Bufalin increases sensitivity to AKT/mTOR-induced autophagic cell death in SK-HEP-1 human hepatocellular carcinoma cells

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Abstract. Bufalin is the major component of Chan-Su (a traditional Chinese medicine, TCM) extracts from the venom of Bufo bufo gargarizan. In the present study, we investigated the pharmacological mechanisms of cell cycle arrest and autophagic cell death induced by bufalin in SK-HEP-1 human hepatocellular carcinoma cells in vitro. Bufalin inhibited cell survival by MTT assay and increased cell death by trypan blue exclusion assay in a concentration-dependent manner. In addition, bufalin induced G2/M phase arrest by reducing CDK1 activity. Bufalin triggered DNA fragmentation and apoptotic cell death in SK-HEP-1 cells by DNA gel electrophoresis, TUNEL and caspase-3 activity assay, while bufalin induced autophagic cell death by double-membrane vacuoles (transmission electron microscopy, TEM), acidic vesicular organelles (acridine orange staining) and cleavage of microtubule-associated protein 1 light chain 3 (LC3). Protein expression levels of cyclin A and B, CDK1, phospho-CDK1 (Thr161), Cdc25c, phospho-Cdc25c (Ser198), phospho-AKT (Thr308), phospho-AKT (Ser473), phospho-mTOR (Ser2481) were downregulated. In contrast, protein expression levels of the Chk1, Weel, LC3-II, Beclin-1, Atg 5, Atg 7 and Atg 12 were upregulated in SK-HEP-1 cells after bufalin treatment. Inhibition of autophagy by 3-methyl-adenine (an inhibitor of class III phosphatidylinositol-3 kinase; 3-MA) or bafilomycin A1 (an inhibitor of the vacuolar proton pump of lysosomes and endosomes) reduced the effect of bufalin on cell viability and enhanced the effect of bufalin on apoptosis. In conclusion, bufalin triggered autophagic cell death and G2/M phase arrest through the AKT/mTOR signaling pathway in SK-HEP-1 cells. Our findings showed that bufalin may be potentially efficacious in the treatment of human hepatocellular carcinoma.

Introduction

In Taiwan, 33.5 per 100,000 people die from liver cancer each year according to the Department of Health, Executive Yuan in 2010s (http://www.doh.gov.tw/EN2006/index EN.aspx/). Hepatocellular carcinoma (HCC) is one of the most common primary cancers of the liver in the world and in Taiwan, and accounts for more than 90% of all primary liver tumors (1). HCC is the sixth most common cancer and the third most common cause of death from cancer worldwide (2,3). HCC is a high degree of malignancy but poor prognosis disease, and most patients with HCC are not curable (4). Chemotherapy is one of the treatment options in HCC, but these outcomes are not fully satisfactory (5). Multidrug resistance gene is overexpressed in HCC, which results in un-sensitizing chemotherapeutic agents (6–8). Therefore, discovery to more effective chemotherapeutic agents for HCC is urgently needed.

The most effective strategy for curing HCC is to induce cell cycle arrest and cell death (9,10). The promotion of cell cycle progression at G2/M phase has been intensively investigated. Cyclin-dependent protein kinase 1 (CDK1)/cyclin B complex plays a major role in the regulation of the cell cycle in G2/M phase. When the cells progress from S phase into G2/M phase, the CDK1/cyclin B1 complex becomes active, suggesting
that CDK1 has undergone an activating phosphorylation (on Thr161), and the inhibitory phosphorylation (on Thr14 and Tyr15) removed by active Cdc25c phosphatase (11,12). The Weel kinase is regulating G2/M phase transition by phosphorylation of CDK1 at Tyr15. In addition, Chk1 is the essential kinase in the G2/M checkpoint by phosphorylating Cdc25c in response to cellular damaging and antimitic agents (13-15).

Three major morphologically processes lead to cell death, apoptosis, necrosis and autophagic cell death (16,17). Autophagic cell death, or cellular self-digestion, occurs in multi-cellular organisms and plays an important role in normal physiology in animals (18). When the cells undergo nutrient starvation, cellular damage, pathogen infection, aging and degenerative processes, autophagic cell death is required for the promotion of cellular survival (19,20). Autophagy (self-eating), causes specific morphological and biochemical modification, and is considered as programmed cell death type II (apoptosis, programmed cell death type I), which occurs in some situations and then induces cell death (21,22). The cytoplasm and double smooth membrane (a phagophore) of various organelles such as mitochondria, endoplasmic reticulum (ER) and peroxisomes are sequestered by a membrane to form an autophagosomes and then the autophagosome fuses with the lysosome, forming autophagolysosome, finally resulting in degradation of the captured proteins/organelles by lysosomal enzymes (18,23-25). Many studies have demonstrated that a group of autophagy-related proteins (Atg) is involved in autophagic cell death. The membrane nucleation is mediated by a class III phosphatidylinositol 3-kinase and Beclin 1. The production of autophagosome is necessary for the recruitment of Atg12-Atg7-Atg5 complex and microtubule-associated protein 1 light chain 3 (LC3) (26-29). There are two major forms of LC3, type I is cytosolic and type II is membrane-bound. When cells undergo autophagic cell death, LC3-II, an autophagosomal marker, increases from the conversion of LC3-I (30). Promotion of autophagic cell death from cancer cells is one of the best strategies in chemotherapy (31-33).

Bufalin, a digoxin-like chemical reagent, is one of the major Chinese tradition medicine Chan-Su components extracted from dried toad venom from the skin glands of Bufo bufo gargarizan and Bufo melanostictus. It has been demonstrated that bufalin exhibits significant anticancer activities against many human tumor cells in vitro and in vivo. The anticancer activities by bufalin are involved in the induction of cell differentiation, cell cycle arrest, apoptosis and inhibition of cell metastasis. Xie et al demonstrated that bufalin induces autophagic cell death through reactive oxygen species (ROS) generation and JNK activation in HT29 human colon cancer cells (34). The anticancer activities of bufalin have been reported, however, no comprehensive studies have been reported on the effects of bufalin on human hepatocellular carcinoma cells. The goal of this study is to explore whether the antitumor activity of bufalin mediates through the direct cytotoxic effect and to understand the molecular mechanisms in human hepatocellular carcinoma SK-HEP-1 cells. The present study is focused on the cell cycle arrest and autophagy-induced by bufalin in the SK-HEP-1 cells. Our results demonstrate that bufalin inhibits cells viability, induces autophagic cell death, and simultaneously causes cell cycle arrest in G2/M phase through the AKT/mTOR signaling pathway in SK-HEP-1 cells.

Materials and methods

Chemicals and reagents. Acidine orange (AO), agarose, bafilomycin, bufalin, dimethyl sulfoxide (DMSO), 3-methyladenine (3-MA), propidium iodide (PI), RNase A, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), proteinase K, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), L-glutamine, LC3B antibody kit for autophagy, penicillin/streptomycin, RPMI-1640 medium, trypan blue solution and Tryptsin-EDTA were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). AKT kinase assay Kit was purchased from Cell Signaling Technology (Danvers, MA, USA). Caspase-3 activity assay kit was purchased from R&D Systems Inc. (Minneapolis, MN, USA). CDK1 kinase activity kit was purchased from Medical & Biological Laboratories International (Nagoya, Japan). Tdt-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay kit was purchased from Roche Diagnostics (GmbH, Mannheim, Germany). Primary antibodies (anti-cyclin A, anti-cyclin B, anti-CDK1, anti-Cdc25c, anti-Chk1, anti-Weel, anti-PARP, anti-mTOR and anti-GAPDH), and second antibodies for western blot analysis were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The primary antibodies [anti-phospho-CDK1 (Thr161), phospho-Cdc25c (Ser198), anti-phospho-AKT (Thr308), anti-phospho-AKT (Ser473), anti-phospho-mTOR (Ser2481) and anti-caspase-3, anti-LC3-II, anti-Beclin-1, anti-Atg 5, anti-Atg 7 and anti-Atg 12] were obtained from Cell Signaling Technology. Antibody against β-actin was purchased from Sigma Chemical Co. All peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. The enhanced chemiluminescence (ECL) detection kit was purchased from Pierce Chemical (Rockford, IL, USA).

Cell culture. The human hepatocellular carcinoma cell line (SK-HEP-1) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in 75-cm² tissue culture flasks at 37°C under a humidified 5% CO2 atmosphere in RPMI-1640 medium containing 10% FBS, 2 mM of L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

Cell viability and morphology. Cell viability was evaluated using the MTT assay (35). SK-HEP-1 cells (1x10⁴ cells) in 96-well plates were incubated with 0, 25, 50, 100 and 200 nM of bufalin or 0.1% DMSO as a vehicle control for 24 h. For incubation with the inhibitors, cells were pretreated with 3-MA (10 mM) or bafilomycin (10 nM) for 1 h, followed by treatment with or without bufalin (100 nM). After washing the cells, RPMI-1640 medium containing MTT (0.5 mg/ml) of was added to each well. The cells were incubated for 4 h at 37°C the supernatant was removed. The formed formazan crystals in viable cells were dissolved with isopropanol in 0.04 N HCl. The absorbance of each well was measured at 570 nm with ELISA reader with a reference wavelength of 620 nm. All experiments were performed in triplicate and the cell viability was expressed as percentage of the control as previously described. Cell morphological examination of autophagic vacuoles was determined utilizing a phase-contrast microscope.
Assessment for cell death. Cell death was evaluated using a trypan blue assay (36,37). SK-HEP-1 cells (2.5x10^4 cells) in 24-well plates were incubated with 0, 25, 50, 100 and 200 nM of bufalin for 24 h. At the end of incubation, cells were stained in the 0.25% trypan blue solution and then determined cell number by Countess Automated Cell Counter (Invitrogen/Life Technologies) as previously described.

Cell cycle progression. The 2.5x10^5 cells of SK-HEP-1 cells in 24-well plate were exposed to bufalin (50 and 100 nM) for 24 h. At the end of incubation, cells were then collected, fixed in 70% ethanol overnight, then washed in PBS once, and then resuspended in 500 µl of Na_2HPO_4 (192 mM), citric acid (4 mM) pH 7.8 at 25°C for 30 min. The cells were stained with 0.5 ml of PBS containing RNase (1 mg/ml), PI (10 µg/ml) for 30 min in the dark, and then analyzed by flow cytometry as previously described (38,39).

CDK1 kinase activity. CDK 1 kinase activity was performed according to the manufacturer's protocols (CycLex Cdc2-Cyclin B Kinase Assay Kit; MBL International Corporation, Japan) (40). SK-HEP-1 cells were seeded into 75-T flasks (1x10^6/each). Bufalin (100 nM) were added and incubated for 0, 6, 12 and 24 h. At the end of incubation, cells were harvested, washed twice with ice-cold PBS, and then resuspended the cell pellet with 500 µl of extraction buffer (20 mM Tris-HCl, pH 8.5, 150 mM NaCl, 0.2% NP-40, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 µg/ml pepstatin, 0.5 µg/ml leupeptin, 5 mM β-glycerophosphate, 5 mM NaF, 1 mM Na_3VO_4, 5 mM β-mercaptoethanol). Cell extracts were diluted in a 1:5 ratio with Q-buffer (20 mM Tris-HCl, pH 8.5, 0.2 mM EDTA, 1 mM EGTA, 1 µg/ml pepstatin, 0.5 µg/ml leupeptin, 0.2 mM Na_3VO_4, 5 mM β-mercaptoethanol). The CDK1 activity was measured absorbance using ELISA reader at OD450. All results were performed in three independent experiments.

DNA gel electrophoresis. SK-HEP-1 cells were seeded into 75-T flasks (1x10^6/each). Bufalin (0, 50 and 100 nM) were added and incubated for 24 h. At the end of incubation, cells were harvested and washed twice with ice-cold PBS. The cell pellets were re-suspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.2% Triton X-100). Cell lysates were treated with 0.1 µg/ml proteinase K at 50°C for 12 h, followed by 50 µg/ml RNase A at 37°C for 30 min. After precipitation, the DNA was subjected to electrophoresis in a 1.5% agarose gel (Sigma-Aldrich Corp.). DNA fragmentation on agarose gel was taken (44).

Caspase-3 activity assay. SK-HEP-1 cells (1x10^7 cells/75-T flask) were treated with bufalin (25, 50 and 100 nM) or paclitaxel (100 nM) for 24 h. At the end of incubation, cells were harvested and re-suspended in a lysis buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM DTT]. Cell lysates (50 µg protein) were incubated with caspase-3 specific substrates (Ac-DEVD-pNA) (R&D Systems Inc.) for 1 h at 37°C. The caspase-3 activity was determined by measuring OD405 of the released pNA (42,43).

Transmission electron microscopy. SK-HEP-1 cells (1x10^6 cells/6-well) were treated with bufalin (100 nM) for 24 h. At the end of incubation, cells washing three times with PBS, cells were fixed for 30 min at room temperature in 2% paraformaldehyde and 2.5% glutaraldehyde in PBS buffer. The cells were rinsed twice in the same buffer and subsequently post fixed in 1% osmium tetroxide. After rinsing followed by dehydration in graded alcohol series, the cells were embedded in LR white resin and polymerized at 70°C overnight. Ultrathin sections were then cut with a diamond knife and loaded onto TEM grids. The sections were examined by a Philips CM10 electron microscope at accelerating voltage of 120 kV and micrographs were taken (44).

Detection of acidic vesicular organelles (AVO) with acridine orange (AO). SK-HEP-1 cells (1x10^6 cells/6-well) were treated with bufalin (100 nM) for 24 h. At the end of incubation, cells were harvested. To detect and quantify acidic vesicular organelles (AVO), cells were stained with acridine orange (AO). The number of acidine orange positive cells was analyzed by flow cytometry using a FACSCalibur (Becton Dickinson). Cell morphology was examined using phase-contrast and fluorescence microscopy (Nikon, Melville, NY, USA). All results were performed in three independent experiments (45).

Immunofluorescence analysis. LC-3 staining was performed according to the manufacturer's protocol (LC3 antibody kit for autophagy; Invitrogen/Life Technologies) (46,47). SK-HEP-1 cells (1x10^6 cells/6-well) in 6-well flask were treated with bufalin (100 nM) for 24 h. At the end of incubation, cells were harvested then fixed with 3.7% formaldehyde in PBS for 15 min. Cells were washed three times with PBS, added 0.2% Triton X-100 in PBS to the cells, and incubated for 15 min at room temperature. Permeabilized cells were incubated with an anti-LC3 antibody (1:50) at 4°C overnight, and followed by incubation with an Alexa Fluor 488-conjugated secondary antibody (1:200; Invitrogen) for 1 h. The images were taken under a fluorescence microscope (Nikon). The number of LC-3 positive cells was analyzed by flow cytometry using a FACSCalibur (Becton Dickinson). All results were performed in three independent experiments (48).

Western blot analysis. SK-HEP-1 cells (1x10^7 cells/75-T flask) were treated with bufalin (100 nM) for 0, 2, 4, 6, 8, 12, 18 and 24 h. At the end of incubation, cytosolic or total proteins were prepared and determined as previously described. Protein lysates were sonicated and the supernatants were boiled in SDS sample buffer
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for 5 min. The protein concentration was measured by using a BCA assay kit (Pierce Chemical). Equal amounts of cell lysates were run on 10 to 12% SDS-polyacrylamide gel electrophoresis and electro-transferred to a nitrocellulose membrane by using the iBot Dry Blotting System (Invitrogen/Life Technologies). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in Tris buffered saline/Tween-20 and incubated with primary antibodies at 4˚C overnight. Membranes were washed three times with Tris-buffered saline/Tween-20 for 10 min and incubated with secondary HRP-conjugated antibody. The blots were developed by using an ECL kit and Kodak Bio-MAX MR film (Eastman Kodak, Rochester, NY, USA) (35).

**Statistical analysis.** All the statistical results were expressed as the mean ± SEM of triplicate samples. Statistical analyses of data were done using one-way ANOVA followed by Student’s t-test and *P<0.05* were considered significant.

**Results**

**Bufalin inhibits cell proliferation, promotes G<sub>2</sub>/M phase arrest in SK-HEP-1 cells.** SK-HEP-1 cells were treated with bufalin (0, 25, 50, 100 and 200 nM) for 24 h. Bufalin reduced cell viability of SK-HEP-1 cells in a concentration-dependent manner as measured by the MTT assay (Fig. 1A). The half maximal (50%) inhibitory concentration (IC<sub>50</sub>) for a 24-h treatment of bufalin in SK-HEP-1 cell was 110.33±5.32 nM. Fig. 1B shows that bufalin increased cell death in a concentration-dependent manner by the trypan blue exclusion assay. We then examined cell cycle distribution in SK-HEP-1 cells exposed to 100 nM of bufalin for 24 h. These results showed G<sub>2</sub>/M accumulation after SK-HEP-1 cells were treated with 100 nM of bufalin (Fig. 1C). Interestingly, SK-HEP-1 cells exposed to 50 and 100 nM of bufalin have undergone cell death, but did not detect significant sub-G<sub>1</sub> DNA content, a typical apoptosis. Our results suggested that bufalin effectively caused G<sub>2</sub>/M arrest and then induced cell death.

**Effects of bufalin on G<sub>2</sub>/M phase-associated protein levels in SK-HEP-1 cells.** We investigated the G<sub>2</sub>/M phase specific protein expression levels by western blot analysis. As shown
in Fig. 2A, bufalin (100 nM) caused an increase in the protein level of Chk1 and Wee1, and a decrease in the protein levels of Cyclin A, Cyclin B, CDK1, phospho-CDK1 (Thr161), Chk1, Wee1, Cdc25c, phospho-Cdc25c (Ser198) and β-actin in bufalin-treated SK-HEP-1 cells. We examined the CDK1 activity in bufalin-treated SK-HEP-1 cells. Fig. 2B depicted that bufalin (100 nM)
caused a significant decrease in CDK1 activity for 0, 6, 12 and 24-h treatment in SK-HEP-1 cells. Our data indicated that bufalin-treated SK-HEP-1 cells downregulated CDK1 activity and caused G2/M phase arrest.

Bufalin induces caspase-independent cell death in SK-HEP-1 cells. To examine whether bufalin induced apoptosis in SK-HEP-1 cells, cells were treated with bufalin (0, 50 and 100 nM) for 24 h, and analyzed DNA fragmentation by DNA gel electrophoresis. Fig. 3A showed that no DNA fragmentation was observed in SK-HEP-1 cells treated with bufalin for 24 h. This result suggested that bufalin did not induce apoptosis in SK-HEP-1 cells. Similar result was obtained from TUNEL staining (Fig. 3B) when SK-HEP-1 cells were treated with bufalin (0, 50 and 100 nM) or paclitaxel (100 nM; an apoptotic agent) for 24 h. Fig. 3B showed that the percentage of TUNEL positive cells in bufalin-treated SK-HEP-1 cells was less than 5% compared with the paclitaxel-treated cells (37.26±4.34%). To investigate whether bufalin-induced cell death is mediated through caspase-3 activation, SK-HEP-1 cells were treated with bufalin (0, 50 and 100 nM) or paclitaxel (100 nM; an apoptotic reagent) for 24 h and then analyzed the protein levels of cleaved caspase-3 and poly (ADP-ribose) polymerase (PARP), downstream target of caspase-3. As shown in Fig. 3C, the protein levels of cleaved caspase-3 or cleaved PARP were not increased in bufalin-treated SK-HEP-1 cells. In addition, the caspase-3 activity showed no change in bufalin-treated SK-HEP-1 cells. Our results indicated that bufalin-induced cell death did not proceed through apoptosis in SK-HEP-1 cells.

Bufalin induces autophagic cell death in SK-HEP-1 cells. No apoptotic feature was detected in SK-HEP-1 cells. Next, we examine whether autophagic cell death is involved in bufalin-induced cell death. SK-HEP-1 cells were treated with bufalin (0,
25, 50 and 100 nM) for 24 h and the formation of autophagic vacuoles was examined by the phase microscopy. As shown in Fig. 4A, bufalin induced the formation of autophagic vacuoles in SK-HEP-1 cells in concentration-dependent manner. We further confirmed the formation of autophagosome vesicles in bufalin-treated SK-HEP-1 cells using transmission electron microscopic (TEM) analysis (Fig. 4B). Double- or multi-membrane structure characteristics of autophagosomes and autolysosomes were present in bufalin-treated SK-HEP-1 cells. The cytosolic acidic vesicular organelles (AVOs) are one of the hallmarks of autophagic cell death. In Fig. 5A, acridine orange (AO) staining of bufalin-treated SK-HEP-1 cells clearly showed AVOs within the cytoplasm compared to control by fluorescence microscopy. This observation was confirmed by FACS analysis, which showed a clear increase in red fluorescence (FL-3 positive) in concentration-dependent manner on bufalin-treated SK-HEP-1 cells (Fig. 5B). Furthermore, it has been shown that microtubule-associated protein 1 light-chain 3 (LC3) is an autophagic membrane marker for the detection of early autophagosome formation (50). We examined the LC3 distribution in bufalin-treated SK-HEP-1 cells by fluorescence microscopy and FACS analysis. As shown in Fig. 5C, bufalin treatment enhanced the punctate pattern of LC3-GFP in autophagic SK-HEP-1 cells. In addi-
an increase in the green fluorescence (FL-1 positive) cells by FACS analysis showed that bufalin-treated SK-HEP-1 cells underwent autophagic cell death in a concentration-dependent manner (Fig. 5C, right). Our results suggested that bufalin-induced cell death in SK-HEP-1 cells is dependent on the induction of autophagy.

Bufalin upregulates the autophagy-associated protein levels and blocks the AKT/mTOR signaling in SK-HEP-1 cells. Induced autophagic cell death is associated with the elevated protein levels of autophagic proteins LC-3, Atg complex (Atg 5, Atg 7 and Atg 12), Beclin-1, and β-actin in bufalin-treated SK-HEP-1 cells. We investigated the autophagy-associated protein levels in bufalin-treated SK-HEP-1 cells by western blot analysis. As shown in Fig. 6A, bufalin (100 nM) increased the protein levels of LC-3 II, Atg 5, Atg 7 and Atg 12 and Beclin-1 in SK-HEP-1 cells. It is also reported that the AKT activity contributed to autophagic cell death.
To examine whether bufalin-induced autophagic cell death is through the inhibition of AKT in SK-HEP-1 cells, cells were harvested after treatment with 100 nM of bufalin for 2, 4, 6 and 8 h, and then determined the protein levels by western blot analysis. Fig. 6B showed that bufalin (100 nM) significantly decreased the phospho-AKT (Thr308), phospho-AKT (Ser473), phospho-mTOR (Ser2481) protein levels in SK-HEP-1 cells. Bufalin decreased the AKT activity and this effect is time-dependent (Fig. 6C). Our results implied that bufalin induced autophagic cell death in SK-HEP-1 cells through interfering with the AKT/mTOR signaling pathway.

3-Methyladenine and bafilomycin protect against autophagy and induce apoptosis in bufalin-treated SK-HEP-1 cells. 3-Methyladenine (3-MA), an inhibitor of class III phosphatidylinositol-3 kinase, is a reagent potently inhibiting autophagy-dependent protein degradation and suppressing the formation of autophagosomes (51), whereas bafilomycin A1, an inhibitor of the vacuolar proton pump of lysosomes and endosomes, appears to block the fusion of autophagosomes (52). In the present study, SK-HEP-1 cells were pretreated with 3-MA (10 mM) or bafilomycin A1 (10 nM), and then exposed to 100 nM of bufalin before harvested for measuring the levels of autophagic vacuoles and cell viability by MTT assay. Both 3-MA (10 mM) and bafilomycin A1 (10 nM) inhibit autophagic vacuoles (Fig. 7A) and reduce cell viability (Fig. 7B) in bufalin-treated SK-HEP-1 cells. Furthermore, we examined effects of 3-MA (10 mM) and bafilomycin A1 on apoptosis by TUNEL assay (Fig. 7C). The results showed both 3-MA (10 mM) and bafilomycin A1 (10 nM) increased TUNEL positive apoptotic cells in bufalin-treated SK-HEP-1 cells. Taken together, inhibiting autophagic cell death by 3-MA or bafilomycin A1 facilitates bufalin-induced apoptosis in SK-HEP-1 cells.

Discussion

Traditional Chinese medicines (TCM) powerful for prevention or therapy of cancer are marked with their high anticancer activity and low toxicity in normal cells (53,54). Bufalin has been used in clinical trials for cancer therapy in China (55). Han et al has demonstrated that bufalin has significant anti-human hepatocellular carcinoma activity in orthotopic transplantation nude mouse model in vivo and possesses no marked toxicity (56). Although bufalin upregulates Bax protein and induces tumor cell apoptosis in vivo, the signaling pathways underlying bufalin-induced cell death have not been elucidated. Arsenic trioxide, a TCM, has been reported to induce autophagic cell death without activation of caspase-dependent apoptotic cell death (57). The current study focused on exploring molecular mechanisms of bufalin-induced cell death in human hepatocellular carcinoma SK-HEP-1 cells. Bufalin induced autophagic cell death through inhibiting the
AKT/mTOR signaling pathway in SK-HEP-1 cells. Our results suggested that bufalin may be used as a novel therapeutic reagent for the medical treatment and/or prevention of human hepatocellular carcinoma.

In addition, we demonstrated that bufalin induced growth inhibitory effects through G2/M arrest (Fig. 1C) and autophagic cell death in SK-HEP-1 cells. Our results indicated that bufalin induced G2/M phase arrest (Fig. 1C). Cyclin A, cyclin B, CDK1, phospho-CDK1 (Thr161), Cdc25c, phospho-Cdc25c (Ser198) were decreased, while Chk1, Wee1 was increased by bufalin treatment in a time-dependent manner (Fig. 2A). It was reported in the G2/M phase progression, which is regulated with CDK1 and CDK2 kinases that are activated primarily in association with cyclins A and B. Furthermore, bufalin also inhibited the CDK1 activity (Fig. 2B). Previous studies have demonstrated that bufalin inhibited cell proliferation through induction of G2/M arrest of T24 human bladder cancer cells (46), human endometrial stromal cells, and ovarian cancer cells (58), but our findings indicated that bufalin induced different cell cycle phase arrest dependent on the cell types. The results are in agreement with previous studies indicated that bufalin inhibited cell proliferation through induction of G2/M arrest of endothelial cells (59), leukemia ML1 cells (60) and human osteosarcoma U-2OS and U-2OS methotrexate 300-resistant cells in vitro (61).

Autophagy plays two physiologic roles: one is protective role allowing cell survival and generating nutrients and energy; the other promotes cell death (62). When the cells undergo starvation, endoplasmic reticulum (ER) stress stimulation, ROS production and hypoxia, autophagy protects cells from the damage, which leads to cell survival (63). However, autophagy is observed to be induced by cytotoxic chemotherapeutic reagents such as paclitaxel (Taxol) (64), chloroquine, arsenic trioxide (65,66), sorafenib (67,68) and imiquimod (69). Bufalin has been demonstrated to induce apoptosis and cell cycle arrest in leukemia (70), prostatic cancer (71), gastric cancer (72), ovarian cancer (58), osteosarcoma (61) and bladder cancer cells (59). In the present study, apoptotic characteristics such as DNA fragmentation, and cleavages of caspase-3 and PARP were not found in bufalin-treated SK-HEP-1 cells (Fig. 3C and D). Our data indicated that bufalin-induced cell death is caspase-independent apoptosis in SK-HEP-1 cells. Intriguingly, bufalin-induced autophagic cell death in SK-HEP-1 cells was demonstrated by several lines of evidence including autophagosome formation (Fig. 4A), double-membrane vacuoles (Fig. 4B), acidic vesicular organelles (Fig. 5A), cleavage of microtubule-associated protein 1 light chain 3 (LC3) (Fig. 5C) and elevated protein levels of autophagic proteins, Atg complex (Atg 5, Atg 7 and Atg 12) and Beclin-1 (Fig. 6). When SK-HEP-1 cells were pre-treated with 3-MA or bafilomycin A1 followed by treating with bufalin, growth inhibitory effects and apoptosis were significantly enhanced compared with the bufalin alone treatment group by TUNEL assay (Fig. 7C). Our results suggested autophagy protects SK-HEP-1 cells from undergoing apoptosis in the early stage through antagonizing bufalin induced apoptosis. Our results did not rule out bufalin might be involved in apoptotic signaling pathways after longer exposure (more than 24 h), but showed that SK-HEP-1 cells induced autophagic cell death after the 24 h treatment with bufalin. This is in agreement with the finding of Xie et al that human colorectal cancer HT-29 and Caco-2 cells treated with bufalin for 24 h and induced autophagy (34).

Previous studies demonstrated that autophagic cell death is triggered by multiple signaling pathways such as the adenosine monophosphate-activated protein kinase (AMPK) pathway (73,74), the AKT/mammalian target of rapamycin (mTOR) pathway (75), the MAPK (ERK, p38 and JNK) pathway (34,76), the BCL2 and its family members involved pathway (77), the death-associated protein kinase (DAPK) pathway and the death-associated related protein kinase 1 (DRP1) pathways (78). The AKT/mTOR pathway is involved in regulating cell survival and cell death. In the current study, bufalin inhibited protein levels of phospho-AKT (Thr308), phospho-AKT (Ser473), phospho-mTOR (Ser2481) and decreased the activity of AKT in SK-HEP-1 cells (Fig. 6B and C). Liu et al reported that AKT gene was overexpressed in hepatocellular carcinoma HCC and suggested that AKT activation participates in the pathogenesis and progression of HCC (79). Recently, our preliminary result demonstrated that human oral squamous cell carcinoma CAL 27 cells stably expressed constitutively active AKT (CA-AKT) increased AKT activity and attenuated bufalin-induced growth inhibition and cell death (data not shown). In the present study, the result showed that the AKT activity and AKT/mTOR pathway are associated with the induction of autophagic cell death in bufalin-treated SK-HEP-1 cells.

Overall, the molecular signaling pathways are summarized in Fig. 8. Our results demonstrated that the AKT/mTOR signaling pathway promotes autophagy in bufalin-treated SK-HEP-1 cells. These findings implied that bufalin may be used as a novel therapeutic reagent for the treatment of hepatocellular carcinoma.

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