Andrographolide induces autophagic cell death in human liver cancer cells through cyclophilin D-mediated mitochondrial permeability transition pore

Wei Chen1,*, Lina Feng1, Hao Nie1 and Xiaodong Zheng2
1College of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou 310035, China, and 2Department of Food Science and Nutrition, Zhejiang University, Hangzhou 310058, China

Liver cancer is the third most leading cause of cancer death worldwide and about half of the patients with liver cancer require adjuvant therapy after surgical resection. Therefore, development of novel agents to eradicate cancer cells may constitute a viable approach to treat patients with liver cancer. Andrographolide, a diterpenoid lactone isolated from Andrographis paniculata, is known to possess potent antioxidant, anti-inflammatory, antineoplastic and antiviral properties. In this study, we investigated the cytotoxic effect of andrographolide on human liver cancer cells and explored the cell death mechanism. Andrographolide induced a cell death distinct from apoptosis in multiple human liver cancer cells. The death was characterized by autophagy as evidenced by the accumulation of LC3 II and autophagosomes, and the formation of puncta GFP-LC3. This autophagy as well as cytotoxicity caused by andrographolide could be effectively prevented by 3-methyladenine (a chemical inhibitor of autophagy). Mechanistic study indicated that andrographolide induced autophagic cell death by disruption of mitochondrial membrane potential and elevation of reactive oxygen species, which were correlated with mitochondrial permeability transition pore (MPTP). Inhibition of cyclophilin D (a component of MPTP) by cyclosporin A or abrogation of its expression by small interfering RNA significantly suppressed the cytotoxicity of andrographolide, suggesting that cyclophilin D may play an important role in mediating andrographolide-induced cytotoxicity. Taken together, our findings unveil a novel mechanism of drug action by andrographolide in liver cancer cells and suggest that andrographolide may represent a promising novel agent in the treatment of liver cancer.

Introduction
Liver cancer is the fifth most frequently diagnosed cancer and the third leading cause of cancer-related death in the world (1). This aggressive disease is a major global threat to public health, with an estimated 748,300 new cases and 695,900 cancer deaths occurred worldwide in 2008. Most of the cases and deaths occur in developing countries and half in China alone (2). Of patients presenting with earlier stages of the disease, >50% undergo surgical resection, but even after a curative resection, 80% of these patients are reported to develop new tumors in the residual liver within 2 years and eventually die due to this disease (3). In addition, conventional chemotherapy is not very effective for liver cancer (3). Therefore, development of novel agents to enhance the effectiveness of treatment is urgently needed.

Andrographis paniculata, a traditional medicinal herb, has been widely used in China, India and other Southeast Asian countries for the treatment of various diseases, including fever, cold, inflammation, diarrhea and other infectious diseases (4). Andrographolide, a diterpenoid lactone isolated from A. paniculata, has a broad range of pharmacological effects, such as antioxidant, anti-inflammatory, anti-HIV, immunomodulatory and hepatoprotective activities (5–8). Recent studies indicated that andrographolide possesses potent anticancer property in various types of cancer cells. Andrographolide could induce apoptosis through mitochondrial-mediated pathway in human leukemic HL-60 cells (9). In addition to apoptosis, andrographolide could inhibit human colorectal carcinoma Lovo cells migration and invasion via downregulation of MMP-7 expression (10). Andrographolide also downregulated hypoxia-inducible factor-1α in human non-small cell lung cancer A549 cells (11). Further, andrographolide has been reported to enhance chemosensitivity of cancer cells to doxorubicin through inhibition of the JAK-STAT3 pathway in human colorectal cancer HCT116 cells (12). Taken together, these findings suggest that andrographolide might be a multitargeted inhibitor that performs its functions in a cell type-dependent manner.

Autophagy, a physiologic process that occurs in all eukaryotic cells, is characterized by the sequestration of bulk cytosolic components including unfolded proteins and membranous organelles, and delivery of the enclosed components to lysosomes for degradation (13). This serves as a temporary survival mechanism and favor tumor development (14). However, recent studies have shown that autophagy also has an active role in cell death (15,16). Autophagy or autophagic cell death, known as type II programmed cell death, is a response to various anticancer therapies in many kinds of cancer cells (17). Recently, several groups reported that some natural compounds could stimulate excess autophagy in cancer cells, which ultimately led to cell death (18–20). Therefore, natural compounds from medicinal herbs that trigger autophagic cell death may represent potent anticancer agents for the treatment of liver cancer.

In this study, we investigated the effects of andrographolide on human liver cancer cells and further examined the cell death mechanism. Our observations demonstrated that andrographolide-induced cytotoxicity was attributed to autophagy but not apoptosis in human liver cancer cells and that this autophagy-inducing activity was closely associated with the cyclophilin D-mediated mitochondrial permeability transition pore (MPTP). These preclinical studies suggest that andrographolide could be useful for the treatment of liver cancer.

Materials and methods

Reagents
Andrographolide (with >98% purity) was obtained from National Institutes for Food and Drug Control, China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamin123 (Rh123), cyclosporin A (CsA), bongkrekic acid (BA), 3-methyladenine (3-MA), dichlorodihydrofluorescein diacetate (DCFH-DA) and N-acetyl-L-cysteine (NAC) were purchased from Sigma. Pan-caspase inhibitor, z-VAD-fmk (z-VAD), was from R&D.

Cell lines and cell culture
The human liver cancer cell lines Huh-7, QGY-7703 and Bel-7402 were obtained from CBTCCCAS (the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China). The above cell lines were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Gibco), 100 units/ml penicillin, and 100 units/ml streptomycin in a humidified incubator with an atmosphere of 5% CO2 at 37°C.

Cell viability assay
Cell viability was measured by the MTT method as described previously (21). Briefly, cells were seeded into 96-well microtiter plates at a density of 5 × 103 cells/well. After 24h of incubation, cells were treated with various concentrations of andrographolide. When incubated for the indicated times, cells were incubated with MTT (0.5 mg/ml) for 4h. The formazan precipitate was dissolved in 150 μl dimethyl sulfoxide, and the absorbance was detected at 570 nm with a model E19800 microplate reader (Bio-Tek Instruments). Each test was performed in triplicate experiments.

Abbreviations:
ANT, adenine nucleotide translocase; BA, bongkrekic acid; CsA, cyclosporin A; CyDp, cyclophilin D; DCFH-DA, dichlorodihydrofluorescein diacetate; DOX, doxorubicin; 3-MA, 3-methyladenine; MMP, mitochondrial membrane potential; MPTP, mitochondrial permeability transition pore; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-L-cysteine; Rh123, rhodamine 123; ROS, reactive oxygen species; siRNA, small interfering RNA.
**Andrographolide induces autophagic cell death**

**Apoptosis assays**

Apoptotic rates were analyzed by flow cytometry using an APO-BUGE™ kit (BD Biosciences) according to the manufacturer’s instructions. Briefly, cells were treated with andrographolide for 24 h, washed twice with ice-cold phosphate-buffered saline, and then fixed and permeabilized by 4% paraformaldehyde and 70% ethanol, followed by incubation with a mixture of fluorescein isothiocyanate–deoxyuridine triphosphate and TdT for 1 h at 37°C. Stained cells were analyzed with a FACSCalibur flow cytometer.

**Caspase-3 activity** was measured using a colorimetric assay kit (Biovision) according to the manufacturer’s instructions. Briefly, cell lysate from 1 x 10⁶ cells was incubated at 37°C for 2 h with 200 μM DEVD-pNA (caspase-3 substrate). Samples were read at 405 nm in a microplate reader (Bio-Tek Instruments) and expressed as fold increase on the basal level (dimethyl sulfoxide-treated cells).

**Western blot analysis**

Western blot was conducted as described previously with some modifications (21). Briefly, about 30 μg total protein was separated by electrophoresis on 10–15% standard sodium dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad). After blocking with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20 (Bio-Rad), the membrane was incubated with the primary antibody (1:1000 dilution) overnight at 4°C. After 3–4 washes, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000 dilutions) for 1 h. After sequential washes, the immunoreactive protein bands were visualized by the use of the Enhanced Chemiluminescence kit (GE Healthcare) according to the manufacturer’s instructions. The following antibodies were used: anti-LC3B, anti-ATG5, anti-p-AKT (Cell Signaling Technology), anti-cyclophilin D (Calbiochem). The secondary antibodies were horseradish peroxidase-conjugated anti-rabbit, anti-mouse and anti-goat IgG (Bio-Rad).

**GFP-LC3 transfection and fluorescence**

Huh-7 cells were transfected with GFP-LC3-expressing plasmid (Cell Biolabs) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Twenty-four hours after transfection, cells were treated with andrographolide for 24 h, the fluorescence of GFP-LC3 was viewed and the rate of GFP-LC3 vacuoles (autophagosomes) was counted under a fluorescent microscope.

**Transmission electron microscopy**

Cell pellets were fixed in 2% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium cacodylate for 2 h, postfixed with 1% OsO4 for 1 h, washed and finally stained for 1 h in 3% aqueous uranyl acetate. The samples were then washed again, dehydrated with graded alcohol and embedded in Epon-Araldit resin (Canemco). Ultrathin sections were cut on a Reichert ultramicrotome, counterstained with 0.3% lead citrate and examined on a FEI Tecnai 12 transmission electron microscope.

**Detection of mitochondrial membrane potential**

Mitochondrial membrane potential (MMP) was measured according to the previously reported method (21). Briefly, after treatment, cells were collected and incubated with 10 μg/ml of Rh123 for 30 min and analyzed by flow cytometry.

**Permeability transition pore activity in isolated mitochondria**

Mitochondria extracts were isolated from Huh-7 cells using a mitochondria isolation kit for cultured cells (Pierce) according to the manufacturer’s instructions. MPTP opening was assayed spectrophotometrically as described previously (22). Briefly, mitochondria (protein concentration of 0.5 mg/ml) were incubated at 25°C in 125 mM KCl, 20 mM HEPES (pH 7.4), 2 mM K₂HPO₄, 1 mM EGTA, 1 mM MgCl₂, 5 mM malate and 5 mM glutamate. Changes in absorbance at 540 nm, indicating mitochondrial swelling as a result of MPTP opening, were determined after addition of 16 μg/ml andrographolide in the presence or in the absence of 5 μM CsA (a MPTP inhibitor) using a microplate reader.

**Determination of cellular reactive oxygen species**

Cellular reactive oxygen species (ROS) contents were measured by incubating the control or drug-treated cells with 10 μM DCFH-DA at 37°C for 30 min. After incubation with the fluorochrome, cells were washed with phosphate-buffered saline and immediately analyzed by flow cytometry (21).

**Silencing ATG5 or cyclophilin D by siRNA**

Human ATG5 small interfering RNA (siRNA) (sense, 5-GCCUGU AUGUCAGCUUUA-3; antisense, 5-CAGGCTUCUCGUGGC-3), 10 μM siRNA, was purchased from Cell Signaling Technology. Human Cyclophilin D siRNA (sense, 5-UUUGACGGUACGACGAAAC-3; antisense, 5-CAGUUGUUGUUGCGUACGAAAA-3) was purchased from Dharmacon. Briefly, 2 x 10⁶/well cells were cultured in 6-well plates with 2 ml antibiotic-free growth medium at 37°C in a CO₂ incubator for 24 h.

siRNA duplex−lipofectamine was prepared by 6.0 μl of 10 μmol/l ATG5 siRNA, Cyclophilin D siRNA or control siRNA and 3.0 μl lipofectamine RNAiMAX (Invitrogen) in 500 μl siRNA Transfection Medium (Santa Cruz Biotechnology) according to the manufacturer’s instructions, and directly added into the media. Forty-eight hours after transfection, cells were collected. The knockdown of ATG5, or Cyclophilin D siRNA was measured by quantitative reverse transcription-PCR (qRT-PCR) and western blot.

**Statistical analysis**

Unless otherwise stated, data were expressed as mean ± SD. Student’s t-test was used to evaluate the statistical differences between the experimental values of two samples being compared. P< 0.05 was considered statistically significant.

**Results**

**Andrographolide inhibited growth of Huh-7 cells independent of apoptosis**

To identify the therapeutic potential of andrographolide, human liver cancer cell line Huh-7 was cultured with different concentrations of andrographolide for 24, 48 and 72 h, and then cell viability was determined by MTT assay. Andrographolide inhibited the growth of Huh-7 cells in a dose- and time-dependent manner (Figure 1A), with 50% inhibition (IC₅₀) at 24, 48 and 72 h of 17.6, 11.3 and 8.1 μg/ml, respectively.

Because andrographolide has been reported to induce apoptosis in some cancer cells (23–25), we investigated whether andrographolide could induce apoptosis in Huh-7 cells by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. As shown in Figure 1B, there was no significant increase of apoptotic cells detected after 24 h andrographolide treatment. This finding was further confirmed by caspase-3 activity analysis (Figure 1C). Similar results were also observed in other liver cancer QGY-7703 and Bel-7402 cells treated with andrographolide for 24 h (Figure 1D and 1E). Further studies revealed that there was no significant apoptosis observed after 48–72 h andrographolide treatment in Huh-7 (Supplementary Figure 1A and B, available at Carcinogenesis Online), QGY-7703 (Supplementary Figure 1C and D, available at Carcinogenesis Online) or Bel-7402 cells (Supplementary Figure 1C and D, available at Carcinogenesis Online).

Both TUNEL assay (Figure 1B and 1D; Supplementary Figure 1A and 1C, available at Carcinogenesis Online) and caspase-3 activity analysis (Figure 1C and 1E; Supplementary Figure 1B and D, available at Carcinogenesis Online) showed that doxorubicin did induce apoptosis in Huh-7, QGY-7703 and Bel-7402 cells, indicating that the pathway of apoptosis is intact in these cells. Taken together, our results indicated that andrographolide did not induce apoptosis in Huh-7, QGY-7703 or Bel-7402 cells.

**Andrographolide induced autophagy in Huh-7 cells**

Accumulating evidence indicates the importance of autophagy in tumor suppression (26,27). Autophagy or autophagic cell death, is a response to various anticancer therapies in many kinds of cancer cells (28,29). To determine whether autophagy is involved in andrographolide-induced growth inhibition of Huh-7 cells, western blotting was first performed to detect the conversion of cytosolic LC3-I to lipidated, autophagosome-membrane-bound LC3-II, which is a specific marker for autophagosome formation (30). As shown in Figure 2A, compared with untreated group, andrographolide induced significantly higher amount of LC3-II expression in Huh-7 cells after 24 h of treatment. Similar phenomena were also observed in QGY-7703 and Bel-7402 cells (Figure 2A). To confirm the characteristics of autophagy elicited by andrographolide, puncta GFP-LC3 and accumulation of autophagosomes in Huh-7 cells were investigated by fluorescence microscopy and transmission electron microscopy. Fluorescence microscopy analysis showed that a punctate pattern of LC3 was observed in andrographolide-treated Huh-7 cells, whereas a diffused distribution of LC3 was found in control cells (Figure 2B).

Moreover, a significant increase in the percentage of autophagic cells (cells with GFP-LC3 dots) was found in 8 and 16 μg/ml andrographolide-treated Huh-7 cells (Figure 2C). Similar results were
also found in QGY-7703 and Bel-7402 cells (Figure 2C). In addition, Huh-7 cells showed cytoplasmic accumulation of autophagosomes, a morphological correlate of autophagy, as determined by transmission electron microscopy, after andrographolide treatment (Figure 2D). Taken together, these results demonstrated that andrographolide induced autophagy in Huh-7 cells.

Autophagy inhibition attenuated andrographolide-induced cytotoxicity
To determine whether the cytotoxicity of andrographolide toward Huh-7 cells was caused by autophagy, we further used 3-methyladenine (3-MA, a chemical inhibitor of autophagy) to examine the effect of andrographolide on autophagy and cell viability in Huh-7 cells. As illustrated in Figure 3A, andrographolide-induced accumulation of GFP-LC3 vacuoles (cells with GFP-LC3 dots) was markedly reduced by pretreatment with 2 mM 3-MA for 2 h, whereas the caspase inhibitor z-VAD-fmk (z-VAD) failed to change GFP-LC3 vacuoles formation in Huh-7 cells (Figure 3A). To investigate whether inhibition of autophagy affected the cytotoxicity of andrographolide, Huh-7 cells were treated with 16 μg/ml andrographolide for 24 h in the presence of 2 mM 3-MA. As shown in Figure 3B, 3-MA significantly attenuated andrographolide-induced cytotoxicity in Huh-7 cells. In contrast, the caspase inhibitor z-VAD failed to reverse andrographolide-induced cytotoxicity. ATG5 has been characterized as a ubiquitin-like protein involved in autophagosome formation (31). We hypothesized that the suppression of ATG5 expression will decrease andrographolide-induced autophagy and cytotoxicity. In Huh-7 cells, the expression of ATG5 was reduced following transfection of siRNA against ATG5 (Figure 3C). The level of andrographolide-induced cell death was also attenuated by ATG5 siRNA (Figure 3D). Altogether, these data indicated that andrographolide induced cytotoxicity of Huh-7 cells through an autophagic mechanism.
Fig. 2. Andrographolide-induced autophagy. (A) Western blotting was performed to determine LC3II accumulation in Huh-7, QGY-7703 and Bel-7402 cells after treatment with 0–16 μg/ml andrographolide for 24 h. Actin (β-actin) was used as internal control to ensure that equal amounts of proteins were loaded in each lane. (B) GFP-LC3 puncta was detected in Huh-7 cells after treatment with 0–16 μg/ml andrographolide for 24 h as described in Materials and methods. (C) The percentage of cells with GFP-LC3 dots (punctate GFP-LC3) was determined in Huh-7, QGY-7703 and Bel-7402 cells after treatment with 0–16 μg/ml andrographolide for 24 h as described in Materials and methods. Cells were visualized by fluorescence microscopy, and the GFP-expressing cells were counted as punctate or diffuse. The results are means ± SD of three independent experiments (*P < 0.05 versus control). (D) Autophagic vesicles or autophagosomes in control or 16 μg/ml andrographolide-treated Huh-7 cells were observed by electron microscopy after 24 h. The bottom picture enlargement showing clearer autophagic vesicles. A0, andrographolide 0 μg/ml; A4, andrographolide 4 μg/ml; A8, andrographolide 8 μg/ml; A16, andrographolide 16 μg/ml. Data of column represent means ± SD of three independent experiments (*P < 0.05 versus control).
Andrographolide-induced autophagy is associated with a loss of MMP and an increased ROS production

Recent studies reported that mitochondrial events were involved in the autophagy process (32,33). Therefore, we next investigated the effect of andrographolide on the integrity of mitochondria. As shown in Figure 4A, incubation of Huh-7 cells with andrographolide caused a substantial decrease of MMP in a time-dependent manner, as revealed by staining of the cells with a transmembrane potential-sensitive dye rhodamine-123 (Rh 123) and analyzed with flow cytometry analysis. To further determine the relationship between autophagy and disruption of MMP, we used an autophagy inhibitor 3-MA to examine the effect of andrographolide on MMP in Huh-7 cells. As illustrated in Figure 4A, the autophagy inhibitor 3-MA failed to reverse andrographolide-induced MMP decrease in Huh-7 cells. Moreover, ATG5 siRNA also failed to attenuate andrographolide-induced MMP loss (Figure 4A). Opening of the MPTP may result in dissipation of MMP (34), therefore we next used CsA (a MPTP inhibitor) to determine the effect of andrographolide on MMP in Huh-7 cells. Interestingly, andrographolide-induced collapse of MMP was largely prevented by a 2h pretreatment with 5 μM CsA (Figure 4A). To elucidate if this phenomenon was due to a direct effect on mitochondria, we isolated mitochondria from Huh-7 cells and measured changes in absorbance at 540 nm, which is an indicator of mitochondrial swelling as a result of MPTP opening (22). Andrographolide induced a significant decrease in the absorbance at 540 nm (Figure 4B), which can be largely blocked by CsA, indicating that andrographolide induced CsA-sensitive mitochondrial permeability transition. Since CsA can specifically interact with cyclophilin D (the MPTP component) and affects the pore permeability (35,36), these data suggested a possibility that cyclophilin D might be a target of andrographolide.

Recent investigations have reported that generation of ROS is associated with disruption of MMP (37). Therefore, we analyzed the production of intracellular ROS in andrographolide-treated Huh-7 cells by flow cytometry using DCFH-DA fluorescence dye. As shown in Figure 4C, compared with untreated cells (the control group), andrographolide caused a dramatic increase of ROS generation in Huh-7 cells in a time-dependent manner. This ROS increase could be largely suppressed by 2h pretreatment with 5 μM CsA, whereas the autophagy inhibitor 3-MA (Figure 4C) or ATG5 siRNA (Figure 4D) failed to suppress andrographolide-induced ROS increase, suggesting that the increase of ROS generation might be a consequence of andrographolide-induced disruption of MMP.
leading to respiratory dysfunction and electron leakage from the respiratory chain.

Cyclophilin D played an important role in andrographolide-induced autophagic cell death. Because andrographolide caused a significant increase of ROS, which can be suppressed by CsA (the cyclophilin D targeted compound), we further determined the role of ROS generation and the significance of cyclophilin D in mediating andrographolide-induced autophagy and cytotoxicity. As illustrated in Figure 5A, treatment with 16 μg/ml andrographolide resulted in a significant increase of autophagy in Huh-7, QGY-7703 and Bel-7402 cells (Figure 5A). Accordingly, a dramatic cell death was also observed in these andrographolide-treated cells (Figure 5B). This autophagy increase and massive cell death could be largely prevented by 2h pretreatment with 5 μM CsA (Figure 5A and 5B). Interestingly, preincubation of cells with antioxidant NAC (1 mM) failed to suppress andrographolide-induced autophagy (Figure 5C; Supplementary Figure 2A, available at Carcinogenesis Online) or cell death (Figure 5D; Supplementary Figure 2B, available at Carcinogenesis Online), although ROS generation caused by andrographolide was reduced by the antioxidant NAC (Figure 4C; Supplementary Figure 2C, available at Carcinogenesis Online). These results suggest that the increase of cellular ROS was not a critical event for andrographolide-induced autophagic cell death and might be merely an indication of mitochondrial dysfunction caused by andrographolide. Moreover, pretreatment of cells with 5 μM BA, an inhibitor of adenine nucleotide translocase (ANT, also a component of MPTP) (38), did not suppress andrographolide-induced autophagy (Figure 5E) or cell death (Figure 5F). Taken together, these data suggest that cyclophilin D might play a key role in mediating andrographolide-induced autophagic cell death.

Fig. 4. Effect of andrographolide on mitochondrial permeability transition (A and B) and ROS production (C and D) in Huh-7 cells. (A) After treatment with 16 μg/ml andrographolide for 0–24 h, Huh-7 cells were incubated with Rh123 (10 μg/ml) for 30 min, and then immediately subjected to flow cytometric analysis. CsA (5 μM) or 3-MA (2.0 mM) was preincubated for 2 h before the addition of andrographolide. ATG5 was silenced by siRNA as described in Materials and methods. Results were expressed as mean Rh123 fluorescence (means ± SD of three independent experiments). (B) Mitochondria were incubated as indicated under Materials and methods. Changes in absorbance at 540 nm were recorded after addition of 16 μg/ml andrographolide in the presence or in the absence of 5 μM CsA. (C and D) After treatment with 16 μg/ml andrographolide for 0–24 h, Huh-7 cells were incubated with 10 μM DCFH-DA for 30 min and then immediately subjected to flow cytometric analysis. CsA (5 μM), 3-MA (2.0 mM) or NAC (1.0 mM) was preincubated for 2 h before the addition of andrographolide. ATG5 was silenced by siRNA as described in Materials and methods. Results were expressed as mean DCF-fluorescence (means ± SD of three independent experiments). Data of column represent means ± SD of three independent experiments, *P value represents significant difference between conditions where P < 0.05.
To further determine the critical role of cyclophilin D, we used siRNA to knock down the endogenous cyclophilin D and then evaluated its effect on andrographolide-induced autophagic cell death. As shown in Figure 6A, transfection of cells with Cyclophilin D siRNA led to a dramatic reduction of cyclophilin D protein levels in Huh-7, QGY-7703 and Bel-7402 cells, whereas the scrambled RNA did not alter cyclophilin D expression. Importantly, andrographolide-induced autophagy (Figure 6B) and cell death (Figure 6C) were largely suppressed by knockdown of Cyclophilin D, further confirming the key role of cyclophilin D in mediating andrographolide-induced autophagic cell death.

**Discussion**

This study showed that treatment with andrographolide-induced autophagy but not apoptosis in human liver cancer Huh-7, Bel-7402 and QGY-7703 cells. Moreover, andrographolide-induced autophagy as well as cytotoxicity was largely attenuated by autophagy inhibitor 3-MA or ATG5 siRNA. We further demonstrated that MPTP played a critical role in andrographolide-induced autophagy. More importantly, cyclophilin D probably mediated the cytotoxic action of andrographolide by triggering the opening of MPTP.
Andrographolide has been shown to induce apoptosis in various types of cancer cells (24,25,39,40). However, we did not detect any increase of apoptosis signal (TUNEL or caspase-3 assay) in andrographolide-treated human liver cancer Huh-7, QGY-7703 or Bel-7402 cells. The pan-caspase inhibitor z-VAD did not attenuate andrographolide-caused cell death. Taken together, these results indicated that andrographolide-induced cell death is not via caspase-dependent apoptosis in these cells. Rather, andrographolide was found to trigger autophagy instead of apoptosis in Huh-7, QGY-7703 and Bel-7402 cells. Autophagy is a process by which subcellular constituents are degraded in autophagosomes/autolysosome in response to stress (41). The significance of autophagy in antitumor therapeutics has not been clearly elucidated. To adapt adverse conditions induced by stress from anticancer therapies, cancer cells may trigger an autophagic response that promotes a portion of the cytoplasm and organelles into autophagic vesicles as part of the survival response to stress (14,42).

In this case, autophagy may be a survival mechanism of cancer cells. On the other hand, some anticancer agents, including arsenic trioxide and rapamycin, have been reported to stimulate excess autophagy, which ultimately led to a caspase-independent cell death (17). In this regard, autophagy might be a crucial mechanism of cancer cell death by these agents. Whether autophagy triggered by andrographolide in Huh-7 cells is a survival mechanism or a cell death mechanism was elucidated in this study by applying 3-MA, a chemical inhibitor of autophagy. Our results indicated that 3-MA could reverse the cytotoxic effect of andrographolide, suggesting that andrographolide induces an autophagic cell death. These findings further validated by silencing of ATG5, a key molecule involved in autophagosome formation. The ability of andrographolide to trigger multiple death pathways suggests its effectiveness and versatility in killing cancer cells.

Recently, caspase-independent autophagic cell death has been reported to associate with alteration of ROS (43). Consistent with these findings, intracellular ROS was elevated after being exposed to andrographolide. Furthermore, this study suggests that the mechanism of andrographolide-induced cytotoxicity appeared not directly through ROS caused oxidative damage, but probably through activating MPTP, leading to mitochondrial dysfunction and autophagic cell death. Several evidence could support this conclusion. First, antioxidant NAC effectively blocked cellular ROS production, but did not prevent andrographolide-triggered autophagy or cell death, suggesting ROS may not play a critical role in andrographolide-induced cytotoxicity. Second, andrographolide caused a dramatic decrease of MMP, which could not be blocked by 3-MA or ATG5 siRNA, whereas CsA, an agent that prevents the opening of MPTP by binding to cyclophilin D (36), could effectively block the collapse of MMP, ROS elevation, autophagy and cell death. Third, knockdown of Cyclophilin D by siRNA abrogated andrographolide-induced autophagy and cell death. Together, these findings indicated that the cytotoxicity of andrographolide was closely associated with cyclophilin D, which seemed to play an important role in andrographolide-induced autophagic cell death. Moreover, the observation that BA (a chemical inhibitor of ANT, another MPTP component) (38) failed to attenuate andrographolide-induced autophagy or cell death further suggests that cyclophilin D might be specific for andrographolide-induced cytotoxicity.

In summary, our study revealed that andrographolide induced a death pathway characterized by autophagy, in which cyclophilin D mediated the cytotoxic action by triggering the opening of the mitochondrial permeability transition pore. Thus, andrographolide may represent a promising novel targeted agent in the prevention and treatment of liver cancer. Further investigation of andrographolide in mouse models will contribute to the additional understanding of its in vivo activity toward malignant cells.

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Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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