行政院國家科學委員會專題研究計畫 期末報告

中草藥萃取物誘發肝癌細胞發生自噬反應（autophagy）及相關機制之探討

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 101-2320-B-040-008-
執 行 期 間 ：101 年 08 月 01 日至 102 年 07 月 31 日
執 行 單 位 ：中山醫學大學醫學檢驗暨生物技術學系（所）

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報 告 附 件 ：出席國際會議研究心得報告及發表論文

公 開 資 訊 ：本計畫可公開查詢

中 華 民 國 102 年 10 月 31 日

中 文 摘 要 ： 中草藥萃取物或天然食品近年來被廣泛的應用在對各種癌症的治療研究結果成果上，希望具有輔助療效的效果。然而，薯蕷皂甙對於影響腫瘤細胞自體吞噬的研究尚未很清楚。在本研究中，我們針對薯蕷皂甙對引發肝癌細胞的自體吞噬反應的效果進行了進一步的調查。結果發現，薯蕷皂甙可誘導caspase-3和-9依賴型的細胞凋亡。藉由抑制ERK1 / 2的磷酸化可顯著的抑制薯蕷皂甙所誘導的細胞凋亡反應。此外，薯蕷皂甙引發細胞自噬屬於早期的階段。藉由處理自體吞噬抑製劑，得知細胞凋亡反應顯著的增強。而我們也發現抑制caspase蛋白的活化並沒有影響薯蕷皂甙誘導LC3-II蛋白的表達。根據這些研究結果，我們認為，一旦細胞凋亡反應受阻，薯蕷皂甙所誘導的自體吞噬過程也將停止，而薯蕷皂甙會引起細胞自體吞噬反應，而這樣的自體吞噬現象則是屬於細胞本身的保護反應。

中文關鍵詞： 薯蕷皂甙，自體吞噬，細胞凋亡

英 文 摘 要 ： Extensive research results support the application of herbal medicine or natural food as an augment during therapy for various cancers. However, the effect of dioscin on tumor cells autophagy has not been clearly clarified. In this study, the unique effects of dioscin on autophagy of hepatoma cells were investigated. Results found that dioscin induced caspase-3- and -9-dependent cell apoptosis in a dose-dependent manner. Moreover, inhibition of ERK1/2 phosphorylation significantly abolished the dioscininduced apoptosis. In addition, dioscin triggered cell autophagy in early stages.With autophagy inhibitors to hinder the autophagy process, dioscin-induced cell apoptosis was significantly enhanced. An inhibition of caspase activation did not affect the dioscininduced LC3-II protein expression. Based on the results, we believed that while apoptosis was blocked, dioscin-induced autophagy process also diminished in Huh7 cells. In conclusion, this study indicates that dioscin causes autophagy in Huh7 cells and suggests that dioscin has a cytoprotective effect.

英文關鍵詞： dioscin, apoptosis, autophagy, ERK1/2, LC3

附件一

行政院國家科學委員會補助專題研究計畫 □期中進度報告 ■期末報告

中草藥萃取物誘發肝癌細胞發生自噬反應（autophagy）及相關機制之探討

計畫類別：■個別型計畫　□整合型計畫
計畫編號：NSC 101-2320-B-040-008
執行期間：101 年 08 月 01 日至 102 年 07 月 31 日

執行機構及系所：中山醫學大學醫學檢驗暨生物技術學系

計畫主持人：邱慧玲
共同主持人：
計畫參與人員：謝明儒

本計畫除繳交成果報告外，另含下列出國報告，共 _2_ 份：
□移地研究心得報告
■出席國際學術會議心得報告
□國際合作研究計畫國外研究報告

處理方式：除列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，■一年□二年後可公開查詢

中　華　民　國 102 年 08 月 01 日

中文摘要

中草藥萃取物或天然食品近年來被廣泛的應用在對各種癌症的治療研究結果成果上,希望具有輔助療效的效果。然而，薯蕷皂甙對於影響腫瘤細胞自體吞噬的研究尚未很清楚。在本研究中，我們針對薯蕷皂甙對引發肝癌細胞的自體吞噬反應的效果進行了進一步的調查。結果發現，薯蕷皂甙可誘導 caspase-3 和-9 依賴型的細胞凋亡。藉由抑制 ERK1 / 2 的磷酸化可顯著的抑制薯蕷皂甙所誘導的細胞凋亡反應。此外，薯蕷皂甙引發細胞自噬屬於早期的階段。藉由處理自體吞噬抑製劑，得知細胞凋亡反應顯著的增強。而我們也發現抑制 caspase 蛋白的活化並沒有影響薯蕷皂甙誘導 LC3-II 蛋白的表達。根據這些研究結果，我們認為，一旦細胞凋亡反應受阻，薯蕷皂甙所誘導的自體吞噬過程也將停止，而薯蕷皂甙會引起細胞自體吞噬反應，而這樣的自體吞噬現象則是屬於細胞本身的保護反應。

中文關鍵詞：薯蕷皂甙，自體吞噬，細胞凋亡

Abstract

Extensive research results support the application of herbal medicine or natural food as an augment during therapy for various cancers. However, the effect of dioscin on tumor cells autophagy has not been clearly clarified. In this study, the unique effects of dioscin on autophagy of hepatoma cells were investigated. Results found that dioscin induced caspase-3- and -9-dependent cell apoptosis in a dose-dependent manner. Moreover, inhibition of ERK1/2 phosphorylation significantly abolished the dioscininduced apoptosis. In addition, dioscin triggered cell autophagy in early stages. With autophagy inhibitors to hinder the autophagy process, dioscin-induced cell apoptosis was significantly enhanced. An inhibition of caspase activation did not affect the dioscininduced LC3-II protein expression. Based on the results, we believed that while apoptosis was blocked, dioscin-induced autophagy process also diminished in Huh7 cells. In conclusion, this study indicates that dioscin causes autophagy in Huh7 cells and suggests that dioscin has a cytoprotective effect.

Keywords: dioscin, apoptosis, autophagy, ERK1/2, LC3

Introduction

In recent years, products derived from natural plants have been gaining more and more attention for the intervention of malignant invasive progression in the late stage of neoplastic diseases [1] or as potent chemopreventive drugs [2], especially for relatively chemo-refractory tumors such as hepatocellular carcinoma (HCC) [3].

Previous studies have indicated that *Dioscorea nipponica extract* (DNE) could reduce the metastasis of melanoma in vitro and in vivo through inhibited phosphorylation of Akt, activation of NF-κB and increased the expression of IκB [4]. Furthermore, Du et al., have isolated and identified a new water-soluble steroidal saponin from *Dioscorea nipponica* Makino and defined its chemical structure (as shown in Fig. 1A) [5]. This newly isolated plant steroidal saponins named as dioscin. The diosgenyl saponin dioscin is one of the most common steroidal saponins found in plants and exhibits cytotoxicity in several cancer cells. It has been extensively studied on its antitumor effect by antiproliferative activities, cell cycle arrest and induced apoptosis via the mitochondrial and some other pathway [6-9]. Results indicated that dioscin is able to induce Hela cells apoptosis via the inhibition of Bcl-2 and activation of caspases-9 and caspase-3 [10] and cause generation of reactive oxygen species (ROS) in HL-60 cells to induce apoptosis [11]. Furthermore, its capability to decrease the resistance degree of HepG2/adriamycin cells via a significant inhibition of P-glycoprotein expression has been proven, and therefore, was proposed to be a potent multidrug resistance reversal agent [12]. However, the effect of dioscin on tumor cells autophagy has not been clearly clarified.

Motivation

Autophagy is a major intracellular degradation mechanism operating under stress conditions to promote survival during starvation or lead to programmed cell death type II under specific conditions such as the inhibition of apoptosis [13-15]. The process of autophagy is initiated by engulfing large sections of cytoplasm by a crescent-shaped phagophore that elongates to autophagosome, which subsequently fusing with a lysosome and its contents are degraded by lysosomal hydrolases [16-18]. Since autophagy is vital in regulating growth and maintaining homeostasis in multicellular organisms, defective autophagy contributes to pathogenesis of a number of diseases, including myopathies, neurodegenerative diseases, and some forms of cancers [19]. The aim of this study was to characterize the effects of dioscin and underlying molecular mechanism on autophagy and apoptosis in dioscin-induced cytotoxicity.

Materials and methods

Chemicals

Dioscin of ≥98% purity was purchased from China Langchem INC. (St. Caliun, Shanghai). Stock solution of dioscin was made at 10 mM concentration in dimethyl sulfoxide (DMSO) (Sigma, St. Louis Co.) and stored at -20℃. The final concentration of DMSO for all treatments was less than 0.1%. Other chemicals, including 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), paraformaldehyde, Triton X-100, bafilomycin A1 (BafA1), 4'-6-Diamidino-2-phenylindole (DAPI), 3-Methyladenine (3-MA), p38 MAPK inhibitor SB203580, and JNK1/2 inhibitor SP600125 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The ERK1/2 inhibitor U0126, General caspase inhibitor Z-VAD-FMK was purchased from Promega (Madison, WI, USA). Specific caspase inhibitors for caspase 3 (Z-DEVE-FMK), caspase 8 (Z-IETD-FMK) or caspase 9 (Z-LEHO-FMK) were purchased from BioVision (Mountain View, CA). NE-PER Nuclear and Cytoplasmic Extraction Kit and BCA protein assay reagent (Thermo). The FITC Annexin V Apoptosis Detection Kit I was obtained from BD Biosciences, USA. Antibodies for Beclin-1, LC3, cleaved PARP, caspase-8, caspase-9 and Bcl-2 were obtained from Cell Signaling; antibodies for cytochrome c and caspase 3 were obtained from Invitrogen, CA; antibodies for p38, JNK1/2, and β-actin were obtained from BD Biosciences, USA; antibodies for ERK1/2, p-ERK1/2, p-p38 and p-JNK were obtained from Millipore Corporation, Milford, MA, USA.

Cell culture

Huh-7, a human hepatocellular carcinoma cell line, obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS), 1 mM glutamine, 1% penicillin/streptomycin, 1.5 g/L sodium bicarbonate, and 1 mM sodium pyruvate (Sigma, St. Louis, Mo, USA). The cell cultures were maintained at 37℃ in a humidified atmosphere of 5% CO_2.

Cell cytotoxicity and cell count assay

The effect of dioscin on cell growth was assayed by the MTT method, as previously described [4]. Briefly, 2×10^5 cells/well were cultured in 6-well plates and stimulated with different concentrations of dioscin (0, 0.625, 1.25, 2.5, 5 μM). After 24 or 48 hours, MTT was added to each well (at a final concentration of 0.5 mg/ml) and incubated for further 4 hours. The viable cell number was directly proportional to the production of formazan, reflected by the color intensity measured at 570 nm, following the solubilization with isopropanol. Cell proliferation was also evaluated by a cell count assay. Briefly, 2×10^5 cells/well were cultured in 6-well plates and stimulated with different concentrations of dioscin (0, 0.625, 1.25, 2.5, 5 μM). After 24 or 48 hours, the cells were trypsinized and centrifuged, viable and death cells were counted using a hemocytometer after staining with trypan blue. Each condition was performed in 3 replicate wells and data were obtained from at least 3 separate experiments.

Preparation of cell nuclear and cytosolic extracts

Nuclear extracts and cytosolic extracts were prepared essentially as described [20]. NE-PER nuclear and cytoplasmic extraction reagents were used to prepare extracts. Briefly, cells were washed with cold PBS and then harvested with trypsin-EDTA and then centrifuge at 1200 rpm for 5 minutes. After the removal of the supernatant, ice-cold reagents were added to the cell pellet with a volume ratio of CER I: CER II: NER at 200: 11: 100. After a high-speed vortex for 15 seconds to fully suspend, the reactions were incubated on ice for 10 minutes and then ice-cold CER II were added, followed by a 5-second vortex and 1 minute incubation. After a 5-minute centrifugation at the maximum speed (15000 rpm), resultant supernatant (cytoplasmic extract) was

transferred to a clean pre-chilled tube and store at -80℃. Meanwhile, the insoluble (pellet) fraction was resuspended in ice-cold NER by vortexing for 15 seconds. This vortex procedure was repeated for 4 times with a 10-minute incubation on ice between each vortex. A centrifugation at the maximum speed (15000 rpm) was conducted after the final vortex and the resultant supernatant (nuclear extract fraction) was transferred to a clean tube and stored at -80℃.

Western blot analysis

Cell lysates were separated in a 10% or 15% polyacrylamide gel and transferred onto a PVDF membrane (Millipore Corporation, Milford, MA, USA). The blot was subsequently incubated with 3% non-fat milk in PBS for 1 hour to block non-specific binding, and probed with a corresponding antibody against a specific protein for 37℃ at 2 hours or overnight at 4℃, and then with an appropriate peroxidase conjugated secondary antibody for 1 hour. Extensive washing with wash buffer was conducted between each incubation and after the final washing, signal was developed by ECL detection system and relative photographic density was quantitated by a gel documentation and analysis system (Alpha Imager 2000, Alpha Innotech Corporation).

Cell transfection

Cells were grown on 6-well cell culture dish overnight and then transfected with 4 μg of pEGFPC1-LC3 [21] for 6 hours, followed by an indicated treatment. Afterwards, cells were fixed with 2% paraformaldehyde for 12 minutes and then incubated with 0.5% Triton X-100 for 10 minutes. Extensive PBS washing was conducted between each reaction to remove any residual solvent. The dot formation of GFP-LC3 was detected under a fluorescence microscope after drug treatment.

DAPI staining

4×10^5 cells were grown on 6-well cell culture dish overnight and followed treatment drug. After drug treatment, cells were fixed with 4% paraformaldehyde for 12 minutes. Extensive PBS washing was conducted between each reaction to remove any residual solvent. Cells were subjected to DAPI staining for 5 minutes and then observed under fluorescence microscopy equipped with filters for UV.

Mitochondrial membrane potential assay

To measure mitochondrial membrane potential, Huh7 cells were seeded into 6-well cell culture dish containing growth medium at a density of 2×10^5 cells per dish, and treated with dioscin for 24 hours. After the incubation, cells were washed and stained with 5 μg/mL JC-1. The mitochondrial membrane potential collapses, the monomeric JC-1 remains cytosolic and stains the cytosol with a green color in apoptotic cells. On the other hand, in nonapoptotic cells, JC-1 impulsively forms complexes, aggregates with intense red fluorescence. The loss of mitochondrial membrane potential was observed under fluorescence microscopy equipped with filters for Blue 488 nm and Green 543 nm.

Annexin V/PI double staining

To detect apoptosis in Huh7 cells after exposure to dioscin, an FITC Annexin V Apoptosis Detection Kit I was used to quantify cell numbers in different stages of cell death [22]. Briefly, 1×10^5 cells were resuspended in 100 μl 1× binding buffer (0.01 M Hepes/NaOH (pH 7.4), 0.14 M NaCl, 2.5 mM $CaCl_2$). With an addition of 5 μl of FITC Annexin V and 5 μl PI, the cell suspension was gently mixed and then incubated for 15 minutes at room temperature in the dark. Afterwards, 400 μl of 1× binding buffer was added to each tube followed by flow cytometry analysis within 1 hour.

Statistical analysis

Statistical significances of differences throughout this study were analyzed by One-way ANOVA test. A p value <0.05 was considered to be statistically significant. Values represent the means ± standard deviation and the

experiments were repeated three times.

Results

Dioscin induced cell death via apoptosis in Huh7 cells

To assess the effects of dioscin on cell viability, Huh7 cells were treated with dioscin and then analyzed with MTT assay and cell count assay. As shown in Fig. 1B and 1C, after a treatment with dioscin of various concentrations for 24 hours, cell viability were significantly reduced in a dose-dependent manner, as compared with that of untreated cells. Results from MTT assay showed that around 40% of cells survived after a treatment of dioscin at 5 μM. Since trypan blue staining indicating cell death, microscopic cell counting revealed a dramatic decrease in viable cell numbers in dioscin-treated Huh7 cells compared to that of control untreated cells.

Induction of apoptosis is dependent on the activation of caspase-3 and caspase-9 in dioscin-treated Huh7 cell lines

To determine whether dioscin-induced cell death is related to apoptosis, DAPI staining was performed to analyze the changes in nuclear morphology. Results revealed the condensed and fragmented nuclei at a concentration of 2.5 μM or higher of dioscin (Fig. 1D). Further, mitochondrial membrane potentials in dioscin-treated Huh7 cells were measured to discover that the mitochondrial membrane potential of Huh7 cells was decreased by dioscin treatment (Fig. 1E). Annexin V/PI double staining was also determined by flow cytometry and results showed an increased percentage of cells displaying phosphatidyl serine (PS) externalization in dioscin-treated Huh7 cells (Fig. 1F). To investigate underlying mechanisms involved in dioscin-induced apoptosis, apoptosis-related molecules were examined by western blotting. Results indicated that after a 24-hour treatment of dioscin, levels of cytochrome c release, cleaved PARP, activated caspase 3 and 9 were increased, while activated caspase-8 remained unchanged. On the other hand, decreased expression level of Bcl-2, an anti-apoptosis protein, was also detected in Huh7 cells treated with dioscin for 24 hours (Fig. 2A and B). To further confirm the involvement of caspase activation in dioscin-induced apoptosis, caspase specific inhibitors were used. Results shown in Figure 2C indicated that apretreatment with caspase-3 and -9 specific inhibitors both could effectively attenuate dioscin-induced cell apoptosis (Fig. 2C). These data suggest that dioscin-induced apoptosis is dependent on the activation of caspase-3 and caspase-9, but not that of caspase-8.

Dioscin-induced apoptosis was dependent on ERK1/2 activation and subsequent caspase-3/-9 pathway

Previous studies reported that mitogen-activated protein kinase (MAPK) family played as a multi-functional mediator of signal transduction processes, including cell death, differentiation, proliferation, and migration [23] and [24]. To investigate the possible role of MAPK pathways in dioscin-induced apoptosis, the expression levels of the phosphorylated forms of ERK1/2, p38MAPK, and JNK1/2 were examined by western blotting. Results shown that dioscin treatment may lead to an increase in the activation of ERK1/2 in a dose-dependent manner (Fig. 3A). Furthermore, cells were subjected to a pretreatment with MAPK specific inhibitors followed by a 24-hour treatment of 5 μM dioscin. Results from MTT assay and Annexin V/PI double staining showed that dioscin-induced apoptosis was attenuated by inhibiting ERK1/2 activation (Fig. 3B and C).

Induction of autophagy in dioscin-treated Huh7 cell lines

Previous data showed that, dioscin treated cells displayed characteristic apoptotic changes in cell morphology. Moreover, various numbers of vacuoles were observed in the cytoplasm at 12 hours after dioscin treatment (Fig. 4A). The formation of vacuoles in dioscin-treated cells are similar to that in cell autophagy [25], a general phenomenon that occurs when cells response to stress. To determine whether dioscin also

induces autophagy, LC3-II protein and Beclin-1, two autophagy-related proteins, were analyzed. As shown in Fig. 4B and 4C, after a transfection of pEGFPC1-LC3 and a treatment of dioscin for 12 and 24 hours, cytoplasmic LC3 formation was observed, which indicated the formation of autophagosomes, in cells treated with dioscin. A significant change of LC3 puncta formation was found as soon as 12 hours after a treatment of dioscin 2.5 μM while an increased LC3-II protein expression was also observed in at 24 hours at a dose-dependent manner. Compared to that of control, the protein expression of Beclin-1 was up-regulated by dioscin (Fig. 4D).

Dioscin-induced cell death was enhanced by the treatment of autophagy inhibitors

In order to clarify the interaction between dioscin-induced apoptosis and autophagy, two autophagy inhibitors acting at different stages, 3-MA and bafilomycin A1 (BafA1), were used in the following experiments. 3-MA, an autophagy inhibitor that can block autophagosome formation via the inhibition of type III PI-3K, was used as. Huh7 cells were pre-treated with 5 mM 3-MA for 1 hour and then dioscin for 24 hours and then subjected to western blotting. Results as for Fig. 5A indicated that 3-MA pretreatment decreased the protein levels of dioscin-induced LC3-II and Beclin-1 and enhanced the expression levels of cleaved PARP and cleaved caspase-3. Meanwhile, the percentage of annexin V-positive cells was also higher in cells pretreated with 3-MA (Fig. 5B). As for BafA1, an inhibitor of vacuolar ATPase to prevent the fusion between lysosomes and autophagosomes, cells were pretreated with BafA1 for 1 hour and then dioscin for 24 hours. As shown in Fig. 5C, cells pretreated with BafA1 were more susceptible to dioscin together with an increased percentage of annexin V-positivity (Fig. 5D).

Furthermore, a co-treatment of dioscin and Z-VAD-FMK, a broad-spectrum caspase inhibitor, was conducted to show that dioscin-induced increase of cleaved PARP and cleaved caspase-3, as well as the percentage of annexin V-positivity were abolished by Z-VAD-FMK, but dioscin-induced LC3-II and Beclin-1 expression remained unaltered (Fig. 5E and F). A similar result was obtained in the MTT assay (Fig. 5G). Clearly, inhibition of autophagy did not hinder dioscin-induced cell death, even further enhanced the cell toxicity of dioscin. Therefore, these results indicate that autophagy had a cytoprotective effect in dioscin-induced Huh7 cell death.

Discussion

Natural herbal products provide one of the most important sources for the development of novel chemotherapeutics, which have been practiced traditionally in various ethnic societies worldwide. Extensive studies indicate that these herbal may arrest the tumor promotion and progression in various human cancer cell lines by controlling cell proliferation, invasion or apoptosis. Our results show that dioscin may induce death of hepatic cancer cells. While dioscin-treated Huh7 cells showed significant changes in nuclei condense and mitochondrial membrane potential, the amounts of cleaved caspase-3, -9 and PARP were increased after treatment with dioscin together with a decreased expression of antiapoptotic proteins Bcl-2. These results indicated that dioscin may induce apoptosis of Huh7 cells through an activation of ERK1/2 signal pathway. The findings are in agreement with quercetin-induced apoptosis in A549 cells, and similar to the effects of saponins of 20-O-(beta-D-glucopyranosyl)-20(S)-protopanaxadiol (IH-901) [26] on ERK1/2 and subsequently cell death.

Autophagy is an important cellular response for various environmental stimuli, diseases and even cancers [27-29]. Many anticancer agents, including tamoxifen, rapamycin, arsenic trioxide and temozolomide were reported to induce autophagy [30]. Further investigation revealed that sulforaphane causes autophagy as a defense mechanism against apoptosis in PC3 and LNCaP prostate cancer cells [31] and 7,7″-Dimethoxyagastisflavone (DMGF) induced autophagic cell death in HepG2 cells [34]. In this study, dioscin resulted in apparent apoptosis at 24 hours, autophagy was observed as soon as 12 hours after dioscin was added to the culture medium (Fig. 4A and B) and the expression of LC3-II indicated that the induction of autophagy was dose-dependent (Fig. 4D).

Previous studies have suggested that autophagy can be induced by various compounds and involved in cell death or cytoprotection in HCC cell lines [17, 33, 34]. To further investigate the role of autophagy in dioscin-induced cell death, an autophagy inhibitor, 3-MA to show that 3-MA inhibited maturation of LC3-II, but could not decrease dioscin-induced cell death rather enhanced. Previous study has reported that inhibition of autophagy at different stages has opposite effects on cell survival [35] and in this study, inhibition of autophagy leads to enhanced apoptosis at early stages. Treatment with caspase inhibitor alone may restore a small percentage of cell viability. Neither inhibitor showed any protective effect suggesting that autophagy is an important mechanism in dioscin-induced apoptosis in Huh7 cell lines. Autophagy also serves as a critical defensive mechanism against common chemotherapeutic agents.

Autophagy is suppressed by functional p53 and certain cytoplasmic p53 mutants [36] and a previous study pointed out that LC3-II formation was not detected in DU145 cells treated with Zoledronic Acid [37]. Tasdemir et al. suggest that cytoplasmic p53 suppresses autophagy and p53 inhibition induces autophagy [38]. Furthermore, nuclear expression of p53 may stimulate autophagy by DRAM upregulation and mTOR inhibition [39, 40]. This suggests that p53 has the opposite effect on autophagy regulation. Therefore, dioscin-induced autophagy and apoptosis in Huh7 cells may be partially attributed to the lack of functional p53, which could be verified by additional experiments exploring the role of p53 in dioscin-induced autophagy. Furthermore, more experiments conducted in other types of cancer cells may provide more data to identify signaling pathways involved in dioscin-induced autophagy. Autophagic agonists have been described as anticancer drugs [41-43] with various compounds being shown to induce death in tumor cells with defective apoptotic machinery [44, 45]. Thus, with the capability to induce both the apoptotic and autophagic pathways, dioscin may be a good candidate for antitumor treatment.

In conclusion, this study is the first to demonstrate that dioscin suppressed cell growth and induced apoptosis in Huh7 cells through the activation of ERK1/2 signal pathway. By inhibiting cell growth and inducing autophagy in the early stage of dioscin-induced apoptosis, the anticancer properties of dioscin is quite promising. We also found that combination of dioscin with autophagy inhibitors may strengthen the efficiency of proapoptotic chemotherapeutic strategies, suggesting that autophagy protects cancer cells from the anticancer activity of dioscin in Huh7 cells.

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Figure 1

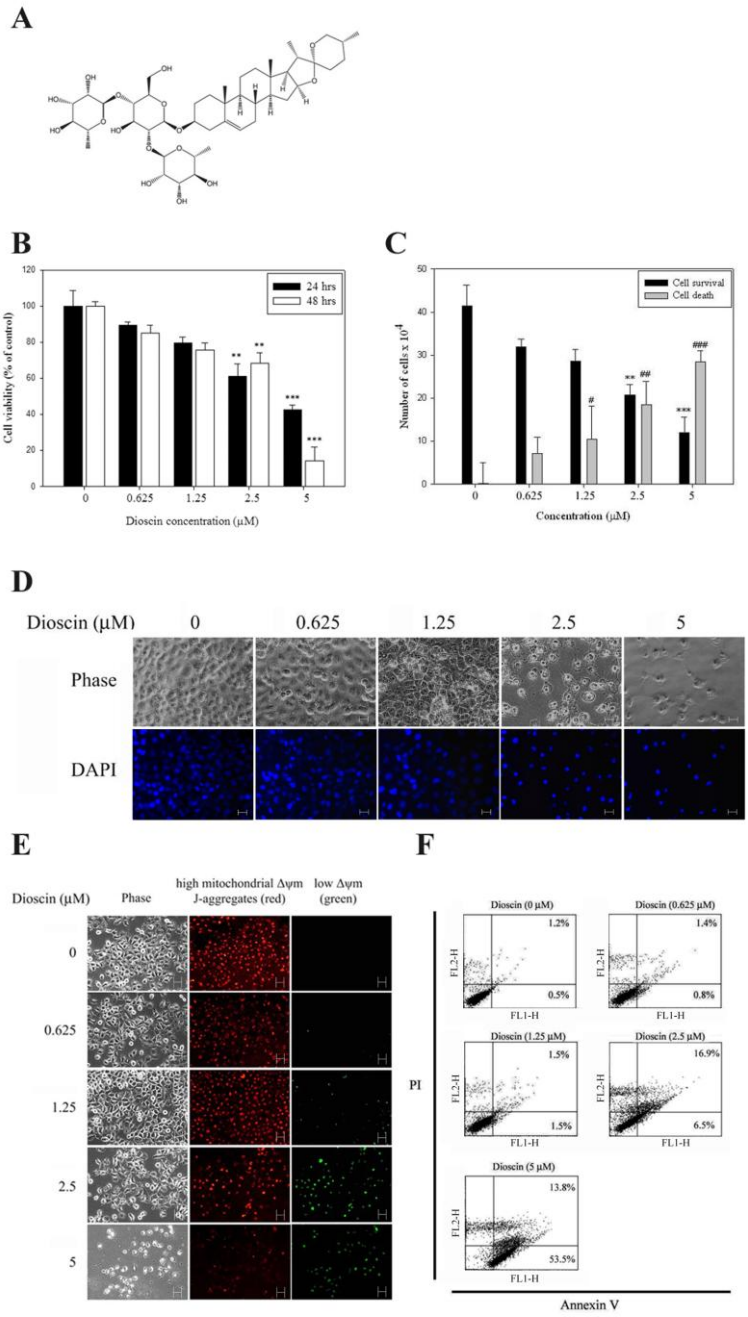


Figure 1. Dioscin exerts apoptotic effect on Huh7 cells. (A) Structure of dioscin. (B) Cell viability of Huh7 cells cultured in presence of dioscin for 24 and 48 hours, as analyzed by MTT assay. (C) Cell survival and cell death, determined by cell count, of Huh7 cell treated with dioscin for 24 hours. Cells were treated with an indicated concentration of dioscin for 24 hours and then subjected to DAPI staining (D) and JC-1 (E) followed by an observation under fluorescence microscopy. For quantitative analysis of apoptosis, dioscin-treated cells were harvested and then subjected to Annexin-V and PI double-stained flow cytometry (F). Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. **P<0.01; ***P<0.001, compared with the control (0 μM); #P<0.05; ##P<0.01; ###P<0.001, compared with the control (0 μM). Scale bars = 100 μm.

Figure 2

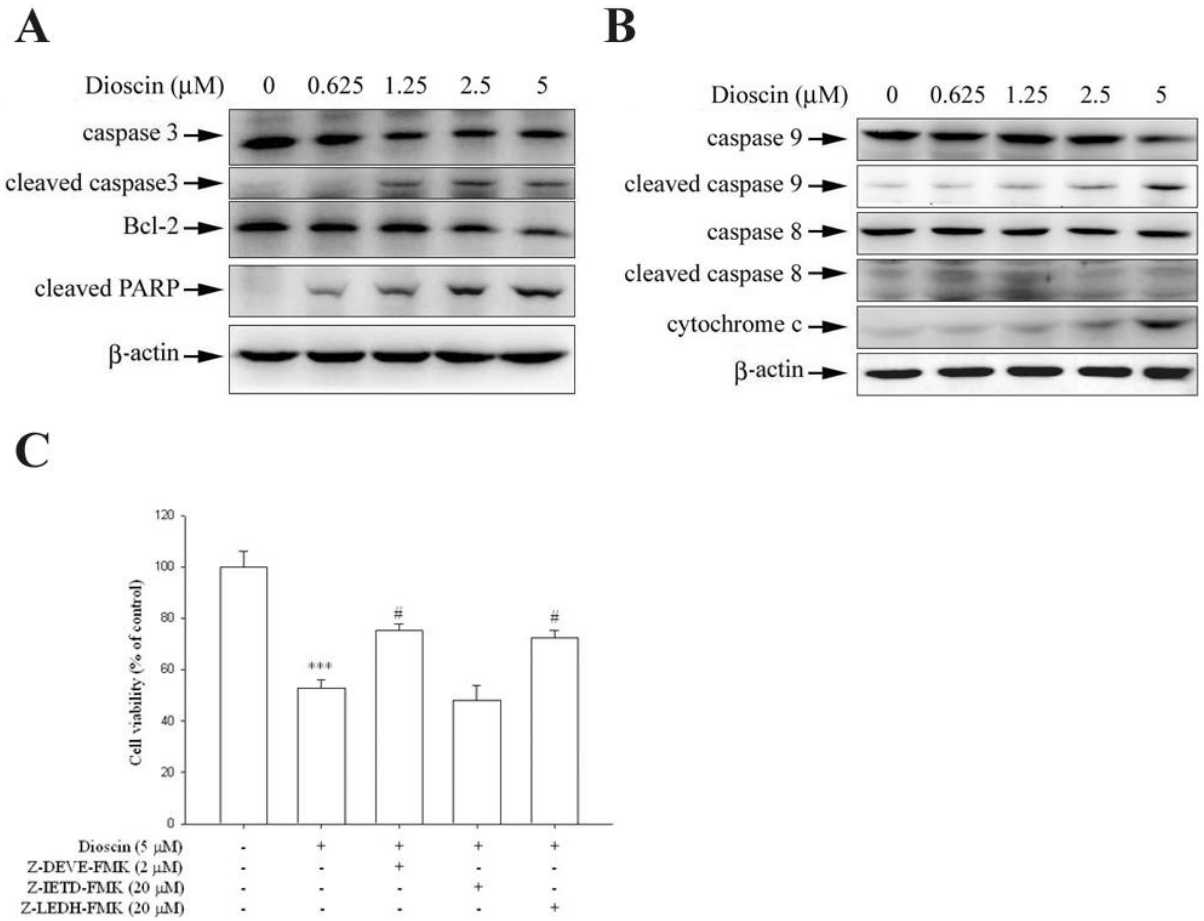


Figure 2. Dioscin may induce the activation of caspases in Huh7 cells. Cells were treated with an indicated concentration of dioscin for 24 hours and then analyzed by western blotting with an antibody against Bcl-2, PARP or caspase-3 (A). Meanwhile, the expression of cleaved caspase-8, -9 and cytochrome c release was also analyzed with that of β-actin as an internal control (B). Furthermore, Cell were treated with 5 μM dioscin for 24 hours in the presence or absence of 2 μM Z-DEVE-FMK, 20 μM Z-IETD-FMK, and 20 μM Z-LEHD-FMK and then subjected to MTT assay for cell viability (C). Results are shown as mean ± SD from 3 determinations per condition repeated 3 times. ***P<0.001, control versus dioscin; #P<0.05, dioscin versus Z-DEVE-FMK, Z-IETD-FMK, and Z-LEHD-FMK plus dioscin.

Figure 3

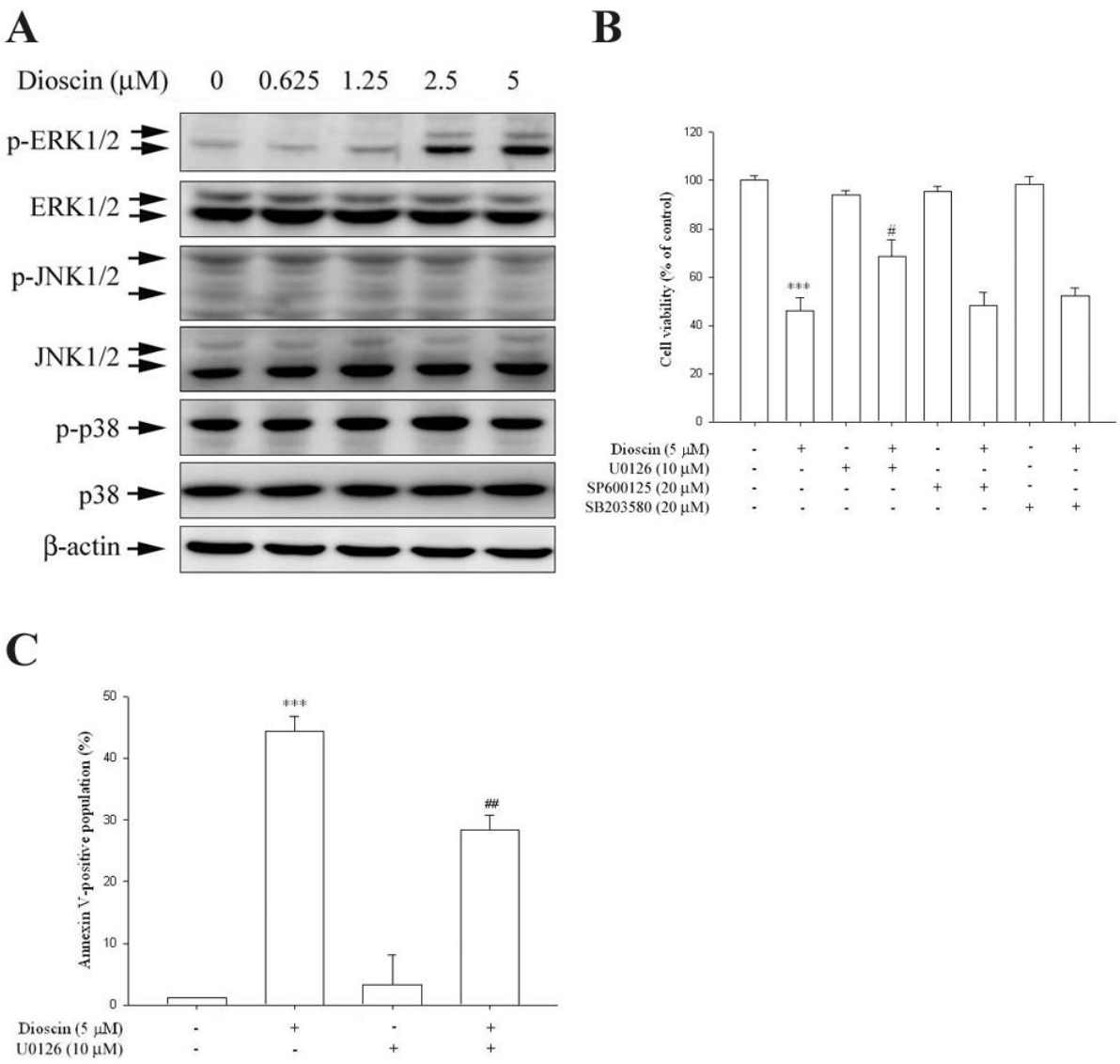


Figure 3. Dioscin may enhance the activation of MAPK in Huh7 cells. Cells were treated with an indicated concentration of dioscin for 24 hours and then analyzed by western blotting with an appropriate antibody to investigate the expression of phosphorylation of ERK1/2, JNK1/2, and p38 with β-actin acting as an internal control (A). Cells were treated with 5 μM dioscin for 24 hours in the presence or absence of 10 μM U0126, 20 μM SB203580, and 20 μM SP600125 and then subjected to MTT assay for cell viability Results are shown as mean ± SD from 3 determinations per condition repeated 3 times (B). Huh7 cells were treated with 5 μM dioscin for 24 hours in the presence or absence of 10 μM U0126 and then subjected to Annexin-V and PI double-stained flow cytometry (C). Results are shown as mean ± SD. ***P<0.001, control versus dioscin; #P<0.05, dioscin versus MAPK inhibitor plus dioscin; ##P<0.01, dioscin versus U0126 plus dioscin.

Figure 4

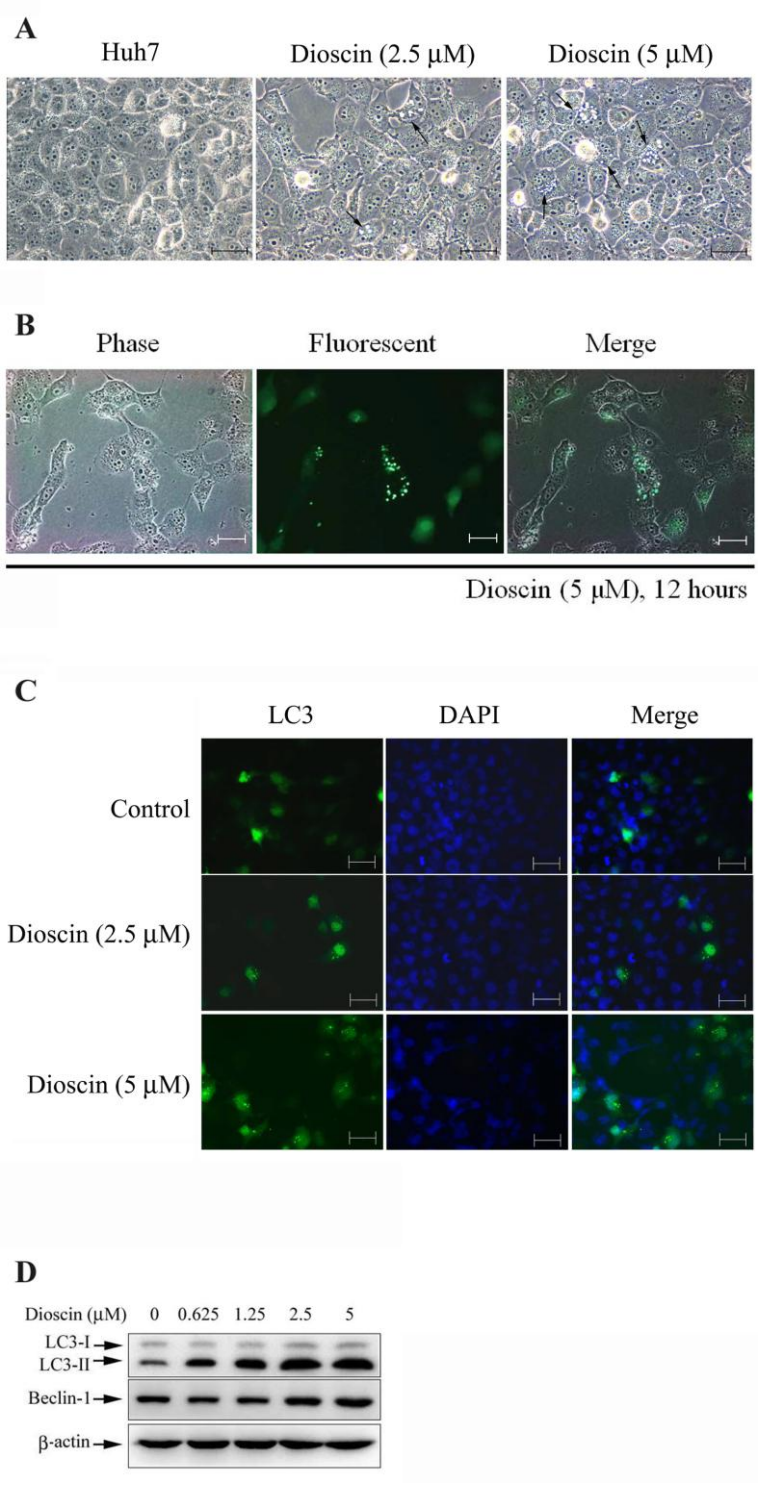


Figure 4. Autophagy was induced in dioscin-treated cells. Huh7 cells were treated with 2.5 and 5 μM dioscin for 12 hours and an microscopic observation revealed the formation vacuoles in cytoplasms of treated cells (A). After a successful transfection with pEGFPC1-LC3, Huh7 cells were treated with dioscin of 5 μM for 12 hours, followed by an observation for LC-3 (green fluorescence) under fluorescence microscopy (B). After a 24-hour treatment with dioscin, DAPI staining and subsequent fluorescence microscopy for LC-3 and DAPI (blue fluorescence) were conducted (C), as well as western blotting with antibodies against LC3 and beclin-1 (D). Scale bars = 100 μm.

Figure 5

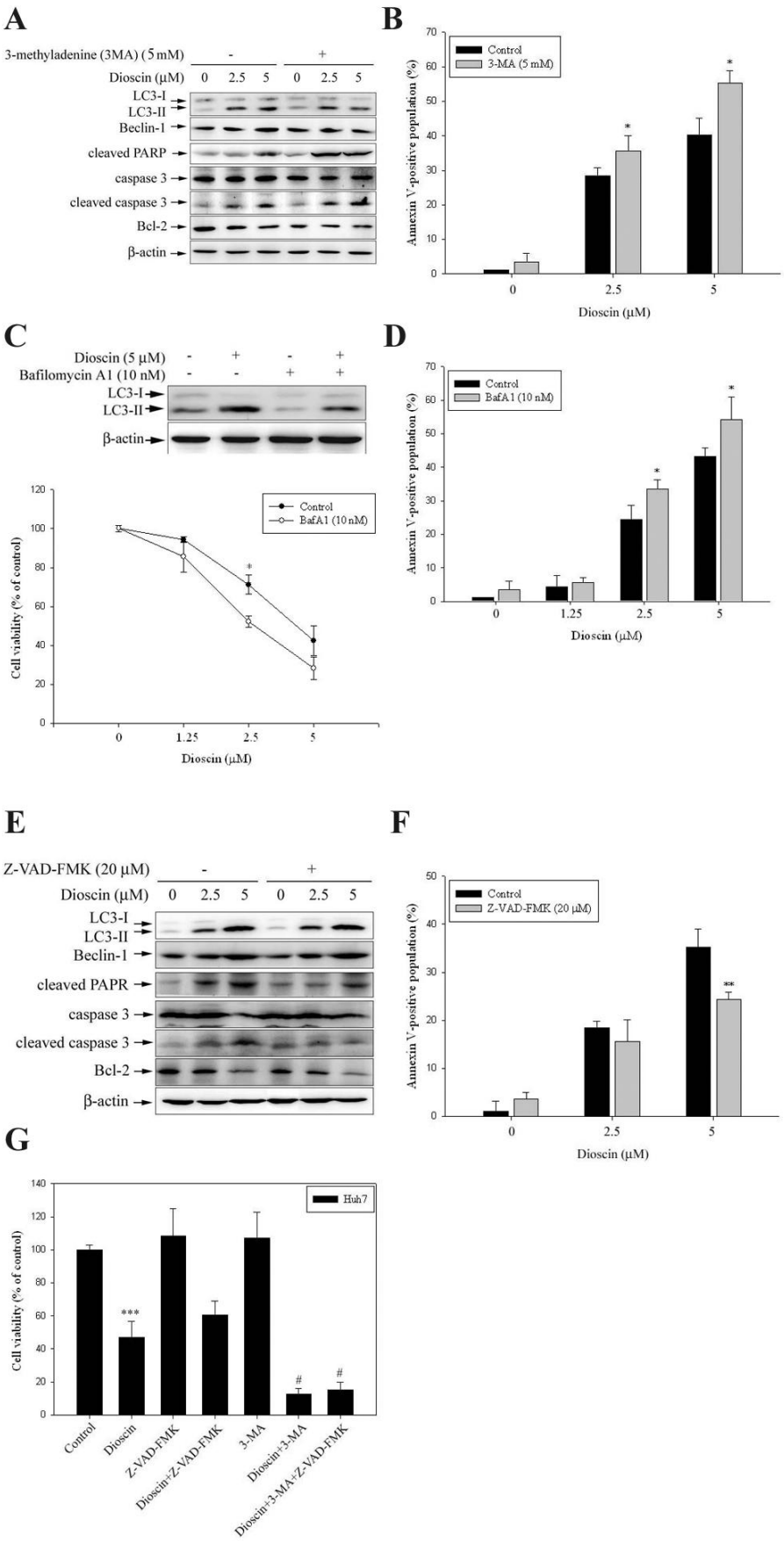

A
3-methyladenine (3MA) (5 mM)
Dioscin (μM)
LC3-I
LC3-II
Beclin-1
cleaved PARP
caspase 3
cleaved caspase 3
Bcl-2
β-actin
B
Control
3-MA (5 mM)
Annexin V-positive population (%)
Dioscin (μM)
C
Dioscin (5 μM)
Bafilomycin A1 (10 nM)
LC3-I
LC3-II
β-actin
Control
BafA1 (10 nM)
Cell viability (% of control)
Dioscin (μM)
D
Control
BafA1 (10 nM)
Annexin V-positive population (%)
Dioscin (μM)
E
Z-VAD-FMK (20 μM)
Dioscin (μM)
LC3-I
LC3-II
Beclin-1
cleaved PAPR
caspase 3
cleaved caspase 3
Bcl-2
β-actin
F
Control
Z-VAD-FMK (20 μM)
Annexin V-positive population (%)
Dioscin (μM)
G
Huh7
Cell viability (% of control)
Control
Dioscin
Z-VAD-FMK
Dioscin+Z-VAD-FMK
3-MA
Dioscin+3-MA
Dioscin+3-MA+Z-VAD-FMK

Figure 5. Autophagy inhibitors have effects on dioscin-induced cell death. Cells were treated with dioscin of 2.5 and 5 μM for 24 hours in the presence or absence of autophagy inhibitor 3-MA of 5 mM and then subjected to western blotting for LC3-II, beclin-1, cleaved PARP, caspase 3, Bcl-2 and β-actin (A). Subsequently, these treated cells were double-stained with Annexin-V and PI and then analyzed by flow cytometry (B). For another inhibitor BafA1, cells were treated with an indicated concentration of dioscin for 24 hours in the presence or absence of 10 nM BafA1 and then subjected to MTT assay for cell viability. The expression of LC3-II formation was investigated by western blotting with β-actin being an internal control (C). These treated cells were double-stained with Annexin-V and PI and subsequently analyzed by flow cytometry (D). Results are shown as mean ± SD. *P<0.05, dioscin versus BafA1 plus dioscin. Cells were treated with dioscin of 2.5 and 5 μM for 24 hours in the presence or absence of caspase inhibitor Z-VAD-FMK of 20 μM and then subjected to western blotting for LC3-II, beclin-1, PARP, caspase 3, Bcl-2 and β-actin (E). Subsequently, these treated cells were double-stained with Annexin-V and PI and then analyzed by flow cytometry (F). Furthermore, cells were treated with various combinations for 24 hours, including 20 μM Z-VAD-FMK, 5 μM dioscin plus 20 μM Z-VAD-FMK, 5 mM 3-MA, 5 μM dioscin plus 5 mM 3-MA, 5 μM dioscin plus 20 μM Z-VAD-FMK and 5 mM 3-MA, and then subjected to MTT assay for cell viability (G). Results are shown as mean ± SD (G). ***P<0.001, control versus dioscin; #P<0.05, dioscin versus 3-MA plus dioscin or Z-VAD-FMK and 3-MA plus dioscin.

附件二

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■ 達成目標

□ 未達成目標（請說明，以 100 字為限）

□ 實驗失敗

□ 因故實驗中斷

□ 其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

探討中草藥萃取物或是天然物的成分與效果，並進一步的應用到營養保健或是臨床癌症的輔助治療等方面，一直是目前許多研究機構努力追求的方向與目標，近年來本實驗室積極地開發新的研究方向與領域，並且在目前有新的研究成果與斬獲，而成果也持續地發表在 SCI 等國際期刊上與獲得國科會計畫的逐年補助，因此，我們認為這樣的研究成果不論在學術成就或是對於往後的癌症醫學治療上，能夠提供新的研究產物，並且也可進一步與產業界合作開發，若能夠持續地進行相關的研究，相信對於未來的應用價值上，將有無限的潛能值得開發。

國科會補助專題研究計畫項下出席國際學術會議心得報告

日期：102 年 7 月 20 日

計畫編號	NSC 101－2320 － B － 040 － 008 －		
計畫名稱	中草藥萃取物誘發肝癌細胞發生自噬反應（autophagy）及相關機制之探討		
出國人員 姓名	邱慧玲	服務機構 及職稱	中山醫學大學 教授兼任教務長
會議時間	101 年 8 月 18 日至 101 年 8 月 22 日	會議地點	德國・柏林
會議名稱	(中文) (英文) 2012 IFBLS World Congress		
發表論文 題目	(中文)補骨脂 (*Psorlaea corylifolia*)萃取物可增加具 DOC 抗藥性肺癌細胞的 ABCB1 表現量 (英文) Psorlaea Corylifolia Extract May Enhance the Expression Level of ABCB1 in DOC-resistant A549 Cells		

一、 參加會議經過

此次參與的國際會議是由 International Federation of Biomedical Laboratory Sciences 所主辦的二年一次國際會議。此次國際會議輪值到德國的柏林舉行，參加學者來自多國，主要來自歐洲及非洲各國，因台灣醫事檢驗學會及中華民國醫事檢驗師公會全國聯合會聯手爭取到 2014 年在台北主辦的緣故，此次台灣地區特別組了一團學者參加，除了觀摩會議舉辦方式外，也藉此宣傳下次在台北舉辦的訊息，希望屆時能衝高 2014 年到台灣參加的人數及地區。當天抵達柏林後即前往會議中心報到及領取大會議程及摘要手冊，聆聽多場特別演講，並於大會指定時段以電子壁報方式展示本實驗室的近期結果。有多位外國研究人員對實驗結果及假設表示高度興趣，也有多位學者提出問題討論。在閉幕式中，台灣團隊以精采的宣傳影片及高度團隊精神讓他國與會人員留下深刻印象。

二、 與會心得

本次大會的主題涵蓋很廣，從血清、生化到細菌及病毒等傳染疾病，還涵蓋實驗室管理及品質管理等等重要臨床檢驗領域。除了本身研究領域 C 型肝炎病毒以外，我也對肺結核桿菌的專題很有興趣。在參觀壁報論文及聆聽口頭報告過程中，與其他相關研究人員的諸多討論，也獲得很多寶貴的意見及想法。此次會議與多位非洲及亞洲地區研究學者及臨床檢驗人員討論，瞭解其他地區的研究現況，由於地域及就醫習慣的差異，有許多臨床檢驗分析上的相似及相異之處，尤其過去一般認為相對落後的東南亞及非洲地區的檢驗品質及研究正在急起直追，值得注意及探討。

三、 發表論文全文或摘要

Psorlaea corylifolia extract may enhance the expression level of ABCB1 in docetaxel-resistant A549 cells

Ming-Shih Lee, Ming-Ju Hsieh, Hui-Ling Chiou
School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Department of Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan, ROC

Abstract

Chemotherapy is suggested for treatment of advanced-stage cancers. However, the ability of cancer cells to become simultaneously resistant to different drugs, a trait known as multidrug resistance (MDR), limits the efficacy of chemotherapy. Our previous study has shown that extracts of Psorlaea corylifolia, a traditional Chinese herbal medicine, were capable of reversing the resistance in drug-resistant A549/D16. This study was conducted to further investigate the underlying mechanisms. Results from RT-PCR and quantitative real-time PCR showed that ABCB1 gene expression is decreased by a combination treatment of PCE (100 μg/ml) with DOC (16 nM). In addition, results from western blots and flow cytometry also revealed that protein expression level and activity of ABCB1 are decreased concurrently. These data showed that PCE may inhibit ABCB1 expression, at the transcriptional and translational levels, to affect ABCB1 activity and decreased anti-cancer drug efflux result, which eventually result in drug resistance reverse.

四、 建議

過去在台灣能爭取到大型國際會議的舉行相當不容易，此次國內醫檢相關學會及公會攜手合作下爭取到 2014 年的主辦權實屬不易。除了能提升在相關領域的國際能見度外，也能藉此宣傳台灣的觀光及知名度，對於外交及國際研究均有很大幫助。建議應多加舉辦如此大型會議或增加補助出國額度，讓年輕研究學者有機會與大師級學者學習。藉由此類國際會議出席，除了能瞭解國際相關領域之研究現況外，也能增進彼此之瞭解，進而增加國際合作之可能性。

五、 攜回資料名稱及內容

會議議程手冊及會議摘要手冊

30TH WORLD CONGRESS OF BIOMEDICAL LABORATORY SCIENCE

18-22 August, 2012 | Berlin, Germany

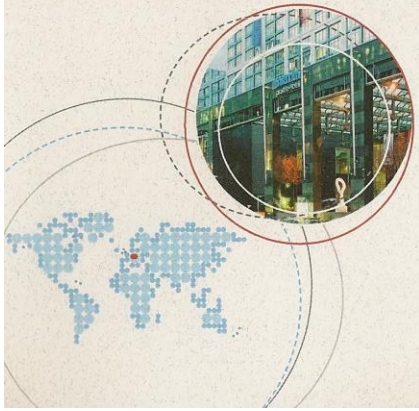
dvta IFBLS

Certificate of Attendance

This is to certify that

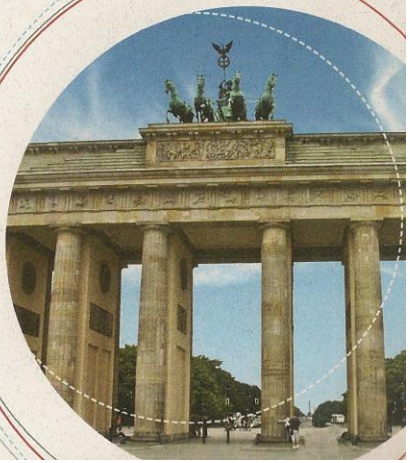
Hui Ling Chiou

Participated in
The 30th World Congress of Biomedical Laboratory Science
18-22 August, 2012 | Berlin, Germany



Annette Artelt
Chair of Organizing Committee

Vincent S. Gallicchio Ph.D
IFBLS President



Abstract number: 1902729

Psorlaea Corylifolia Extract May Enhance the Expression Level of ABCB1 in DOC-resistant A549 Cells

Chiou, Hui-Ling; Lee, Ming-Shih; Hsieh, Ming-Ju
School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taiwan

Chemotherapy is suggested for treatment of advanced-stage cancers. However, the ability of cancer cells to become simultaneously resistant to different drugs, a trait known as multidrug resistance (MDR), limits the efficacy of chemotherapy. Our previous study has shown that extracts of Psorlaea corylifolia, a traditional Chinese herbal medicine, were capable of reversing the resistance in drug-resistant A549/D16.
This study was conducted to further investigate the underlying mechanisms. Results from RT-PCR and quantitative real-time PCR showed that ABCB1 gene expression is decreased by a combination treatment of PCE (100 gg/ml) with DOC (16 nM). In addition, results from western blots and flow cytometry also revealed that protein expression level and activity of ABCB1 are decreased concurrently. These data showed that PCE may inhibit ABCB1 expression, at the transcriptional and translational levels, to affect ABCB1 activity and decreased anti-cancer drug efflux result, which eventually result in drug resistance reverse.

國科會補助計畫衍生研發成果推廣資料表

日期:2013/06/24

國科會補助計畫	計畫名稱: 中草藥萃取物誘發肝癌細胞發生自噬反應（autophagy）及相關機制之探討
	計畫主持人: 邱慧玲
	計畫編號: 101-2320-B-040-008- 學門領域: 寄生蟲學、醫事技術及實驗診斷

無研發成果推廣資料

101 年度專題研究計畫研究成果彙整表

計畫主持人：邱慧玲	計畫編號：101-2320-B-040-008-
計畫名稱：中草藥萃取物誘發肝癌細胞發生自噬反應（autophagy）及相關機制之探討	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	1	1	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

探討中草藥萃取物或是天然物的成分與效果，並進一步的應用到營養保健或是臨床癌症的輔助治療等方面，一直是目前許多研究機構努力追求的方向與目標，近年來本實驗室積極地開發新的研究方向與領域，並且在目前有新的研究成果與斬獲，而成果也持續地發表在 SCI 等國際期刊上與獲得國科會計畫的逐年補助，因此，我們認為這樣的研究成果不論在學術成就或是對於往後的癌症醫學治療上，能夠提供新的研究產物，並且也可進一步與產業界合作開發，若能夠持續地進行相關的研究，相信對於未來的應用價值上，將有無限的潛能值得開發。