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Berberine Induces Autophagic Cell Death and Mitochondrial Apoptosis in Liver Cancer Cells: The Cellular Mechanism

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ABSTRACT

Extensive studies have revealed that berberine, a small molecule derived from *Coptidis rhizoma* (Huanglian in Chinese) and many other plants, has strong anti-tumor properties. To better understand berberine-induced cell death and its underlying mechanisms in cancer, we examined autophagy and apoptosis in the human hepatic carcinoma cell lines HepG2 and MHCC97-L. The results of this study indicate that berberine can induce both autophagy and apoptosis in hepatocellular carcinoma cells. Berberine-induced cell death in human hepatic carcinoma cells was diminished in the presence of the cell death inhibitor 3-methyladenine, or following interference with the essential autophagy gene Atg5. Mechanistic studies showed that berberine may activate mitochondrial apoptosis in HepG2 and MHCC97-L cells by increasing Bax expression, the formation of permeable transition pores, cytochrome C release to cytosol, and subsequent activation of the caspases 3 and 9 execution pathway. Berberine may also induce autophagic cell death in HepG2 and MHCC97-L cells through activation of Beclin-1 and inhibition of the mTOR-signaling pathway by suppressing the activity of Akt and up-regulating P38 MAPK signaling. This is the first study to describe the role of Beclin-1 activation and mTOR inhibition in berberine-induced autophagic cell death. These results further demonstrate the potential of berberine as a therapeutic agent in the emerging list of cancer therapies with novel mechanisms. J. Cell. Biochem. 111: 1426–1436, 2010.

KEY WORDS: BERBERINE; AUTOPHAGY; APOPTOSIS; BCL-2/BECLIN-1; MTOR

Hepatocellular carcinoma (HCC), accounting for 80–90% of liver cancer, has become one of the most common and prevalent human malignancies in the world [Budhu et al., 2008]. However, effective therapy for this malignancy remains elusive. One of the primary approaches for the treatment of liver cancer is chemotherapy, in which the chemotherapeutic agents act by inducing cancer cell death. Several types of cell death have been classified and defined by the Nomenclature Committee on Cell Death (NCCD), including apoptotic and autophagic cell death. These two types of cell death, also known as Type I and Type II Programmed Cell Death (PCD), respectively, differ in their morphological features and signal transduction but share the same result of cell death [Kroemer et al., 2009]. Apoptosis is considered a conventional type of cell death whereas autophagic cell death is simply defined as cell death with autophagy, a process that is also thought to be important for cell survival [Galluzz et al., 2008]. Recently, extensive studies have reported that apoptosis and autophagy were both involved in chemotherapeutic agent-induced cancer cell death [Cheng et al., 2009; Kim et al., 2009], suggesting that apoptosis and autophagy are important target mechanisms for novel therapeutic agents.

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Berberine is a natural product derived from several herbs, such as *Coptidis rhizoma* (Huanglian in Chinese), *Hydrastis canadensis*, *Berberis aquifolium*, *Berberis aristata*, and *Berberis vulgaris* (barberry). Many studies have reported the in vitro and in vivo anti-cancer effect of *Huanglian* and berberine through different mechanisms [Tang et al., 2009]. Berberine may suppress cancer cell growth and proliferation by regulating the cell cycle [Serafim et al., 2008], inhibiting ATP generation [Pereira et al., 2007], or inducing cancer cell apoptosis [Pandey et al., 2008]. Berberine has also been reported to suppress cancer invasion and metastasis by regulating HIF-1α activity in SC-M1 cells [Lin et al., 2004]. Recently, we found that the anti-invasion activity of berberine may act by inhibiting the RhoA-signaling pathway at low dosages, but berberine induces apoptosis through G2 arrest at high doses resulting from dose-dependent berberine distribution in the cell nucleus and cytoplasm [Tsang et al., 2009]. These points illustrate the potential application of berberine for cancer therapy. A previous study reported a synergistic induction of autophagic cell death by combined treatment with radiation and berberine in lung cancer [Peng et al., 2008], but no reports have been published on the induction of autophagy by berberine treatment alone. Moreover, the contribution of berberine's anti-cancer effects and the underlying signaling mechanism for the different types of cell death remain unknown.

This study reports for the first time that berberine induces both autophagic cell death and mitochondrial apoptosis in the human hepatic carcinoma cell lines HepG2 and MHCC97-L. Berberine shows potent cytotoxicity to various cancer cell lines including HepG2, MHCC97-L, HONE1, HeLa, and HK1. Berberine can induce mitochondrial apoptosis in HepG2 and MHCC97-L cells, indicated by a decrease in mitochondrial membrane potential and an increase in Bax expression as well as activation of caspases 3 and 9. Berberine can also induce autophagy by activating Beclin-1 and inhibiting the mTOR-signaling pathway in the same cancer cell lines. Berberine-induced inhibition of mTOR activity may result from both the activation of P38 MAPK and the suppression of Akt signaling. Inhibition of autophagy by 3-methyladenine (3-MA) treatment or by silencing the autophagy protein 5 (Atg5) gene attenuates cell death induced by berberine, indicating that berberine-induced autophagy contributes to its anti-cancer effect. By integrating the underlying mechanism of cell death, our findings suggest that berberine, which initiates both apoptotic and autophagic cell death (Type I and Type II cell death), has potential for anti-cancer therapy.

MATERIALS AND METHODS

CHEMICALS, PLASMIDS, AND ANTIBODIES

Berberine hydrochloride, the autophagy-apoptosis inhibitor 3-MA and the autophagosome–lysosome fusion inhibitor bafilomycin A1 were purchased from Sigma–Aldrich (USA). The pcDNA3 plasmid encoding human LC3 was a kind gift from Professor Yoshimori. Anti-Bcl-2, anti-Bax, anti-caspase-3, anti-caspase-9, anti-Beclin-1, anti-β-actin, anti-phosphorylated mTOR, anti-mTOR, anti-phosphorylated AKT, anti-AKT, and anti-COX-IV were purchased from Abcam (UK). Anti-LC3B was purchased from Novus Biologicals (USA). Anti-P38 MAPK, anti-phosphorylated P38 MAPK (Thr180/Tyr182), and anti-Atg5 were purchased from Cell Signaling Technology (USA).

CELL LINES AND CELL CULTURE

The human hepatocarcinoma cell line HepG2 and the human nasopharyngeal carcinoma cell lines HONE1 and HK1 were purchased from ATCC (USA). The highly metastatic human liver cancer cell line MHCC97-L was a gift from Professor Tang and was used in our previous study [Lee et al., 2005]. Cells were maintained in Dulbecco's Modified Eagle Medium with high glucose levels and incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

CELL VIABILITY ASSAY

The MTT assay was used to detect the cytotoxicity of berberine against different cancer cells. In brief, cells were seeded in a 96-well plate with DMEM medium (high glucose, supplemented with 10% FBS) at a density of 10,000 cells/well. A series of berberine concentrations were added followed by a 48-h incubation. All experiments were conducted in parallel with controls (0.1% DMSO). Fifteen microliters of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml; Sigma, USA) were added to each well at the end of the treatment period and incubated at 37°C for 4 h. Then, the medium was removed, and 200 μl DMSO were added to each well. The absorbance of formazan was measured at 595 nm by a Multiskan MS microplate reader (Labsystems, Finland).

MONODANSYLCADAVERINE AND ACRIDINE ORANGE STAINING

Cells were seeded on cover slips and exposed to different concentration of berberine (50, 100, or 200 μM in HepG2 cells and 100, 200, or 400 μM in MHCC97-L cells) for 6 h. For monodansylcadaverine (MDC) staining, cells were stained with 0.05 mM MDC in PBS at 37°C for 10 min [Munafó and Colombo, 2001]. After incubation, cells were washed with PBS four times, and the cover slips were placed on slides and visualized under a fluorescence microscope (Carl Zeiss, USA, 63×, CCD camera). For Acridine Orange (AO) staining, cells were stained by 1 μg/ml AO in PBS at 37°C for 15 min [Yang et al., 2008] and then washed. The cover slips were then placed on slides and visualized under a fluorescence microscope (Carl Ziess, USA, 63×, CCD camera).

QUANTIFICATION OF GFP-LC3 PUNCTA

HepG2 cells were transfected with GFP-LC3 in the pcDNA3 plasmid using Lipofectamine 2000 (Invitrogen, USA) in serum- and antibiotic-free medium for 6 h, followed by a 72-h incubation in growth medium. Afterward, cells were selected with 1 mg/ml G418 (Gibco, USA) to establish a cell line stably expressing the GFP-LC3 fusion protein. Selected cells were seeded onto 35 mm confocal dishes and treated with 50, 100 or 200 μM berberine for 6 h. The accumulation of GFP-LC3 was examined by fluorescence microscopy (Carl Ziess, 63×, CCD camera). Autophagy was quantified by counting the percentage of cells showing an accumulation of GFP-LC3 puncta, analyzing 100 cells per preparation in three independent experiments. Cells containing several intense punctate GFP-LC3 aggregates were classified as autophagic cells [Shen et al., 2010].

ANNEXIN V/PI DOUBLE STAINING

Treated HepG2 and MHCC97-L cells were collected using a micro-scraper (Corning, USA) and then centrifuged. To detect apoptosis in HepG2 and MHCC97-L cells after exposure to berberine, an Annexin V/PI double staining kit (Sigma–Aldrich, USA) was used to quantify cell numbers in different stages of cell death [Vermes et al., 1995]. Briefly, cells were resuspended in 1 ml binding buffer containing 100 mM HEPES/NaOH, 1.4 mM NaCl, and 25 mM $CaCl_2$ at pH 7.5. Then, 5 μl FITC-conjugated Annexin V (50 μg/ml) and 10 μl propidium iodide (100 μg/ml) were added, the solution was incubated in the dark for exactly 10 min at room temperature, and apoptosis was detected by flow cytometry (Epics XL; Beckman Coulter, USA).

DETECTION OF MITOCHONDRIAL MEMBRANE POTENTIAL (MMP)

The mitochondrial membrane potential probe Rhodamine-123 (Rh-123) was used to qualitatively and quantitatively determine the decrease of MMP in HepG2 and MHCC97-L cells treated with high doses of berberine. For qualitative analysis, HepG2 and MHCC97-L cells were seeded onto 30-mm confocal culture dishes at 40% confluence. Cells were treated or not treated with berberine (50, 100, or 200 μM in HepG2 cells and 100, 200, or 400 μM in MHCC97-L cells) for 12 h. After treatment, cells were washed with PBS and stained with 1 μM Rh-123 (Sigma, USA) for 25 min at room temperature in the dark. Then, cells were rinsed and visualized under a fluorescence microscope (Carl Ziess, 63 ×, CCD camera). For the quantitative analysis of MMP in berberine-treated cells, HepG2 or MHCC97-L cells were cultured in a 96-well black-bottomed plate and treated with a series of concentrations of berberine for 12 h. Cells were washed once with PBS and then incubated with Rh-123 (1 μM) in PBS for 25 min. The fluorescence intensity in each well was then measured using a fluorescence microplate spectrometer (LS55, PerkinElmer, USA) with excitation and emission wavelengths of 488 and 530 nm, respectively.

MEASUREMENT OF CYTOCHROME C RELEASE

Cells with or without berberine treatment were harvested using a micro-scraper, and cytoplasmic protein was extracted by NP-40 lysis buffer (Invitrogen, USA) supplemented with proteinase inhibitor (1% PMSF, 0.5% aprotinin and 0.5% leupeptin) on ice for 5 min followed by centrifugation at 14,000 rpm at 4°C for 10 min. The supernatant was collected, and the protein concentration was determined. The release of cytochrome C (Cyto C) was monitored by immunoblot, and COX-IV was used to monitor the purity of the mitochondrial fraction.

IMMUNOBLOTTING

Treated cells were harvested using a micro-scraper (Corning), lysed with RIPA buffer supplemented with proteinase inhibitor (1% PMSF, 0.5% aprotinin and 0.5% leupeptin) and phosphatase inhibitor (1 mM Na_3VO_4 and 1 mM NaF) on ice for 30 min and then centrifuged at 14,000 rpm at 4°C for 25 min. The supernatant was transferred to a new tube, and the protein concentration was determined using BSA as a standard. Equal amounts of protein were resolved by SDS–PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF; Biorad, USA). Then, the membrane was blocked with 5% BSA in buffer containing Tris (10 mmol/L, pH 7.4), NaCl (150 mmol/L), and Tween 20 (1%) overnight at 4°C. The membrane was then incubated with primary antibodies at 4°C overnight followed by incubation with the appropriate secondary antibody (Abcam, UK) at room temperature for 2 h. Immunoreactivity was detected using an advanced ECL kit (GE Healthcare, UK) and visualized using a chemiluminescence imaging system (Biorad).

RNA INTERFERENCE

HepG2 cells stably transfected with GFP-LC3 were cultured in 35 mm confocal dishes overnight. Then, the cells were transfected with siRNA against human ATG5 (Santa Cruz, 1.25 μg/ml) using Lipofectamine 2000 (Invitrogen, USA) in serum- and antibiotic-free medium for 6 h, then cultured in normal growth medium for 72 h. Transfected cells were treated with 100 μM berberine for 6 h and then visualized under a fluorescence microscope (Carl Ziess, 63 ×, CCD camera).

STATISTICAL ANALYSIS

Results were analyzed using Student's *t*-test and expressed as the mean ± SD, differences between group means were considered to be statistically significant if values of $P < 0.05$.

RESULTS

BERBERINE INDUCES LIVER CANCER CELL DEATH IN VITRO

MTT assay results demonstrate that berberine treatment results in a significant induction of the death of carcinoma cell lines including HepG2 and MHCC97-L HCC cells, HeLa human cervical epithelioid carcinoma cells, and HONE1 and HK1 nasopharyngeal carcinoma cells. The IC50 of berberine varies between cancer cell lines. The MTT results indicate that MHCC97-L cells exhibit the greatest resistance to berberine. The IC50 of berberine in HepG2 cells was approximately 100 μM after 48 h treatment, whereas MHCC97-L cells had an IC50 of 250 μM (Fig. 1A). The addition of 3-MA reduces berberine's effect on HCC cell viability (Fig. 1B). Consistent with this, silencing Atg5 attenuates berberine-induced hepatoma cell death (Fig. 1C,D), indicating that autophagy may function as one of berberine's anti-cancer mechanisms.

BERBERINE INDUCES AUTOPHAGY AND APOPTOSIS IN LIVER CANCER CELLS

Autophagy is a cellular process that functions in cell survival under certain circumstances or as a type of programmed cell death (Type II PCD) [Hsieh et al., 2009]. In cells undergoing autophagy, cytoplasmic proteins are sequestered into lytic compartments characterized by the formation of autophagosomes and acidification to form the autophagic vacuole/autolysosomes [Gao et al., 2008]. MDC has been widely used as a marker for autophagic vacuoles because of its ability to show the accumulation of autophagic vacuoles in acidic compartments enriched in lipids [Bampton et al., 2005]. In our study, berberine treatment resulted in a dose-dependent increase in staining intensity in the cytoplasm of HepG2 and MHCC97-L cells (Fig. 2A). Similar results were observed when cells treated with berberine were stained with AO, a specific

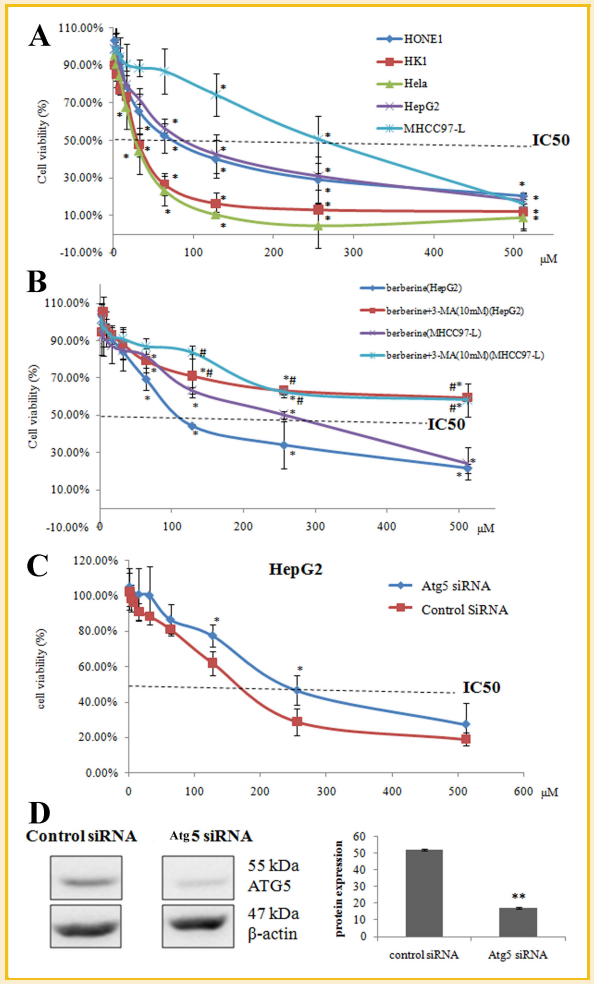

Fig. 1. Cytotoxicity of berberine against different cancer cell lines. A: Cytotoxicity of berberine against HepG2, MHCC97-L, HONE1, HK1, and HeLa cells after 48 h treatment. B: Viability of HepG2 and MHCC97-L cells after treatment with berberine plus or minus 3-MA (10 mM). Berberine induced a dose-dependent decrease in residual cell viability, whereas treatment with berberine plus 10 mM 3-MA significantly increased residual cell viability. C: The cytotoxicity of berberine can be attenuated by introducing siRNA against ATG5 into HepG2 cells. D: Expression of Atg5 in cells transfected with control or Atg5 siRNA by Western blotting (left) and their relative expression levels by quantitative analysis (right) are shown. All experiments were conducted in triplicate and the results were analyzed for statistical significance ($^{*}P < 0.01$ when compared with corresponding control; $\#P < 0.01$ when compared with corresponding cells treated with berberine only).

fluorescent probe frequently used to stain the lysosome, and compared with untreated cells [Moriyama et al., 1982]. Figure 2B shows the accumulation of lysosomes in cells treated with berberine. Moreover, addition of 3-MA suppresses the formation of autophagosomes and autolysosomes (Fig. 2A,B). Increased LC-II expression was detected in cells treated with berberine. These results indicate the induction of autophagy in liver cancer cells exposed to berberine. Moreover, berberine-induced autophagy was further confirmed by the accumulation of expressed GFP-LC3 in HepG2 cells (Fig. 2D). Quantification of GFP-LC3 puncta shows that berberine treatment induces dose-dependent autophagosome puncta, and silencing Atg5 expression blocks these berberine-induced effects (Fig. 2D, right). The combination of berberine with the autophagosome–lysosome fusion inhibitor bafilomycin A1 results in a significant decrease in LC3-I formation (Fig. 2E), indicating that berberine is able to transform the cytoplasmic form of LC3 into the autophagosomal form. These results demonstrate berberine's ability to induce autophagy in HCC cells.

Annexin V/PI double staining was used to assess the other type of programmed cell death, apoptosis, in HepG2, and MHCC97-L cells treated with berberine. Our results show that berberine can significantly induce early and median apoptosis after 12 h treatment (Fig. 3A). The induction of apoptosis by berberine was enhanced in a dose-dependent manner (Fig. 3B). These results suggest that berberine can induce apoptosis but not necrosis in HCC cells.

LOSS OF MITOCHONDRIAL MEMBRANE POTENTIAL ACTIVATES THE MITOCHONDRIAL CELL APOPTOSIS SIGNALING PATHWAY IN CELLS TREATED WITH BERBERINE

Berberine treatment led to a dose-dependent decrease in the fluorescent signal in both HepG2 and MHCC97-L cells, indicating a berberine-induced loss of transmembrane potential (TP; Fig. 4A,B). Immunoblot analysis indicates the overexpression of Bax and cleaved caspases 3 and 9 following berberine treatment (Fig. 4C), indicating that berberine could induce liver cancer cell apoptosis via a mitochondrial cell death pathway in a dose-dependent manner (Fig. 4D).

INHIBITION OF BCL-2 EXPRESSION AND ACTIVATION OF BECLIN-1 IN MITOCHONDRIAL APOPTOSIS AND AUTOPHAGY

Bcl-2 is a member of the Bcl-2 family, the structures of which contain at least one Bcl-2 homology (BH) region. The Bcl-2 protein serves as an anti-apoptotic factor by stabilizing the mitochondrial membrane and preventing pro-apoptotic factors from being released into the cytosol [Levine et al., 2008]. Interestingly, extensive studies have found that Bcl-2 may also serve as an autophagy inhibitor that binds to beclin-1 and thereby blocks its function in autophagy in various kinds of cancer cells [Pattingre and Levine, 2006]. Beclin-1 was initially discovered as a Bcl-2-interacting protein and a positive regulator of autophagy induction in eukaryotic cells [Sun et al., 2009]. Beclin-1 was also identified as a tumor suppressor in various types of cancer [Yue et al., 2003; Liang et al., 1999; Qu et al., 2003], indicating its important role in autophagic cell death during cancer therapy. In this study, the expression of Bcl-2 was inhibited by berberine in HepG2 and MHCC97-L cells, and an increase in Beclin-1 expression was observed in cells exposed to different concentrations of berberine (Fig. 5). Moreover, the overexpression of Bax, a pro-apoptotic factor important for PT pore formation, has an apparent effect on mitochondrial apoptosis in HepG2 and MHCC97-L cells treated with berberine. These results suggested that berberine could serve to activate Beclin-1 and Bax by suppressing Bcl-2 expression. The activation of Beclin-1 and Bax could then induce both apoptotic and autophagic cell death in HCC cells.

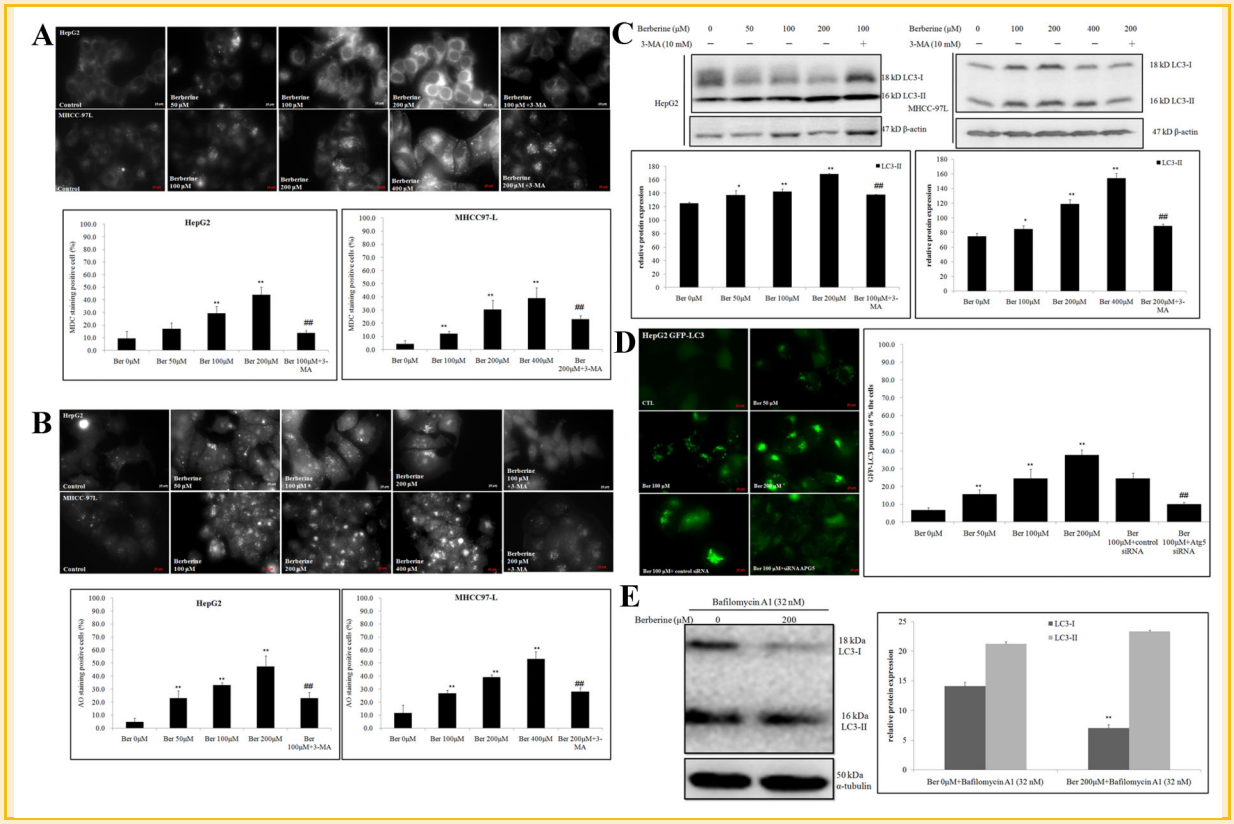

Fig. 2. Berberine induces autophagy in hepatocellular carcinoma cells. A: Autophagosome formation after 6 h treatment of berberine or berberine plus 3-MA using MDC staining. Microscopic photos were taken at 63× magnification (upper panel) and the percentage of cells with positive staining was calculated (lower panel). B: Lysosome accumulation after 6 h treatment with berberine or berberine plus 3-MA visualized using AO staining. Microscopic photos were taken at 63× magnification (upper panel) and the percentage of cells with positive staining was calculated (lower panel). C: Immunoblot analysis of LC3-I (18 kDa) and LC3-II (16 kDa). Fifteen micrograms of cell lysate from HepG2 (left) or MHCC97-L (right) cells were subjected to Western blot analysis using an LC3B antibody (upper panel) and the relative expression of LC3-II protein was calculated (lower panel). D: Berberine can induce GFP-LC3 accumulation in nucleofected HepG2 cells (63× magnification; left) and increase the percentage of cells with autophagy puncta (right). Relative expression of Atg5 in cells transfected with control or Atg5 siRNA was shown in Figure 1D. E: The expression of LC3-I and LC-II in HepG2 cells exposed to berberine (200 μM) or vehicle in the presence of bafilomycin A1 (32 nM; $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with corresponding control; $\#P < 0.05$, $\#\#P < 0.01$ when compared with corresponding berberine-treated only cells).

ACTIVATION OF P38 MAPK AND INHIBITION OF AKT SIGNALING CONTRIBUTE TO BERBERINE-INDUCED MTOR INHIBITION AND INDUCE AUTOPHAGY IN HEPATOCELLULAR CARCINOMA CELLS

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase involved in the regulation of many cellular processes including cell proliferation, migration, and protein synthesis [Hay and Sonenberg, 2004; Beevers et al., 2006]. mTOR signaling has been reported to negatively regulate autophagy, and inhibition of mTOR activity has been reported by some to induce autophagy [Wang et al., 2009; Tang et al., 2008]. In this study, a significant dose-dependent inhibition of mTOR activity by berberine treatment was observed in both HepG2 and MHCC97-L cells, indicating that mTOR suppression contributes to the induction of autophagy by berberine (Fig. 5A,B). Moreoever, multiple signaling pathways have been found to regulate mTOR activity, including P38 MAPK, MEK/Erk, and Akt [Tang et al., 2008; Bommareddy et al., 2009; Wang et al., 2009]. In this study, Akt inhibiton was observed to depend on the dose of berberine (Fig. 5A,B), revealing that Akt functions as a regulator of mTOR and autophagy.

DISCUSSION AND CONCLUSIONS

With a long history of clinical use in both Chinese and Ayurvedic medicine, berberine has conventionally been used for the treatment of infectious diseases including bacterial diarrhoea, intestinal parasite infections, and ocular trachoma infections because of its strong anti-microbial activity against bacteria, viruses, fungi, protozoans, helminths, and chlamydia [Birdsall and Kelly, 1997]. Although many studies have also reported berberine's action on cancer in vitro and in vivo, including anti-proliferation, G2/M cell cycle arrest, and apoptosis in HepG2 cells, the underlying mechanism of berberine-induced cancer cell death is not fully understood [Tang et al., 2009]. The non-steroidal anti-inflammatory drug (NSAID)-activated gene (NAG-1) was reported to be the molecular target of berberine in HepG2 cells [Auyeung and Ko, 2009]. In this study, we tried to produce a more comprehensive illustration of berberine-induced cell death including apoptosis and autophagy in cancer cells using HCC cell models. Berberine induces Bax activation in HepG2 cells and MHCC97-L cells followed by the

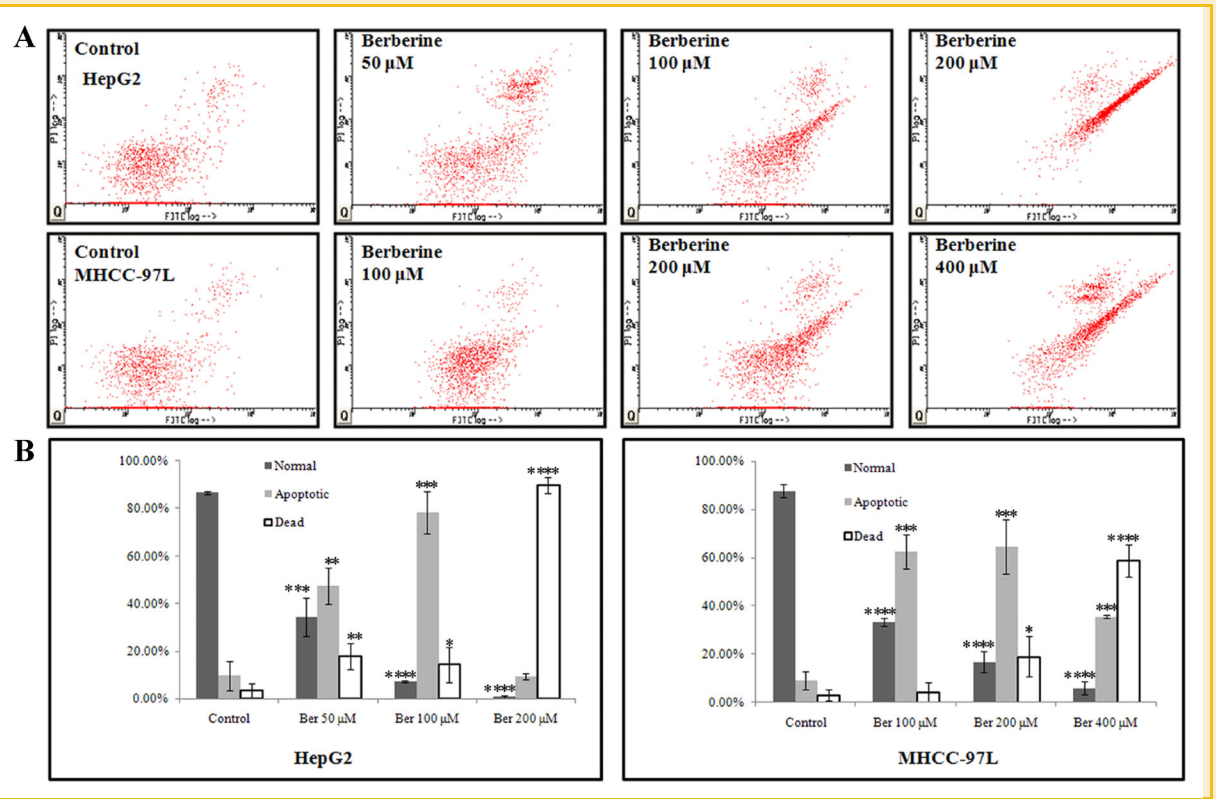

Fig. 3. Berberine induces apoptosis in hepatocellular carcinoma cells. A: HepG2 and MHCC97-L cells were stained with Annexin V (0.5 μg/ml) and PI (2 μg/ml) after 12 h treatment with berberine for FACS analysis. B: Quantitative data show that berberine induces apoptosis in HepG2 (left) and MHCC97-L (right) cells in a dose-dependent manner. All experiments were conducted in triplicate, and the results were analyzed for statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ when compared with corresponding controls).

formation of a PT pore and a reduction of mitochondrial membrane potential, and allows Cyto C to be released from the mitochondria to the cytosol. The subsequent activation of caspase-signaling pathways results in apoptosis. Additionally, berberine inhibits the expression of anti-apoptotic factor Bcl-2 but enhances Beclin-1 expression, releasing Beclin-1 from the Bcl-2/Beclin-1 complex and eventually inducing autophagy. These results demonstrate that berberine induces both apoptotic and autophagic cell death in liver cancer cells. The role of autophagy in cancer cell death is controversial and needs classification in some cases. Some studies report that autophagy is involved in chemotherapeutic agent-induced cancer cell death as a pro-survival approach for the cancer cell [Sy et al., 2008] because autophagy is considered a self-defense mechanism when cancer cells undergo nutrient starvation, hypoxia, and ionizing radiation [Ito et al., 2005]. In other cases, however, autophagy may be self-destructive and enhance cancer cell death [Kanzawa et al., 2003]. In this study, 3-MA potently attenuated the cytotoxicity of berberine to HepG2 and MHCC97-L liver cancer cells (Fig. 1B), indicating that both apoptosis and autophagy contributed to cell death in this case. Consistent palliative effects on HepG2 cell death resulted from blocking autophagosome formation with Atg5 siRNA (Fig. 1C,D). Because 3-MA is considered an inhibitor of apoptosis and autophagy, the contribution of berberine-induced autophagic cell death versus apoptosis was calculated by comparing the rate of inhibition of 3-MA and Atg5 siRNA on cell death. Up to 30% of cell death was found to result from autophagy induced by berberine in HepG2 cells at 250 μM (Fig. 1C), and apoptosis made the greatest contribution to berberine-induced cell death at 250 μM (Fig. 1B).

Note that this study used berberine at a relatively high dose. In some cases, the IC50 of berberine was lower than 4 μg/ml, which is below the safe limit established by the National Cancer Institute [Letasiová et al., 2006]. The high IC50 of berberine in this study needs further exploration in the future. However, berberine is a natural product that has been widely used for many years. As an anti-microbial agent, it has been used to cure microbe-related gastric diseases. The dose varies and can be very high, up to 1 g per day [China Pharmacopeia, 2005 edition]. Our other studies have shown that berberine causes very little cytotoxicity to rat hepatocytes (data not shown) and has a liver-protective effect in an animal model of liver damage [Ye et al., 2009]. These data suggest that berberine has a low toxicity and indicate that berberine can be used at high dosages. Memorial Sloan-Kettering Cancer Center has conducted a phase I dose escalation study of *Huanglian* (a Chinese medicine in which the main active compound is berberine) in patients with advanced solid tumors (http://www.cancer.gov/clinicaltrials/MSKCC-00061) based on the potential exhibited by berberine and *Huanglian* in vitro results and Chinese cancer

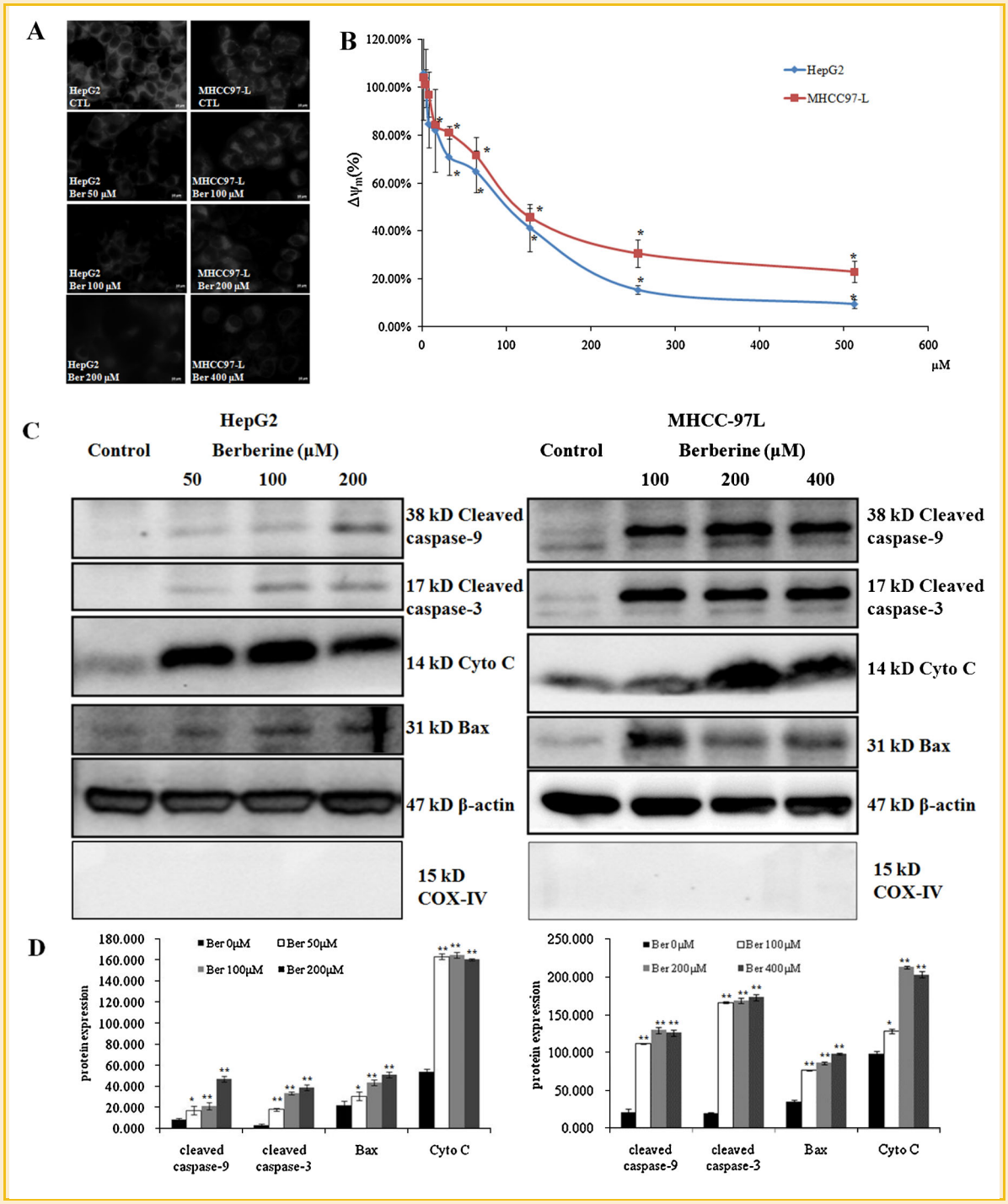

Fig. 4. Berberine induces mitochondrial apoptosis in hepatocellular carcinoma cells. A: Mitochondrial membrane potential after 12 h treatment with berberine or vehicle in HepG2 and MHCC97-L cells. Microscopic photos were taken at 63× magnification. B: Quantitative analysis of the decrease in fluorescent Rh-123 signal with increasing berberine concentration in HepG2 and MHCC97-L cells. C: Mitochondrial apoptosis pathway-related protein expression in HepG2 and MHCC97-L cells with treatment by berberine or vehicle by Western blot analysis. D: Quantitative analysis of relative protein expression in C. All experiments were conducted in triplicate ($^{*}P < 0.05$, $^{**}P < 0.01$ when compared with the appropriate control).

treatment practices [Tang et al., 2009]. The current study reveals new molecular targets of berberine, characterizes the effective berberine dosage, and studies its effectiveness on new cell lines, providing useful information for further study.

Several signaling pathways are involved in the regulation of autophagy in cancer cells, including the Beclin-1, PI3K/Akt/mTOR, death-associated protein kinase (DAPK), and Bcl-2 pathways [Kondo et al., 2005]. Exogenous expression of Beclin-1 in MCF-7 cells does

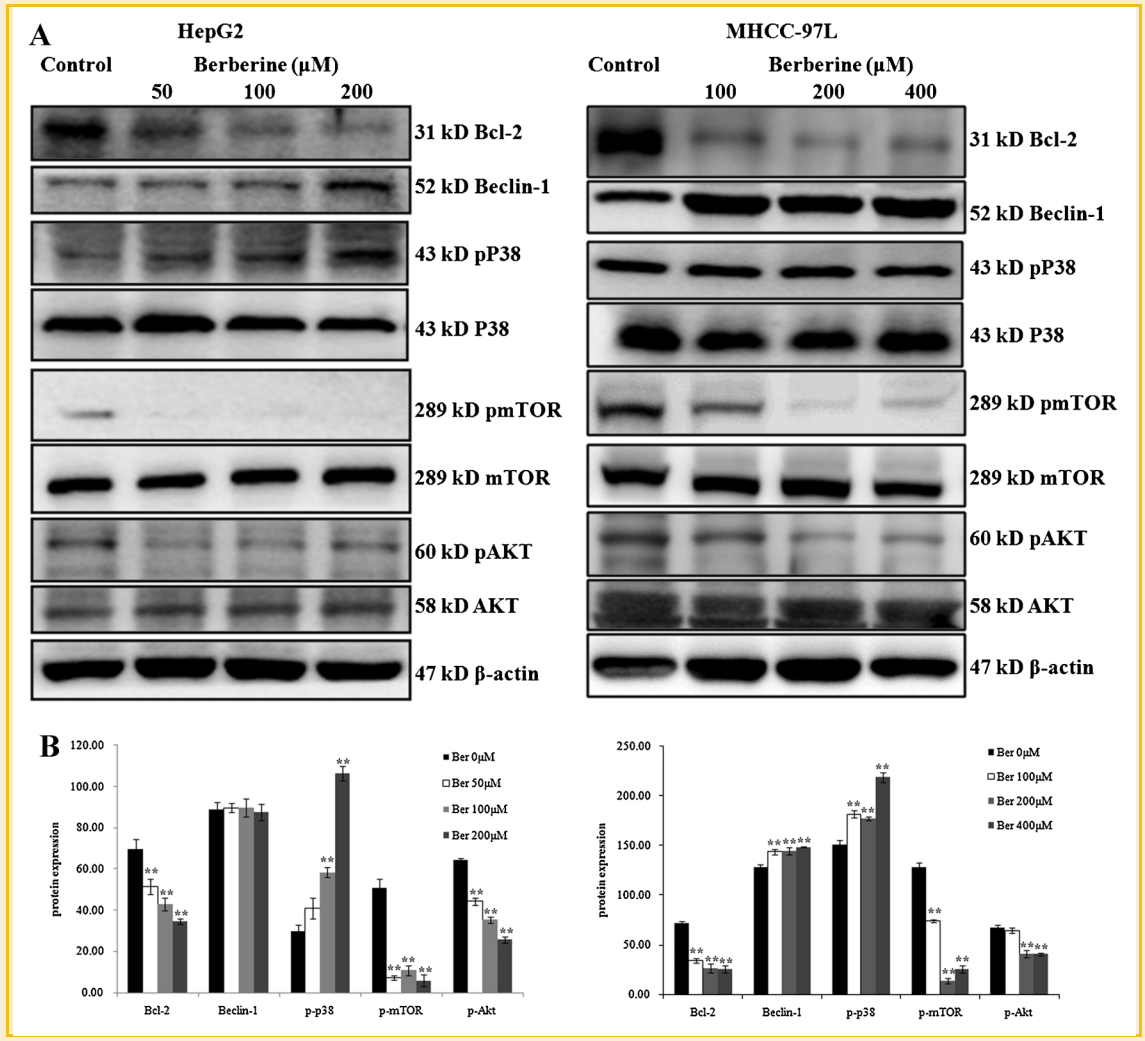

Fig. 5. Autophagy-related signal transduction in hepatocellular carcinoma cells with or without berberine treatment. A: HepG2 and MHCC97-L cells were treated with the indicated doses of berberine for 6 h, and 15 μg of cell lysates from HepG2 cells (left) and MHCC97-L cells (right) were subjected to Western blot analysis using related antibodies. B: Quantitative analysis of relative protein expression in A. All experiments were conducted in triplicate ($^{*}P < 0.05$, $^{**}P < 0.01$ when compared with corresponding controls).

not endogenously generate Belcin-1-initiated autophagy, and results in cell death [Liang et al., 1999]. Additionally, some studies report that Bcl-2 can negatively regulate the ability of Beclin-1 to induce autophagy by binding to its BH3 domain [Ciechomska et al., 2009]. The current study suggests that berberine can activate autophagy by releasing Beclin-1 from its interaction with Bcl-2 via inhibition of Bcl-2 expression (Fig. 5A,B). Another important mechanisms of autophagy is the mTOR-signaling pathway, which is constitutively activated in different kinds of cancers [Vivanco and Sawyers, 2002]. TOR kinase was demonstrated to be an upstream negative regulator for all autophagy-associated genes in yeast [Wang and Klionsky, 2003]. Using rapamycin to inhibit this signaling pathway can induce autophagy and suppress the proliferation of cancer cells [Takeuchi et al., 2005], indicating that an inhibitor of this pathway may function in cancer therapy. Because this study observed mTOR inhibition, we examined some up-stream activators of mTOR, including Erk1/2, P38 MAPK, and Akt. Our results indicate that berberine does not affect Erk1/2 signaling (data not shown), but it activates P38 MAPK and inhibits Akt activity (Fig. 5A,B). These results suggest that berberine-induced inhibition of mTOR activity results from a combination of p38 MAPK activation and Akt inhibition. Figure 6 presents a possible mechanism of berberine-induced autophagy in cancer cells.

The relationship between the loss of mitochondrial membrane potential and cell apoptosis has been extensively studied in the past. Some studies suggest that the collapse of the mitochondrial inner membrane potential initiates the opening of a large conductance channel, forming a mitochondrial permeability transition pore (PT pore) and allowing the release of pro-apoptotic signals like Cyto C and caspase-activating proteins into the cytosol [Green and Reed, 1998]. The involvement of Bax has been observed during PT pore formation [Crompton, 2000]. However, recent reports showed that the release of Cyto C and other pro-apoptotic molecules are Bax-dependent but PT pore formation-independent. The release of Cyto C

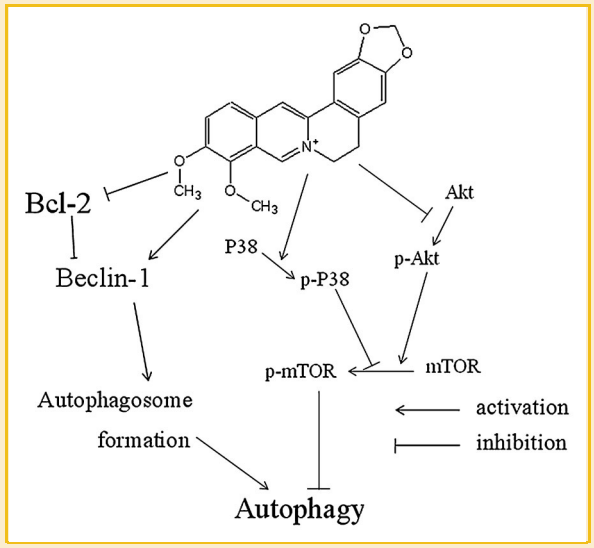

Fig. 6. Proposed mechanism for berberine-induced autophagy in liver cancer cells. The schematic signaling patterns indicate that Bcl-2/Beclin-1 complexes and the PI3K/Akt/mTOR signaling pathway are modulated by berberine-mediated autophagy induction in liver cancer cells. Our study reveals that berberine may release Beclin-1 from the Bcl-2/Beclin-1 complex by inhibiting the expression of Bcl-2. The inhibition of mTOR by berberine leads to the inhibition of Akt, and the activation of P38 MAPK initiates autophagy. Berberine connects the two signaling pathways to enhance authophagy in hepatoma cells.

is actually initiated by the rupture of the outer mitochondrial membranes (OMM) by Bax activation [Knudson and Brown, 2008], and then the apoptosis process begins. Thus, the release of Cyto C and the activation of Bax can be considered indicators of mitochondrial apoptosis. However, the regulation and function of PT pore formation in mitochondria remain subjects of intense debate. Nevertheless, it is clear that PT pore formation is characterized by the loss of MMP and that it plays a key role in determining whether the cell lives or dies [Zorov et al., 2009]. Bax has also been reported to be involved in the formation of the PT pore [Brenner et al., 2000], but others have reported Bax-independent induction of PT pore formation [Mullauer et al., 2009]. We observed Bax activation and Cyto C release, indicating possible mitochondrial apoptosis in berberine-treated hepatic cancer cells. Treatment with berberine also resulted in a sudden loss of MMP along with significant cell death in both HepG2 and MHCC97-L cells. The exact function and mechanism of this abrupt change in the mitochondria induced by berberine require further investigation.

This study systematically examined berberine's role in apoptotic and autophagic cell death, although apoptosis induced by berberine and its underlying mechanism have been previously studied. We observed that apoptotic and autophagic cell death were simultaneously induced by berberine in HCC cells, as indicated by changes in biochemical markers in molecular targeting and cell signal pathways. Based on our results, we found that total cell death is 20–80% when treated with 100–500 μM berberine (Fig. 1B, 3-MA inhibits both apoptotic and autophagic cell death), whereas autophagic cell death may only contribute 10–25% (Fig. 1C,D, Atg5 siRNA only inhibits autophagic cell death). These results demonstrate that berberine has the ability to induce both apoptotic and autophagic cell death, and that apoptosis is the major type of cell death observed. However, the cell death inhibitor (3-MA) could not fully rescue berberine-induced cell death. It remains unclear whether the induction of autophagic cell death by low doses of berberine will be able to make a significant contribution to the treatment of cancer, but in the following specific cases, the combination of berberine with radiotherapy or chemotherapy work additively to produce cancer cell autophagic and apoptotic cell death, and berberine's induction of cell death may increase. Combination of radiation with 5 and 10 μM berberine significantly increases the radio-sensitivity of A549 cells, in which both autophagy and cell-cycle G2/M arrest occur [Peng et al., 2008]. Combined treatment with As_2O_3 and berberine also has an anti-cancer effect in human neuroblastoma SH-SY5Y cells that function through a mitochondria/caspase-3 dependent-signaling pathway [Kim et al., 2007]. Here, we have shown that berberine induces apoptotic and autophagic cell death in HCC cells, but further investigation is required to fully characterize the concomitant mediators and determinant switches of cell death induced by berberine alone or in combination with other therapies in cancer cell models and animal models.

In conclusion, this study has presented a novel combined mechanism of berberine-induced cancer cell death, with concurrent apoptotic and autophagic cell death in which apoptosis was the major mode of cell death. Berberine activates mitochondrial apoptosis in HCC cells by increasing Bax expression, PT pore formation, Cyto C release to the cytosol, and subsequent activation of caspases 3 and 9-signaling pathways. Berberine may also induce autophagic cell death by activating Beclin-1 and inhibiting the mTOR-signaling pathway. We examined multiple avenues of berberine-induced cell death in HCC cells, and we identified the mechanism of berberine-induced autophagic cell death for the first time. These results shed light on berberine's anti-cancer effects and support its potential as a novel anti-cancer therapeutic agent.

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REFERENCES

Auyeung KK, Ko JK. 2009. Coptis chinensis inhibits hepatocellular carcinoma cell growth through nonsteroidal anti-inflammatory drug-activated gene activation. Int J Mol Med 24:571–577.

Bampton ET, Goemans CG, Niranjan D, Mizushima N, Tolkovsky AM. 2005. The dynamics of autophagy visualized in live cells: From autophagosome formation to fusion with endo/lysosomes. Autophagy 1:23–36.

Beevers CS, Li F, Liu L, Huang S. 2006. Curcumin inhibits the mammalian target of rapamycin-mediated signaling pathways in cancer cells. Int J Cancer 119:757–764.

Birdsall TC, Kelly GS. 1997. Berberine: Therapeutic potential of an alkaloid found in several medicinal plants. Altern Med Rev 2:94–103.

Bommareddy A, Hahm ER, Xiao D, Powolny AA, Fisher AL, Jiang Y, Singh SV. 2009. Atg5 regulates phenethyl isothiocyanate-induced autophagic and apoptotic cell death in human prostate cancer cells. Cancer Res 69:3704–3712.

Brenner C, Cadiou H, Vieira HL, Zamzami N, Marzo I, Xie Z, Leber B, Andrews D, Duclohier H, Reed JC, Kroemer G. 2000. Bcl-2 and Bax regulate the channel activity of the mitochondrial adenine nucleotide translocator. Oncogene 19:329–336.

Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, Zanetti KA, Ye QH, Qin LX, Croce CM, Tang ZY, Wang XW. 2008. Identification of metastasis-related MicroRNAs in hepatocellular carcinoma. Hepatology 47:897–907.

Cheng Y, Qiu F, Ikejima T. 2009. Molecular mechanisms of oridonin-induced apoptosis and autophagy in murine fibrosarcoma L929 cells. Autophagy 5:430–431.

Ciechomska IA, Goemans GC, Skepper JN, Tolkovsky AM. 2009. Bcl-2 complexed with Beclin-1 maintains full anti-apoptotic function. Oncogene 28:2128–2141.

Crompton M. 2000. Mitochondrial intermembrane junctional complexes and their role in cell death. J Physiol 529:11–21.

Galluzz L, Vicencio JM, Kepp O, Tasdemir E, Maiuri MC, Kroemer G. 2008. To die or not to die, that is the autophagic question. Curr Mol Med 8:78–91.

Gao M, Yeh PY, Lu YS, Hsu CH, Chen KF, Lee WC, Feng WC, Chen CS, Kuo ML, Cheng AL. 2008. OSU-03012, a novel celecoxib derivative, induces reactive oxygen species-related autophagy in hepatocellular carcinoma. Cancer Res 68:9348–9357.

Green DR, Reed JC. 1998. Mitochondria and apoptosis. Science 281:1309–1312.

Hay N, Sonenberg N. 2004. Upstream and downstream of mTOR. Genes Dev 18:1926–1945.

Hsieh YC, Athar M, Chaudry IH. 2009. When apoptosis meets autophagy: Deciding cell fate after trauma and sepsis. Trends Mol Med 15:129–138.

Ito H, Daido S, Kanzawa T, Kondo S, Kondo Y. 2005. Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. Int J Oncol 26:1401–1410.

Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I. 2003. Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res 63:2103–2108.

Kim DW, Ahan SH, Kim TY. 2007. Enhancement of arsenic trioxide (As(2)O(3))-mediated apoptosis using berberine in human neuroblastoma SH-SY5Y cells. J Korean Neurosurg Soc 42:392–399.

Kim RH, Coates JM, Bowles TL, McNerney GP, Sutcliffe J, Jung JU, Gandour-Edwards R, Chuang FY, Bold RJ, Kung HJ. 2009. Arginine deiminase as a novel therapy for prostate cancer induces autophagy and caspase-independent apoptosis. Cancer Res 69:700–708.

Knudson CM, Brown NM. 2008. Mitochondria potential, bax ''activation,'' and programmed cell death. Methods Mol Biol 414:95–108.

Kondo Y, Kanzawa T, Sawaya R, Kondo S. 2005. The role of autophagy in cancer development and response to therapy. Nat Rev Cancer 5:726–734.

Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nuñez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G Nomenclature Committee on Cell Death 2009. 2009. Classification of cell death: Recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 16:3–11.

Lee TK, Man K, Ho JW, Wang XH, Poon RT, Xu Y, Ng KT, Chu AC, Sun CK, Ng IO, Sun HC, Tang ZY, Xu R, Fan ST. 2005. FTY720: A promising agent for treatment of metastatic hepatocellular carcinoma. Clin Cancer Res 11:8458–8466.

Letasiová S, Jantová S, Cipák L, Múcková M. 2006. Berberine-antiproliferative activity in vitro and induction of apoptosis/necrosis of the U937 and B16 cells. Cancer Lett 239:254–262.

Levine B, Sinha S, Kroemer G. 2008. Bcl-2 family members: Dual regulators of apoptosis and autophagy. Autophagy 4:600–606.

Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B. 1999. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402:672–676.

Lin S, Tsai SC, Lee CC, Wang BW, Liou JY, Shyu KG. 2004. Berberine inhibits HIF-1alpha expression via enhanced proteolysis. Mol Pharmacol 66:612–619.

Moriyama Y, Takano T, Ohkuma S. 1982. Acridine orange as a fluorescent probe for lysosomal proton pump. J Biochem 92:1333–1336.

Mullauer FB, Kessler JH, Medema JP. 2009. Betulinic acid induces cytochrome c release and apoptosis in a Bax/Bak-independent, permeability transition pore dependent fashion. Apoptosis 14:191–202.

Munafó DB, Colombo MI. 2001. A novel assay to study autophagy: Regulation of autophagosome vacuole size by amino acid deprivation. J Cell Sci 114:3619–3629.

Pandey MK, Sung B, Kunnumakkara AB, Sethi G, Chaturvedi MM, Aggarwal BB. 2008. Berberine modifies cysteine 179 of IkappaBalpha kinase, suppresses nuclear factor-kappaB-regulated antiapoptotic gene products, and potentiates apoptosis. Cancer Res 68(13): 5370–5379.

Pattingre S, Levine B. 2006. Bcl-2 inhibition of autophagy: A new route to cancer? Cancer Res 66:2885–2888.

Peng PL, Kuo WH, Tseng HC, Chou FP. 2008. Synergistic tumor-killing effect of radiation and berberine combined treatment in lung cancer: The contribution of autophagic cell death. Int J Radiat Oncol Biol Phys 70:529–542.

Pereira GC, Branco AF, Matos JA, Pereira SL, Parke D, Perkins EL, Serafim TL, Sardão VA, Santos MS, Moreno AJ, Holy J, Oliveira PJ. 2007. Mitochondrially-targetted effects of berberine onK1735-M2mousemelanoma cells comparison with direct effects on isolated mitochondrial Fractions. J Pharmacol Exp Ther 323:636–649.

Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B. 2003. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 112:1809–1820.

Serafim TL, Oliveira PJ, Sardao VA, Perkins E, Parke D, Holy J. 2008. Different concentrations of berberine result in distinctcellular localization patterns and cell cycle effects in a melanoma cell line. Cancer Chemother Pharmacol 61:1007–1018.

Shen S, Kepp O, Martins I, Vitale I, Souquère S, Castedo M, Pierron G, Kroemer G. 2010. Defective autophagy associated with LC3 puncta in epothilone-resistant cancer cells. Cell Cycle 9:377–383.

Sun Q, Fan W, Zhong Q. 2009. Regulation of Beclin 1 in autophagy. Autophagy 5:713–716.

Sy LK, Yan SC, Lok CN, Man RY, Che CM. 2008. Timosaponin A-III induces autophagy preceding mitochondria-mediated apoptosis in HeLa cancer cells. Cancer Res 68:10229–10237.

Takeuchi H, Kondo Y, Fujiwara K, Kanzawa T, Aoki H, Mills GB, Kondo S. 2005. Synergistic augmentation of rapamycin-induced autophagy in malig-

nant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors. Cancer Res 65:3336–3346.

Tang G, Yue Z, Talloczy Z, Hagemann T, Cho W, Messing A, Sulzer DL, Goldman JE. 2008. Autophagy induced by Alexander disease-mutant GFAP accumulation is regulated by p38/MAPK and mTOR signaling pathways. Hum Mol Genet 17:1540–1555.

Tang J, Feng Y, Tsao S, Wang N, Curtain R, Wang Y. 2009. Berberine and *Coptidis rhizoma* as novel antineoplastic agents: A review of traditional use and biomedical investigations. J Ethnopharmacol 126:5–17.

Tsang CM, Lau EP, Di K, Cheung PY, Hau PM, Ching YP, Wong YC, Cheung AL, Wan TS, Tong Y, Tsao SW, Feng Y. 2009. Berberine inhibits Rho GTPases and cell migration at low doses but induces G2 arrest and apoptosis at high doses in human cancer cells. Int J Mol Med 24(1): 131–138.

Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 184:39–51.

Vivanco I, Sawyers CL. 2002. The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2:489–501.

Wang CW, Klionsky DJ. 2003. The molecular mechanism of autophagy. Mol Med 9:65–76.

Wang J, Whiteman MW, Lian H, Wang G, Singh A, Huang D, Denmark T. 2009. A non-canonical MEK/ERK signaling pathway regulates autophagy via regulating Beclin 1. J Biol Chem 284:21412–21424.

Yang C, Kaushal V, Shah SV, Kaushal GP. 2008. Autophagy is associated with apoptosis in cisplatin injury to renal tubular epithelial cells. Am J Physiol Renal Physiol 294:F777–F787.

Ye X, Feng Y, Tong Y, Ng KM, Tsao S, Lau GK, Sze C, Zhang Y, Tang J, Shen J, Kobayashi S. 2009. Hepatoprotective effects of *Coptidis rhizoma* aqueous extract on carbon tetrachloride-induced acute liver hepatotoxicity in rats. J Ethnopharmacol 124:130–136.

Yue Z, Jin S, Yang C, Levine AJ, Heintz N, 2003. Beclin 1, an autophagy gene essential for early embryonic development, is a haplo insufficient tumor suppressor. Proc Natl Acad Sci USA 100:15077–15082.

Zorov DB, Juhaszova M, Yaniv Y, Nuss HB, Wang S, Sollott SJ. 2009. Regulation and pharmacology of the mitochondrial permeability transition pore. Cardiovasc Res 83:213–225.