Original Article

Autophagy Accompanied with Bisdemethoxycurcumin-induced Apoptosis in Non-small Cell Lung Cancer Cells*

XU Jin Hong, YANG He Ping#, ZHOU Xiang Dong, WANG Hai Jing, GONG Liang, and TANG Chun Lan

Department of Respiratory Medicine, Southwest Hospital, Third Military Medical University, Chongqing 400038, China

Abstract

Objective To investigate the effects of bisdemethoxycurcumin (BDMC) on non-small cell lung cancer (NSCLC) cell line, A549, and the highly metastatic lung cancer 95D cells.

Methods CCK-8 assay was used to assess the effect of BDMC on cytotoxicity. Flow cytometry was used to evaluate apoptosis. Western blot analysis, electron microscopy, and quantification of GFP-LC3 punctuates were used to test the effect of BDMC on autophagy and apoptosis of lung cancer cells.

Results BDMC inhibited the viability of NSCLC cells, but had no cytotoxic effects on lung small airway epithelial cells (SAECs). The apoptotic cell death induced by BDMC was accompanied with the induction of autophagy in NSCLC cells. Blockage of autophagy by the autophagy inhibitor 3-methyladenine (3-MA) repressed the growth inhibitory effects and induction of apoptosis by BDMC. In addition, BDMC treatment significantly decreased smoothened (SMO) and the transcription factor glioma-associated oncogene 1 (Gli1) expression. Furthermore, depletion of Gli1 by siRNA and cyclopamine (a specific SMO inhibitor) induced autophagy.

Conclusion Aberrant activation of Hedgehog (Hh) signaling has been implicated in several human cancers, including lung cancers. The present findings provide direct evidence that BDMC-induced autophagy plays a pro-death role in NSCLC, in part, by inhibiting Hedgehog signaling.

Key words: Bisdemethoxycurcumin; Autophagy; Non-small cell lung cancer; Apoptosis; Hedgehog pathway

Biomed Environ Sci, 2015; 28(2): 105-115 *doi: 10.3967/bes2015.013* *ISSN: 0895-3988*

www.besjournal.com (full text) *CN: 11-2816/Q*

INTRODUCTION

Lung cancer is the leading cause of cancer related mortality worldwide. It has been estimated that more than 221,130 new cases and 156,940 deaths were reported in the United States in 2011[1]. About 600,000 people die of lung cancer each year and the annual lung cancer mortality rate may reach 1 million by 2025 in China[2]. Lung cancers are clinically divided into two major histologic categories, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). And NSCLC accounts for 80% of the cases, presenting as locally advanced disease in approximately 25%-30% of all cases. Despite recent improvements in chemotherapy and surgery, the 5-year survival rate remains less than 15%[1]. NSCLC continues to be a lethal cancer worldwide due to the emergence of drug resistance to conventional chemotherapy. The chemo-resistance of NSCLC largely results from the

*This study was supported by a grant from the National Science Foundation (Grant number 81102857).

#Correspondence author should be addressed to YANG He Ping, chief physician/professor, MD, Tel: 86-23-65318301; Fax: 86-23-65317511; E-mail: heping_yang6@163.com

Biographical note of the first author: XU Jin Hong, female, born in, 1979, attending doctor, MD, major in lung cancer.

failure of apoptotic and non-apoptotic cell death induced by anti-cancer treatments[3]. Therefore, there is an urgent need to identify additional compounds that might overcome the drug resistance, which limits the therapeutic effectiveness of conventional drugs.

Autophagy is an evolutionarily conserved catabolic process in which a cell engulfs long-lived proteins and damaged organelles within the autophagosomes, a double-membrane vacuole, and delivers them to the lysosome for degradation. This promotes the maintenance of cellular homeostasis by sequestering cytosol and organelles[4]. In tumor cells, the role of autophagy may depend on the tumor type and the stage of tumorigenesis. Appropriate modulation of autophagy through the suppression of cytoprotective autophagy or the promotion of cyto-killing autophagy could augment the cytotoxicity caused by anticancer therapy in tumor cells. Thus, in addition to the apoptotic response, it might be beneficial to find antitumor agents that could induce autophagy.

Curcumin (diferuloylmethane), one of the most well-known naturally occurring compounds, is subdivided into three components: Curcumin (Cur), Demethoxycurcumin (DMC), and Bisdemethoxy-curcumin (BDMC). Curcumin possesses a wide variety of biological activities. Curcumin was considered to have anti-inflammatory[5], anti-proliferative[6], anti-carcinogenic[7], anti-angiogenic[8], and anti-oxidant properties[9]. It has been shown that Curcumin exhibits strong anticancer efficacy by modulating a multiple of molecules involved in tumorigenesis of several cancers[10]. However, curcumin-stimulated autophagy has not been fully investigated in lung cancer.

Recently, it has been reported that Curcumin can induce autophagy in hepatoma[11], colon cancer cells[12], osteosarcoma[13], chronic myeloid leukemia cell[14], and prostate cancer cells[15]. CLEFMA, a synthetic analog of Curcumin has been reported to induce autophagy of lung adenocarcinoma H441 cells[16]. These studies suggest that curcumin-induced autophagy might be a therapeutic strategy to suppress growth and induce apoptosis of lung cancer cells. However, little is known about the relationship between autophagy and BDMC in NSCLC. Therefore, we investigated the effects of BDMC on the proliferation and autophagy of NSCLC cells.

The Hedgehog-Gli1 pathway is composed of the signal molecule Hh, membrane receptors patched (Ptch) and smoothened (SMO), and downstream nuclear transcription factor Gli, was considered as a highly conserved signaling pathway. Gli proteins are the effectors of hedgehog signaling. Gli target genes are involved in tumor cell growth, apoptosis, metastasis[17], and epithelial to mesenchymal transition[18-19].

The hedgehog signaling pathway has been implicated in the development of different human cancers including colon cancer, prostate cancer and lung cancer[20-21]. It has been reported that it is also involved in regulating autophagy[22]. In the present study, we investigated the potential effect of BDMC on 95D and A549 NSCLC cells. We observed that BDMC induced autophagy in NSCLC cells, and inhibition of autophagy could lead to suppression of BDMC-mediated cytotoxicity by decreasing the apoptotic activity.

MATERIALS AND METHODS

Reagents and Antibodies

BDMC, Cyclopamine, 3-methyladenine (3-MA), anti-LC3 antibody, and anti-GAPDH antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of BDMC was over 98%, which was dissolved in dimethyl sulfoxide (DMSO) to make a 100 mmol/L stock solution and was diluted with medium to the concentration as needed. The final concentration of DMSO was not more than 0.01%. Antibodies against Bcl-2, Bax, Caspase 3, SMO, and Gli1 were purchased from Santa Cruz Biotechnology (CA, USA). Goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Zhongshan Golden Bridge Biotechnology (Beijing, China). Cell Counting Kit-8 (CCK-8) was bought from Dojindo Laboratories (Kumamoto, Japan). AnnexinV/fluorescein isothiocyanate (FITC) Apoptosis Detection Kit was purchased from Beyotime (Jiangsu, China). Other reagents used in this study were obtained locally.

Cell Lines, Cell Culture, and Transfection

The NSCLC A549 cell line obtained from our laboratory and highly metastatic lung cancer cell line 95D bought from Chinese Academy of Science Cellbank (Shanghai, China) were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) (GIBCO, USA) in a humidified atmosphere at 37 °C with 5% CO_2. The lung small airway epithelial cells (SAECs) from

Clonetics (CA, USA) were maintained in SAGM supplemented with 10% FBS at 37 °C with 5% CO_2. The green fluorescent protein (GFP)-tagged microtubule-associated protein 1 light chain 3 (LC3) expression vector was kindly provided by Dr. Jianhua, LI (Fudan University, Shanghai, China). Transient transfection was performed with GFP-LC3 plasmid using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocols.

CCK-8 Assay

Cell viability was measured using the CCK-8. Briefly, 5×10^3 cells in the logarithmic growth phase in 100 µL complete medium were seeded into a 96-well plate and incubated at 37 °C in a humidified, 5% CO_2 atmosphere overnight. The cells were then treated with various concentrations of BDMC (10-80 µmol/L) for 24 h, 48 h, and 72 h, into each well of the plate, 10 µL of the CCK-8 reagent was added and incubated at 37 °C for 1 h. The optical density (OD) at 450 nm was measured using a microplate spectrophotometer (Bio-Rad, Segrate, Italy). Cell viability was calculated using the data and presented as percentage of the control. The 50% inhibitory concentration (IC50) values were calculated using probit analysis (SPSS software version 16.0).

Quantification of GFP-LC3 Punctuates

GFP-LC3-A549 and GFP-LC3-95D cells were cultured overnight. The cells were treated with 35 µmol/L BDMC for 24 h, fixed with 4% paraformaldehyde for 1 h, and then examined under a confocal microscope. To quantify the autophagic cells after BDMC treatment, the number of autophagic cells demonstrating GFP-LC3 dots (≥10 dots/cell) among 200 GFP-positive cells was counted as previously described[23].

Electron Microscopy

A549 and 95D cells treated with or without BDMC were washed and fixed with 2.5% glutaraldehyde at 4 °C. The cells were then post-fixed in 1.5% osmium tetroxide for 1 h, dehydrated with cold acetone, and embedded in Durcupan resin. The fragment was cut into thin sections, which were then stained with lead citrate before being examined with the TECNAI 10 electron microscope (Philips, Holland) at 80 kV.

Detection of Apoptosis by Flow Cytometry

Cells were seeded in six-well plates and incubated overnight. The cells were treated with the indicated concentrations of BDMC in the presence or absence of 3-MA. After 24 h of treatment, the cells were harvested by trypsinization and washed twice in phosphate-buffered saline (PBS). After staining with the combination of Annexin V-FITC and propidium iodide (PI), the cells were immediately analyzed by flow cytometer (Becton Dickinson).

Real-time Polymerase Chain Reaction

Total RNA was extracted from NSCLC cells using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara Biotechnology Co. Ltd, Dalian, China) according to the manufacturer's instructions. The following primers were used in this study: 5'-TCCATTCCCATTCCAGCTGT-3' and 5'-TCCCT-GGTAACCTTTGGAACAC-3' for Gli1, 5'-TGGCACACTT-CCTTCAAAGC-3' and 5'-CTCCCTCAAACTCACCCCATL-3' for SMO, 5'-CGGGAAATCGTGCGTGAC-3' and 5'-TGGAAGGTGGACAGCGAGG-3' for β-actin. PCR was performed under the following conditions: 95 °C for 30 s, 40 cycles of 96 °C for 45 s, 57 °C (Gli1), 56 °C (SMO) or 54 °C (β-actin*)* for 40 s, and 72 °C for 1 min followed by 10 min at 72 °C. All the PCR reactions were repeated at least three times. β-actin was amplified as an internal control. The intensity of each band amplified by RT-PCR was analyzed using Image J software.

Western Blot Analysis

To determine the levels of protein expression, cells were lysed using RIPA buffer containing protease inhibitor. Briefly, the cells were lysed in RIPA buffer on ice for 30 min. The supernatant was collected after the cell lysates were centrifuged at 12,000 g for 20 min. The protein concentrations were determined using the BCA Protein Assay Kit (Beyotime, Jiangsu, China). After separation by electrophoresis, the proteins were electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). The membranes were blotted with the appropriate primary antibodies at 4 °C overnight after blocking with 5% non-fat milk in Tris-buffered saline (TBS) for 1 h, and then incubated with Goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (diluted 1:2000) for 2 h at room temperature. After washing with TBS containing 0.1% Tween-20, the protein bands were visualized using enhanced chemiluminescence Western blotting system (Beyotime, Jiangsu, China). The relative amount of protein was quantified by

densitometry using Image J software. GAPDH was used as the loading control.

RNA Interference

Double-stranded small interference RNA (siRNA) was obtained from Sangon Biotech Co. Ltd (Shanghai, China). The sequences for the control siRNA were 5'-AACGUA-CGCGGAAUACAACGA-3' and the GLi1 siRNA sequence used was 5'-AACUC-CACAGG-CAUACAGGAU-3', which were used with reference to an earlier study[18].

Statistical Analysis

All experiments were performed in triplicate. Data are expressed as means±standard deviation (SD). The Student's *t* test was used for paired comparisons, and the one-way ANOVA test was used for multiple comparisons. $P<0.05$ was considered to be statistically significant.

RESULTS

BDMC Inhibited Cell Viability in NSCL Cells in a Dose- and Time-Dependent Manner

To investigate the cytotoxic effect of BDMC on NSCLC cells, we treated 95D and A549 cells with different concentrations of BDMC (0, 10, 20, 40, and 80 μmol/L) for various time points (24, 48, and 72 h) and followed by CCK-8 assay. As shown in Figure 1, the viability of 95D and A549 was reduced with with increasing BDMC concentration and longer incubation time. After being treated with 35.7 μmol/L and 34.4 μmol/L BDMC for 24 h, respectively, the 95D and A549 cells viability declined to around 50%. We then treated 95D and A549 cells with 35 μmol/L of BDMC for increasing amounts of time. These results suggested that BDMC suppressed the growth of cells in a dose- and time-dependent manner.

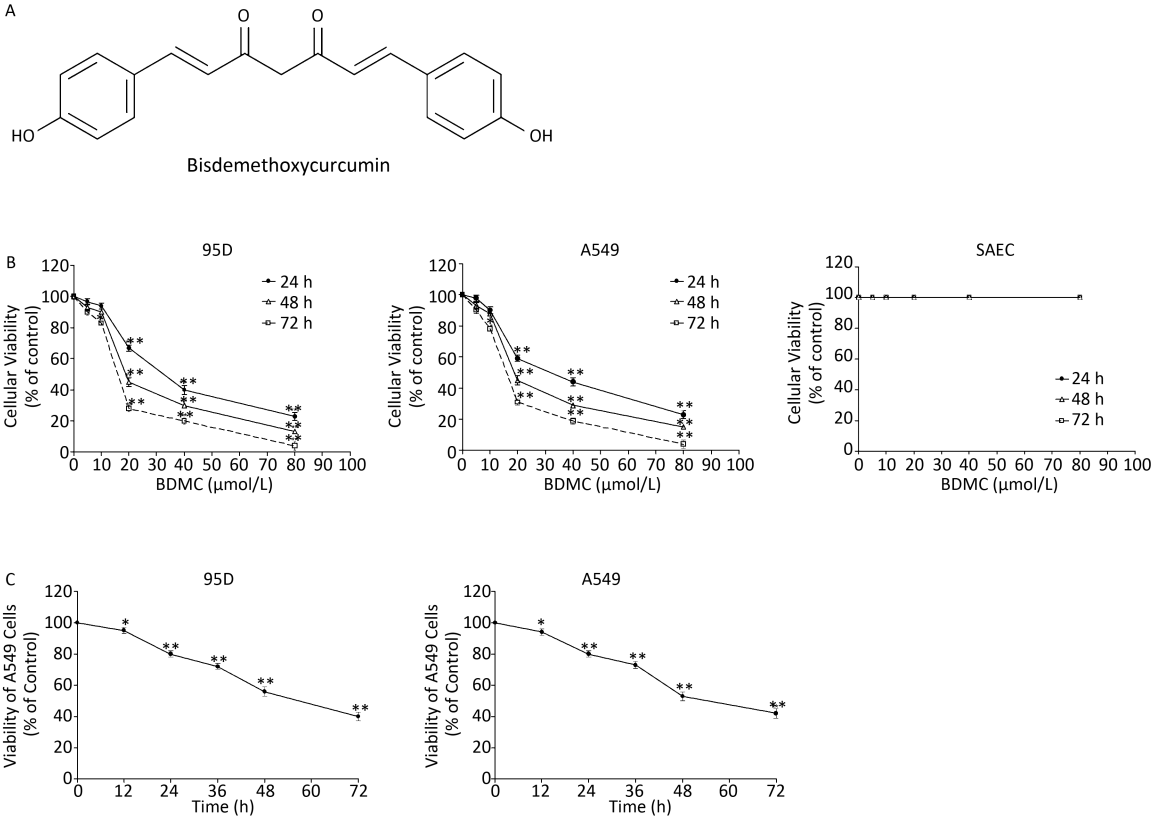

Figure 1. BDMC inhibited cell viability in 95D and A549 cells in a dose- and time-dependent manner. A, Schematic representation of the BDMC. B, 95D and A549 cells were treated with varying doses of BDMC (10, 20, 40, and 80 μmol/L) for different time points. Cell viability was measured by CCK-8 assay. C, 95D and A549 cells were treated with 35 μmol/L of BDMC for increasing amounts of time. Cell viability was determined using CCK-8 assay. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* controls.

BDMC Induces Autophagy in NSCLC Cells

To assess whether BDMC triggers autophagy in NSCLC cells, 95D and A549 cells were treated with BDMC (35 μmol/L) for 24 h and the levels of LC3-I and LC3-II were determined by Western blot analysis. We observed that BDMC significantly increased LC3-II conversion in 95D and A549 cells (Figure 2A). LC3-II conversion in BDMC-treated 95D and A549 cells was 17-fold and 3-fold higher than vehicle (DMSO)-treated cells, respectively. To confirm autophagy induction in BDMC-treated cells, we assessed the formation of punctate LC3-GFP in LC3-GFP transgene-expressing 95D and A549 cells. BDMC treatment caused a significant increase in LC3 punctate formation, which is indicative of autophagy in these cells (Figure 2B). Furthermore, the occurrence of autophagy by BDMC was confirmed by direct observation of the formation of autophagosomes by electron microscopy (Figure 2C). Taken together, these observations indicate that BDMC could stimulate autophagy in NSCLC cells.

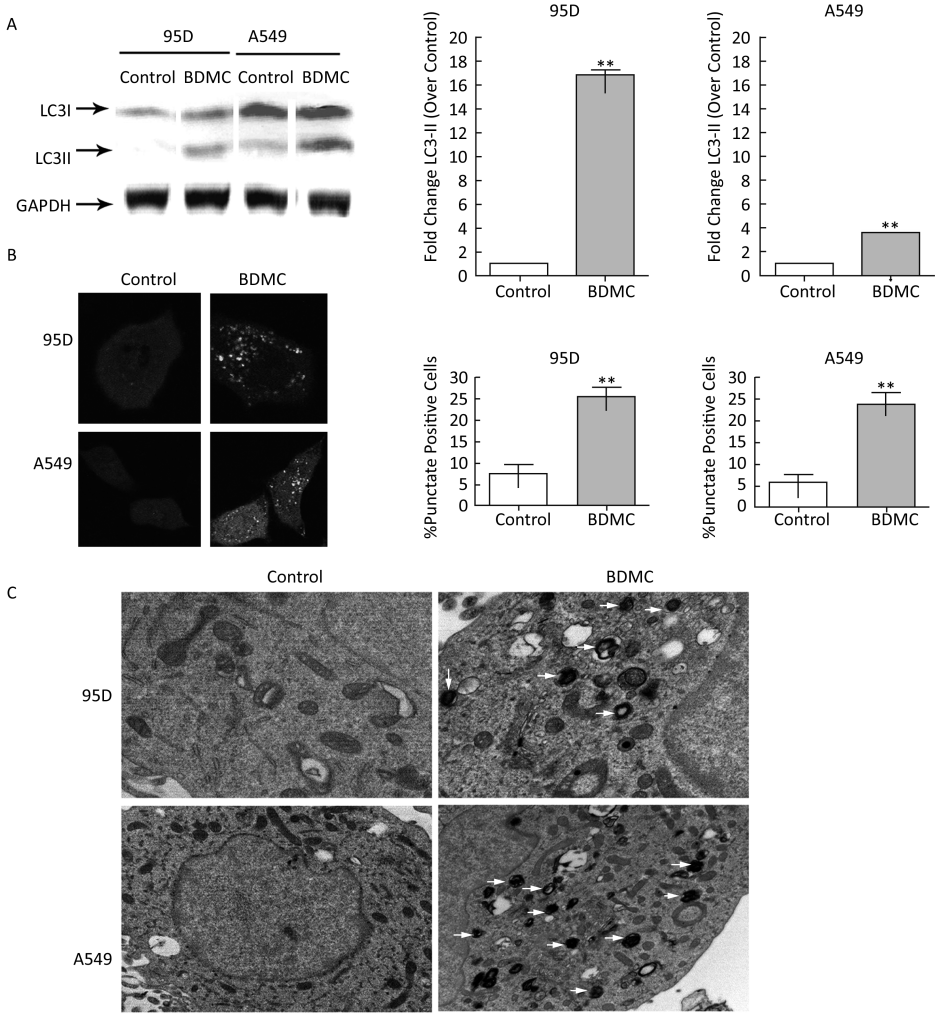

Figure 2. BDMC induces tumor cell autophagy. A, 95D and A549 cells were treated with BDMC (35 μmol/L) for 24 h. LC3-I to LC3-II conversion was determined by Western blot analysis. GAPDH was used as loading control. Bar graphs indicate fold change of LC3-II (normalized to GAPDH) over untreated cells. B, Accumulation of LC3-II-GFP punctate. 95D and A549 cells transiently expressing LC3-GFP (95D-LC3-GFP) were treated with BDMC (35 μmol/L) for 24 h. Formation of punctate was determined by fluorescence microscopy, and the proportion of punctate-positive cells per field of view was determined. Representative pictures of untreated and BDMC-treated cells. C, 95D and A549 cells treated with or without BDMC for 24 h were analyzed on electron microscopy. Arrows indicate autophagosomes. Scale bar, 2 μm. Data are from three independent experiments. $^{**}P<0.01$ *vs.* controls.

Autophagy Induced by BDMC Decreased Cell Viability

To understand the impact of autophagy on BDMC-induced growth inhibition, we determined the effect of BDMC on lung cancer cells before and after the suppression of autophagy by 3-MA (3 mmol/L). While 3-MA alone had no significant effect on cellular proliferation, the growth inhibitory effect of BDMC was significantly lowered after 3-MA treatment (Figure 3), indicating that autophagy in response to BDMC treatment inhibited cancer cells growth.

Inhibition of Autophagy by 3-MA Inhibits BDMC-induced Apoptotic Cell Death

Apoptosis was detected using flow cytometry (Figure 4A). Compared with control group, cells treated with 3-MA group showed no change in the apoptotic rate. The apoptotic rate in the BDMC combined with 3-MA groups was significantly lower than that in the BDMC alone groups, which indicates that autophagy played a positive role in the process of promoting apoptosis by BDMC. To prove that inhibition of autophagy by 3-MA inhibits BDMC-induced apoptotic cell death. We performed Western blot analysis to analyze the expression of apoptosis related proteins Bcl-2, Bax, and Caspase 3 (Figure 4B). These results indicate that autophagy positively regulated BDMC-induced apoptosis.

BDMC Induces Cell Autophagy through Down-regulation of the Hedgehog-Gli1 Signaling Pathway

We investigated the mechanisms underlying the inhibitory effects of BDMC on NSCLCS. The Hedgehog pathway is known to be an important regulator of carcinogenisis. Emerging data from many human tumors have suggested that aberrant activation of SMO and Gli1 are critical regulators of the Hedgehog-Gli1 pathway. As shown in Figure 5A, 35 μmol/L BDMC significantly decreased the mRNA level of SMO by 60%, 65% and of Gli1 by 62%, 70% in 95D and A549 cells compared with control, respectively, which was further confirmed with western blot analysis (Figure 5B). To investigate whether Gli1 and SMO are involved in autophagy, 95D and A549 cells were treated with Gli1 small interfering RNA (siRNA) and cyclopamine (a specific SMO small molecule inhibitor) for 24 h, then LC3-II production was determined by Western blot analysis (Figure 6A). Cyclopamine induced a 2.3-fold increase in LC3-II in Cyclopamine-treated 95D and A549 cells. Down-regulation of Gli1 induced a 5-fold, 14-fold increase in LC3-II in Gli1 siRNA-treated 95D and A549 cells, respectively. To confirm down-regulation of Gli1 and Cyclopamine can induce autophagy in 95D and A549 cells, we assessed the formation of punctate LC3-GFP in LC3-GFP transgene-expressing 95D cells and A549 cells, which were modified by the presence of the SMO inhibitor and the siRNA of Gli. SMO inhibitor and Gli1 down-regulation by siRNA caused a significant increase in LC3 punctate formation. Collectively, these results showed that SMO loss and Gli1 down-regulation can initiate autophagy, and that SMO down-regulation is responsible, in part, for the BDMC-induced autophagy in lung cancer cells.

DISCUSSION

Lung cancer is one of the most common malignancies and remains the leading cause of cancer

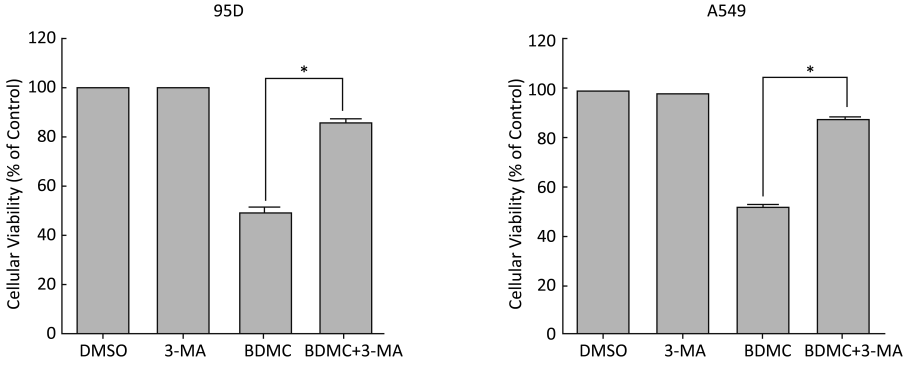

Figure 3. 3-MA desensitizes 95D and A549 cells to cytotoxic actions of BDMC. CCK-8 analysis of cell viability in cells treated with 35 μmol/L BDMC in the absence or presence of 3-MA (3 mmol/L, pretreated for 2 h). $^{*}P<0.05$ *vs.* controls.

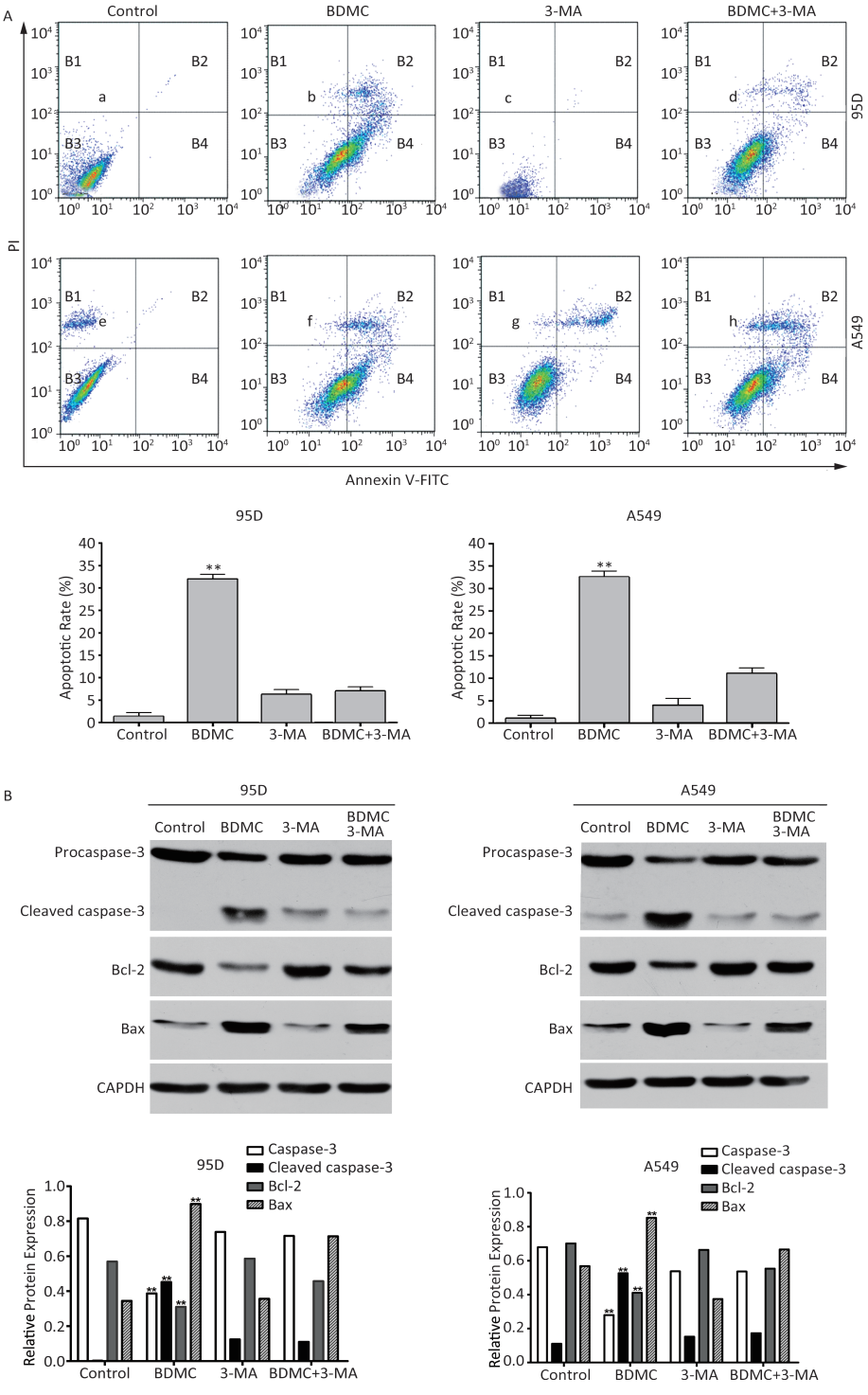

Figure 4. BDMC induced apoptosis in 95D and A549 cells *in vitro*. A, Apoptosis was detected by flow cytometry for Annexin V-FITC and PI dual labeling. 95D and A549 cancer cells were treated with a 0.1% DMSO control, 35 μmol/L of BDMC for 24 h in the absence or presence of 3-MA (5 mmol/L, pretreated for 2 h). The population of apoptotic cell was calculated. 'PI' denotes propidium iodide staining. 'B4' denotes the apoptotic rate, and 'B2' denotes the proportion of dead cells. B, Correlative changes of molecules in apoptosis after treatment with BDMC. Twenty-four hours after BDMC treatment, Western blot analysis was performed for Bcl-2, Bax, and Caspase 3. $^{**}P<0.01$ *vs.* controls.

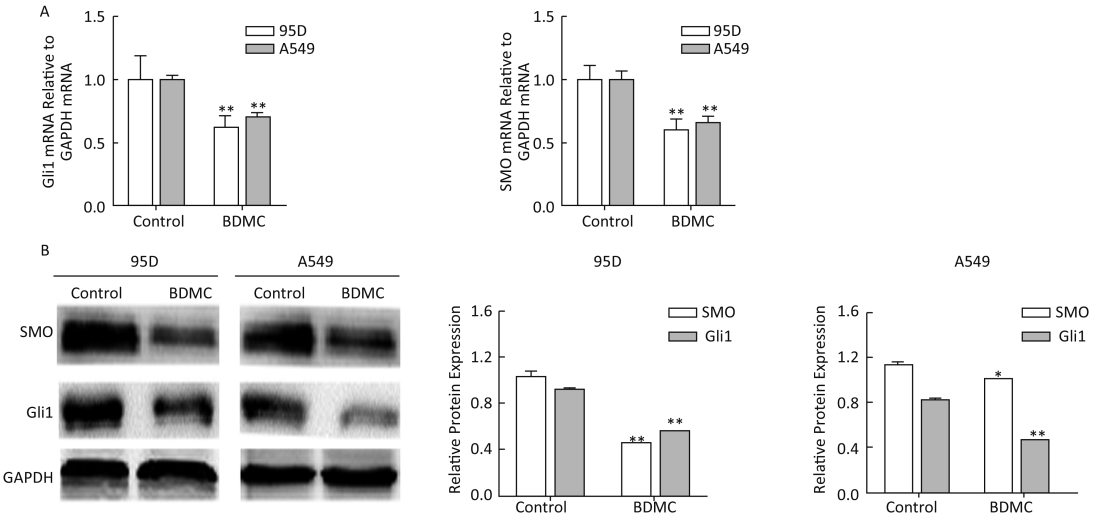

Figure 5. BDMC inhibits Hedgehog-GLi1 pathway *in vitro*. A, Real-time polymerase chain reaction analysis of human SMO and GLI1 in 95D and A549 cells. The cells were incubated with BDMC (35 μmol/L) for 24 h. B, Protein levels of SMO and Gli1 were measured by Western blot analysis in 95D and A549 cells and accompanied by a quantitative bar chart. As an internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* controls.

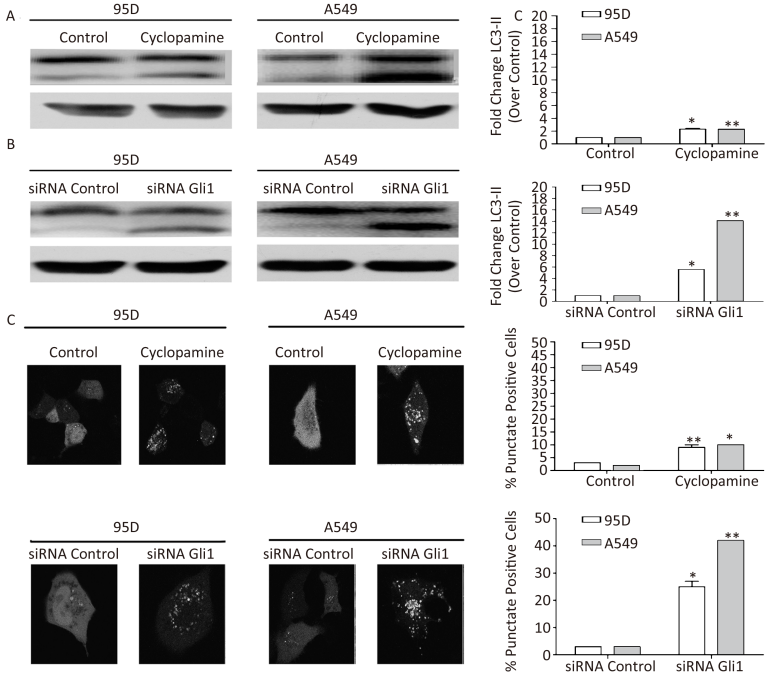

Figure 6. Gli1 down-regulation and cyclopamine induced autophagy. A, 95D and A549 cells were treated with cyclopamine (10 μmol/L) for 24 h. DMSO was used as the control group. The expression of LC3-II was determined by Western blotting. GAPDH was used as the loading control. B, 95D and A549 cells were transfected with control siRNA or Gli1 siRNA for 24 h. The expression of LC3-II was determined by western blotting. GAPDH was used as the loading control. C, Accumulation of LC3-II-GFP punctate. 95D and A549 cells expressing LC3-GFP fusion protein were treated with Cyclopamine or transfected with siRNA of Gli1 for 24 h. Formation of punctate was determined by fluorescence microscopy, and the proportion of punctate-positive cells per field of view was determined, which are described in the Materials and Methods. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* controls.

related deaths worldwide[24]. Drug resistance often leads to the failure of chemotherapy. Autophagy, a lysosome-dependent degradation pathway in eukaryotic cells, has been implicated in many physiological and pathological processes[25]. In the cancer cells, autophagy plays a dual role, as a mechanism responsible for protecting or killing the cell[26]. That is, autophagy acts as a process which could result in both cell survival and cell death and its relationship with the lung cancer therapy has attracted increasing attention in cancer fields[25]. Disorder of autophagic regulatory mechanisms plays an important role in the development of malignant tumor. However, the specific function of autophagy in tumorigenesis and tumor development remains to be clarified. It has been demonstrated that autophagy can be modulated by many frontline anticancer agents including Paclitaxel[27], arsenic trioxide[28], tamoxifen[29], and imatinib[30] in tumors. Curcumin has been reported to induce apoptosis and inhibit cell growth by down-regulating proteins of the Shh pathway in medulloblastoma[31]. Moreover, curcumin inhibited prostate cancer cell growth through the Shh pathway[32]. Curcumin can induce autophagy in a majority of cancers including esophageal cancer[33], malignant gliomas[23], leukemia[34], and prostate cancer[35]. The mechanisms consist of inhibition of the Akt/mTOR/p70S6K pathway and activation of the ERK1/2 pathways[23], but other pathways may also be involved, which are yet to be identified. Autophagy is a catabolic process involving the degradation of long-lived proteins and organelles through the lysosomal machinery. The hedgehog (Hh) signaling pathway is an important therapeutic target in cancer and is implicated in a variety of cancers including basal cell carcinoma[36], medulloblastoma[37], and leukemia[38], and in gastrointestinal[39], breast[40], prostate[41], lung[18], and pancreatic cancers[42]. BDMC, as one of the derivatives of Curcumin, has a similar biological activity to Curcumin. The anticancer mechanisms of BDMC are complex and remain largely unknown. In this study, we showed that BDMC decreased the Hedgehog-Gli1 signaling pathway (Figure 5) and also inhibited it by siRNA Gli1 or using cyclopamine induced autophagy (Figure 6). BDMC induced autophagy as well as apoptosis. And BDMC-induced cell death was selective for cancer cells because normal lung small airway epithelial cells (SAECs) were resistant to treatment with BDMC. Additionally, we found that inhibition of autophagy using 3-MA in combination with BDMC significantly decreased tumor cell viability compared with BMDC treatment alone.

In our study, to clarify the contributions of autophagy in BDMC-induced cell death, we dissected the role of autophagy to the survival of 95D and A549 cells during BDMC-induced apoptosis using autophagy inhibitor 3-MA. As expected, 3-MA significantly inhibited BDMC-induced autophagy and attenuated BDMC-induced cell apoptosis in 95D and A549 cells. These data suggest that BDMC-induced autophagy might provide a self-destructive mechanism for cancer cells and inhibition of autophagy may diminish the therapeutic efficacy of BDMC in the treatment of cancer. Curcumin modulates multiple molecular pathways. The clinical application of Curcumin is currently compromised by its poor bioavailability. Meanwhile, the limitations in using BDMC in NSCLC therapy still need for further study and investigation. For example, depletion of specific modulators of autophagy through RNA interference studies is needed to further elucidate the mechanisms underlying BDMC-induced apoptosis. In addition, further studies in mouse models of cancer should also be performed.

In the present study, we also demonstrated that BDMC significantly inhibited the growth of A549 and 95D cells in a dose- and time-dependent manner, but had little effect on normal lung epithelial cell line SAEC (Figure 1). And BDMC can simultaneously induce autophagy and apoptosis in 95D and A549 cells. Our results have shown that autophagic form of LC3-II was significantly increased after BDMC treatment in 95D and A549 cells. Meanwhile, we have also provided evidence that BDMC was able to increase the activity of double-membrane vacuole (named autophagosome). All these results indicate that autophagy was induced by BDMC. And currently results are consistent with the previous report that BDMC can suppress cancer cell growth. Although autophagy was able to induce apoptosis when cells are not viable, it is also a temporary survival mechanism under stress conditions[43]. In that study, we consider that autophagic cell death was induced rather than cell survival because administration of 3-MA attenuated the reduction in cell viability. To our knowledge, the present study is the first report of induction of autophagy in lung cancer cells by BDMC. The effects of Curcumin may differ depending on cell types. Further studies *in vivo* and on the potential mechanisms of autophagy induction are necessary. More recently accumulating evidence demonstrated that the anti-cancer activities of

Curcumin are related to multiple biological pathways involved in cell apoptosis[12], tumor growth[23], oncogene expression, cell cycle regulation, tumorigenesis[15], and metastasis[44]. Although multiple anti-cancer mechanisms of Curcumin are revealed, the underlying mechanism by which BDMC induced autophagy of lung cancers is not well understood. And the efficacy of BDMC on the autophagy has not been fully investigated. Several studies have shown that Curcumin could induce cell autophagy in many cancers. In agreement with previous studies, we also observed BDMC induced autophagy in NSCLC cells.

There is emerging evidence that autophagy plays critical roles in the generation of antineoplastic responses and mediates caspase-independent malignant cell death. On the other hand, autophagy represents a protective mechanism against apoptotic cell death under starvation as well as contributes to resistance against therapy-induced apoptosis in cancer cells. It has been shown that autophagy is activated as a killing mechanism against BDMC-induced apoptosis, autophagy blockade desensitizes lung cancer cells towards BDMC, autophagy serves a killing role in BDMC mediated cell killing, and autophagy inhibition attenuates the anticancer activity of the BDMC. In our study, 3-MA inhibits BDMC-induced autophagy and attenuated BDMC-induced cell apoptosis in 95D and A549 cells. These findings suggest that BDMC-induced autophagy might provide a self-destructive mechanism for cancer cells, and inhibition of autophagy may diminish the therapeutic efficacy of BDMC in the treatment of cancer. In our study, we also analyzed the contributions of autophagy to the survival of 95D and A549 cells during BDMC-induced apoptosis using autophagy inhibitor 3-MA.

However, the mechanism of autophagy inhibition desensitizes 95D and A549 cell to apoptotic cell death induced by BDMC needs to be further investigated. At present, evidence indicates that Hedgehog signaling pathway is an important regulators for autophagy and apoptosis[18-22]. During the process of chemotherapy, the apoptotic rate would be increased when autophagy was inhibited to reduce the scavenging of damaged cells. Numerous documented studies have indicated that suppression of autophagy enhances the sensitivity of cancer cells to chemotherapeutic drugs[45]. Our results were contrary to the previous studies, which indicated that the chemotherapeutic drugs induce autophagy and inhibition of autophagy weakens the effect of chemotherapy.

Taken together, we demonstrate for the first time that BDMC induces autophagy in NSCLC cells 95D and A549 *in vitro*, along with BDMC-induced apoptosis. And inhibition of autophagy was able to attenuate the toxicity of BDMC. Therefore, autophagy is involved in the antitumor of BDMC in NSCLC. And the possible anti-cancer mechanism may include the down-regulation of the Hedgehog-Gli1 pathway. However, further study is required to determine whether autophagic enhancement could be utilized *in vivo.*

Revieved: September 28, 2014;
Accepted: December 19, 2014

REFERENCES

1. Siegel R, Ward E, Brawley O, et al. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA: a cancer journal for clinicians, 2011; 61, 212-36.
2. She J, Yang P, Hong Q, et al. Lung cancer in China: challenges and interventions. Chest, 2013; 143, 1117-26.
3. Okouoyo S, Herzer K, Ucur E, et al. Rescue of death receptor and mitochondrial apoptosis signaling in resistant human NSCLC *in vivo*. Int J Cancer, 2004; 108, 580-7.
4. Tang D, Kang R, Zeh HJ 3rd, et al. High-mobility group box 1, oxidative stress, and disease. Antioxid Redox Signal, 2011; 14, 1315-35.
5. Wu J, Zhang Y, Cai Y, et al. Discovery and evaluation of piperid-4-one-containing mono-carbonyl analogs of curcumin as anti-inflammatory agents. Bioorg Med Chem, 2013; 21, 3058-65.
6. Ravindran J, Subbaraju GV, Ramani MV, et al. Bisdemethylcurcumin and structurally related hispolon analogues of curcumin exhibit enhanced prooxidant, anti-proliferative and anti-inflammatory activities *in vitro*. Biochem Pharmacol, 2010; 79, 1658-66.
7. Heng MC. Curcumin targeted signaling pathways: basis for anti-photoaging and anti-carcinogenic therapy. Int J Dermatol, 2010; 49, 608-22.
8. Gururaj AE, Belakavadi M, Venkatesh DA, et al. Molecular mechanisms of anti-angiogenic effect of curcumin. Biochem Biophys Res Commun, 2002; 297, 934-42.
9. Aldebasi YH, Aly SM, Rahmani AH. Therapeutic implications of curcumin in the prevention of diabetic retinopathy via modulation of anti-oxidant activity and genetic pathways. International journal of physiology, pathophysiology and pharmacology, 2013; 5, 194-202.
10. Devipriya B, Kumaradhas P. Molecular flexibility and the electrostatic moments of curcumin and its derivatives in the active site of p300: a theoretical charge density study. Chem Biol Interact, 2013; 204, 153-65.
11. Qian H, Yang Y, Wang X. Curcumin enhanced adriamycin-induced human liver-derived Hepatoma G2 cell death through activation of mitochondria-mediated apoptosis and autophagy. Eur J Pharm Sci, 2011; 43, 125-31.
12. Basile V, Belluti S, Ferrari E, et al. bis-Dehydroxy-Curcumin triggers mitochondrial-associated cell death in human colon

cancer cells through ER-stress induced autophagy. PLoS One, 2013; 8, e53664.
13. Ma D, Tremblay P, Mahngar K, et al. Selective cytotoxicity against human osteosarcoma cells by a novel synthetic C-1 analogue of 7-deoxypancratistatin is potentiated by curcumin. PLoS One, 2011; 6, e28780.
14. Jia YL, Li J, Qin ZH, et al. Autophagic and apoptotic mechanisms of curcumin-induced death in K562 cells. J Asian Nat Prod Res, 2009; 11, 918-28.
15. Teiten MH, Gaascht F, Cronauer M, et al. Anti-proliferative potential of curcumin in androgen-dependent prostate cancer cells occurs through modulation of the Wingless signaling pathway. Int J Oncol, 2011; 38, 603-11.
16. Lagisetty P, Vilekar P, Sahoo K, et al. CLEFMA-an anti-proliferative curcuminoid from structure-activity relationship studies on 3,5-bis(benzylidene)-4-piperidones. Bioorg Med Chem, 2010; 18, 6109-20.
17. Lu JT, Zhao WD, He W, et al. Hedgehog signaling pathway mediates invasion and metastasis of hepatocellular carcinoma via ERK pathway. Acta Pharmacol Sin, 2012; 33, 691-700.
18. Ahmad A, Maitah MY, Ginnebaugh KR, et al. Inhibition of Hedgehog signaling sensitizes NSCLC cells to standard therapies through modulation of EMT-regulating miRNAs. J Hematol Oncol, 2013; 6, 77.
19. Lei J, Ma J, Ma Q, et al. Hedgehog signaling regulates hypoxia induced epithelial to mesenchymal transition and invasion in pancreatic cancer cells via a ligand-independent manner. Mol Cancer, 2013; 12, 66.
20. Chi S, Huang S, Li C, et al. Activation of the hedgehog pathway in a subset of lung cancers. Cancer Lett, 2006; 244, 53-60.
21. Yuan Z, Goetz JA, Singh S, et al. Frequent requirement of hedgehog signaling in non-small cell lung carcinoma. Oncogene, 2007; 26, 1046-55.
22. Jimenez-Sanchez M, Menzies FM, Chang YY, et al. The Hedgehog signalling pathway regulates autophagy. Nat Commun, 2012; 3, 1200.
23. Aoki H, Takada Y, Kondo S, et al. Evidence that curcumin suppresses the growth of malignant gliomas *in vitro* and *in vivo* through induction of autophagy: role of Akt and extracellular signal-regulated kinase signaling pathways. Mol Pharmacol, 2007; 72, 29-39.
24. Zaric B, Stojsic V, Tepavac A, et al. Adjuvant chemotherapy and radiotherapy in the treatment of non-small cell lung cancer (NSCLC). J Thorac Dis, 2013; 5, S371-S7.
25. Polewska J. Autophagy-molecular mechanism, apoptosis and cancer. Postepy Hig Med Dosw (Online), 2012; 66, 921-36.
26. Martinet W, Agostinis P, Vanhoecke B, et al. Autophagy in disease: a double-edged sword with therapeutic potential. Clin Sci (Lond), 2009; 116, 697-712.
27. Veldhoen RA, Banman SL, Hemmerling DR, et al. The chemotherapeutic agent paclitaxel inhibits autophagy through two distinct mechanisms that regulate apoptosis. Oncogene, 2013; 32, 736-46.
28. Chai CY, Huang YC, Hung WC, et al. Arsenic salts induced autophagic cell death and hypermethylation of DAPK promoter in SV-40 immortalized human uroepithelial cells. Toxicol Lett, 2007; 173, 48-56.
29. de Medina P, Silvente-Poirot S, Poirot M. Tamoxifen and AEBS ligands induced apoptosis and autophagy in breast cancer cells through the stimulation of sterol accumulation. Autophagy, 2009; 5, 1066-7.
30. Shingu T, Fujiwara K, Bogler O, et al. Inhibition of autophagy at a late stage enhances imatinib-induced cytotoxicity in human malignant glioma cells. Int J Cancer, 2009; 124, 1060-71.
31. Lim SH, Choi SA, Lee JY, et al. Therapeutic targeting of subdural medulloblastomas using human neural stem cells expressing carboxylesterase. Cancer Gene Ther, 2011; 18, 817-24.
32. Mimeault M, Batra SK. Potential applications of curcumin and its novel synthetic analogs and nanotechnology-based formulations in cancer prevention and therapy. Chin Med, 2011; 6, 31.
33. O'Sullivan-Coyne G, O'Sullivan GC, O'Donovan TR, et al. Curcumin induces apoptosis-independent death in oesophageal cancer cells. Br J Cancer, 2009; 101, 1585-95.
34. Wu JC, Lai CS, Badmaev V, et al. Tetrahydrocurcumin, a major metabolite of curcumin, induced autophagic cell death through coordinative modulation of PI3K/Akt-mTOR and MAPK signaling pathways in human leukemia HL-60 cells. Mol Nutr Food Res, 2011; 55, 1646-54.
35. Teiten MH, Gaascht F, Cronauer M, et al. Anti-proliferative potential of curcumin in androgen-dependent prostate cancer cells occurs through modulation of the Wingless signaling pathway. Int J Oncol, 2011; 38, 603-11.
36. Walls B. Basal cell carcinoma: hedgehog pathway inhibitors and beyond. Cutis, 2013; 91, E1-4.
37. Chen ZQ, Han XH, Cao X. [Sonic Hedgehog signaling pathway and regulation of inner ear development]. Yi Chuan, 2013; 35, 1058-64.
38. Lu FL, Yu CC, Chiu HH, et al. Sonic hedgehog antagonists induce cell death in acute myeloid leukemia cells with the presence of lipopolysaccharides, tumor necrosis factor-alpha, or interferons. Invest New Drugs, 2013; 31, 823-32.
39. Biau S, Jin S, Fan CM. Gastrointestinal defects of the Gas1 mutant involve dysregulated Hedgehog and Ret signaling. Biology Open, 2013; 2, 144-55.
40. Goel HL, Pursell B, Chang C, et al. GLI1 regulates a novel neuropilin-2/alpha6beta1 integrin based autocrine pathway that contributes to breast cancer initiation. EMBO Mol Med, 2013; 5, 488-508.
41. Gonnissen A, Isebaert S, Haustermans K. Hedgehog signaling in prostate cancer and its therapeutic implication. Int J Mol Sci, 2013; 14, 13979-4007.
42. Xu Y, An Y, Wang X, et al. Inhibition of the Hedgehog pathway induces autophagy in pancreatic ductal adenocarcinoma cells. Oncol Rep, 2014; 31, 707-12.
43. Chen HY, White E. Role of autophagy in cancer prevention. Cancer Prev Res, 2011; 4, 973-83.
44. Wilken R, Veena MS, Wang MB, et al. Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. Mol Cancer, 2011; 10, 12.
45. Shimizu S, Takehara T, Hikita H, et al. Inhibition of autophagy potentiates the antitumor effect of the multikinase inhibitor sorafenib in hepatocellular carcinoma. Int J Cancer, 2012; 131, 548-57.