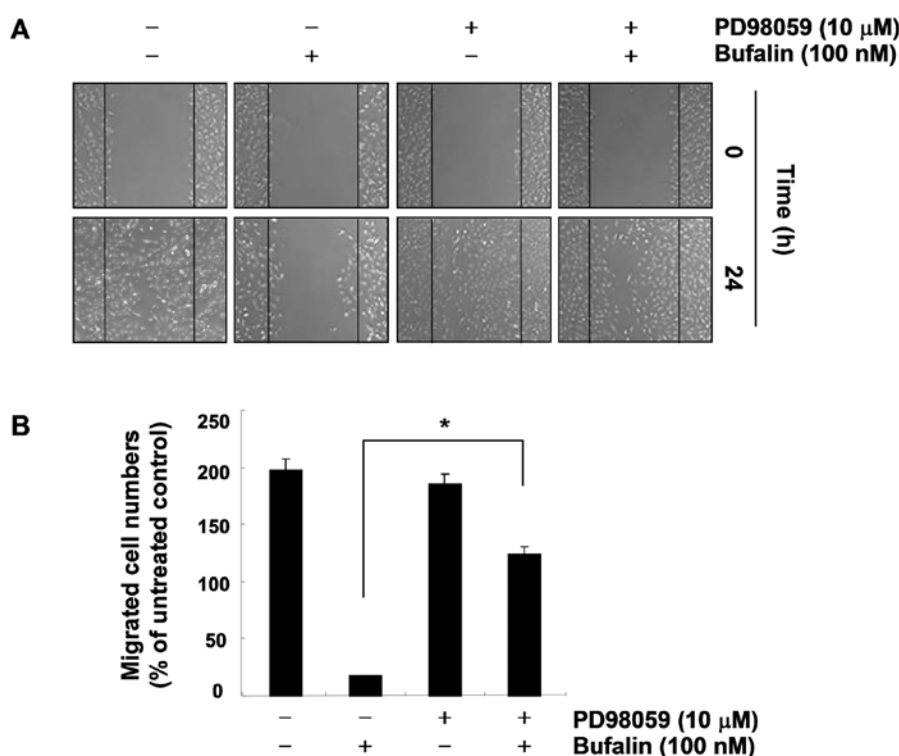

Figure 8. Suppression of bufalin-induced inhibition of cell motility by inhibition of ERK signaling in T24 cells. Cells were treated with 100 nM bufalin for 24 h or the ERK inhibitor, PD98059 (10 μM), was added to T24 cells and incubated for 2 h before treatment with bufalin for 24 h. (A) At indicated times, wound closures were photographed under a microscope (x50). (B) Migrated cell numbers within the black line were counted. The assays were repeated twice and each sample was observed in triplicate. Values marked as (*) indicate significant differences from other treatments (*p<0.05).

bufalin-induced anti-invasive activity. In the present study, we observed that bufalin induced the activation of ERK, but not that of JNK and p38 MAPK, and that the inhibition of ERK suppressed the bufalin-induced inhibition of cell migration. These results suggest that the bufalin inhibition of cell invasion of T24 cells may partly occur through the activation of the ERK pathway.

In view of the results thus far achieved, bufalin suppresses the attachment to the ECM, cell migration and invasion in T24 cells. Although further controlled trials are warranted, these results provide evidence that bufalin inhibits the metastasis of human bladder cancer *in vitro*. Overall, the present study suggests that bufalin is a promising therapeutic agent for cancer therapy with low toxicity and few side-effects.

Acknowledgements

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