Diosmin induces cell apoptosis through protein phosphatase 2A activation in HA22T human hepatocellular carcinoma cells and blocks tumour growth in xenografted nude mice

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Abstract

This study investigated the diosmin effect on HA22T human hepatocellular carcinoma cells in vitro and in an in vivo mouse xenograft model. HA22T cells were treated with different concentrations of diosmin and analysed with Western blot analysis, TUNEL, JC-1 staining and siRNA transfection assays. Additionally, the HA22T-implanted xenograft nude mice model was applied to confirm the cellular effects. Diosmin induced apoptosis, up-regulated death receptor apoptotic pathway markers as well as mitochondrial proteins. Pro-survival Bcl-2 family proteins were inhibited and the pro-apoptotic ones were increased. Protein phosphatase 2A (PP2A) siRNA or okadaic acid reversed the diosmin effects, confirming the role of PP2A in diosmin-induced HA22T apoptosis. The HA22T-implanted nude mice model revealed that diosmin inhibited tumour cell proliferation and enhanced tumour cell apoptosis. All our experimental evidence indicates that diosmin significantly promotes HA22T apoptosis and reduces tumour sizes in xenograft nude mice via PP2A in a dose-dependent manner.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for nearly 600,000 deaths per year (Palmer, 2008). Until now, apoptosis induction using various cytotoxic anti-cancer agents has been one of the most effective cancer therapy methods (Kim & Han, 2001). Alternative treatment options for HCC, along with other types of cancers, are in great demand. Comparatively, phytochemicals with fewer side effects at lower cost from natural resources have opened new avenues for the treatment of various diseases. Phytochemicals therefore stand out as promising candidates for cancer therapy.

Diosmin (3,5,7-trihydroxy-4′-methoxyflavone 7-rutinoside, C28H32O15) is a flavone (Fig. 1) (Yoo, Lee, Chung, Lee, & Kim, 2007) used for its phlebotonic properties as a vascular protector (Hitzenberger, 1997) and as a chemopreventive agent in urinary-bladder (Yang et al., 1997) and colon carcinogenesis (Tanaka et al., 1997). Diosmin has certain biological activities, it also exhibits an anti-inflammatory effect, and inhibition of prostaglandin synthesis (Lonchampt et al., 1989). It has been shown to possess antihyperglycaemic activity by stimulating insulin production from the existing β-cells of the pancreas (Pari & Srinivasan, 2010). Diosmin shows very good tolerability and is considered a safe, non-toxic drug (Hitzenberger, 1997) for the treatment of haemorrhoids and venous leg ulcers (El-Shafae & El-Domiaty, 2001).

Protein phosphatase 2A (PP2A), a phosphatase regulating many cellular functions, is genetically inactivated in many types of cancer.
There are links between an oncogenic kinase and a phosphatase with tumour suppressor activity, indicating that pharmacologic enhancement of PP2A represents a possible therapeutic strategy (Neviani et al., 2005). However, the anti-tumour and pro-apoptotic mechanisms of diosmin on human HCC are still not clearly understood. This study was designed to investigate whether diosmin could induce apoptosis in HA22T cells and investigate the molecular mechanisms of its anti-cancer properties in *in vitro* and *in vivo* models.

2. Materials and methods

2.1. Materials

Antibodies against AIF, Bad, Bak, Bax, Bcl-2, Bcl-xl, caspase-3, caspase-9, cytomegoc c, PP2A-Cα, p-PI3K, PI3K, t-BID, α-tubulin, donkey anti-goat IgG, goat anti-mouse IgG, and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibodies against p-Akt and p-Bad were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against IGF-I, p-IGF1R, IGF1R were purchased from Abcam, Ltd. (Cambridge, UK). Dulbecco’s modified Eagle’s medium (DMEM), Phenoll red-free DMEM, Foetal bovine serum (FBS) were purchased from Hyclone (Logan, UT). Okadaic acid (OA) (PP2A inhibitor, Cat. No. 495604) was purchased from Alexis Biochemicals (San Diego, CA). Diosmin (CAS, 520-27-4; ≥95% pure) was purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Reagents

Stock solutions were prepared in dimethyl sulfoxide (DMSO) and appropriately diluted in culture medium. The final DMSO concentration never exceeded 0.1%. According to the experimental design, the HA22T cell line was incubated with 0, 5, 20, 40, 80, and 120 μM of diosmin for 24 h. The dose for the animal model experiment was 15 and 30 mg/kg diosmin.

2.3. Cell culture

HA22T cells (BCRC No. 60168) were obtained from Bio-resources Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. Cells were seeded in cell culture flasks and maintained in a humidified incubator at 37 °C with 5% CO₂.

2.4. Animals

About 20 male NU/NU nude mice of 20–22 g in weight, 5 weeks in age, were obtained from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan) and given food and water *ad libitum*. The NU/NU nude mice were maintained at the China Medical University Animal Center for 2 weeks under guidelines for the use of animals before grouping and initiating the experiments. Mice were housed in a room maintained at 25 ± 1 °C with 55% relative humidity.

2.5. Cell morphological changes determinations

HA22T cells were grown in 6-well plates (at a density of 5 × 10⁴ cells/well) containing DMEM for 24 h. For dose-response experiments, different concentrations of diosmin (0, 5, 20, 40, 80, and 120 μM) were incubated in humidified air with 5% CO₂ at 37 °C for 24 h. At the end of each incubation period, cells were fixed with 4% paraformaldehyde for 20 min. After washing with PBS, photographs were taken to record the morphological changes in the HA22T cells, using an inverted fluorescent microscope.

2.6. TUNEL assay

The HA22T cells were cultured in a 24-well plate (5 × 10⁴ cells/well) with different concentrations of diosmin (0, 5, 20, 40, 80, and 120 μM) at 37 °C for 24 h. The cells were rinsed five times with PBS and fixed in 4% paraformaldehyde solution at room temperature for 1 h. The fixed cells were pre-treated with blocking buffer (3% H₂O₂ in methanol) for 10 min at room temperature. After a rinse with PBS, cells were treated with permeation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min at 4 °C. Following washing with PBS, 10× diluted TUNEL reagent (enzyme solution and label solution) (In Situ Cell Death Detection kit, Fluorescein; Roche, Mannheim, Germany) was added and the plate was placed in humidified air at 37 °C for 1 h for the reagent to react with cell nuclei. 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma–Aldrich, Cat. No. D9564) diluted 10,000× per well was added and the plate was covered with tin foil and rested for 25 min. The cells were finally observed with fluorescence microscopy.

2.7. Western blot analysis

HA22T cells were scraped and washed once with phosphate-buffered saline (PBS). Cell pellets were lysed for 30 min in lysis buffer (50 mM Tris, 0.5 M NaCl, 1.0 mM EDTA, 1% glicerol, 1 mM β-mercaptoethanol, 1% NP40, and proteinasinhibitor cocktail tablet) and spun down at 12,000g for 10 min. Tissue samples were homogenised with ice-cold PBS and then subjected to lysis in a solution containing 20 mM Tris, 2 mM ethylenediaminetetraacetic acid (EDTA) and 1% glicerol. Supernatants were obtained after centrifugation at 12,000g for 40 min. After boiling, proteins were separated on a 12% SDS–PAGE gel with a constant voltage of 75 V for 2.5 h. Proteins were then transferred to a polyvinylene difluoride (PVDF) membrane (0.45-μm pore size, IPVH00010, Millipore, Bedford, MA), with a transfer apparatus, at a constant voltage of 100 V for 2 h. The membranes were then blocked with 5% non-fat milk in 0.05% Tween-20 in PBS for 1 h at room temperature and then incubated with primary antibodies. The membranes were washed with 10 mM Tris, 150 mM NaCl, and 0.05% Tween-20 and then incubated with goat anti-mouse IgG, goat anti-rabbit IgG, or donkey anti-goat IgG secondary antibodies at 1:2000. The immunoblotted proteins were visualised using an ECL Western blot analysis luminal reagent and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Fujifilm, Tokyo, Japan).

2.8. JC-1 staining

JC-1 (5,5’,6,6’-tetrachloro-1,1’,3,3’-tetracyethylbenzimidazolocarbocyanine iodide) (Sigma–Aldrich, Cat. No. C50390) is a lipophilic fluorescent cation that can incorporate into the mitochondrial membrane, where it can form aggregates, due to the mitochondria physiological membrane potential state. Briefly, HA22T cells were cultured in a 6-well plate (2 × 10⁴ cells/well) with different concentrations of diosmin (0, 5, 20, 40, 80, and 120 μM) at 37 °C for 24 h. The cells were washed three times with PBS and incubated with medium containing JC-1 staining reagent at 37 °C for 20 min.
followed by washing with PBS. The stained cells were examined under an Olympus CKX41 fluorescence microscope. JC-1 formed aggregates in the apoptotic mitochondria while it remained a monomer in the normal mitochondria.

2.9. Gene knockdown using siRNA

HA22T cells were seeded into 6-well plates and grown to 80% siRNA transfection was carried out with DharmaFECT Duo transfection reagent (Dharmacon, Inc., Lafayette, CO). The 100–μL PP2A-Cx siRNA (A: Sense: GCAAAUCACCAUAACAAAtt, Antisense: UUUGUAUCUGGUAUUUCUt; B: Sense: GAACUGAGCAUCUCUCAtt, Antisense: UCAUAAUUUGAGUGGUGt and C: Sense: GGAUACGACGAAACAAUCAtt, Antisense: AUAAU GAUGUGGCAAU UCtt) and 100 μL negative control transfect Non-Targeting Pool (NT) (Dharmacon, Inc.) were mixed with 100 μL serum-free medium. At the same time, a sufficient amount of DharmaFECT Duo reagent was diluted to 1:50 in serum-free medium and incubated for 5 min at room temperature. The two mixtures were combined by careful pipetting and the plates were incubated at 37 °C for 20 min, to allow for transfection complex formation. After adding sufficient serum-free medium for 24 h, the serum-free medium was removed and then cultured with a medium containing 10% fortified bovine calf serum. Cells were incubated at 37 °C in 5% CO₂, and then harvested 48–96 h post-transfection for protein expression analysis. Specific silencing was confirmed by immunoblotting with cellular extracts, 72 h after transfection.

2.10. Liver cancer tumour model

The NU/NU nude mice were divided into three groups with each group containing six animals. In group I, HA22T cells (1 × 10⁶ in 100 μL DMEM) were subcutaneously injected into the left flank of NU/NU mice as a control. Group II was injected with HA22T cells and orally treated with 15 mg/kg diosmin. Group III was injected with HA22T cells and orally treated with 30 mg/kg diosmin. Four days after tumour inoculation the mice were given daily oral treatment with diosmin. Tumour volumes were measured every three days after tumour inoculation. The tumours were excised and weighed.

2.11. Immunohistochemistry

Tissue specimens from sacrificed NU/NU mice were collected and immediately fixed in 10% buffered formalin overnight, embedded in paraffin, and sectioned to 4-μm thicknesses. The tumour sections were immobilised and deparaffinised by immersing in xylene, dehydrated in a graded series of ethanol and washed with distilled water. For antigen retrieval, the tumour sections were boiled in 0.1% so- dium citrate (in 0.1% Triton X-100) for 10 min. Next, following washing twice with PBS, endogenous peroxidase activity was blocked by incubation in 3% H₂O₂-methanol for 10 min at room temperature. The sections were stained with antibodies for proliferating cell nu-

clear antigen PCNA and caspase-3 overnight at 4 °C. Terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining was performed using a TACS™ 2 TdT-DAB In Situ Apoptosis Detection kit (Gaithersburg, MD, USA). Images were taken using an inverted Olympus cxa 41 fluorescence microscope.

Proliferative index (%) = (No. of PCNA positive cells/total cells) × 100.

Apoptotic index (%) = (No. of caspase-3 positive cells/total cell) × 100 = (No. of TUNEL positive cells/total cell) × 100.

2.12. Statistical analysis

Each sample was analysed based on results that were repeated at least three times using SigmaPlot 10.0 software. The results were presented as mean ± SE, and statistical comparisons were made using the Student’s t-test. In all cases, differences at p < 0.05 were regarded as statistically significant.

3. Results

3.1. Induction of apoptosis by diosmin in HA22T cells

HA22T cells were treated with various doses of diosmin. As shown in Fig. 2A, untreated HA22T cells grew well with clear skeletons, while cells treated with diosmin, were distorted and some became rounded. The floating cells increased with increasing drug concentrations.

To confirm the morphological changes in the apoptosis data, DNA fragmentation was then detected using the in situ DNA labelling TUNEL assay, which is a very sensitive indicator of apoptosis. As shown in Fig. 2B, approximately 0.67%, 10.02%, 14.58%, 32.17%, 49.14% and 77.34% TUNEL-positive cells (apoptotic cells) were observed after 24 h of diosmin treatment. The data from the TUNEL assay also support that treatment with diosmin induced apoptotic cell death in human hepatoma cell lines.

3.2. Diosmin induces apoptosis in HA22T cells through the mitochondria-dependent apoptotic pathway

We further examined the diosmin effect on the Bcl-2 family proteins, essential components of the apoptotic pathways. As shown in Fig. 3A exposure of HA22T cells to diosmin for 24 h markedly increased the protein expression of the pro-apoptotic Bax, Bak, and Bad, with a concomitant decrease in the anti-apoptotic Bcl-2, Bcl-xL expression, p-Bad proteins dose-dependently, compared with the untreated control, thereby the Bax/Bcl-2 ratio would be increased (Fig. 3B). Based on these findings, it can be concluded that diosmin induced apoptosis in HA22T cells through Bcl-2 family modulation.

We also examined whether the mitochondrial-mediated apoptotic pathway was involved in diosmin-induced apoptosis. As shown in Fig. 3C, the expression levels of cytochrome c, caspase-9, and caspase-3 in HA22T cells were enhanced by diosmin in each of groups 1–5, as compared to the control group.

Since the apoptosis-inducing factor (AIF) protein is one of the death protein candidates that serves in the mitochondrial-related caspase-independent pathway (Espinosa et al., 2006), we investigated whether the AIF protein was involved in cell death induced by diosmin. In our experiments, the level of AIF protein expression was significantly increased after the treatment of HA22T cells with 0, 5, 20, 40, 80, and 120 μM diosmin for 24 h (Fig. 3C).

These observations imply that translocation of AIF from the mitochondria into the cytosol plays an important role in the early stages of the apoptotic process and confirm that this apoptosis pathway is involved in mitochondrial function. Moreover, it is possible that the caspase-independent apoptotic pathway via AIF protein translocation protein from the mitochondria into the cytosol may also be activated by diosmin treatment. Mitochondria permeability transition results in cytochrome c release from the mitochondrial intermembrane space into the cytoplasm (Hengartner, 2000). Treatment with diosmin at concentrations greater than 5 μM induced the cytoplasmic release of cytochrome c, confirming mitochondria-dependent pathway activation. This result suggests that diosmin induces apoptosis in human liver cancer cells through the mitochondria-dependent pathway.
Fig. 2. Diosmin induces apoptosis in human HA22T liver cancer cells. The HA22T cells were treated with different concentrations of diosmin (0, 5, 20, 40, 80, and 120 µM) for 24 h. (A) The morphological changes of viable HA22T cells after treatment with diosmin. Photographs were taken on the morphological changes in HA22T cells and then observed under an inverted light microscope. (B) DAPI was used to label nuclei (upper panels) and apoptotic cells nuclei were labelled by TUNEL stain (lower panels). (C) Partition of positive apoptotic cells was based on percentages calculated from three sections of each treatment as described in Section 2. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 values based on comparisons with untreated controls.

Fig. 3. The diosmin effects on mitochondria-dependent apoptotic pathways in human hepatoma cell line HA22T. The HA22T cells were treated with different concentrations of diosmin and the cells were incubated for 24 h then the total protein samples were obtained. (A) Regulation of the Bcl-2 family proteins by diosmin. (B) Bars represent the relative quantification of Bcl-2, Bax, and p-Bad on the basis of control level. (C) The protein expression levels of cytochrome c (cyt c), caspase-9, caspase-3, and apoptosis inducing factor (AIF) were measured by Western blotting. (D) Bars represent the relative quantification of cyt c, caspase-9, caspase-3, and AIF compared to the control. Equal loads were assessed with an anti-α-tubulin antibody. Cells cultured without treatments were used as control. The quantitative results are expressed as the mean value ± SE (n = 3). *p < 0.05, **p < 0.01 and ***p < 0.001 versus the control group (line 1). (E) The diosmin effects on the mitochondrial outer membrane permeability of HA22T cells. FITC (green) signals represent unstable mitochondrial outer membrane permeabilisation (MOMP) and Cy3 (red) signals represent normal mitochondrial membrane permeability. Comparison of the ratio of green and red images implies the damage degree of MOMP on HA22T cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
The mitochondrial membrane depolarisation and the associated mitochondria damage was evaluated using JC-1 staining. When the electron transport chain (ETC.) functions abnormally, the mitochondrial membrane potential becomes unstable and JC-1 appears as a JC-1 monomer, which emits at 530 nm and can be detected with FITC (green). This indicates a mitochondrial outer membrane permeabilisation (MOMP) event in a cell. Changes in Cy3 (red) and FITC emission intensities can be used to detect changes in the mitochondrial membrane potential and therefore changes in the cellular metabolic state. In control cells (i.e., no drug treatment), there were only strong Cy3 signals and no FITC (Fig. 3E). Administration of 5, 20, 40, 80, and 120 μM diosmin resulted in weak Cy3 signals and an in-
crease in FITC signals. It is thus concluded that diosmin treatment significantly decreased the mitochondrial membrane potential of HA22T cells in a dose-dependent manner.

3.3. Okadaic acid (OA) inhibits diosmin-induced apoptosis mediated through the down-regulating of PP2A-Cx in HA22T cells

We further determined the roles of PP2A on the diosmin-inhibited HA22T cell proliferation. HA22T cells were pre-treated with OA, a pharmacological inhibitor of PP2A, followed by treatment with 80 μM diosmin for 24 h.

Diosmin (80 μM) treatment significantly inhibited the expression of Bcl-2, and is accompanied with an increase in PP2A-Cx, t-BID, Bax, cytochrome c, caspase-9, and caspase-3 expression. However, this situation was totally reversed after treatment with OA in the presence of diosmin (Fig. 4A).

3.4. PP2A-Cx siRNA blocks diosmin-induced apoptosis in HA22T cells

To further confirm that diosmin induces HA22T cell apoptosis through PP2A, we transfected HA22T cells with PP2A-Cx siRNA. The Western blot analysis assay results showed a significant reduction in PP2A-Cx, t-BID, Bax, cytochrome c, caspase-9, and caspase-3 proteins level in HA22T cells, accompanied with an increased Bcl-2 protein level (Fig. 4B). Therefore, our results strongly suggested that PP2A is an important mediator of diosmin-induced HA22T cell apoptosis.

3.5. Diosmin suppresses tumour growth in vivo

We further examined whether the strong diosmin anti-proliferative and apoptosis effects detected in cell culture experiments could be observed in an in vivo model.

NU/NU mice bearing subcutaneously implanted HA22T cells were given a daily oral dose of diosmin. In a pilot study, we used 15 and 30 mg/kg of diosmin in an in vivo study. The HA22T xenograft growth was monitored every three days for 2 weeks. Side effects, such as body weight loss, mortality, and lethargy were not observed in mice treated with diosmin for 2 weeks. The final tumour size, as shown in Fig. 5A and B, was markedly smaller in the majority of mice treated with 15 or 30 mg/kg of diosmin. The tumour weight was significantly suppressed by treatment with diosmin at these doses (Fig. 5C and D), and the overall data suggests a dose–response trend. The most effective anti-hepatoma response was elicited at 30 mg/kg of diosmin. Only one nude mouse in the negative control group (mice injection of HA22T cells and without diosmin treatments) died before sacrifice, due to the highly proliferative tumour and probably massive invasion. These results strongly indicate that diosmin suppresses tumour growth in vivo.

3.6. Diosmin inhibits tumour cell proliferation and increase tumour cell apoptosis in vivo

To assess the antiproliferative effect of diosmin in vivo, paraffin-embedded tumour sections were immunohistochemically stained for PCNA, to estimate the proliferation index (Fig. 6A). Diosmin significantly decreased PCNA expression; the magnitude of change was 67.83% and 81.31%, respectively, in the 15 and 30 mg/kg diosmin-treated groups (Fig. 6C). The detection of caspase-3 (Fig. 6A and D) and TUNEL (Fig. 6B and E) positive cells for the apoptosis indices indicated more than 3.04- and 8.86-fold increase in apoptosis due to 15 and 30 mg/kg diosmin treatment. These data therefore support the in vivo involvement of caspase-mediated apoptosis as a key contributor in tumour growth suppression induced by diosmin, which is consistent with the in vitro results.

Fig. 5. Diosmin effect on the growth of xenografted HA22T tumour tissue in nude mice. (A) Representative pictures of drug-treated hepatomas. Red arrows indicate tumour mass. (B) Diosmin effect on final tumour volumes. (C) Photographic records of final harvested tumours. (D) Effect of diosmin on final tumour weight. Data were expressed as means ± SE, n = 5. The statistically significant differences compared with control were calculated by the Student’s t-test (*p < 0.05, **p < 0.01 and ***p < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Western blot analysis of the PP2A-Cα, t-BID, Bax, cytochrome c, caspase-9, and caspase-3 expression levels in the tumour cells of mice treated with different doses of diosmin are shown in Fig. 6F. In our experiments, the PP2A-Cα, t-BID, Bax, cytochrome c, caspase-9, and caspase-3 expression levels in the tumour cells of the mice in group 2 (positive control) were lower than those in group 1 (negative control; mice without injection of HA22T cells and without diosmin treatments). However, the expression levels of PP2A-Cα, t-BID, Bax, cytochrome c, caspase-9, and caspase-3 significantly increased when the diosmin concentration was increased in groups 3 and 4 (Fig. 6F). The expression level of Bcl-2 in the tumour cells in mice in group 2 was higher than that in the tumour cells of mice in group 1 (negative control; mice without injection of HA22T cells and without diosmin treatments). We investigated whether the apoptotic pathway is involved in HA22T cell death caused by diosmin. Our results confirmed apoptosis induction, as evident by the presence of membrane blebbing and apoptotic bodies (Figs. 2A, B and 3E).

The apoptotic signalling pathway that leads to caspase activation can be subdivided into two major categories: death receptor-mediated and mitochondria-mediated pathways (Jin & El-Deiry, 2005). The tumour necrosis factor (TNF) family death receptors, such as Fas and TNF-R1, are the best understood death pathways. Recruitment of procaspase-8 through FADD leads to suppression of the apoptotic machinery is a hallmark of cancer development and, thus, apoptosis induction in cancer cells is a useful method for treatment (Kornblau, 1998). The aim of this study was to evaluate diosmin, a compound newly developed, as a therapeutic agent for malignant human liver cancer. We found that diosmin significantly induced apoptosis in HA22T cells both in vitro and in vivo.
its auto-cleavage and activation, which in turn, activates effector caspases, such as caspase-3 (Thorburn, 2004). Many investigators have demonstrated that mitochondria are the key regulators of apoptosis (Green & Reed, 1998). Mitochondria have been shown to be involved in integrating different pro-apoptotic pathways via cytochrome c release into cytosol (Green & Reed, 1998), which is associated with mitochondrial membrane potential loss (MMP) and an increased production of reactive oxygen species (Xiang, Chao, & Korsmeyer, 1996). The released cytochrome c induces caspase family activation (aspartate-specific cystein proteases) (Kroemer & Reed, 2000).

There is accumulated evidence that cytochrome c release from mitochondria is an important step in apoptosis (Reed, 1997). Alteration in the mitochondrial function in general and mitochondrial permeability transition induction in particular plays a key part in apoptosis regulation (Green & Reed, 1998). In this study, diosmin disrupted the mitochondrial membrane potential (Fig. 3E) and caused the release of cytochrome c as well as proteolytic activation of caspase-9 and caspase-3 in a dose-dependent manner (Fig. 3C). These results provide evidence that diosmin caused apoptosis in a human liver cancer cell line through the mitochondria-dependent apoptotic pathway.

AIF is a caspase-independent apoptosis effector that can be released from the mitochondria into the cytosol (Kagan et al., 2009) and is believed to be involved in caspase-independent cell death (Espinosa et al., 2006). Correspondingly, our Western blot analysis revealed that HA22T cells treated with 0, 5, 20, 40, 80, and 120 μM diosmin for 24 h resulted in increased AIF production (Fig. 3C). These findings indicate that Bax translocation allows the release of AIF protein from the mitochondria into the cytosol by diosmin, which means diosmin induced apoptosis through a caspase-independent pathway as well.

The Bcl-2 family proteins (pro-apoptotic and anti-apoptotic proteins) and caspases are critical regulators of the apoptotic pathway (Antonsson & Martinou, 2000). Reports have demonstrated that translocation of the pro-apoptotic Bax into the mitochondria can alter the permeability of cytochrome c, followed by activation of the post-mitochondrial caspase cascade e.g., caspase-9, caspase-8, and caspase-3, leading to apoptotic cell death (Kluck, Bossy-Wetzel, Green, & Newmeyer, 1997).

Consistent with this process, our present study revealed that HA22T cells treated with diosmin (0, 5, 20, 40, 80, and 120 μM) for 24 h, markedly decreased Bcl-2, Bcl-xL, and p-Bad proteins expression, whereas Bax, Bak, and Bad proteins expression was increased (Fig. 3A). Overexpression of Bax has been reported to accelerate apoptosis, whereas Bcl-2 represses the death function of Bax (Zhao, Guo, You, Wu, & Gu, 2004). Thus, an increased Bax/Bcl-2 ratio is observed in apoptotic cells (Fan et al., 2007). Our results indicated that diosmin promoted pro-apoptotic Bax and Bad levels and inhibited the levels of anti-apoptotic Bcl-2 and Bcl-xL; and, hence, an increase in the Bax/Bcl-2 ratio occurred (Fig. 3A and B), which may lead to the release of cyt c, procaspase-9 and AIF from the mitochondria to the cytosol, inducing cell apoptosis.

PP2A is a key enzyme in this regulatory network. It is widely distributed throughout the animal and plant kingdoms and appears to be critical in regulating a number of physiological processes, via dephosphorylation of a cohort of specific target proteins (Kinoshita, Ohkura, & Yanagida, 1990). Inhibition of PP2A by OA results in major cytotoxic effects (Cohen, Holmes, & Tsukitani, 1990) and the inhibition of PP2A activity at later stages causes mitotic defects (Cohen, 1991). Consequently, the specific inhibition of PP2A may promote the cell cycle progression at the start/restriction point (Cohen, 1991). Therefore, we further applied PP2A inhibitor and siRNA assay to examine the role of PP2A involved in diosmin-induced HA22T cell apoptosis. In this study OA totally reversed the diosmin-mediated up-regulation of PP2A-Cα, t-BID, Bax, cytochrome c, caspase-9, and caspase-3. In addition, OA reversed the diosmin mediated down-regulation of Bcl-2 as well (Fig. 4A). These findings suggest that targeting PP2A may be a feasible way to affect the pivotal apoptotic signal pathway. Furthermore, silencing PP2A by RNA-interference blocked the diosmin-mediated HA22T apoptotic effect, confirming that PP2A is indispensable for mediating the effects of diosmin (Fig. 4B). Taken together, the in vitro findings suggest that diosmin-induced HA22T apoptosis in a dose-dependent manner mediated through PP2A. Our current data may provide some significant information for future clinical applications.

Moreover, we found that diosmin dramatically suppressed tumour cell proliferation and induced tumour cell apoptosis in nude mice model (Figs. 5 and 6). Diosmin at a concentration of 30 mg/kg showed a strong effect and selectively triggered cancer cell death via inducing the classical apoptotic pathway and suppressing the proliferation of human hepatoma cells in vivo. Based on our findings, diosmin was able to induce human hepatocellular carcinoma HA22T cell apoptosis, which was confirmed using in vitro and in vivo systems. The involved signal pathways are sketched and shown in Fig. 7.

![Fig. 7. A schematic representation shows the molecular mechanism of diosmin-induced human HA22T hepatocellular carcinoma cell apoptosis and inhibition of xenografted HA22T tumour growth in nude model. Diosmin induces cell apoptosis in a dose-dependent manner through PP2A, enhances the death receptor and mitochondrial-dependent apoptotic pathway, and expression of the pro-apoptotic Bcl-2 family members. These results present the molecular mechanism behind diosmin-induced HA22T cell apoptosis and xenografted HA22T tumour suppression.](image314x115 to 541x484)
5. Conclusions

All of our experimental evidence indicates that diosmin significantly promotes HA22T apoptosis and reduces tumour sizes in xenograft nude mice via PP2A in a dose-dependent manner. In the near future we would like to further investigate the diosmin anti-cancer effect using preclinical studies and clinical trials. Efforts aimed at enhancing diosmin function and/or activity may provide an alternative therapy against liver cancer.

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