

RESEARCH ARTICLE

Parthenolide-Induced Apoptosis, Autophagy and Suppression of Proliferation in HepG2 Cells

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Abstract

Purpose: To investigate the anticancer effects and underlying mechanisms of parthenolide on HepG2 human hepatocellular carcinoma cells. **Materials and Methods:** Cell viability was assessed by MTT assay and cell apoptosis through DAPI, TUNEL staining and Western blotting. Monodansylcadaverin (MDC) and AO staining were used to detect cell autophagy. Cell proliferation was assessed by Ki67 immunofluorescence staining. **Results:** Parthenolide induced growth inhibition in HepG2 cells. DAPI and TUNEL staining showed that parthenolide could increase the number of apoptotic nuclei, while reducing the expression of the anti-apoptotic protein Bcl-2 and elevating the expression of related proteins, like p53, Bax, cleaved caspase9 and cleaved caspase3. Parthenolide could induce autophagy in HepG2 cells and inhibited the expression of proliferation-related gene, Ki-67. **Conclusions:** Parthenolide can exert anti-cancer effects by inducing cell apoptosis, activating autophagy and inhibiting cell proliferation.

Keywords: Parthenolide - apoptosis - autophagy - hepatocellular carcinoma - human HepG2 cells

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Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant diseases which have the characteristics of strong malignance, easy invasion and metastasis, bad prognosis and so on. HCC therapy has become increasingly important all over the world (Gomaa et al., 2008). There are many methods in the treatment of HCC such as chemotherapy by using new anti-tumor drugs, operation, intervene therapy, liver transplantation, and so on. Currently, most of drugs used for HCC, such as doxorubicin, fluorouracil, cisplatin, and mitomycin, are non-selective cytotoxic molecules and have significant side effects (Avila et al., 2006; Kaseb et al., 2013). Therefore, it is a permanent subject to find safe and effective new agents for the treatment of HCC.

Natural product substances have historically served as the most significant source of new leads for pharmaceutical development, and several plant-derived compounds are currently successfully employed in cancer treatment (Rocha et al., 2001), which has provided a novel opportunity to improve the existing standard of care for HCC and other cancers (Newman 2008). Parthenolide is the principal component of sesquiterpene lactones present in medical plants such as feverfew (*Tanacetum parthenium*) (Knight 1995). Besides its immunomodulating effect, parthenolide has been used in anti-cancer treatment. It has been reported that parthenolide induces proliferation

inhibition and apoptosis of human cancer cells (Parada et al., 2007; Zunino et al., 2007).

Although the anticancer property of parthenolide has been demonstrated in a number of human cancer cells (Zhang et al., 2004; Zunino et al., 2007), its anticancer potential in human hepatocellular carcinoma HepG2 cells and the anticancer mechanism of parthenolide is still not fully understood, especially, the effect of parthenolide on cell autophagy has not been reported. In the present study, we investigated the effects of parthenolide on cell apoptosis, autophagy and proliferation in human hepatocellular carcinoma HepG2 cells.

Materials and Methods

Cell Culture. Human liver cancer cell line HepG2 and human normal liver cell line L02 were cultured at 37°C in 5% CO₂, in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Invitrogen, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (Sijiqing, Hangzhou, China), 100 U/mL penicillin, and 100 mg/mL streptomycin (Ameresco, USA).

Cell Viability Assay. Cell viability was determined by measuring the metabolism of a tetrazolium substrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were plated on 96-well microplates at a density of 6×10³ (HepG2) and 8×10³ (L02). On the

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following day, the culture medium was removed and cells were exposed to serial dilutions of parthenolide prepared in the fresh medium with 3% FBS. The plate was incubated for an additional 48 h. Subsequently, 20 μ L of 5 mg/mL MTT solution was added to each well and the plate incubated in the dark for 4 h. Formazan crystals were dissolved in 100 μ L/well of DMSO. The absorbance at 560 nm was read on a spectrophotometric plate reader (Bio-Rad, CA, USA) and the proportion of surviving cells was calculated by dividing the average absorbance of the treated wells by the average of nontreated wells.

Immunofluorescence Assay. Cells were treated with parthenolide (6 μ g/mL) for 24 h and fixed in 4% paraformaldehyde for 30 min at room temperature. Fixed cells were penetrated with 0.5% Triton X-100 for 5 min and blocked with 5% BSA. Cells were incubated overnight at 4°C with specific primary antibodies. The following primary antibodies were used: anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), anti-survivin (Santa Cruz, CA, USA) and anti-Ki-67 (Santa Cruz, CA, USA). Antibodies were diluted with PBS contains 5% BSA. After washing, cells were incubated with Cy3-conjugated goat anti-mouse IgG (Beyotime, Shanghai, China) for 1 h at room temperature and examined using a fluorescence microscope (Olympus, Tokyo, Japan).

DAPI Staining. Cells treated with parthenolide (6 μ g/mL) for 24 h were fixed in 4% paraformaldehyde and incubated in 0.5 μ g/mL 4, 6-Diamidino-2-phenylindole (DAPI) solution for 30 min in the dark at room temperature. The cells were examined by fluorescence microscope.

TUNEL Assay. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed according to the kit manufacturer's protocol (Roche, Swiss). HepG2 cells exposed to parthenolide were fixed in 4% paraformaldehyde at room temperature. Fixed cells were penetrated with 0.5% Triton X-100 for 2 min on the ice and then incubated with TUNEL reaction mixture for 1 h at 37°C. The cells were then examined using a fluorescence microscope.

Western Blot Analysis. HepG2 cells were plated in a 6-well plate in a density of 5×10^5 cells in a volume of 2 mL and incubated for 24 h at 37°C. After incubation with fresh medium of parthenolide for another 24 h, cells were harvested and rinsed twice with PBS. Cell extracts were lysed with lysis buffer (1% Nonidet P-40; 50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM NaF; 1 mM phenylmethylsulfonyl fluoride; 4 mg/mL leupeptin; 1 mg/mL aprotinin) for 30 minutes, with occasional rocking, followed by centrifugation at 12,000 rpm, for 5 minutes at 4°C. Identical amounts (100 mg of protein) of sample lysate were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto PVDF membrane. The membrane was blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.02% Tween-20), and incubated overnight at 4°C with specific primary antibodies. The following primary antibodies were used: anti-Bax, anti-Bcl-2, anti-p53 (Santa Cruz, CA, USA), anti-caspase-9 (Cell Signaling Technology, Beverly, MA) and anti-GAPDH (Kangcheng Biological

Company, Shanghai, China). Antibodies were diluted with TBST buffer. Subsequently, the membrane was washed with TBST buffer and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature, and washed extensively before detection. The membranes were subsequently developed using enhanced chemiluminescence (ECL) reagent (Beyotime, Shanghai, China) and exposed to film according to the manufacturer's protocol.

MDC Staining. HepG2 cells cultured on glass coverslips were treated with parthenolide (6 μ g/mL) for 24 h at 37°C. Subsequently, the cells were incubated with 50 μ M monodansylcadaverine (MDC) (Sigma, USA) in medium at 37°C for 1 h. Intracellular MDC was measured by fluorescence photometry.

Acridine Orange Staining. Cell staining was performed according to published procedures (Mains et al., 1988). HepG2 cells cultured on glass coverslips and then exposed to parthenolide (6 μ g/mL) for 24 h in the fresh medium with 3% FBS. After incubated with parthenolide, the Acridine orange (Sigma, USA) was added at a final concentration of 10 μ M for a period of 15 min at 37°C. It's examined by fluorescence microscope.

Statistical Analysis. Experiments were repeated at least three times. Statistical analysis was performed with Student's t-test to determine the significant differences ($p < 0.01$).

Results

Parthenolide Induces Cell Death In HepG2 Cells. MTT assays were performed to investigate the effects of parthenolide on the cells viability. We treated the human hepatocellular carcinoma HepG2 cells with six concentrations of parthenolide (0, 2.5, 5, 10, 20 and 40 μ g/mL) for 48 h. As shown in Figure 1A, parthenolide inhibited the HepG2 cells proliferation effectively in a concentration dependent manner. In addition, the IC₅₀ (50% inhibitory concentration) value of HepG2 cells was 23.23 μ g/mL, and the IC₁₀ (10% inhibitory concentration) value was 1.78 μ g/mL (Figure 1B). To text the cytotoxicity of parthenolide to normal liver cells, we also performed MTT assay in human embryonic hepatocytes L02 cells. At the same concentration, the cell viabilities of L02 cells

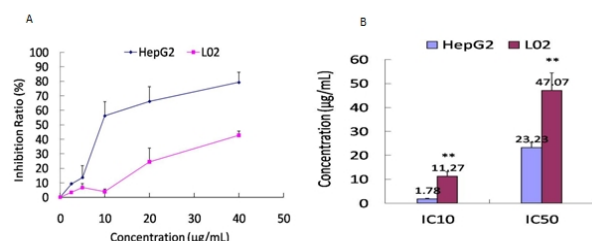


Figure 1. Parthenolide Suppresses the Viability of HepG2 Cells. HepG2 cells were treated with 0, 2.5, 5, 10, 20 and 40 μ g/mL parthenolide in the fresh medium with 3% FBS for 48 h. After treatment, 20 μ L MTT was added to each well for an additional 4 h and incubated in the dark at 37°C. The data are presented as the relative proportion of viable cells (%) by comparing the parthenolide- treated group with the untreated cells, the viability of which was assumed to be 100%. ** $p < 0.01$ was determined by Student's t-test

were much higher than HepG2 cells (Figure 1A). The IC₅₀ and IC10 value of parthenolide for L02 cells were 47.07 µg/mL and 11.27 µg/mL, respectively (Figure 1B). These results suggest that parthenolide has the advantage to induce cancer cells death, while without cytotoxic effects on normal liver cells. Thus, it is a potential compound for drug development against liver cancer.

Parthenolide Induces Apoptosis In HepG2 Cells. Cancer cells exposed to chemotherapy typically die through a process of programmed cell death or apoptosis. Apoptosis is marked by cellular shrinking, condensation and margination of the chromatin and ruffling of the plasma membrane, called budding. Eventually the cell becomes divided in apoptotic bodies which consist of cell organelles and/or nuclear material surrounded by an intact plasma membrane. After treatment with parthenolide, the cell morphological changes were observed under an inversion microscope (vv 2A). These changes include blebbing, loss of cell membrane asymmetry and attachment and cell shrinkage. The mechanism of cell killing by parthenolide was determined by a variety of assays for apoptosis include DAPI staining and TUNEL assay. Apoptotic bodies were observed through fluorescence microscope in HepG2 cells treated with parthenolide (6 µg/mL, 24 h). DAPI staining indicated that nuclear condensation and perinuclear apoptotic bodies were induced by parthenolide while control cells showed round and homogeneous nuclei (Figure 2B). The presence of TUNEL-positive cells (stained green), showing that DNA strand breaks had occurred, also indicated that parthenolide had induced apoptosis in HepG2 cells (Figure 2C).

Subsequently, we investigated the molecular mechanisms of parthenolide-induced apoptosis in HepG2 cells. The role of the tumor suppressor p53 is studied extensively and p53 could regulate cell death under various anticancer drug treatments (Shu et al., 2007). In our study, the expression of p53 in cytoplasm decreased

and increased in nucleus in a dose-dependent manner following treatment with parthenolide in HepG2 cells (Figure 3A). Mitochondria are critical for p53-dependent death (Wang et al., 2001). The outer mitochondrial membrane (OMM) undergoes permeabilization, which causes the release of potent death factors from the intermembrane space into cytosol when mitochondrial receive a death signal (Green et al., 2002). These apoptogenic factors can activate caspase-9. OMM permeabilization is regulated by the opposing actions of pro- and antiapoptotic Bcl-2 proteins, such as Bcl-2 and Bax. In this study, we detected the level of activated caspase-9, the result as shown in Figure 3B, treatment with parthenolide induced a dramatic increase of cleaved caspase-9 in a dose-dependent manner in HepG2 cells. The expression of Bcl-2 was decreased, whereas the expression of Bax was increased after treatment by parthenolide for 8 h (Figure 3C). The activation of caspase-3 plays a central role in apoptosis and survivin is a new inhibitor of apoptosis protein (IAP-family member) which suppresses cell apoptosis by the inhibition of effective caspase-3 (Obiol et al., 2008). So we examined the expression of cleaved caspase-3 and survivin by an immunofluorescence assay in HepG2 cells. The result shown that the caspase-3 was activated after treatment with parthenolide (Figure 3D), whereas the expression of survivin was decreased obviously when treatment with parthenolide (Figure 3E).

Parthenolide Induces Autophagy In HepG2 Cells. Autophagy, is a cellular pathway involved in protein and organelle degradation, with connections to human disease and health (Du et al., 2013). Cancer is one of the first diseases genetically linked to autophagy malfunction (Levine et al., 2008). Many anticancer agents have been reported to induce autophagy, leading to the suggestion that autophagic cell death may be an important mechanism for tumor cell killing by these agents (Kondo et al., 2008).

Monodansylcadaverine (MDC) has been proposed as

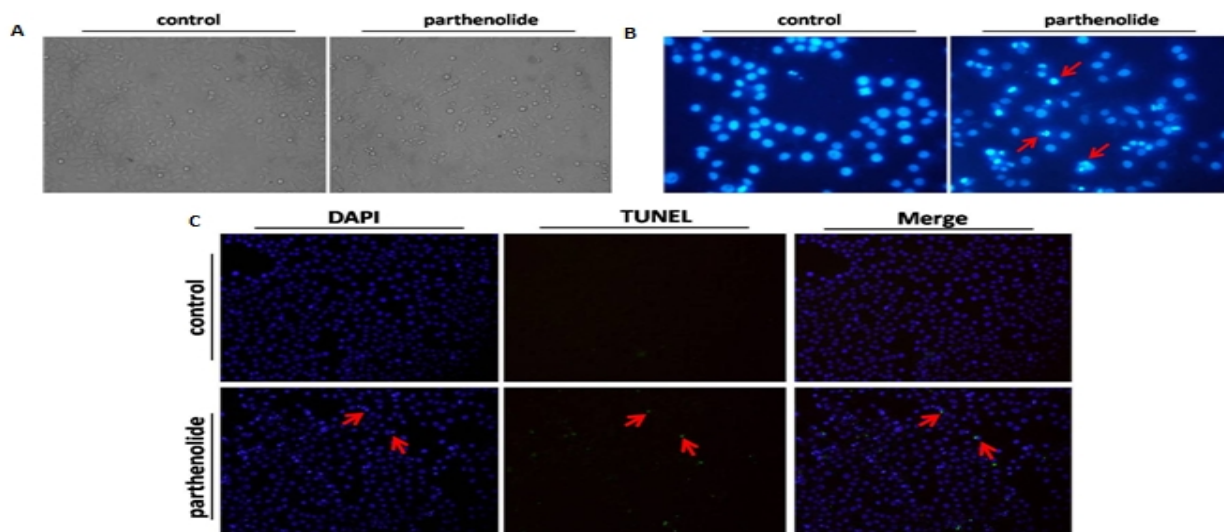


Figure 2. Parthenolide Induces Apoptosis of HepG2 Cells. A) HepG2 cells were treated with 6 µg/mL parthenolide for 24 h and then the morphology changes were observed with a microscope. B) HepG2 cells were treated with 6 µg/mL parthenolide for 24 h and stained with DAPI. The morphological changes in nuclear chromatin were then photoed with a fluorescence microscope. The arrow points to the apoptotic body in apoptotic HepG2 cells. The results were from one experiment representative of three experiments. (magnification, 350×). C) Cells apoptosis observed by TUNEL assay. Parthenolide-treated HepG2 cells (6 µg/mL, 24 h) were stained with TUNEL and viewed under a fluorescence microscope (magnification, 200×). The arrow points to DNA strand breaks in apoptotic HepG2 cells

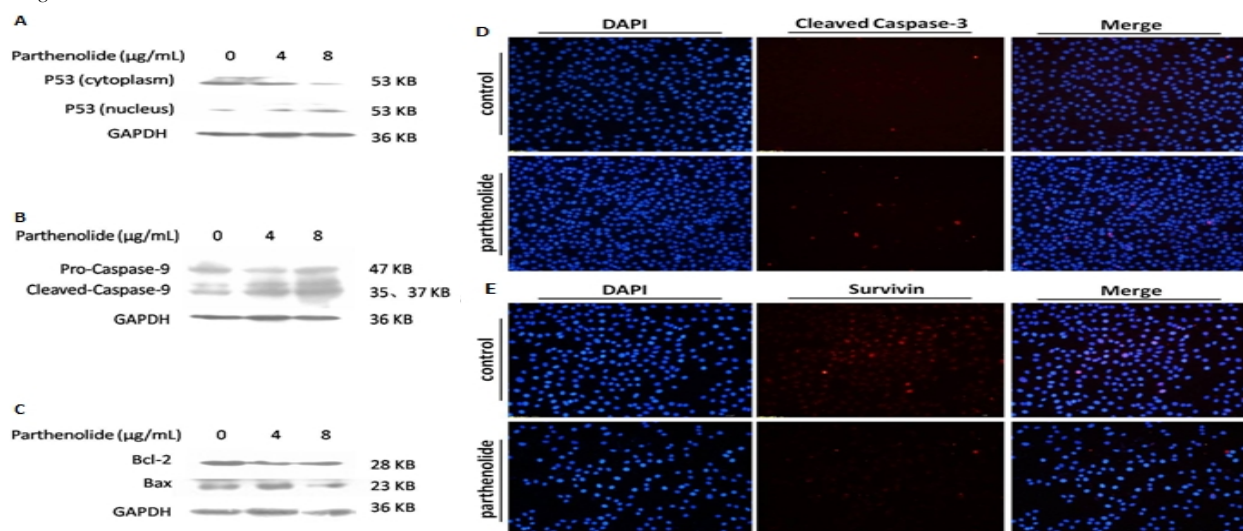


Figure 3. Parthenolide Affected the Expression of Apoptotic-Related Protein. A-C) HepG2 cells were incubated with 0, 4 and 8 μg/mL parthenolide. Cell lysates were prepared, and subjected to SDS-PAGE. Proteins were determined by Western blotting using specific antibodies. **A)** The expression of p53 in cell cytoplasm and nucleus after treatment with parthenolide for 24 h. **B)** The level of active caspase-9 in HepG2 cells after treatment with parthenolide for 24 h. **C)** The expression of anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein after treatment with parthenolide for 8 h (6 μg/mL). **D)** HepG2 cells were incubated with 6 μg/mL parthenolide for 24 h. Caspase-3 activation was detected by immunofluorescence assay. Cleaved caspase-3 expressed after treatment with parthenolide (magnification, 200×). **E)** The expression of anti-apoptotic survivin protein in HepG2 cells after incubated with 6 μg/mL parthenolide for 24 h (magnification, 200×)

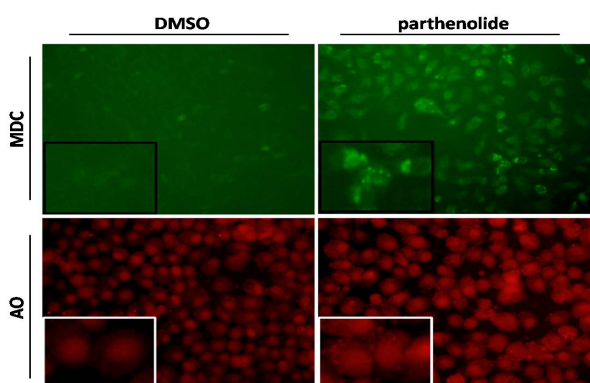


Figure 4. Parthenolide Induces Autophagy in HepG2 Cells. HepG2 cells cultured on glass coverslips were treated with parthenolide (6 μg/mL) for 24 h at 37°C, then the cells autophagy were detected by MDC and AO staining assay (magnification, 400×)

a tracer for autophagic vacuoles and MDC is a specific marker for autolysosomes (Biederbick et al., 1995; Mizushima et al., 2004). Autophagy is also characterized by acidic vesicular organelle (AVO) formation, which is detected and measured by vital staining of acridine orange (AO) (Paglin et al., 2001). In this study, the effect of parthenolide on autophagy was detected by MDC and AO staining assay. Exposed to 6 μg/mL parthenolide for 24 h can increase MDC uptake evidently compared with control cells (Figure 4). Vital staining of HepG2 cells with acridine orange showed the accumulation of AVO in the cytoplasm of cells exposed to 6 μg/ml parthenolide. These results suggest that parthenolide can induce autophagy obviously in HepG2 cells.

Parthenolide Inhibits Cell Proliferation In HepG2 Cells. The number of mitoses and proliferative capacity are crucial factors for tumor grading and metastasis. Ki-67 antigen is a nonhistone protein that was first described

in 1983 and present in proliferating cells, but absent in resting cells. Because the Ki-67 nuclear antigen is present throughout the cell cycle, immunostaining with monoclonal antibody Ki-67 is a reliable means of rapidly evaluating the proportion of proliferating cells (Gerdes et al., 1984).

We further investigated the effects of parthenolide on proliferation by immunofluorescence assay. HepG2 cells were incubated in medium with 6 μg/ml of parthenolide for 24 h and labeled by specific primary antibody Ki-67 and secondary antibody of CY3-conjugated goat anti-mouse IgG. To exclude the impact of DMSO solution, we used equal volume of DMSO as a negative control. Figure 5 showed that parthenolide can decrease the expression of Ki-67, but there is little influence of DMSO compared to the untreated control. This result suggests that cell proliferation inhibition may be also involved in the anticancer activity of parthenolide.

Discussion

Parthenolide is a kind of sesquiterpene lactone with a traditional reputation of treating a variety of disorders. Parthenolide has analgesic, anti-microbial, anti-inflammatory and anti-cancer effect, which may depend on a wide range of parthenolide-stimulated intracellular signals (Zhang et al., 2004). Parthenolide play its anti-tumor role through different molecular mechanisms (Mathema et al., 2012). Parthenolide can bind directly to and inhibit IκB kinaseβ (IKKβ) with the sustained cytoplasmic retention of NF-κB (Kwok et al., 2001). Parthenolide also can efficiently inhibit the IL-6-induced up-regulation of ACT (α1-antichymotrypsin) mRNA. Further study revealed that parthenolide effect was achieved by blocking of STAT-3 and STAT-1 binding to the regulatory elements in DNA (Sobota et

al., 2000). Parthenolide activated caspases, dissipated the mitochondrial membrane potential and released the mitochondrial proapoptotic proteins in colorectal cancer cells (Zhang et al., 2004). All of these lead to mitochondrial dysfunction and apoptosis induction. Therefore, parthenolide induces apoptosis in various cancer cells via different mechanisms, yet its anti-cancer mechanism in hepatic carcinoma is still elusive. The effect on autophagy of parthenolide has not been reported. In this study, we studied the mechanisms underlying parthenolide-induced cell death in human hepatocellular carcinoma HepG2 cells. We clarified the effects of parthenolide on apoptosis, autophagy and proliferation.

Cell proliferation and cell death are carefully and continuously balanced in multi-cellular organisms. Imbalances in the coordination of these processes can result in diseases such as cancer due to over proliferation and reduced cell death (Overholtzer et al., 2007). Anti-proliferation is an anti-tumor mechanism which applied in cancer therapy. Ginkgo biloba extract kaempferol inhibits cell proliferation in pancreatic cancer cells (Zhang et al., 2008) and berberine has an anti-proliferation effect on human esophageal cancer cell lines (Iizuka et al., 2000). In this study, the result revealed that parthenolide can inhibit cell proliferation in HepG2 cells.

Apoptosis, also termed programmed cell death, is an elaborate cellular homeostasis mechanism that ensures correct organ development, tissue remodeling, immune response, and tumor suppression. Cancer-associated defects in apoptosis play important roles in tumor pathogenesis. Defects in apoptosis also increased the threshold for cell death and thereby requiring higher doses for tumor killing (Leong et al., 2012). Thus, the activation of apoptosis in tumor cells is a promising strategy for the treatment of cancer. In the present study, parthenolide has been found to induce apoptosis in HepG2 cells (Du et al., 2013). Further study revealed that the expression of p53 has changed both in cytoplasm and nucleus, suggested that apoptosis induced by parthenolide in a p53-dependent pathway. Our results also indicated that the expression of anti-apoptotic protein Bcl-2 was decreased, whereas the apoptotic protein Bax was increased, which result in the activation of caspase-9 and caspase-3.

Cancer cells often acquire alterations in the mechanisms of apoptosis so cancer therapy has largely focus on apoptosis (Riedl and Shi, 2004). More recently, non-apoptotic mechanisms of cell death have emerged as potential alternative mechanisms for effective cancer therapies, such as autophagy (Lockshin et al., 2004). Autophagy is a conserved response to stress that facilitates cell survival in some contexts and promotes cell death in others (Cui et al., 2013). We revealed for the first time that treatment with parthenolide induced autophagy in HepG2 cells.

In conclusion, this study has evaluated various effects of parthenolide in hepatocellular carcinoma cell line in vitro. Our result revealed that parthenolide induces apoptosis in HepG2 cells via a mitochondrial- dependent apoptotic pathway. p53, survivin, Bax and Bcl-2 may also involved in this process. In addition, parthenolide also induces autophagy and inhibits proliferation in HepG2

cells, these results suggest that both apoptosis induction and proliferation inhibition contribute to the anticancer effect of parthenolide in HepG2 cells. Furthermore, autophagy induction may also involved in this process.

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