Bufalin induces G2/M phase arrest and triggers autophagy via the TNF, JNK, BECN-1 and ATG8 pathway in human hepatoma cells

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Abstract. Liver cancer is the fifth most common cause of cancer death worldwide. The study of more effective anti-hepatoma drugs is urgently required. Bufalin has been isolated from a traditional Chinese medicine and possesses less toxicity to normal cells. However, it has been found to inhibit growth of cancer cells. In this study, we aimed to investigate the efficacy and mechanism of bufalin in Huh7, Hep3B and HA22T human hepatoma cells. The three cell lines were treated with bufalin, the proliferation was detected by WST-1 assay and cell cycle was detected by flow cytometry analysis. The results showed that bufalin inhibited the proliferation of hepatoma cells and regulated the hepatoma cell death program in a dose- and time-dependent manner without typical features of apoptosis. RT-PCR arrays were used to investigate the autophagy transcriptional response triggered by bufalin and 13 genes were altered and further confirmed by real-time PCR. The translation levels of selected genes were examined by western blot analysis to reveal the bufalin-induced autophagy cascade. Bufalin synergized with the JNK pathway to induce autophagy of hepatoma cells and is closely associated with the upregulation of TNF, BECN-1, MAPK and ATG8, together with the downregulation of Bcl-2 and Bid. Our study provided a multi-angle evaluation system for anti-hepatoma pharmacology for pre-clinical drug investigation. In this case, bufalin was capable of inducing hepatoma cell autophagy, suggesting a potential regimen for single or combined chemotherapy to overcome hepatoma in clinical practice.

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Abbreviations: Bid, BH3 interacting-domain death agonist; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2

Key words: bufalin, autophagy, hepatoma cell, G2/M phase arrest, PCR array

Introduction

Liver cancer shows high incident and mortality all over the world. It ranks the fifth and seventh most diagnosed cancer worldwide for male and female, respectively (1), and is the second and sixth leading cause of cancer death for man and women, respectively (1). In 2008, almost 750,000 new cases were recorded and 700,000 cancer deaths occurred worldwide (1). Geographically, the regions of highest liver cancer rates in the world are East and South-East Asia, Middle and Western Africa (1,2). In America and Europe, over the past 10 years, incidence rates of liver cancer among men and women increased and cancer mortality trends (death rates) increased for liver cancer in all age groups and among white, black and Hispanic men and white women (3). The differential trend is found in Japan, where the incidence and mortality of liver cancer is declining, indicated that there may be a difference in Eastern and Western countries (4). This may be due to variations in genetic background, environmental exposure, diet habits and hepatitis B virus (HBV, for the Eastern) or hepatitis C virus (HCV, for USA and Europe) infection (2).

The therapeutic strategies of liver cancer include surgical treatments and chemotherapy. Radiation is not considered since the liver is the largest organ with the endocrinic and detoxicificative functions. Liver surgical treatments are resection, liver transplantation and chemoembolization. In chemotherapy for liver cancer, the commonly used drugs include cisplatin, epirubicin, etoposide and 5-fluorouracil, all have an unsatisfactory therapeutic efficacy in single drug treatment (5). The most promising agent for treatment of liver cancer is the oral multikinase inhibitor, sorafenib. However, it is reported that its efficacy in cancer cell killing is not satisfactory enough without the combination of other drugs, such as fluvastatin (6). Therefore, a more effective anticancer drug is urgently needed for its development and revealing its efficacy mechanism.

Bufalin, a bufadienolide derivative, is the active compound of cinobufacini (Fig. 1). In literature, cinobufacini is water soluble and extracted from the dried toad skin of traditional Chinese medicine with a variety of biological activities (7,8). Bufalin is effective in cardiotonic and anaesthetic treatment, blood pressure controlling and promoting antineoplastic activities (9). In addition, bufalin was reported to exhibit significant antitumor activity in hepatocellular carcinoma, non-small cell lung cancer, pancreatic cancer and gallbladder carcinoma, with low toxicity and few side effects (8,10,11). Moreover, bufalin can inhibit cell growth and proliferation in various human cells, including colon cancer, hepatoma, leukemia, endometrial cancer and ovarian cancer (12-15). However, the detail mechanism is not well understood.

Autophagy is a mechanism of cell suicide which eliminates malignant cells, supports the surviving cells promoting the viability of the whole population. In programmed cell death, the process of autophagy is regulated by multiple autophagy-related (Atg) genes and correlated with cell cycle arrest (16). Mounting evidence is revealing the pathways responsible for autophagy in different cells under various environmental stresses (17). Only one report exists on investigation of the mechanism of bufalin-induced autophagy, which reported that reactive oxygen species (ROS) and c-Jun NH_2-terminal kinase (JNK) pathways were involved (12). In addition to apoptosis, autophagy is a novel cell death type in cancer cell killing for potential anticancer drug candidates (18,19). It is more beneficial for the anticancer drugs to induce autophagy rather than apoptosis in the cancer patients and the prognosis may be better in the patients treated by drugs with the former efficacy.

In this study, we examined the chemotherapeutic efficacy of bufalin in inhibition of the proliferation in human hepatoma cell lines, Huh7, Hep3B and HA22T. Our results unveiled that bufalin could arrest cell cycle at G2/M phase and induce cell death by autophagy instead of apoptosis in hepatoma cells.

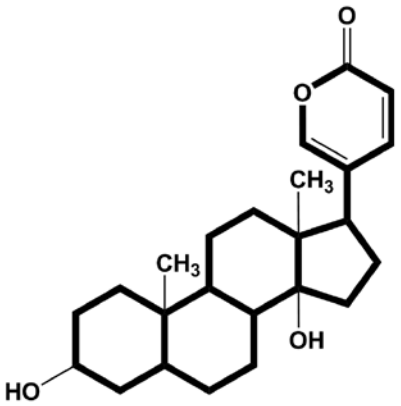

Figure 1. Molecular structure of bufalin.

Materials and methods

Cell culture. Three hepatoma cancer cell lines, Huh7, Hep3B and HA22T, were obtained from the Bioresource Collection and Research Center (BCRC, Taiwan) and cultured in DMEM with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin; PS; Gibco). Culture medium was replenished every 3-4 days and grown at 37˚C in a humidified atmosphere containing 5% CO_2. Bufalin was purchased from Phytomarker Ltd. (Tianjin, China) and was dissolved in DMSO and maintained at -20˚C as a 20 mM stock.

Cell viability and cell death assay. For cell viability assay, the cells were seeded in 96-well plates at a density of 3,000 cells/well overnight, treated with the respective agents (bufalin) for 72 h and then exposed to WST-1 reagent (Roche, Mannheim, Germany) for 4 h at 37˚C according to the manufacturer's instructions. Absorbance was measured at 450 nm on a microplate Reader (iMark™ Microplate Absorbance Reader, Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was also evaluated by counting cells that excluded trypan blue. All experiments were done at least three times.

Analysis of cell cycle by flow cytometry. The cell cycle analysis by flow cytometry as previous descripted after 24 h of culture either with or without bufalin (20). Briefly, Huh7, Hep3B and HA22T cells were cultured at $1x0^6$ cells for different time courses with or without the presence of bufalin (0.04 μM). Then the cells were trypsinized, washed in PBS, fixed in 70% methanol and incubated for 30 min at 4˚C in the dark with a PBS solution of 5 μg/ml propidium iodide (Sigma), 1 mg/ml RNase (Sigma) and 0.1% Nonidet P-40 (Sigma). Stained cells were immediately analyzed using a FACSCanto flow cytometry system (BD Biosciences, San Jose, CA). Flow cytometry analysis of the cell cycle was performed immediately using the ModFit LT 3.0 program (Verity Software House, Inc., ME).

Gene expression profiling by PCR array. To examine the effects of bufalin treatment on gene expression in hepatoma cells, Huh7 cells were treated with bufalin (0.04 μM) for 12 h. At the termination of an experiment, total RNA was extracted by using an RNeasy Mini kit (Qiagen, Valencia, CA) and processed for PCR array analysis. Quantification and quality control of total RNAs have been performed by the measurement of optical densities at 260 and 280 nm. Reverse transcriptions have been performed on 1 μg of total RNA in a 20-μl final volume using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA). Expression of genes involved in the bufalin treatment was studied by using 96-well RT^2 Profiler PCR Arrays-Human Autophagy (Qiagen, Frederick, MD, USA) in a LightCycler 480 PCR system (Roche, Germany).

Quantitative real-time PCR. Relative real-time PCR was performed on a LightCycler 480 PCR system (Roche, Germany) with gene-specific primers and TaqMan probes protocol to confirm the gene expression changes observed by using PCR array. Total RNA (2 μg) from each pool was reverse transcribed to cDNA in the presence of random primer sequences in total volume of 20 μl. After dilution of the cDNA with 80 μl of water, 2 μl of this cDNA was used as template in the real-time PCR. Relative expression ratios were normalized to glyceraldehydes 3-phosphate dehydrogenase (GAPDH). The PCR primers used in this study are available upon request and in Table III. All PCRs were performed in triplicates.

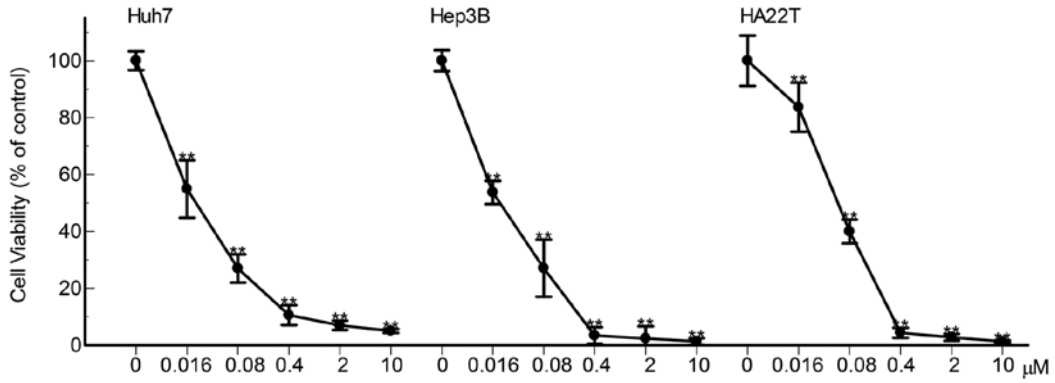

Figure 2. Bufalin-induced cytotoxicity in hepatoma cells. Cells were seeded in 96-well plates 24 h before treatment and Huh7, Hep3B and HA22T cells were treated with bufalin at indicated doses. Cells were harvested after 72-h treatment and WST-1 assays were performed immediately. The percentage of cell viability is shown for each treatment compared to the sham-treated cells, which were considered as 100% control. Experiments were conducted in triplicate.

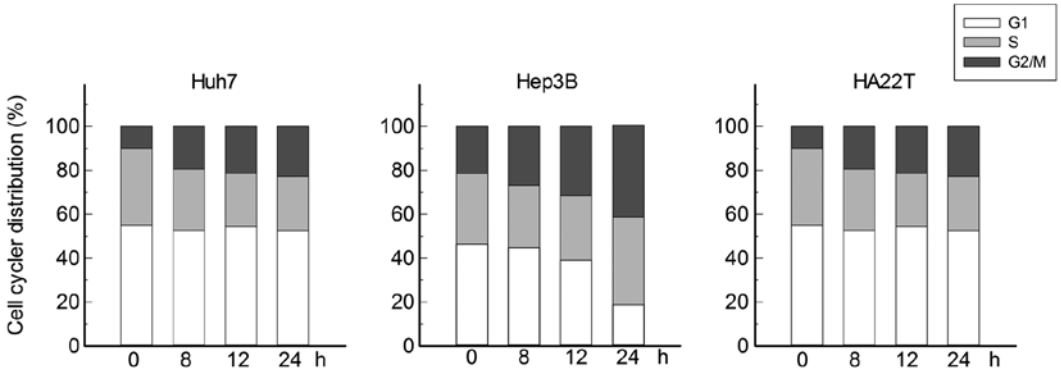

Figure 3. The G2/M phase cell cycle arrest induced by bufalin in hepatoma cells at 0, 8, 12 and 24 h post-treatment. Huh7, Hep3B and HA22T cells were collected at 0, 8, 12 and 24 h after 0.04 μM bufalin treatment and subjected to flow cytometry. Percentages of cells distributed in the G1, S and G2/M phases of the cell cycle were determined by Mitfit LC 3.0.

Western blot analysis. Cells were treated with bufalin (0.04 μM) for 0, 4, 8 and 12 h. After treatment, total cell lysates were prepared and 30 μg protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblot analysis. Primary antibodies used included anti-PARP, cytochrome C, cdc25c, cyclin B and cdc2/cdk1 (Cell Signaling, Beverly, MA); Bid (BD Biosciences, San Diego, CA) and GABARAPL1 (GeneTex, San Antonio, TX). Anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase was also used (GE Healthcare, Piscataway, NJ). Immunoreactive bands were detected by enhanced chemiluminescence kit (ECL, Pierce, Thermo Fisher Scientific, Pittsburgh, PA, USA) for western blotting detection by using a ChemiGenius bioimaging system (Syngene, USA). Equal loading was confirmed via probing the blots with β-actin antibody (Abcam, Cambridge, MA, USA).

Statistical analysis. Statistical analysis was performed using Student's t-test for comparison of two groups or one-way analysis of variance for comparison of more than two groups followed by Tukey's multiple comparison test. Statistical calculations were performed using the software from SPSS (SPSS Institute, Chicago, IL, USA). Data were expressed as means ± SEM of at least three independent experiments. A $p<0.05$ was considered statistically significant.

Results

Effects of bufalin on the proliferation and viability of human hepatic cell lines in vitro. To investigate the anticancer efficacy of bufalin on three hepatoma cell lines, Huh7, Hep3B and HA22T cells were treated with physiological achievable concentrations of bufalin for 72 h. As shown in Fig. 2, the overall cytotoxicity of bufalin in Huh7, Hep3B and HA22T cells are presented and the growth of Huh7, Hep3B and HA22T were inhibited by bufalin dose-dependently. The absent of floating cells may indicate that there were no dying cells at these concentrations. Approximately 95% of cell proliferation of the three cell lines was inhibited by bufalin at 10, 2 and as low as 0.4 μM after 72-h bufalin treatment. The IC_{50} values were 0.034-0.04 μM for each cell line (Fig. 2). The results suggested that the micromolar level of bufalin can inhibit cell proliferation of hepatoma cells.

Bufalin arrested the cell cycle of hepatoma cells at G2/M phase. To further investigate the effect of bufalin on the cell cycle regulation of hepatoma cells, Huh7, Hep3B and HA22T cells was treated with 0.04 μM of bufalin for 0, 8, 12 and 24 h. Then the cells were collected and fixed with 70% methanol overnight and then stained with PI to detect the distribution of cell cycle by flow cytometry. As shown in Fig. 3, hepatoma

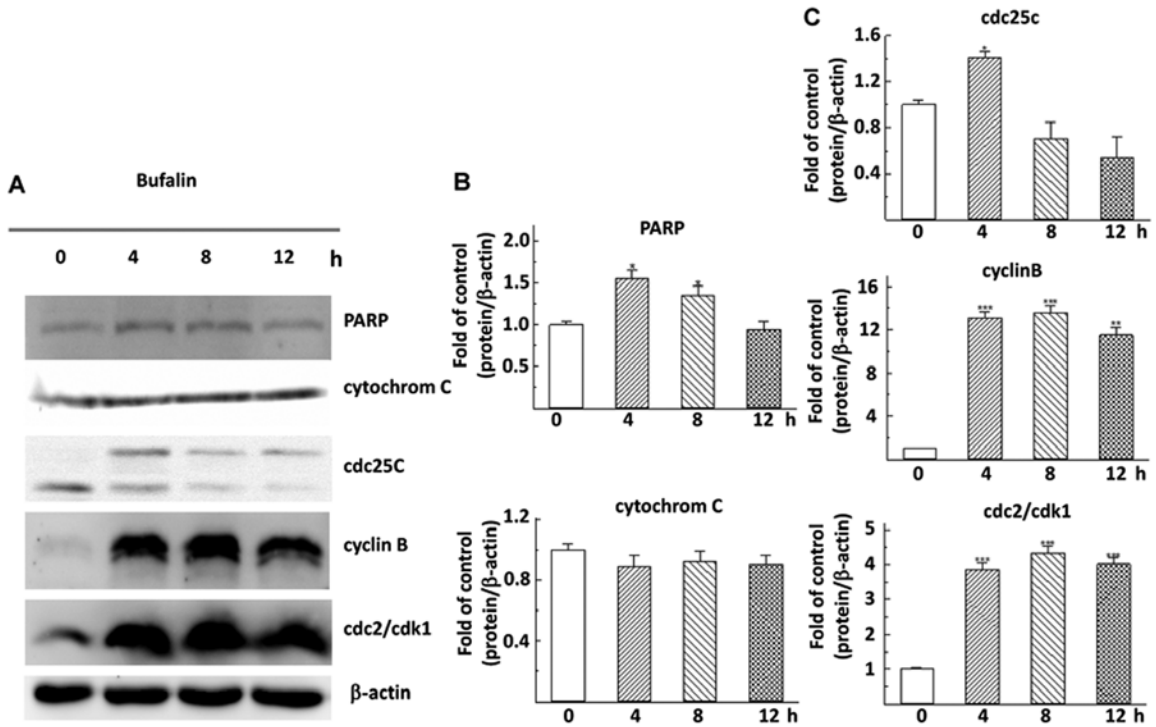

Figure 4. Bufalin affected the expression levels of G2/M phase cell cycle-associated proteins but not apoptosis-associated proteins in Huh7 cells. (A) Cells were exposed to 0.04 μM bufalin for 0, 4, 8 and 12 h and then harvested and lysed for the detection of protein levels of PARP, cytochrome C, cdc25c, cyclin B and cdc2 by western blot analysis. β-actin was used as an internal control. (B) Quantitative analysis of expression of PARP and cytochrome C (n=3 in each group). (C) Quantitative analysis of expression of cdc25c, cyclin B and cdc2 (n=3 in each group, *P<0.05; **P<0.01; ***P<0.001 compared with internal group).

cells were increased at the G2/M phase by 0.04 μM of bufalin time-dependently, with a concomitant decrease in the proportion of those in the S and G1 phase (Fig. 3).

Effects of bufalin on the expression level of G2/M phase-related and apoptosis-related proteins in hepatoma cells. To investigate if the arrest in G2/M phase of cell cycle and the relationship between cell cycle, apoptosis and autophagy, we evaluated the expression of G2/M phase of cell cycle-related and apoptosis-related proteins in hepatoma cells. In Fig. 4 (A and C), bufalin time-dependently upregulated the expression of the cyclin B, cdc2 and cdc25c protein, the check-point of G2/M phase of cell cycle, in hepatoma cells (Fig. 4A and 4C). At the same time, the levels of cytochrome C and PARP proteins, which closely associated with apoptosis in hepatoma cells, were unchanged after bufalin treatment (Fig. 4B).

Effects of bufalin on autophagy-associated proteins at transcriptional level by PCR array analysis. To assess the overall gene expression alteration pattern by bufalin in the hepatoma cells, differential patterns of sham-treated and 0.04 μM bufalin-treated Huh7 cells were compared by a cDNA PCR array. Samples were processed and up to 84 of annotated human cDNAs were analyzed and compared in the array system. Table I shows that 14 of the 84 detected genes were significantly altered by 2-fold (either up- or downregulated) after 0.04 μM bufalin treatment for 12 h in the Huh7 cells. The genes encoding co-regulators of autophagy and apoptosis (CXCR4, TNF, IFNG, IFNA2, PIK3CG), autophagy induction by intracellular pathogens (IFNA2) and co-regulators of autophagy and the cell cycle (IFNG) were upregulated. The expression level of GABARAPL1 and RPS6KB1, which encoded an autophagic vacuole formation and autophagy in response to other intracellular signals, respectively, were also increased. On the contrary, the translational level of the other 7 genes was significantly downregulated. Four main subgroups could be identified: genes encoding co-regulators of autophagy and apoptosis (IGF1, BID, TNFSF10), chaperone-mediated autophagy (HSP90AA1, HSPA8), autophagic vacuole formation (RGS19) and autophagy in response to other intracellular signals (ULK2).

Confirmation of the PCR array data with real-time PCR of the transcriptional changes for a selected subset of 14 genes in Huh7 cells. A subset of Huh7 genes potentially involved in autophagy was selected for further analysis by real-time PCR to confirm and to more precisely quantify the changes at the mRNA expression levels. As shown in Table II, these data synchronized with the results of the real-time PCR array analysis; the upregulated genes of CXCR4, 13.61x; TNF, 17.43x; IFNG, 6.96x; IFNA2, 3.83x; GABARAPL1, 3.43x and RPS6KB1, 1.35x; and repressed genes of HSPA8, HSP90AA1, BID, RGS19, ULK2 and TNFSF10 (HSPA8, 16.03x; HSP90AA1, 1.61x; BID, 1.3x; RGS19, 2.41x; ULK2, 4.18x and TNFSF10, 8.55x repression) were confirmed. IGF1 mRNA level was not altered and the transcriptional level of PIK3CG was undetectable.

Table I. Gene expression pattern modulated by bufalin in Huh7 cells.

Gene name/symbol	Fold alternation	Description
Co-regulators of autophagy and apoptosis		
CXCR4	11.63	Chemokine (C-X-C motif) receptor 4
TNF	7.67	Tumor necrosis factor
IFNG	4.72	Interferon γ
IFNA2	3.51	Interferon α2
PIK3CG	2.23	Phosphoinositide-3-kinase, catalytic, γ polypeptide
IGF1	-2.00	Insulin-like growth factor 1 (somatomedin C)
BID	-2.03	BH3 interacting domain death agonist
TNFSF10	-4.69	Tumor necrosis factor (ligand) superfamily, member 10
Genes involved in autophagic vacuole formation		
GABARAPL1	2.64	GABA(A) receptor-associated protein like 1
RGS19	-2.07	Regulator of G-protein signaling 19
Chaperone-mediated autophagy		
HSP90AA1	-2.75	Heat shock protein 90-kDa α (cytosolic), class A member 1
HSPA8	-10.70	Heat shock 70-kDa protein 8
Autophagy induction by intracellular pathogens		
IFNA2	3.51	Interferon α2
Co-regulators of autophagy and the cell cycle		
IFNG	4.72	Interferon γ
Autophagy in response to other intracellular signals		
ULK2	-2.50	Unc-51-like kinase 2 (*C. elegans*)
Autophagy in response to other intracellular signals		
RPS6KB1	2.08	Ribosomal protein S6 kinase, 70-kDa, polypeptide 1

Table presents the genes regulated with bufalin treatment with ≥2-fold alteration (up- or downregulated) by PCR array. GAPDH was used as an internal comparing standard. In the first column, the abbreviated gene names are listed; second column displays the alternated folds after bufalin 12-h treatment compare with sham-treated control group; third column briefly describes the function, family or known domains of the genes listed in the first column.

Time course of bufalin-regulated gene expression in three hepatoma cells. A 2-fold alteration in expression was defined as the minimum cut-off for the significant alteration in PCR array analysis; we selected 13 genes (Table III) for further analysis to see the time-course differences of the transcription level in Huh7, Hep3B and HA22T cells after bufalin treated. We found 7 genes to be upregulated in their expression at 4, 8 and 12 h in Huh7 cells. Moreover, the transcriptional level of ATG8, which was reported to closely relate to autophagy, was activated in all the three cell lines time-dependently. In contrast, six genes were found to be decreased at their mRNA expression levels at 4, 8 and 12 h in the three cell types. The results of quantitative real-time PCR analysis for all the 13 selected genes functionally related to hepatoma cell autophagy were in agreement with the PCR array findings for each gene detected (Fig. 5).

Bufalin inhibits BID and activates GABARAP (ATG8) family proteins in Huh7 cells. To examine whether BID and GABARAP family proteins were involved in bufalin-induced autophagy in hepatoma cells as reported in other cancer cells, the effects of bufalin on their expression levels were detected by western blot assay in Huh7 cells. Cells were harvested at different times after treatment with 0.04 μM bufalin and then

Table II. The synchronization of PCR array and real-time PCR in Huh7 cell after 12-h bufalin treatment.

Gene name	Fold alternation		Synchronize
	PCR array	RT-PCR	
CXCR4	11.63	13.61±4.84	Both positive
TNF	7.67	17.43±2.83	Both positive
IFNG	4.72	6.96±3.63	Both positive
IFNA2	3.51	3.83±1.42	Both positive
GABARAPL1	2.64	3.43±1.05	Both positive
PIK3CG	2.23	No on QRT-PCR	Only PCR array
RPS6KB1	2.08	1.35±0.26	Both positive
IGF1	-2	4.24±1.89	Not altered
BID	-2.03	-1.30±0.27	Both negative
RGS19	-2.07	-2.41±0.19	Both negative
ULK2	-2.5	-4.18±0.11	Both negative
HSP90AA1	-2.75	-1.61±0.63	Both negative
TNFSF10	-4.69	-8.55±0.01	Both negative
HSPA8	-10.7	-16.08±0.01	Both negative

Real-time PCR analysis of the expression of the selected set of genes closely associated with autophagy. Real-time PCR analysis was used to confirm and further quantity the expression changes showed in the PCR array experiment (Table I). The abbreviated gene names are listed in the first column; second column displays the alternated folds after bufalin 12-h treatment compared with sham-treated control group based on the microarray experiment; third column shows the alternated folds based on real-time PCR and the last column presented the synchronized results of PCR array and real-time PCR.

determined the BID and GABARAP family-related protein levels by western blotting. The results shown in Fig. 6 revealed that bufalin decreased BID after 12-h treatment. The data also showed that bufalin increased the levels of GABARAP family proteins (GABARAP and GABARAPL1) from 4- to 8- and 12-h treatment in Huh7 cells (Fig. 6). Based on these findings, it was suggested that bufalin-induced autophagy in Huh7 cells was mainly mediated through the downregulation of BID and upregulation of GABARAP family proteins.

Discussion

Bufalin has been demonstrated to have therapeutic effect in cancer cells through apoptosis mechanisms, but the signaling pathways of autophagy underlying bufalin-induced cell death in hepatoma cells have not been elucidated (21). In this study, we examined the effects of bufalin on hepatoma cell lines and aimed at unveiling the inhibition of cell growth with bufalin treatment and the molecular mechanism of bufalin-induced cell death in hepatoma cells. At the dosage of 0.4 μM or above, bufalin was effective in decreasing the percentage of cell viability for all the three hepatoma cell lines examined, Huh7, Hep3B and HA22T cells, to <10%. Also, the calculated IC_{50} was as low as 0.04 μM in these examined cells, strength-

Table III. Primer sets for real-time PCR.

Genes	Forward primer	Reverse primer	Annealing temperature (˚C)	GenBank accession no.
BID	TGTGAACCAGGAGTGAGTGC	GGCTGGAACCGTTGTTGA	60	NM_001196.2
CXCR4	ATTGGGATCAGCATCGACTC	CAAACTCACACCCTTGCTTG	60	NM_003467.2
GABARAPL1	TGGGCCAACTGTATGAGGA	CTACCCCCAAGTCCAGGTG	60	NM_031412.2
HSP90AA1	GGGCAACACCTCTACAAGGA	CTTGGGTCTGGGTTTCCTC	60	NM_001017963.2
HSPA8	TTTTTGTGGCTTCCTTCGTT	TCCCTTGGACATGGTTGC	60	NM_006597.3
IFNA2	AATGGCCTTGACCTTTGCTT	CACAGAGCAGCTTGACTTGC	60	NM_000605.3
IFNG	GGCATTTTGAAGAATTGGAAAG	TTTGGATGCTCTGGTCATCTT	60	NM_000619.2
IGF1	TGTGGAGACAGGGGCTTTTA	ATCCACGATGCCTGTCTGA	60	NM_000618.3
RGS19	GTGAGGGTCTGAGAGCTGGT	TCTGGCCCTGTGATCTGC	60	NM_005873.2
RPS6KB1	AGTGGCCACAATCGTGCT	TTTCTTTCTATTCTCCCCAGTGA	60	NM_003161.2
TNF	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA	59	NM_000594.2
TNFSF10	CCTCAGAGAGTAGCAGCTCACA	GCCCAGAGCCTTTTCATTC	59	NM_003810.2
ULK2	TTTAAATACAGAACGACCAATGGA	GGAGGTGCCAGAACACCA	60	NM_014683.3

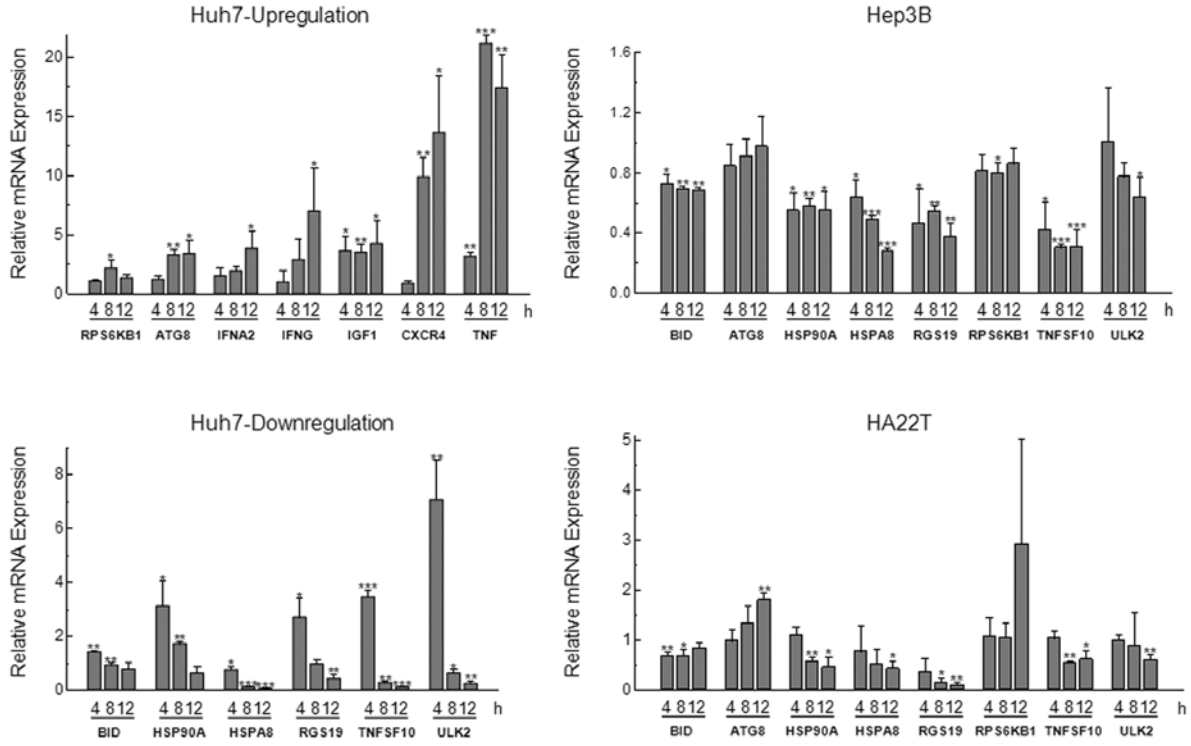

Figure 5. Confirmation of the PCR array data with real-time PCR of the transcriptional changes for a selected subset of 14 genes in Huh7 cells. Real-time PCR was performed on cDNA prepared from RNA isolated from Huh7, Hep3B and HA22T cells at 4, 8 and 12 h after bufalin treatment using the TaqMan probe. GAPDH was used as an internal control. Results are representative of three independent experiments.

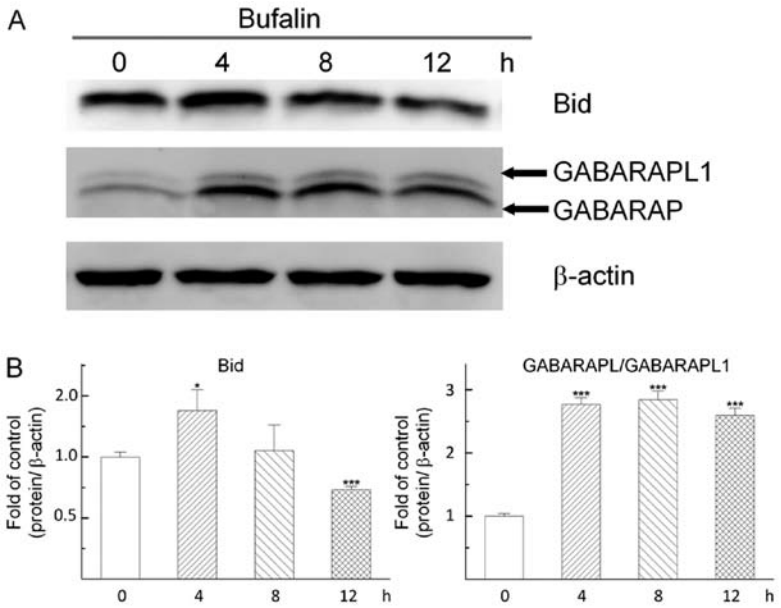

Figure 6. Bufalin induced autophagy related proteins in hepatoma cells. Western blot analysis of BID and GABARAPL1 expression in Huh7 cells treated with bufalin at 0.04 μM for various time intervals. The amount of β-actin served as the loading control. Each image is representative of at least three independent experiments (n=3 in each group, *P<0.05; **P<0.01; ***P<0.001 compared with internal group.

ening the potential of bufalin to be exploited as a therapeutic agent in the therapy of liver cancer (Fig. 2).

In the cell cycle analysis, Huh7, Hep3B and HA22T hepatoma cells were treated with bufalin and the alterations of cell cycle distributions by flow cytometry aided with propidium iodide staining were investigated. Bufalin did not induce the typical sub-G1, a close indicator for programmed cell death. Instead, bufalin increased the G2/M phase accumulation time-dependently in all three cell types, suggesting G2/M phase arrest and a non-apoptotic mechanism (Fig. 3). The alterations of G2/M phase markers, such as cdc25C, cyclin B and cdc2/cdk1, also demonstrated the similar results at their

Table IV. PCR array of gene expression pattern modulated by bufalin in Huh7 cells.

Gene name	Description	Fold Alternation
CXCR4	Chemokine (C-X-C motif) receptor 4	11.63
TNF	Tumor necrosis factor	7.67
IFNG	Interferon γ	4.72
IFNA2	Interferon α2	3.51
GABARAPL1	GABA(A) receptor-associated protein like 1	2.64
PIK3CG	Phosphoinositide-3-kinase, catalytic, γ polypeptide	2.23
RPS6KB1	Ribosomal protein S6 kinase, 70-kDa, polypeptide 1	2.08
MAP1LC3A	Microtubule-associated protein 1 light chain 3α	1.72
SNCA	Synuclein α (non-A4 component of amyloid precursor)	1.72
NFKB1	Nuclear factor of κ light polypeptide gene enhancer in B-cells 1	1.69
MAP1LC3B	Microtubule-associated protein 1 light chain 3β	1.68
UVRAG	UV radiation resistance associated gene	1.66
BCL2L1	BCL2-like 1	1.56
PRKAA1	Protein kinase, AMP-activated, α1 catalytic subunit	1.56
DRAM1	DNA-damage regulated autophagy modulator 1	1.51
EIF2AK3	Eukaryotic translation initiation factor 2-α kinase 3	1.48
CTSS	Cathepsin S	1.48
APP	Amyloid β (A4) precursor protein	1.47
INS	Insulin	1.46
TGFB1	Transforming growth factor β1	1.36
ATG12	ATG12 autophagy related 12 homolog (S. *cerevisiae*)	1.36
PRKAA2	Protein kinase, AMP-activated, α2 catalytic subunit	1.33
ATG4A	ATG4 autophagy related 4 homolog A (S. *cerevisiae*)	1.32
ATG9A	ATG9 autophagy related 9 homolog A (S. *cerevisiae*)	1.26
GABARAPL2	GABA(A) receptor-associated protein-like 2	1.24
ATG4D	ATG4 autophagy related 4 homolog D (S. *cerevisiae*)	1.23
BECN1	Beclin-1, autophagy related	1.16
ATG16L1	ATG16 autophagy related 16-like 1 (S. *cerevisiae*)	1.13
MAPK8	Mitogen-activated protein kinase 8	1.13
TGM2	Transglutaminase 2 (C polypeptide, protein-glutamine-γ-glutamyltransferase)	1.10
CTSB	Cathepsin B	1.04
BAK1	BCL2-antagonist/killer 1	1.04
AMBRA1	Autophagy/beclin-1 regulator 1	1.01
GABARAP	GABA(A) receptor-associated protein	-1.03
TMEM74	Transmembrane protein 74	-1.04
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	-1.06
RAB24	RAB24, member RAS oncogene family	-1.07
ATG16L2	ATG16 autophagy related 16-like 2 (S. *cerevisiae*)	-1.08
MAPK14	Mitogen-activated protein kinase 14	-1.09
ATG5	ATG5 autophagy related 5 homolog (S. *cerevisiae*)	-1.09
CASP8	Caspase 8, apoptosis-related cysteine peptidase	-1.09
TP73	Tumor protein p73	-1.13
ARSA	Arylsulfatase A	-1.14
HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate	-1.19
ATG3	ATG3 autophagy related 3 homolog (S. *cerevisiae*)	-1.19
CASP3	Caspase 3, apoptosis-related cysteine peptidase	-1.21
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	-1.22
FAM176A	Family with sequence similarity 176, member A	-1.23
SQSTM1	Sequestosome 1	-1.25

Table IV. Continued.

Gene name	Description	Fold alteration
RB1	Retinoblastoma 1	-1.28
TP53	Tumor protein p53	-1.28
CLN3	Ceroid-lipofuscinosis, neuronal 3	-1.29
EIF4G1	Eukaryotic translation initiation factor 4 γ1	-1.32
HDAC1	Histone deacetylase 1	-1.33
ESR1	Estrogen receptor 1	-1.33
GAA	Glucosidase α; acid	-1.34
IFNA4	Interferon α4	-1.34
FAS	Fas (TNF receptor superfamily, member 6)	-1.35
BCL2	B-cell CLL/lymphoma 2	-1.36
DRAM2	DNA-damage regulated autophagy modulator 2	-1.39
ATG4B	ATG4 autophagy related 4 homolog B (S. *cerevisiae*)	-1.39
PIK3C3	Phosphoinositide-3-kinase, class 3	-1.42
BAX	BCL2-associated X protein	-1.43
PIK3R4	Phosphoinositide-3-kinase, regulatory subunit 4	-1.44
IRGM	Immunity-related GTPase family, M	-1.44
ULK1	Unc-51-like kinase 1 (*C. elegans*)	-1.46
AKT1	V-akt murine thymoma viral oncogene homolog 1	-1.58
DAPK1	Death-associated protein kinase 1	-1.55
ATG9B	ATG9 autophagy related 9 homolog B (S. *cerevisiae*)	-1.64
HTT	Huntingtin	-1.66
ATG7	ATG7 autophagy related 7 homolog (S. *cerevisiae*)	-1.69
PTEN	Phosphatase and tensin homolog	-1.71
ATG10	ATG10 autophagy related 10 homolog (S. *cerevisiae*)	-1.74
BAD	BCL2-associated agonist of cell death	-1.75
BNIP3	BCL2/adenovirus E1B 19-kDa interacting protein 3	-1.78
ATG4C	ATG4 autophagy related 4 homolog C (S. *cerevisiae*)	-1.82
FADD	Fas (TNFRSF6)-associated via death domain	-1.89
IGF1	Insulin-like growth factor 1 (somatomedin C)	-2.00
BID	BH3 interacting domain death agonist	-2.03
RGS19	Regulator of G-protein signaling 19	-2.07
ULK2	Unc-51-like kinase 2 (*C. elegans*)	-2.50
HSP90AA1	Heat shock protein 90-kDa α (cytosolic), class A member 1	-2.75
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	-4.69
HSPA8	Heat shock 70-kDa protein 8	-10.70

Genes (84) regulated with bufalin treatment by PCR array. GAPDH was used as an internal comparing standard. In the first column, the abbreviated gene names are listed; second column briefly describes the function, family or known domains of the genes listed in the first column; third column displays the fold-change after 12-h bufalin treatment compared with sham-treated control group.

protein level (Fig. 4). Consistent to our findings in hepatoma cells, mounting reports have shown that bufalin can arrest the cell cycle of gastric cancer cells, leukemia cells, bladder carcinoma cells at G2/M phase (22-25).

Although the cell cycle arrest at G2/M phase and the mechanisms of bufalin-induced apoptosis (type I cell death) were investigated by several previous studies (21-26), but the autophagy program (type II cell death) induced by bufalin in human cancer cells has very few reports (12,27). We were interested in revealing whether bufalin induced cell death via apoptosis or autophagy after cell cycle arrest. In literature, bufalin induced apoptosis via Fas through caspase-3 and -8 and increased the level of cleaved-PARP in hepatoma cancer cells and prostate cancer cells, upregulating the expression of downstream Bax *in vitro* and *in vivo* (10,28,29). In our study, however, we did not find increase in cytochrome C and PARP during bufalin treatment in Huh7 cells (Fig. 4). Instead, the mRNA levels of Fas, caspase-3 and -8 and Bax was decreased (Table IV). These results may indicate that bufalin-induced cell death is not by the typical apoptosis pattern in Huh7

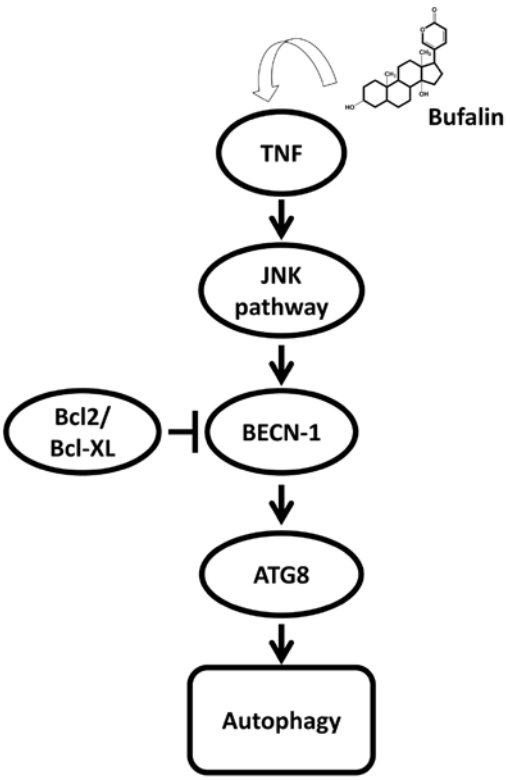

Figure 7. A proposed model of bufalin induced autophagy in hepatoma cells. This model summarizes the findings in this study, showing that bufalin induced autophagy was mainly via the regulation of TNF, JNK pathway, BECN-1 and the ATG8 novel pathway.

cells. Many anticancer agents, including fangchinoline and berberine, have been reported to induce autophagy without activation of caspase-dependent apoptosis (30,31). Bid, Bcl-2 and Beclin-1 (Vps30/Atg6), were reported to be specific regulators for apoptosis and autophagy, which could determine the cell fate of apoptosis or autophagy (32-35). In our results, decreased Bcl-2 and increased Beclin-1 was found in the PCR array (Table IV). Bid was found to decrease at the mRNA level time-dependently in bufalin-treated Huh7, Hep3B and HA22T cells (Table I and Fig. 5). The protein level of Bid was also found to decrease time-dependently in Huh7 cells (Fig. 6). The above evidence collectively suggested that Bid, Bcl-2 and Beclin-1 may play the role in shifting the bufalin-treated hepatoma cells to undergo autophagy instead of apoptosis. Specific cytokines such as IFNA2/IFNA and IFNG/interferon γ, were reported to be important modulating factors to induce autophagy of the cells in interferon resistant bladder cancer and osteoposis (36,37). In our data collected from PCR array and real-time PCR-time course (Table I, Table II and Fig. 5), the increased mRNA levels of IFNA2/IFNA and IFNG/interferon γ in Huh7 cells supported that autophagy was indeed induced by bufalin treatment in hepatoma cells.

After knowing that bufalin induced cell death of hepatoma cells was mainly by autophagy but not apoptosis, we are interested to reveal the mechanisms of bufalin-induced autophagy in hepatoma cells. Autophagic cell death has been reported to associate with the alterations in class III PI3K, ROS and the activation of JNK signaling pathway (38-40). However, our results (Table IV) showed that PIK3C3 (Vps34), a kinase in charge of triggering the class III PIK3 pathway, was decreased in its mRNA level in bufalin-treated Huh7 cells. The data suggested that the class III PIK3 pathway seems not to involve in bufalin-induced autophagy, even in the case that the mRNA level of Beclin-1, which was essential for Vps34 activation was indeed increased. Our results showed that TNFα/TNF, JNK/MAPK8, beclin-1 (BECN-1) and ATG8 family (GABARAP/ATG8A, GABARAPL1/ATG8B, GABARAPL2/ATG8C, MAP1LC3A/ATG8E, MAP1LC3B/ATG8F) were activated in PCR array (TNF, MAPK8, BECN-1 and ATG8 in Table IV), in real-time PCR (TNF and GABARAPL1 in Table I, Table II and Fig. 5) and in western blotting (GABARAP and GABARAPL1 in Fig. 6). GABARAP and GABARAPL1 (ATG8A and ATG8B) interacted with the GABA receptor and high homology sequences with gabarap gene (41). Both of the proteins participate in the autophagy together with other members (LC3, GATE-16 and ATG8). In our data, both GABARAP and GABARAPL1 were highly expressed during bufalin treatment at transcriptional and translational levels. These findings supported that the JNK pathway was involved in bufalin-induced autophagy. Kawazoe *et al* found that the JNK pathway is one of the signaling pathways involved in bufalin-induced apoptosis in leukemia U937 cells (42). In this study, we have proven that the JNK pathway was also associated with bufalin-induced autophagy in human hepatoma cells. Consistent with our findings, JNK-mediated upregulation of Beclin-1 and ATG8 was found to play a causal role in autophagy-mediated cell death (Fig. 7) (43,44).

The search for useful chemotherapy agents is valuable for treatment of hepatocellular carcinoma. To our knowledge, this is the first study to report on hepatoma cells that could be effectively killed by bufalin associated with cell cycle arrest at G2/M phase and through autophagy, not apoptosis. The JNK pathway was demonstrated to be mainly involved in the bufalin-induced autophagy in the hepatoma cells we investigated. Our study provides a platform for potential anti-hepatoma drug screening and mechanism determination. Bufalin was found to induce autophagy in hepatoma cells and to show potential for further pre-clinical investigations.

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