Effects of bufalin on the proliferation of human lung cancer cells and its molecular mechanisms of action

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Received: 31 July 2010 / Accepted: 26 September 2010 / Published online: 21 October 2010
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Abstract Bufalin, a naturally occurring small-molecule compound from Traditional Chinese Medicine (TCM) *Chansu* showed inhibitory effects against human prostate, hepatocellular, endometrial and ovarian cancer cells, and leukemia cells. However, whether or not bufalin has inhibitory activity against the proliferation of human non-small cell lung cancer (NSCLC) cells is unclear. The aim of this study is to study the effects of bufalin on the proliferation of NSCLC and its molecular mechanisms of action. The cancer cell proliferation was measured by MTT assay. The apoptosis and cell cycle distribution were analyzed by flow cytometry. The protein expressions and phosphorylation in the cancer cells were detected by

Western blot analysis. In the present study, we have demonstrated that bufalin suppressed the proliferation of human NSCLC A549 cell line in time- and dose-dependent manners. Bufalin induced the apoptosis and cell cycle arrest by affecting the protein expressions of Bcl-2/Bax, cytochrome *c*, caspase-3, PARP, p53, p21WAF1, cyclinD1, and COX-2 in A549 cells. In addition, bufalin reduced the protein levels of receptor expressions and/or phosphorylation of VEGFR1, VEGFR2, EGFR and/or c-Met in A549 cells. Furthermore, bufalin inhibited the protein expressions and phosphorylation of Akt, NF- κ B, p44/42 MAPK (ERK1/2) and p38 MAPK in A549 cells. Our results suggest that bufalin inhibits the human lung cancer cell proliferation via VEGFR1/VEGFR2/EGFR/c-Met–Akt/p44/42/p38-NF- κ B signaling pathways; bufalin may have a wide therapeutic and/or adjuvant therapeutic application in the treatment of human NSCLC.

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Keywords Bufalin · Non-small cell lung cancer ·
Proliferation · Apoptosis · Cell cycle arrest · VEGFR/
EGFR/c-Met · MAPKs · Akt-NF- κ B pathways

Abbreviations

BF	Bufalin
MAPKs	Mitogen-activated protein kinases
ERK $\frac{1}{2}$	Extracellular signal-regulated kinase $\frac{1}{2}$
NF- κ B	Nuclear factor κ B
VEGFR	Vascular endothelial growth factor receptor

EGFR	Epidermal growth factor receptor
LY	Ly294002
Bay	Bay 11-7082
SB	SB203580
PD	PD98059
PARP	Poly(ADP-ribose) polymerase
Cyto C	Cytochrome <i>c</i>
COX-2	Cyclooxygenase-2
NSCLC	Non-small cell lung cancer

Introduction

Lung adenocarcinoma is the leading cause of cancer-related deaths among both men and women in the world (Jemal et al. 2008; Parkin et al. 2005). Despite recent advances in diagnosis and treatment, overall 5-year survival of lung cancer patients is only 15%. The non-small cell lung cancer (NSCLC), which constitutes ~80% of lung cancer cases, remains an aggressive lung cancer associated with a poor patient survival. The patients with advanced disease have a median survival of approximately 10 months when treated with standard platinum-based therapy. Tumors depend on complex signaling networks to promote malignant progress and dynamically adapt to stress including chemotherapy drug treatment. Blocking only one of these pathways allows others to act as salvage or escape mechanisms for cancer cells. Therefore, it is necessary to develop new anticancer agents that could produce the most effective targeted cancer therapies by their concomitant inhibition of multiple targets. Bufalin is a naturally occurring small-molecule compound from Traditional Chinese Medicine (TCM) *Chansu* which has been used to treat lung, hepatocellular carcinoma, or pancreatic cancer in human patients (Meng et al. 2009). *Chansu* is the dried venom of toad (*Bufo bufo gargarizans* CANTOR or *Bufo melanostictus* SCHNEIDER) and has been used as an Oriental drug (Yasuharu et al. 2004). The chemical structure of bufalin is shown in Fig. 1. Bufalin isolated from *Chansu* was reported to have the activity of inducing apoptosis in cancer cells such as human prostate, hepatocellular, endometrial and ovarian cancer cells and leukemia cells. (Gu et al. 2007; Takai et al. 2008; Watabe et al. 1996; Yu et al. 2008). However, whether or not bufalin has inhibitory activity against the proliferation of human NSCLC

Fig. 1 Chemical structure of bufalin (BF)

cells and its molecular mechanisms of action are unclear. In the present study, we show that bufalin significantly inhibited the proliferation of human NSCLC A549 cell line by inducing apoptosis and cell cycle arrest. We have demonstrated that the molecular mechanisms of action of bufalin against the cancer cells are associated with suppressing the related receptors-mediated multiple signaling pathways in the cancer cells.

Materials and methods

Chemicals and antibodies

The primary antibodies to human Bcl-2, Bax, pp38 and p38 MAP kinase, pp44/42 and p44/42 MAPK (ERK1/2), p21 Waf1/Cip1, p53, caspase-3, PARP-1, cytochrome *c*, cyclic D1, and β -actin were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The primary antibodies to human pVEGFR2, VEGFR2, VEGFR1, pEGF, EGF, p-c-Met, c-Met, nuclear factor (NF- κ B p-65), p-NF- κ B, Akt, and pAkt were purchased from Santa Cruz Technology Inc. (Beverly, MA, USA). DMEM, Penicillin, streptomycin, fetal bovine serum (FBS), trypsin/EDTA, propidium iodide, gelatin, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), bufalin, Ly294002 (LY), Bay 11-7082 (Bay), SB203580 (SB), and PD98059 (PD) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture and in vitro proliferation assay

The A549 lung adenocarcinoma cell line was obtained from the American Type Culture Collection. The human cancer cell lines A549 was cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine

serum (FBS), glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 °C in a humidified incubator with 95% air/5% CO₂ atmosphere. The *in vitro* assay was performed according to our published methods (Liu et al. 2009; Luan et al. 2010). Cells were plated in 96-well plates (Becton–Dickinson, NJ, USA) at 2×10^3 per well and incubated overnight to allow attachment. The cells in control group were treated with DMSO [0.1% (v/v), final concentration]. The cells were, respectively incubated in RPMI 1640 medium supplemented with 10% FBS containing different concentrations of bufalin (BF, 2.5–10 µM), Ly294002 (LY, 50 µM), Bay (10 µM), PD98059 (PD, 7 µM), and SB203580 (SB, 50 µM). LY, Bay, PD, and SB are the inhibitors of PI3 K/Akt, NF-κB, p44 (ERK1/2), and p38 MAPK, respectively. After 48 or 72 h of treatment, 20 µL of MTT (5 mg/mL) was added to each well of cells, and the plate was incubated for 3 h at 37 °C. One hundred and fifty microliters DMSO were added to each well to lyse the cells. Absorbance was measured at a wavelength of 570 nm and a reference wavelength of 630 nm using a Synergy H4 Hybrid Multi-Mode Microplate Reader (Bio-Tek, Instruments, Inc., Winooski, VT, USA). Absorbance values in each test group were normalized to the values of the control group treated with 0.1% (v/v) DMSO to determine the viability (%) of A549 cells. Here the absorbance values are designated as the viability rate (%) of A549 cells. The viability rate of A549 cells in the control group was designated as 100%. Each experiment was performed in triplicate and repeated at least 3 times.

Flow cytometry for cell cycle analysis and apoptosis

These were done according to our published methods (Wang et al. 2009; Yu et al. 2009; Zhang et al. 2000). In brief, A549 cells were treated for 48 h with bufalin at concentrations of 0, 2.5, 5, and 10 µM. The treated cells were detached in PBS/2 mM EDTA, centrifuged at $300 \times g$ for 5 min, and then gently resuspended in 250 µL of hypotonic fluorochrome solution (PBS, 50 µg propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100) with RNase A (100 units/mL). The function of the fluorochrome solution is to stain cell nuclei. The DNA content was analyzed by flow cytometry (Becton–Dickinson FACS Vantage SE, San Jose, CA, USA). Twenty-thousand events were

analyzed per sample and the cell cycle distribution and apoptosis were determined based on DNA content and the Sub-G₁ cell population, respectively.

Western blot analysis

This was performed according to our published methods with some modifications (Wang et al. 2009; Yu et al. 2009; Zhang et al. 2009). In brief, A549 cells were treated with bufalin (0, 2.5, 5 and 10 µM), or Ly294002 (LY, 50 µM), Bay (10 µM), PD98059 (PD, 7 µM), and SB203580 (SB, 50 µM). The cells in control group were treated with DMSO [0.1% (v/v), final concentration]. The treated cells were collected either at 30 min for detection of phosphorylation ratios of pVEGFR2/pVEGFR2, pEGFR/EGFR, p-c-Met/c-Met, pAkt/Akt, pp38/p38, pp44/42/p44/42, and pNF-κB/NF-κB, or at 48 h for detection of protein expressions of Bcl-2, Bax, p21WAF1, p53, procaspase/caspase-3, PARP-1, cyclin D1, VEGFR1, COX-2, and cytochrome *c*. For detection of mitochondrial and cytosolic cytochrome *c*, both mitochondrial and cytosolic proteins from the cell extracts were used. The method of mitochondrial purification was described below. Equal amounts of cell extracts were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and probed with the primary antibodies to the proteins to be detected as mentioned above, and then with horseradish peroxidase-conjugated secondary antibodies, respectively. Anti-β-actin antibody was used as a loading control. Detection was done using an enhanced chemiluminescence system (GE Healthcare Life Sciences, Piscataway, NJ USA).

Mitochondrial purification

A549 cells were seeded in a 10 cm dish. The cells were treated for 48 h with BF (BF1/2.5, BF5/5, BF10/10 µM), Bay (10 µM), and Ly (50 µM). After the treatment, the cells were harvested and lysed in homogenization buffer (10 mM Tris–HCl, pH 6.7, 10 mM KCl, 0.15 mM MgCl₂, 1 mM PMSF, and 1 mM DTT). After incubating on ice for 15 min, cell membranes were disrupted by more than 10 passes through a 23-gauge needle. The homogenate was centrifuged at $1,200 \times g$ for 5 min, the supernatant was collected and centrifuged at $7,000 \times g$ for 10 min. The mitochondrial pellet obtained was resuspended in

mitochondrial suspension buffer (10 mM Tris–HCl pH 6.7, 0.15 mM MgCl₂, 0.25 M sucrose, 1 mM PMSF, 1 mM DTT). The supernatant was spun at $9,500 \times g$ for 5 min to re-pellet the mitochondria. Protein concentrations were determined by Bio-Rad protein assay and equivalent amounts of protein were resolved by SDS–PAGE and transferred to PVDF membranes. After the membrane was blocked in Tris-buffer saline containing 0.05% Tween 20 (TBST) and 5% non-fat powdered milk, the membranes were incubated with primary antibody specific to cytochrome *c*.

Statistical analysis

The data were expressed as mean $\pm$ standard deviation (S.D.) and analyzed by the SPSS 13.0 software to evaluate the statistical difference. Statistical analysis was done using the ANOVA and Bonferroni test. Values between different treatment groups at different times were compared. The rate (%) of cell viability and mean protein levels are shown for each group. For all tests, *p* values less than 0.05 were considered statistically significant. All statistical tests were two-sided.

Results and discussion

Bufalin shows in vitro inhibition of proliferation and induction of apoptosis and cell cycle arrest in A549 cells

We first confirmed that bufalin reduced the viability rates of human NSCLC A549 cell line in dose- and time-dependent manners after the cells were treated with bufalin at 2.5–10 μ M for 48 and 72 h, respectively (Fig. 2A). In addition, we also showed that the inhibitor of PI3 K/Akt (LY, 50 μ M), NF- κ B (Bay, 10 μ M), ERK1/2 (PD, 7 μ M), and p38 MAPK (SB, 50 μ M) significantly decreased the viability of A549 cells (Fig. 2A). Furthermore, bufalin at concentrations of 2.5–10 μ M induced apoptosis and cell cycle arrest in G₁ phase in A549 cells after the cells were treated for 48 h (Fig. 2B). These results partially explain why bufalin has therapeutic and/or adjuvant therapeutic effects on treatment of some patients with lung cancer.

Bufalin reduces the protein levels of Bcl-2/Bax, cyclin D1 and COX-2 and enhances the protein levels of cytosolic cytochrome *c*, caspase-3, PARP-1, p53 and p21WAF1 in A549 cells

To understand the mechanisms of action of bufalin against proliferation and on induction of apoptosis and cell cycle arrest in A549 cells, we studied the effects of bufalin on related protein expressions. Our results showed that bufalin at 2.5–10 μ M reduced the ratios of Bcl-2/Bax proteins (Fig. 3A) and cyclin D1 (Fig. 4C) protein expression as well as the COX-2 protein level (Fig. 4D) in A549 cells. In addition, bufalin at these concentrations also enhanced the protein expressions of cytosolic/mitochondrial cytochrome *c* (ratio) (Fig. 3B), caspase-3 (Fig. 3C), the cleavage of poly (ADP-ribose) polymerase-1 (PARP-1) (Fig. 3D), tumor suppressor p53 (Fig. 4A) and endogenous cyclin-dependent kinase inhibitor p21 WAF1/CIP1 (Fig. 4B) in A549 cells. In addition, the inhibitor of PI3 K/Akt (LY) and NF- κ B (Bay) showed significant suppression of protein expressions of Bcl-2, cyclin D1 and COX-2 and increase in the protein levels of Bax, cytosolic cytochrome *c*, caspase-3, the cleavage of PARP, p53 and p21 WAF1 in A549 cells (Figs. 3 and 4).

One of the main regulatory steps of apoptotic cell death is controlled by the ratio of antiapoptotic and proapoptotic members of the Bcl-2 family of proteins, which determines the susceptibility to apoptosis. Bax, a proapoptotic factor of the Bcl-2 family, is found in monomeric form in the cytosol or is loosely attached to the membranes under normal conditions. Following a death stimulus, cytosolic and monomeric Bax translocates to the mitochondria where it becomes an integral membrane protein and cross-linkable as a homodimer allowing for the release of factors from the mitochondria, such as cytochrome *c*, to propagate the apoptotic pathway (Oltvai et al. 1993). Bcl-2 and its related proteins control the release of cytochrome *c* from the mitochondria (Reed 1997). Hallmarks of the apoptotic process include the activation of cysteine proteases, which represent both initiators and executors of cell death. In the cytosol, cytochrome *c* activates caspase-9, which in turn activates the effector caspases such as caspase-3 and caspase-7 (Stennicke and Salvesen 2000). In the present study, we observed that bufalin reduced the antiapoptotic/proapoptotic Bcl-2/Bax ratio (Fig. 3A).

Fig. 2 Effects of bufalin (BF) on proliferation and induction of apoptosis and cell cycle arrest in A549 cells. **A** The cells were treated for 48 and 72 h with the indicated concentrations of bufalin (BF1/2.5, BF5/5, BF10/10 μ M), LY/50 μ M, Bay/10 μ M, PD/7 μ M, and SB/50 μ M. The cells in control group were treated with DMSO [0.1% (v/v), final concentration]. The rate of cell viability was determined by the MTT assay. LY, Bay, PD, and SB are the inhibitors of PI3 K/Akt, NF- κ B, p44 (ERK1/2) and p38 MAPK, respectively. **B** Induction of apoptosis (cells in Sub-G₁ phase) and cell cycle arrest (in G₁ phase) in A549 cells by BF was analyzed by flow cytometry. The cells were treated for 48 h with BF at concentrations of 2.5, 5 and 10 μ M. The data are presented as the mean $\pm$ SD (Bar) for each group ($n = 6$). The figures (A, B) are the representative of 3 similar experiments performed. Values with different letters (*a–e*) differ significantly ($p < 0.05$)

In addition, bufalin caused the release of cytochrome c from the mitochondria (Fig. 3B) and subsequently increased caspase-3 activity (Fig. 3C). Caspase-3 is synthesized as a 35-kDa inactive precursor (procaspase-3), which is proteolytically cleaved to produce a mature enzyme composed of 17-kDa fragments. The procaspase-3 protein was reduced and the caspase-3 in cleaved form was increased in the apoptotic A549

cells after the cells were treated with bufalin at the concentrations of 2.5–10 μ M (Fig. 3C). In addition, we observed the cleavage of PARP-1, substrate of caspase-3, in A549 cells treated with 2.5–10 μ M of bufalin (Fig. 3D).

The tumor suppressor protein p21 WAF1/CIP1 acts as an inhibitor of cell cycle progression. It functions in stoichiometric relationships forming

Fig. 3 Effects of bufalin (BF) on protein expressions of Bcl-2/Bax (A), cytosolic and mitochondrial cytochrome *c* (cyto *c*) (B), caspase-3/procaspase-3 (C), and PARP-1 (D) in A549 cells. The cells were treated for 48 h with BF (BF1/2.5, BF5/5, BF10/10 μ M), Bay (10 μ M) and LY (50 μ M). The protein expressions were analyzed by Western Blotting. The optical density (OD) of the band in each panel is normalized to β -actin, respectively. The OD value in each panel is relative to that of control [0.1% (v/v) DMSO vehicle] which is designated

as 100%. The average OD value and standard deviation from three assays were calculated. The OD value of the band (normalized to β -actin) in each panel shown as mean $\pm$ SD is relative to that of the control designated as 100%. The ratios of each band relative to the control are designated as the ratios (%) of protein expression. LY and Bay are the inhibitors of PI3 K/Akt and NF- κ B, respectively. For one experiment, 3 assays were carried out and only one set of gels is shown. Values with different letters (*a–e*) differ significantly ($p < 0.05$)

heterotrimeric complexes with cyclins and cyclin-dependent kinases. In association with CDK2 complexes, it serves to inhibit kinase activity and block progression through G₁/S (Pestell et al. 1999). When p53 is phosphorylated under UV damage or the treatment with anticancer agents, it upregulates p21 transcription via a p53 responsive element (Wang and Prives 1995). Activation of p53 can lead to either cell cycle arrest and DNA repair or apoptosis (Levine 1997). In the present study, we observed that the protein levels of p53 (Fig. 4A) and p21 WAF1 (Fig. 4B) increased by more than 4 and twofold, respectively and Cyclin D1 (Fig. 4C) decreased by 50% in A549 cells by bufalin treatment at 10 μ M. In addition, we also observed that bufalin at 2.5 to 10 μ M displayed a strong effect on suppression of the COX-2 protein expression in A549 cells (Fig. 4D).

Taken together, all these results show that bufalin inhibits the proliferation of A549 cells and induces apoptosis and cell cycle arrest by reducing Bcl-2/Bax ratio and activating the mitochondrial and caspase-3 pathway as well as affecting the cell cycle regulators such as p53, p21 WAF1 and cyclin D1 in A549 cells.

Bufalin reduces the receptor phosphorylation and/or protein expression of VEGFR2, VEGFR1, EGFR, and c-Met in A549 cells

The activated receptors such as VEGFR, EGFR, and c-Met play very important roles in the proliferation of cancer cells including lung cancer cells (Colon et al. 2009; Kim et al. 2008; Morelli et al. 2006; Naumov et al. 2009; Puri and Salgia 2008). In order to clarify the bufalin-targeted receptors in A549 cells, we studied the effects of bufalin on the related receptor

Fig. 4 Effects of bufalin (BF) on protein expressions of p53 (A), p21WAF1 (B), cyclin D1 (C) and COX-2 (D) in A549 cells. The cells were treated for 48 h with BF (BF1/2.5, BF5/5, BF10/10 μ M), Bay (10 μ M) and LY (50 μ M). The protein expressions were analyzed by Western Blotting. The optical density (OD) of the band in each panel is normalized to β -actin, respectively. The OD value in each panel is relative to that of control [0.1% (v/v) DMSO vehicle] which is designated as 100%. The average OD value and standard deviation from

three assays were calculated. The OD value of the band (normalized to β -actin) in each panel shown as mean $\pm$ SD is relative to that of the control designated as 100%. The ratios of each band relative to the control are designated as the ratios (%) of protein expression. LY and Bay are the inhibitors of PI3 K/Akt, and NF- κ B, respectively. For one experiment, 3 assays were carried out and only one set of gels is shown. Values with different letters (a–e) differ significantly ($p < 0.05$)

phosphorylation and expressions in A549 cells. We have confirmed that bufalin at 2.5–10 μ M significantly inhibited the phosphorylation and expressions of VEGFR2 (Fig. 5A), EGFR (Fig. 5C), and c-Met (Fig. 5D) in A549 cells. Bufalin at the concentration of 10 μ M reduced the levels of phosphorylation of VEGFR2, EGFR, and c-Met by 50%, 40% and 22% and the protein expressions of VEGFR2, EGFR, and c-Met by 18%, 33% and 17%, respectively. Bufalin at 10 μ M reduced the VEGFR1 (Fig. 5B) protein expression by 72% in A549 cells. In addition, the inhibitor of PI3 K/Akt (LY) and NF- κ B (Bay) also showed inhibitory effects on the phosphorylation and expressions of these receptors (Fig. 5) in A549 cells. Down-regulation of the receptor phosphorylation and protein expressions in A549 cells by bufalin could greatly contribute to the suppression of their downstream targets and the receptors-mediated PI3 K/Akt

and NF- κ B as well as the related signaling pathways, and thus leads to inhibition of the proliferation in A549 cells.

Bufalin reduces the phosphorylation and protein expression of Akt, NF- κ B, ERK1/2, and p38 MAPK in A549 cells

In order to further confirm the inhibitory effects of bufalin on the receptors-mediated signaling pathways related to the proliferation of A549 cells, we investigated the effects of bufalin on the phosphorylation and expressions of Akt, MAP kinases p44/42 (ERK1/2) and p38 as well as NF- κ B in A549 cells. Our results show that bufalin at 2.5–10 μ M significantly suppressed the phosphorylation and expressions of Akt (Fig. 6A), ERK1/2 (Fig. 6B), p38 MAPK (Fig. 6C), and NF- κ B (Fig. 6D) in A549 cells.

Fig. 5 Effects of bufalin (BF) on receptor protein expressions and phosphorylation of pVEGFR2/VEGFR2 (A), VEGFR1 (B), pEGFR/EGFR (C), and p-c-Met/c-Met (D) in A549 cells. The cells were treated for 30 min with BF (BF1/2.5, BF5/5, BF10/10 μ M), Bay (10 μ M) and LY (50 μ M). The protein expressions and phosphorylation were analyzed by Western Blotting. The optical density (OD) of the band in each panel is normalized to β -actin, respectively. The OD value in each panel is relative to that of control [0.1% (v/v) DMSO vehicle] which is designated as 100%. The average OD value and

standard deviation from three assays were calculated. The OD value of the band (normalized to β -actin) in each panel shown as mean $\pm$ SD is relative to that of the control designated as 100%. The ratios of each band relative to the control are designated as the ratios (%) of protein expression. LY and Bay are the inhibitors of PI3 K/Akt and NF- κ B, respectively. For one experiment, 3 assays were carried out and only one set of gels is shown. Values with different letters (a–d) differ significantly ($p < 0.05$)

In addition, the inhibitor of PI3 K/Akt (LY), ERK1/2 (PD), p38 MAPK (SB) and NF- κ B (Bay) significantly suppressed the phosphorylation and protein expressions of Akt, ERK1/2, p38 MAPK and NF- κ B in A549 cells, respectively (Fig. 6A–D).

Akt is a cytosolic signal transduction protein kinase that plays an important role in cell survival pathways (West et al. 2002). Induction of Akt activity is primarily dependent on the PI3 K pathway. Akt can be activated by many forms of cellular stress, such as those observed under treatment with anticancer agents (West et al. 2002). Once activation, Akt controls cellular functions such as apoptosis, cell cycle, gene transcription, and protein synthesis through the phosphorylation of downstream substrates such as NF- κ B, p53 and COX-2 (West et al. 2002). NF- κ B is a nuclear transcription regulator with a specific motif for Bcl-2 transcription (Marsden et al. 2002; Wang et al. 1996).

Activation of p-Akt and the NF- κ B/Bcl-2 pathway leads to inhibition of chemotherapy-induced apoptosis, which results in treatment resistance (Wang et al. 1996). Our previous results confirmed that suppression of Akt and the NF- κ B/Bcl-2 pathway by anticancer agents inhibited growth and induced apoptosis and cell cycle arrest in A549 cells and breast cancer MDA-MB-231 cells (Wang et al. 2009; Yu et al. 2009). In the present study, we indicated that bufalin and the inhibitor of PI3 K/Akt (LY294002) and of NF- κ B (Bay) inhibited the A549 cell proliferation and the PI3 K/Akt and NF- κ B pathway by suppressing the phosphorylation and expressions of Akt and NF- κ B and activating the apoptotic pathway of Bcl-2/Bax-mitochondrial-caspase-3 in A549 cells.

The p53 is the most extensively studied tumor suppressor gene in human cancer. The p53 gene product is involved in multiple pivotal cellular

Fig. 6 Effects of bufalin (BF) on protein expressions and phosphorylation of pAkt/Akt (A), pp44(42)/p44(42) (B), pp38/p38 (C), and p-c-Met/c-Met (D) in A549 cells. The cells were treated for 30 min with BF (BF1/2.5, BF5/5, BF10/10 μ M), Bay (10 μ M), LY (50 μ M), PD (7 μ M) and SB (50 μ M). The protein expressions and phosphorylation were analyzed by Western Blotting. The optical density (OD) of the band in each panel is normalized to β -actin, respectively. The OD value in each panel is relative to that of control [0.1% (v/v) DMSO vehicle] which is designated as 100%. The average OD value

and standard deviation from three assays were calculated. The OD value of the band (normalized to β -actin) in each panel shown as mean $\pm$ SD is relative to that of the control designated as 100%. The ratios of each band relative to the control are designated as the ratios (%) of protein expression. LY, Bay, PD, and SB are the inhibitors of PI3 K/Akt, NF- κ B, p44 (ERK1/2), and p38 MAPK, respectively. For one experiment, 3 assays were carried out and only one set of gels is shown. Values with different letters (a–d) differ significantly ($p < 0.05$)

processes as a potent transcriptional regulator, and one of its most important roles is the regulation of apoptosis (Vousden and Lu 2002). Up-regulation of wild type (WT) of p53 in human NSCLC is a favorable prognostic factor and is associated with a significantly longer survival (Lee et al. 1995). Rescue of p53-induced apoptosis by survival factors has been associated with the activation of Akt kinase (Sabbatini and McCormick 1999). Akt can phosphorylate and activate MDM2, thereby enhancing the degradation of p53 (Mayo and Donner 2001; Zhou et al. 2001). Thus, up-regulation of WT p53 in A549 cells after treatment with either bufalin or inhibitor of PI3 K/Akt (LY), as observed in our study, could be explained by down-regulation of the pAkt/MDM2 pathway. In addition to its interplay in the regulation of apoptosis, WT p53 has been shown to directly

affect Bax and Bcl-2 expressions. Thus, WT p53 is able to mediate repression of the Bcl-2 gene and the transactivation of Bax (Miyashita et al. 1994). Therefore, up-regulation of WT p53 could be responsible for the decrease in the Bcl-2/Bax ratio in A549 after treatment with bufalin.

COX-2, a marker of poor prognosis in stage I NSCLC, also is a promising target in treating NSCLC (Khuri et al. 2001). Inhibition of p44/42 MAPK-dependent pathway (Chang et al. 2002), p38 MAPK, or pAkt and NF- κ B pathways (Lin et al. 2002; Shishodia et al. 2004) led to down-regulation of synthesis of COX-2 in A549 cells. In the present study, suppression of the pathways of p44/42 and p38 MAPKs, pAkt and p-NF- κ B by bufalin could be responsible for the reduction of COX-2 expression in A549 cells.

The ERK1/2 and p38 MAPK signaling pathways can be activated in response to a diverse range of extracellular stimuli including mitogens, growth factors, and cytokines (Baccarini 2005; Meloche and Pouyssegur 2007; Roux and Blenis 2004) and are important targets in the diagnosis and treatment of cancer (Roberts and Der 2007). Suppression of the activated EGFR and ERK1/2 and p38 MAPKs as well as Akt signaling pathways led to inhibition of the proliferation of A549 cells (Baldys et al. 2007; Nguyen et al. 2004; Recchia et al. 2009; Su et al. 2010). There has been the cross-talk in EGFR and c-Met pathways in A549 cells which is related to the A549 cell proliferation (Puri and Salgia 2008). Suppression of EGFR, VEGFR, and c-Met led to inhibition of A549 cell proliferation in vitro and in vivo (Colon et al. 2009; Kim et al. 2008; Morelli et al. 2006; Naumov et al. 2009). Here we have confirmed that bufalin remarkably suppressed the VEGFR1, VEGFR2, EGFR and c-Met receptors and their downstream targets such as p44/42, p38, Akt and NF- κ B in A549 cells, which could contribute to inhibition of A549 cell proliferation.

In summary, our present results have demonstrated that bufalin suppressed the A549 cell proliferation and induced the apoptosis and cell cycle arrest at G₁ phase by targeting the EGFR, VEGFR1, VEGFR2, and c-Met receptors-mediated signaling pathways of Akt, p44/42 and p38 MAPKs, and NF- κ B in A549 cells. All these findings suggest that bufalin may have the wide therapeutic and/or adjuvant therapeutic application in the treatment of human NSCLC.

Acknowledgments The authors would like to extend our thanks to Drs. Jianyuan Li and Shaohua Jin for FACS analysis. This work is supported in part by grants from the Ministry of Education of the People's Republic of China to G.Z., from the Ministry of Human Resources and Social Security of the People's Republic of China to G.Z., Projects of Yantai University to G.Z., Project from the National Natural Science Foundation of China to G.Z. (No. 30973553), and grants from the Department of Science and Technology of Shandong Province to G.Z. (Y2008C71; 2009GG10002087).

Conflict of interest The authors declare no conflict of interest.

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