行政院國家科學委員會專題研究計畫 期末報告

探討結構相關之不同黃酮類化合物在骨癌細胞其細胞轉移、增生及細胞凋亡之相關性

計 畫 類 別 ：個別型
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中 華 民 國 102 年 09 月 30 日

中文摘要：類黃酮為多酚類化合物中的一個族群，廣泛存在於蔬菜與水果之中，而類黃酮具有許多生物活性，包含抗氧化、抗微生物與抗發炎，近來越來越多的研究中發現，類黃酮具有抗癌與抗癌化的作用。因此，首先分析幾個黃烷酮類藥物（黃烷酮、2’-羥基黃烷酮、3’ -羥基黃烷酮、4’-羥基黃烷酮、5’-羥基黃烷酮、6’ -羥基黃烷酮、7’ -羥基黃烷酮）對骨肉瘤細胞株（U-2 OS、143 B、MG-63、SaoS2）的影響，MTT 分析細胞存活率的結果發現，黃烷酮、2’ -羥基黃烷酮與 3’ -羥基黃烷酮對骨肉瘤細胞株具有明顯的影響，其中 2’ -羥基黃烷酮對所有的骨肉瘤細胞株最具有明顯的影響力。在死細胞與活細胞數目計算的實驗結果中也指出 2’ -羥基黃烷酮導致所有的骨肉瘤細胞株的死亡細胞數目增加。更進一步，我們發現在加藥之後，143 B 細胞產生染色質聚集、粒線體膜電位改變與二倍體細胞（sub-G1 期）等現象出現。接著以西方墨點法分析細胞凋亡相關的蛋白表現量，結果顯示出半胱胺酸蛋白酶-3、半胱胺酸蛋白酶-8 與半胱胺酸蛋白酶-9 產生活化的現象，因此，半胱氨酸蛋白酶的活化證明了細胞凋亡產生。實驗結果顯示 2’-羥基黃烷酮會增加 TRAIL 與 DR5 的表現量。另一方面，2’-羥基黃烷酮會誘導細胞色素 c 從粒線體中釋放出來，我們也發現到抗凋亡蛋白（Bcl-2、Bcl-xL）會受到抑制，以及促凋亡蛋白（Bid、Bax、Bcl-xS）會被活化。因此，2’-羥基黃烷酮會藉由 TRAIL-DR5 訊息路徑誘導 143 B 細胞凋亡。最後，我們進行 in vivo 實驗（143 B-BALB/c nude mice），結果顯示腫瘤的生長會受到 2’-羥基黃烷酮的影響與抑制。這些結果顯示 2’-羥基黃烷酮會經由 TRAIL-DR5 路徑方式去活化細胞內的外在路徑與內在路徑，並且在 in vivo 實驗中對腫瘤的生長呈現有效的影響，因此 2’-羥基黃烷酮為一個在癌症化學預防發展上呈現有效生物活性的化合物。

中文關鍵詞：黃酮類、2’ -羥基黃烷酮、骨癌細胞、轉移、增生、凋亡

英文摘要：Flavanones possess the propensity to anti-proliferation and induce apoptosis of malignant cells. Among four flavanones, 2’-hydroxyflavanone possessed the most potency to reduce the cell viability of 143 B cells in four osteosarcoma cells. Flow cytometry showed that 2’-hydroxyflavanone increased the hypodiploid cells in sub-G1 phase, but resulted in the reduced DNA content in G0/G1 phase in 143 B cells. The 2’-hydroxyflavanone-induced apoptosis in 143 B cells was confirmed by DAPI

staining and mitochondrial membrane potential (ΔΨm) assay. Increasing expressions of TRAIL and DR5 were found in 2'-hydroxyflavanone-treated cells. Also, 2'-hydroxyflavanone increased the expressions of Bcl-xS, cytochrome c, and cleavage PARP, but down-regulated Bcl-2 expressions in 143B cells. Furthermore, in vivo experiments showed that 2'-hydroxyflavanone inhibits tumor growth of 143 B cells. 2'-hydroxyflavanone induces apoptosis of 143 B cells via the extrinsic TRAIL- and intrinsic mitochondrial-dependent pathways, and it can be used as a candidate to induce cancer apoptosis in osteosarcoma.

英文關鍵詞： flavones, 2'-Hydroxyflavanone, osteosarcoma, metastasis, proliferation, apoptosis

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

探討結構相關之不同黃酮類化合物在骨癌細胞其細胞轉移、增生及細胞凋亡之相關性

計畫類別：■個別型計畫　□整合型計畫

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中　華　民　國 101 年 10 月 2 日

中文摘要

類黃酮為多酚類化合物中的一個族群，廣泛存在於蔬菜與水果之中，而類黃酮具有許多生物活性，包含抗氧化、抗微生物與抗發炎，近來越來越多的研究中發現，類黃酮具有抗癌與抗癌化的作用。因此，首先分析幾個黃烷酮類藥物（黃烷酮、2'-羥基黃烷酮、3' -羥基黃烷酮、4'-羥基黃烷酮、5'-羥基黃烷酮、6' -羥基黃烷酮、7' -羥基黃烷酮）對骨肉瘤細胞株（U-2 OS、143 B、MG-63、SaoS2）的影響，MTT 分析細胞存活率的結果發現，黃烷酮、2' -羥基黃烷酮與 3' -羥基黃烷酮對骨肉瘤細胞株具有明顯的影響，其中 2' -羥基黃烷酮對所有的骨肉瘤細胞株最具有明顯的影響力。在死細胞與活細胞數目計算的實驗結果中也指出 2' -羥基黃烷酮導致所有的骨肉瘤細胞株的死亡細胞數目增加。更進一步，我們發現在加藥之後，143 B 細胞產生染色質聚集、粒線體膜電位改變與二倍體細胞（sub-G_1 期）等現象出現。接著以西方墨點法分析細胞凋亡相關的蛋白表現量，結果顯示出半胱胺酸蛋白酶-3、半胱胺酸蛋白酶-8 與半胱胺酸蛋白酶-9 產生活化的現象，因此，半胱氨酸蛋白酶的活化證明了細胞凋亡產生。實驗結果顯示 2'-羥基黃烷酮會增加 TRAIL 與 DR5 的表現量。另一方面，2'-羥基黃烷酮會誘導細胞色素 c 從粒線體中釋放出來，我們也發現到抗凋亡蛋白 （Bcl-2、Bcl-xL）會受到抑制，以及促凋亡蛋白（Bid、Bax、Bcl-xS）會被活化。因此，2'-羥基黃烷酮會藉由 TRAIL-DR5 訊息路徑誘導 143 B 細胞凋亡。最後，我們進行 *in vivo* 實驗（143 B-BALB/c nude mice），結果顯示腫瘤的生長會受到 2'-羥基黃烷酮的影響與抑制。這些結果顯示 2'-羥基黃烷酮會經由 TRAIL-DR5 路徑方式去活化細胞內的外在路徑與內在路徑，並且在 *in vivo* 實驗中對腫瘤的生長呈現有效的影響，因此 2'-羥基黃烷酮為一個在癌症化學預防發展上呈現有效生物活性的化合物。

關鍵詞: 黃酮類、2' -羥基黃烷酮、骨癌細胞、轉移、增生、凋亡

英文摘要

Flavanones possess the propensity to anti-proliferation and induce apoptosis of malignant cells. Among four flavanones, 2'-hydroxyflavanone possessed the most potency to reduce the cell viability of 143 B cells in four osteosarcoma cells. Flow cytometry showed that 2'-hydroxyflavanone increased the hypodiploid cells in sub-G1 phase, but resulted in the reduced DNA content in G0/G1 phase in 143 B cells. The 2'-hydroxyflavanone-induced apoptosis in 143 B cells was confirmed by DAPI staining and mitochondrial membrane potential ($\Delta\Psi m$) assay. Increasing expressions of TRAIL and DR5 were found in 2'-hydroxyflavanone-treated cells. Also, 2'-hydroxyflavanone increased the expressions of Bcl-xS, cytochrome c, and cleavage PARP, but down-regulated Bcl-2 expressions in 143B cells. Furthermore, *in vivo* experiments showed that 2'-hydroxyflavanone inhibits tumor growth of 143 B cells. 2'-hydroxyflavanone induces apoptosis of 143 B cells via the extrinsic TRAIL- and intrinsic mitochondrial-dependent pathways, and it can be used as a candidate to induce cancer apoptosis in osteosarcoma.

Keyword：flavones, 2'-Hydroxyflavanone, osteosarcoma, metastasis, proliferation, apoptosis

目錄

報告內容

前言及研究目的

Flavonoids are polyphenolic compounds and universally present as constituents of flowering plants in the human diet. The high intake of foods and beverages rich in polyphenols, particularly in flavonoids, has been associated with reduction the risk of neoplasm. Previous research has demonstrated that many flavonoids have cytostatic (1) and apoptotic (2) properties and the activities have been attributed to their modulation of several biological processes and possible pharmaceutical applications. Not only epidemiologic data suggested that flavonoids consumption may protect against cancer induction in several human tissues but also dietary flavonoids inhibit the proliferation of various cancer cells and tumor growth in animal models (3-6). Flavanones are a subclass of flavonoids and rich in seeds, fruit skin, bark, and flowers of numerous plants. Recently, flavonoids have been used as a promising strategy for prevention or treatment of malignant tumors. 2'-hydroxyflavanone is a potent antioxidant that exhibits anti-inflammatory activities and possible cancer preventing activity.

Osteosarcoma is the third most common cancer in childhood and adolescents and the most common histological form of primary bone cancer, comprising approximately 20% of all primary bone cancers (7, 8). With the development of new chemotherapy protocols, surgical techniques and radiological staging, a combination of surgery and chemotherapy has increased the 5-year survival and cure rates to 60-80% in patients with localized disease (9). For overcoming resistance to the chemotherapy agent against osteosarcoma and improving survival and limb-sparing surgery, investigations of molecular mechanisms of cytotoxic drug action have shed light on treatments of osteosarcoma. Novel agents that target particular intracellular pathways that are related to the distinctive properties of cancer cells continue to be developed.

The process of apoptosis is triggered by two different signaling pathways. The extrinsic apoptotic signal involves DRs, which respond mainly to extracellular stimuli, while the intrinsic apoptotic signal involves the mitochondria that are activated by modulators within the cell itself (10, 11). Both pathways continuously collect information on various aspects of signal transduction cascades and cellular metabolism, process this information, and eventually decide on the fate of cells.

Tumor necrosis factor receptor-related apoptosis-inducing ligand (TRAIL) plays an important role in immune surveillance and defense mechanisms against malignant tumor cells due to its selective ability of

triggering apoptosis in transformed cells or malignant cells without any toxicity in normal tissues (12). The extrinsic apoptosis signaling pathway is through its interaction with the death-domain containing receptor TRAIL-R1 (death receptor 4; DR4) and/or TRAIL-R2 (DR5) (13). The ligand binding with its receptor induces enzymatic activation of caspase-8, which in turn triggers intracellular activation of the downstream caspase cascade to result in apoptotic cell death (14), so TRAIL is an attractive molecule for study of the mechanism of cancer adjuvant therapy.

Mitochondrial integrity is regulated by pro- and antiapoptotic B-cell lymphoma/leukemia 2 (Bcl-2) family proteins (15). The proapoptotic members include Bax, Bid, and Bcl-xS, whereas the antiapoptotic members include Bcl-2 and Bcl-xL. Activated Bax, Bid and Bcl-xS induces apoptosis by causing outer mitochondrial membrane permeabilization and release of cytochrome c, leading to cleavage of caspase-9, caspase-3, and eventually poly (ADP-ribose) polymerase (PARP). The activation of Bax, Bid and Bcl-xS is blocked by Bcl-2 and Bcl-xL that function as decoy receptors. Bid indirectly promotes apoptosis by binding and neutralizing Bcl-2/Bcl-xL. Ultimately, it is the net balance between antiapoptotic and proapoptotic proteins in the cell that determines cell fate (15-17).

However, *in vitro* effects of 2'-hydroxyflavanone on the operation of extrinsic DR- and intrinsic mitochondria-dependent signaling pathways and *in vivo* effects in human osteosarcoma are not well understood, although we have known that several polyphenolic compounds regulate the genes that are critical for the control of cell cycle and apoptosis pathway in cancer cells (18), and sensitize *in vitro* cancer cells to induce apoptotic cell death by the mitochondrial membrane potential ($\Delta\Psi m$), TNF-family members (death ligands) (19, 20), Bcl-2 family proteins, and the caspase system. The effects of 2'-hydroxyflavanone on osteosarcoma both *in vitro* and *in vivo* were investigated.

研究方法

Cell Culture. The human osteosarcoma 143 B cells and Saos-2 (Sarcoma Osteogenic) cells (Caucasian girl, 11 years old), obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), and American Type Culture Collection (Manassas, VA), respectively, were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corp., Life Technologies, Carlsbad, California) supplemented with 10% f*etal bovine serum* (FBS) (Hyclone Laboratories, Inc-Logan, UT) and 1% penicillin (100 U/mL)/streptomycin (100 μg/mL) (Sigma, St Louis, MO). U2OS (Osteogenic Sarcoma; human; female; 15 years old) cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and were cultured in DMEM supplemented with 10% *FBS, 1*% *penicillin/streptomycin and 5 mL glutamine*. MG-63 cells (human male, 14 years old), obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), were cultured in minimum essential medium (Gibco BRL, Grand Island, NY) supplemented with 10% FBS, 1 mM glutamine, 1% penicillin/streptomycin, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate (Sigma, St Louis, MO). Human osteoblast hFOB 1.19 cells, obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), were cultured in DMEM and supplemented with F12 (10% *FBS,* 2.5 mM L-glutamine and 0.3 mg/mL G418). The cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 incubator.

Cells and Favanones Treatment. Four human osteosarcoma (U2OS, 143 B, MG-63, SaOS2) cells were plated in 24-well plates at a density of 3×10^4 cells per well and were added at different concentrations (0, 10, 20, 30, 40, 50 μM) of four flavanones (flavanone, 2'-hydroxyflavanone, 4'-hydroxyflavanone, and 6'-hydroxyflavanone) (Sigma, St Louis, MO) at 37°C for 24 hours for the subsequent microculture tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric (MTT) assay, death/live assay, morphological analysis of apoptosis, mitochondrial membrane potential (ΔΨm) assay and flow cytometry.

Microculture Tetrazolium Assay. For cell viability experiment, MTT assay was performed to determine the cytotoxicity of seven flavanones. After the exposure period, the media was removed, and cells were washed with phosphatebuffered saline (PBS). Then, the medium was changed and cells were incubated with microculture tetrazolium (0.5 mg/mL) for 4 hours (21). The viable cell number per dish is directly proportional to the production of formazan, which can be measured spectrophotometrically at 563 nm

following solubilization with isopropanol. The results were rechecked by clonogenic assay, flow cytometry and morphological analysis of apoptosis.

Cell Growth Assay. For counting the death and live cells, the treated media was removed and cells were washed with PBS. Then the cells were treated with diluted trypsin-EDTA (Invitrogen Corp., Life Technologies, Carlsbad, California) for five minutes and mixed with the same amount of trypan blue (22, 23). Finally, the numbers of stained (dead cells) and unstained cells (live cells) were counted using a hemocytometer, respectively.

Flow Cytometry. To estimate the proportion of 143 B cells in different phases of the cell cycle affected by 2'-hydroxyflavanone, cellular DNA contents and cell counts were measured by flow cytometry as described previously (23). Briefly, we plated 1×10^6 143 B cells in 6-well plates and treated with different concentrations of 2'-hydroxyflavanone for 24 hours, and then the cells were fixed gently (drop by drop) by putting 70% ethanol (in PBS) in ice overnight and were then resuspended in PBS containing 50 mg/mL propidium iodide and 0.05 mg/ml RNase (Sigma, St. Louis, MO). After 30 min at 37℃, cells were analyzed on a flow cytometer (Becton-Dickinson, San Jose, CA) equipped with an argon ion laser at 488 nm wavelength, and then the cell cycle was determined.

4'-6-Diamidino-2-Phenylindole (DAPI) Staining and 5, 5', 6, 6'-Tetrachloro-1, 1', 3, 3'-Tetraethylbenzimidazol-Carbocyanine Iodide (JC-1) Staining. To establish the apoptotic effect of 2'-hydroxyflavanone, we plated 1.5×10^5 143 B cells in 6-well plates and treated with different concentrations of 2'-hydroxyflavanone for 24 hours. The next step was to fix the cells with 4% formaldehyde for 30 minutes. Morphological analysis of apoptosis by staining with DAPI (Sigma, St Louis, MO) for 5 minutes was evaluated and the phenomenon of chromatin aggregation was observed in UV model of fluorescence microscope (24). DAPI can pass through an intact cell membrane therefore it can be used to stain both live and fixed cells, though it passes through the membrane less efficiently in live cells and therefore the effectiveness of the stain is lower. The mitochondrial membrane potential ($\Delta\Psi m$) assay was examined after staining with JC-1 for 30 minutes and the performance of fluorescence was observed by using fluorescence microscope (24). JC-1 is a cationic dye that can be used as an indicator of mitochondrial potential. It exhibits mitochondrial potential-dependent accumulation, which can be detected by a fluorescence emission shift from green to red.

Preparation of Cell Lysates and Western Blot Analysis. After treating with different concentrations of 2'-hydroxyflavanone for 24 hours, the total cell lysates or nuclear extracts of 2×10^6 143 B cells were prepared as described previously (22, 23). Western blot analysis was performed using primary antibodies against caspase-8, caspase-9, caspase-3, PARP, TRAIL, DR5, Bcl-2 protein family (Bid, t-Bid, Bax, Bcl-2, BCL-xL and Bcl-xS) or cytochrome c, or with the specific antibodies for cleavaged forms of the corresponding caspase-8, caspase-9, caspase-3 and PARP. The relative photographic densities were quantitated by scanning the photographic negatives using a gel documentation and analysis system (AlphaImager 2000, Alpha Innotech Corporation, San Leandro, CA).

In Vivo Studies. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the institutional animal welfare guidelines of the Chung Shan Medical University (IACUC Approval Number: 822). Immuno-deficient nude mice (BALB/c AnN.Cg*Foxn* ^{nu}/Crl Narl mice) were obtained from National Taiwan University Animal Center (Taiwan). Mice were inoculated s.c. into the right posterior flank with 1.5×10^7 143 B cells in 0.2 mL PBS. Mice were randomized into three groups (six animals per group) and treated with different concentrations (0, 10, 20 mg/kg body weight) of 2'-hydroxyflavanone for 28 days and each tumor volume was measured by the formula $V = (1/2)a \times b \times b$, where a is the longest tumor axis and b is the shortest tumor axis every other day (25). All mice were sacrificed by asphyxiation with CO_2. At that time, tumors were removed, photographed and weighted.

Statistical Analysis. Statistical significances were analyzed by one-way analysis of variance (ANOVA) with post hoc Dunnett's test. *P* value <0.05 was considered statistically significant (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA, USA).

結果與討論

2'-hydroxyflavanone possesses the most potency of viability reduction in osteosarcoma cells

In order to determine the cytotoxicity, we examined the effect of four known components of flavanones (flavanone, 2'-hydroxyflavanone, 4'-hydroxyflavanone and 6'-hydroxyflavanone) on cell viability, cell growth, cell cycle distribution, and apoptotic changes of morphology of four human osteosarcoma (U2OS, 143 B, MG-63, SaOS2) cells. Firstly, four osteosarcoma cells were treated with the same concentration (50 μM) of four flavanones for 24 hours, and their cell viabilities were detected by MTT assay. Among four flavanones, 2'-hydroxyflavanone possessed the most cytotoxic effect in four human osteosarcoma cells and the phenomenon showed the largest potency in 143 B cells **(Figure 1A)** and it was in dose dependent manner ($P < 0.001$; **Figure 1B**). Furthermore, 6'-hydroxyflavanone did not show any cytotoxic effect to four human osteosarcoma cells. After reacting with trypsin-EDTA, 2'-hydroxyflavanone indeed exhibited the greatest effect in reducing the number of viable cells in four human osteosarcoma cells (U2OS, 143 B, MG-63 and SaOS2) dose-dependently ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.01$, respectively), especially in 143 B cells. **(Figure 1C)** Thus, four flavanones have selective cytotoxicities to four human osteosarcoma cells individually. A 24 h treatment with 2'-hydroxyflavanone up to 50 μM, the 143 B cell variability was 37%, so we used this concentration range for 2'-hydroxyflavanone in all subsequent experiments. Nevertheless, the concentration of 2'-hydroxyflavanone up to 50 μM did not affect the cell viability of hFOB 1.19 human fibroblastic cells ($P = 0.06$; **Figure 1D**).

2'-hydroxyflavanone induces apoptosis in 143 B cells

To further known the mechanism of 2'-hydroxyflavanone inhibition of cell proliferation, the cell cycle profile of flow cytometry was quantified and showed that 2'-hydroxyflavanone significantly increased the hypodiploid cells in sub-G1 phase, but resulted in the reduced DNA content in G0/G1 phase in 143 B cells dose-dependently. **(Figure 2)** To further confirm whether the suppressive effect of 2'-hydroxyflavanone on cell viability is due to apoptosis, morphological analysis of apoptosis and mitochondrial membrane potential ($\Delta\Psi m$) assay were investigated. 143 B cells were treated with different concentrations (0, 10, 20, 30, 40, 50 μM) of 2'-hydroxyflavanone for 24 hours, and reacted with DAPI for 5 minutes and JC-1 for 30 minutes, respectively. This DAPI DNA-specific dye could pass through intact, living cell membranes, but apoptosis increased cell membrane permeability and uptake of DAPI, leaving a stronger blue stain **(Figure 3)**. In

addition, the nuclear morphology of normal cells was rounding, clear-edged, and uniformly stained. Apoptotic cells showed irregular edges around the nucleus, chromosome concentration in the nucleus, heavier coloring, and, with nuclear pyknosis, an increased number of nuclear body fragments. JC-1 selectively entered the mitochondria and forms J-aggregates with intense red fluorescence (590 nm) in healthy cells. In apoptotic cells, the altered mitochondrial transmembrane potential caused JC-1 to remain in the cytoplasm in monomeric form, showing green fluorescence (525 nm). The 2'-hydroxyflavanone-induced apoptosis in 143 B cells could be easily distinguished by increasing chromatin aggregation in DAPI staining **(Figure 3)** and also be detected by decreasing the mitochondrial membrane potential in JC-1 staining. **(Figure 4)**

2'-hydroxyflavanone triggers the activation of caspase cascade in 143 B cells

The mitochondrial membrane permeabilization results in the release of numerous apoptogenic proteins from the mitochondria, triggering the activation of caspase-9 and caspase-3, and eventually leading to apoptosis. To investigate the downstream effectors in the apoptotic signaling pathway, caspases-8, -9, -3 and PARP as well as their cleavaged forms were determined by western blot analysis after treatment of different concentrations of 2'-hydroxyflavanone for 24 hours. As shown in **Figure 5A**, the higher concentrations of 2'-hydroxyflavanone were treated, the more expressions of cleavaged-forms of caspases-8, -9,- 3 and PARP combined with the less caspases-8, -9,- 3 and PARP expressions were found, and they were dose-dependently ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively) **(Figure 5B & 5C)**.

2'-hydroxyflavanone increases expressions of TRAIL and DR5 in 143 B cells

To establish whether 2'-hydroxyflavanone increases the expressions of TRAIL and DR5, 143 B cells were treated with different concentrations of 2'-hydroxyflavanone for 24 hours and TRAIL and DR5 expressions were determined by western blot analysis. We observed that 2'-hydroxyflavanone significantly increased TRAIL and DR5 expressions in 143 B cells dose-dependently **(Figure 6A & 6B)**.

2'-hydroxyflavanone decreases expressions of anti-apoptotic proteins and increases expressions of pro-apoptotic proteins and cytochrome c

Bcl-2 family proteins, including anti-apoptotic members, such as Bcl-2, Bid and Bcl-xL, and pro-apoptotic members, such as t-Bid, Bax, Bcl-xS and cytochrome c, play important roles in mitochondrial-mediated apoptosis regulation. To understand whether the apoptotic pathway is associated with Bcl family proteins, their protein expressions in different concentrations of 2'-hydroxyflavanone -treated cells

were evaluated by western blot analysis. The results showed that 2'-hydroxyflavanone treatment increased t-Bid, Bax, and Bcl-xS expressions dose-dependently but decreased Bcl-2 and Bcl-xL expressions in 143 B cells dose-dependently **(Figure 6C & 6D)**. In addition, cytochrome c protein expression was significantly increased by 2'-hydroxyflavanone treatment dose-dependently **(Figure 6E & 6F)**. These suggest that 2'-hydroxyflavanone induces mitochondrial-dependent apoptosis in 143 B cells via regulating the expression of the Bcl-2 family proteins.

2'-hydroxyflavanone inhibits tumor growth of 143 B cells in vivo

We subsequently investigated the effect of 2'-hydroxyflavanone *in vivo* by evaluating the effect of drug treatment on tumor growth using BALB/c nude mice as described under Materials and Methods. As can be seen in **Figure 7A**, tumor growth was significantly slower in 2'-hydroxyflavanone-treated mice than in control animals ($P < 0.01$), as a reflection of the markedly smaller size of individual tumors observed and weighted ($P < 0.01$) after excision **(Figure 7B & 7C)**.

Discussion

The purpose of this study was to investigate the effects of 2'-hydroxyflavanone in osteosarcoma both *in vitro* and *in vivo*. We found that 2'-hydroxyflavanone possesses the most potency in four flavanones to reduce the cell viability of 143 B cells in four osteosarcoma cells. 2'-hydroxyflavanone induces 143 B cells apoptosis and upregulates TRAIL and DR5 expressions and caspase-8 activation; therefore, 2'-hydroxyflavanone induces apoptosis of 143 B cells at least via the extrinsic TRAIL-dependent signaling pathway. Additionally, 2'-hydroxyflavanone upregulates expressions of proapoptotic proteins t-Bid, Bax, Bcl-xS and cytochrome c, and downregulates expressions of antiapoptotic proteins Bcl-2, Bid and Bcl-xL, resulting in activation of caspase-9 and caspase-3. Hence, 2'-hydroxyflavanone -induced apoptosis of 143 B cells is also through the intrinsic mitochondrial-dependent signaling pathway. Finally, our data confirmed that 2'-hydroxyflavanone delays tumor growth of 143 B cells *in vivo*.

Initially, our data showed that flavanones have selective cytotoxicities to four human osteosarcoma cells individually and 2'-hydroxyflavanone has the most potency to reduce the cell viability of four human osteosarcoma cells, especially 143 B cells. Thus, further tests need to exam the effects and possible

mechanism of 2'-hydroxyflavanone on the cell cycle progression and regulatory molecules in 143 B cells. Flow cytometry in the present study showed that 2'-hydroxyflavanone increases the hypodiploid cells in sub-G1 phase, but results in the reduced DNA content in G0/G1 phase in 143 B cells, hence suggesting 2'-hydroxyflavanone reduces the cell viability of 143 cells. Furthermore, we confirmed the apoptotic effect of 2'-hydroxyflavanone in 143 B cells on the phenomenon of chromatin aggregation of apoptosis with DAPI stain and on the mitochondrial membrane potential using the fluorescent potential-sensitive dye JC-1, but how 2'-hydroxyflavanone can trigger apoptosis is not clear. Therefore, western blots were done to determine which pathway is activated by 2'-hydroxyflavanone, because apoptosis can be triggered by external or internal signaling pathways that lead to cleavage and activation of the initiator caspases either caspase-8 or caspase-9, respectively (26).

Upon 2'-hydroxyflavanone treatment for 24 hours in the study, there was significant PARP cleavage indicative of apoptosis in general. Caspase-3 cleavage, also common to both extrinsic and intrinsic pathways, was triggered at 2'-hydroxyflavanone treatment for 24 hours. In addition to t-Bid, both caspase-8 and caspase-9 were activated consistently by 2'-hydroxyflavanone. Although activation of caspase-8 is usually associated with DR–mediated apoptosis, it also feeds into the mitochondrial caspase-9 pathway through activation of t-Bid. Like studies reported by others (27, 28), 2'-hydroxyflavanone may play a role as an upstream mediator of caspases, especially caspases-8, -9 and -3, and activates both intrinsic and extrinsic apoptotic pathways in 143 B cells.

Tumor necrosis factor super-family is a group of cytokines and possesses important functions in immunity, inflammation, differentiation, control of cell proliferation, and apoptosis. These cytokines induce cell death through sequential recruitment by DRs and rapid activation of a cascade of caspases (29, 30). In recent years, TRAIL has been identified as a powerful activator of apoptosis in tumor cells while sparing normal cells (31). A substantial role of endogenously expressed TRAIL has been confirmed as the effective molecule in immunosurveillance that eliminates developing and metastatic tumors (13, 32). Our findings showed that 2'-hydroxyflavanone increases TRAIL and DR5 expressions and caspase-8 activation in 143 B cells. Accordingly, increasing expressions of TRAIL and DR5 should be a target of apoptosis in 143 B cells. After investigating the downstream effectors in the apoptotic signaling pathway, it seems that 2'-hydroxyflavanone-induced apoptosis of 143 B cells is, at least partially, through the extrinsic

TRAIL-mediated apoptosis pathway.

Mitochondria have been shown to play a key role in apoptosis through the release of cytochrome c (33) and collapse the mitochondrial transmembrane potential (34). Mitochondrial-dependent apoptosis is known to require pro-apoptotic Bax-like proteins to regulate the formation of pores in the mitochondria (35), while anti-apoptotic Bcl-2-like proteins in mitochondrial morphogenesis are functionally distinct from their role in apoptosis (15). The ratio of Bax to Bcl-2 is critical for determining the release of many apoptogenic proteins, such as cytochrome c (36), to influence cell survival. Cytochrome c can activate caspase-9 to cleave and to trigger executioner caspases, such as caspase-3, and then drives the caspase cascade and the cell death mechanism (37). Consistent with our findings, 2'-hydroxyflavanone upregulates expressions of the pro-apoptotic proteins Bax and Bcl-xS and downregulates expressions of the anti-apoptotic proteins Bid, Bcl-2 and Bcl-xL in 143 B cells. Our findings provide an extensive understanding of 2'-hydroxyflavanone-induced apoptosis by regulating the expression of the Bcl-2 family proteins and enhancing the expression of cytochrome c and activation of caspase-9 and caspase-3. Collectively, 2'-hydroxyflavanone -induced apoptosis of 143 B cells is via the intrinsic mitochondria-mediated apoptosis pathway that 2'-hydroxyflavanone promotes the mitochondrial membrane permeabilization to result in the release of numerous apoptogenic proteins from the mitochondria, triggering caspase-9 and caspase-3 activation, and eventually leading to apoptosis.

Knowing more about the mechanism of signal transduction and the induced apoptosis may help understand how osteosarcoma cells proliferate, which may lead to new treatment strategies. The present study provides the first description that 2'-hydroxyflavanone significantly induces apoptosis in human osteosarcoma cells via the extrinsic and intrinsic pathways and also confirms the tumor growth delay in 2'-hydroxyflavanone treatment of established BALB/c nude mice with 143 B cells inoculation. Indeed, these give us the molecular basis for the development of 2'-hydroxyflavanone as a novel chemopreventive agent for osteosarcoma in the future. Now how it triggers apoptosis may be clear. Nevertheless, further tests are needed to investigate that the detailed results *in vivo* whether are similar to the *in vitro* results.

Overall, our data provide a new perspective for the use of 2'-hydroxyflavanone in the treatment of human osteosarcoma. 143 B cells are uniformly sensitive to 2'-hydroxyflavanone both *in vitro* and *in vivo* and 2'-hydroxyflavanone may exert anti-cancer activities via extrinsic and intrinsic pathways to trigger the

apoptotic effect. It is of clinical relevance; however, more studies are needed to further justify 2'-hydroxyflavanone as a chemopreventive agent and a promising strategy for prevention or treatment of osteosarcoma tumors.

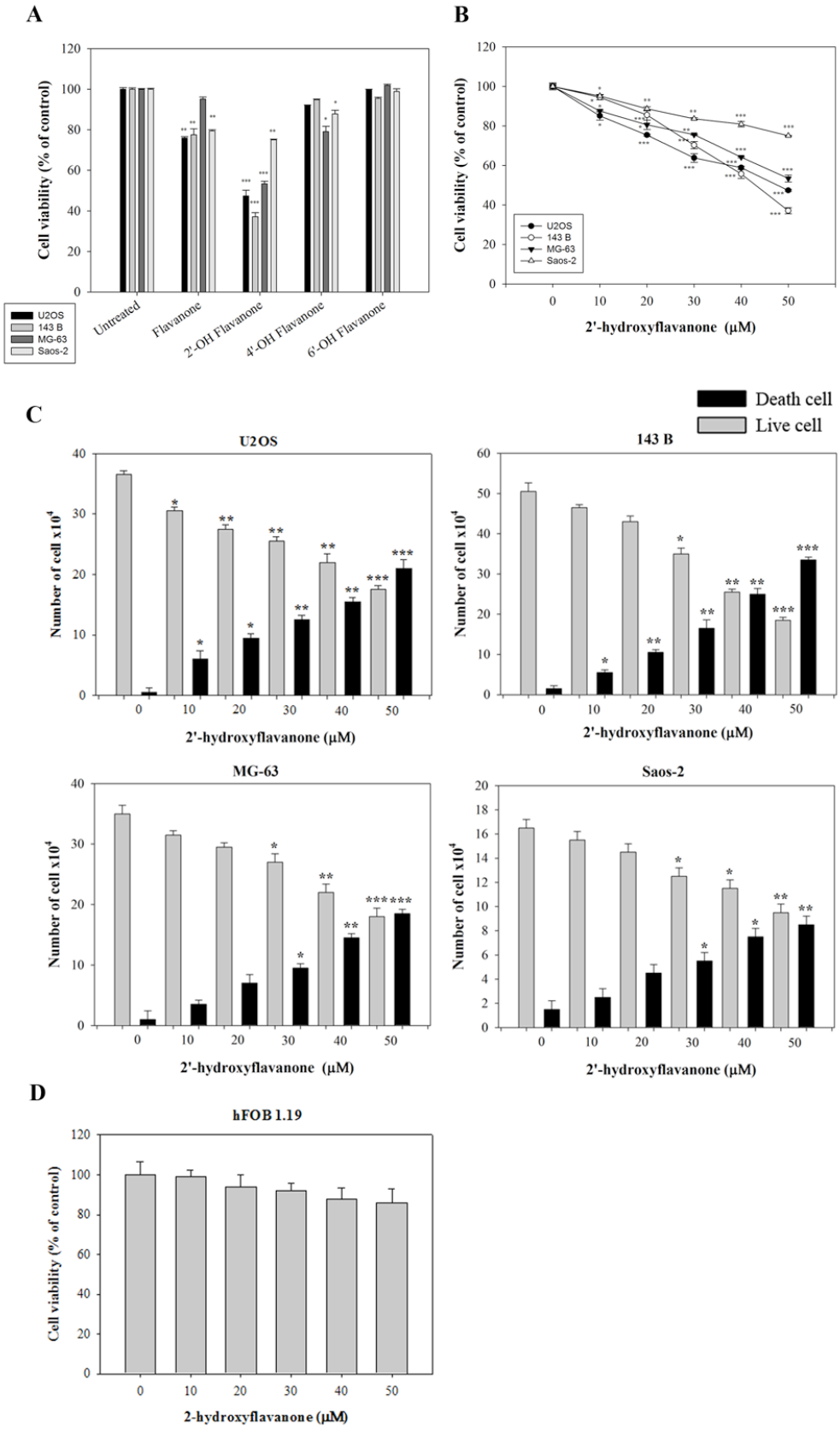

Figure 1. (A) The effect of four flavanones on cell viability of four osteosarcoma cells in MTT assay. (B) The

concentration-dependent effect of 2'-hydroxyflavanone on cell viability of four osteosarcoma cell lines in MTT assay. (C) Four osteosarcoma cells were treated with 2'-hydroxyflavanone and then viable cells were collected and counted using hemocytometer. Each datum represents mean ± standard deviation of at least three quantitative experiments. (D) The concentration-dependent effect of 2'-hydroxyflavanone on cell viability of the human osteoblast hFOB 1.19 cell line in MTT assay. Results were statistically evaluated by using one-way ANOVA with post hoc Dunnett's test (*, p <0.05; **, p <0.01; ***, p <0.001). Results from 3 repeated and separated experiments were similar.

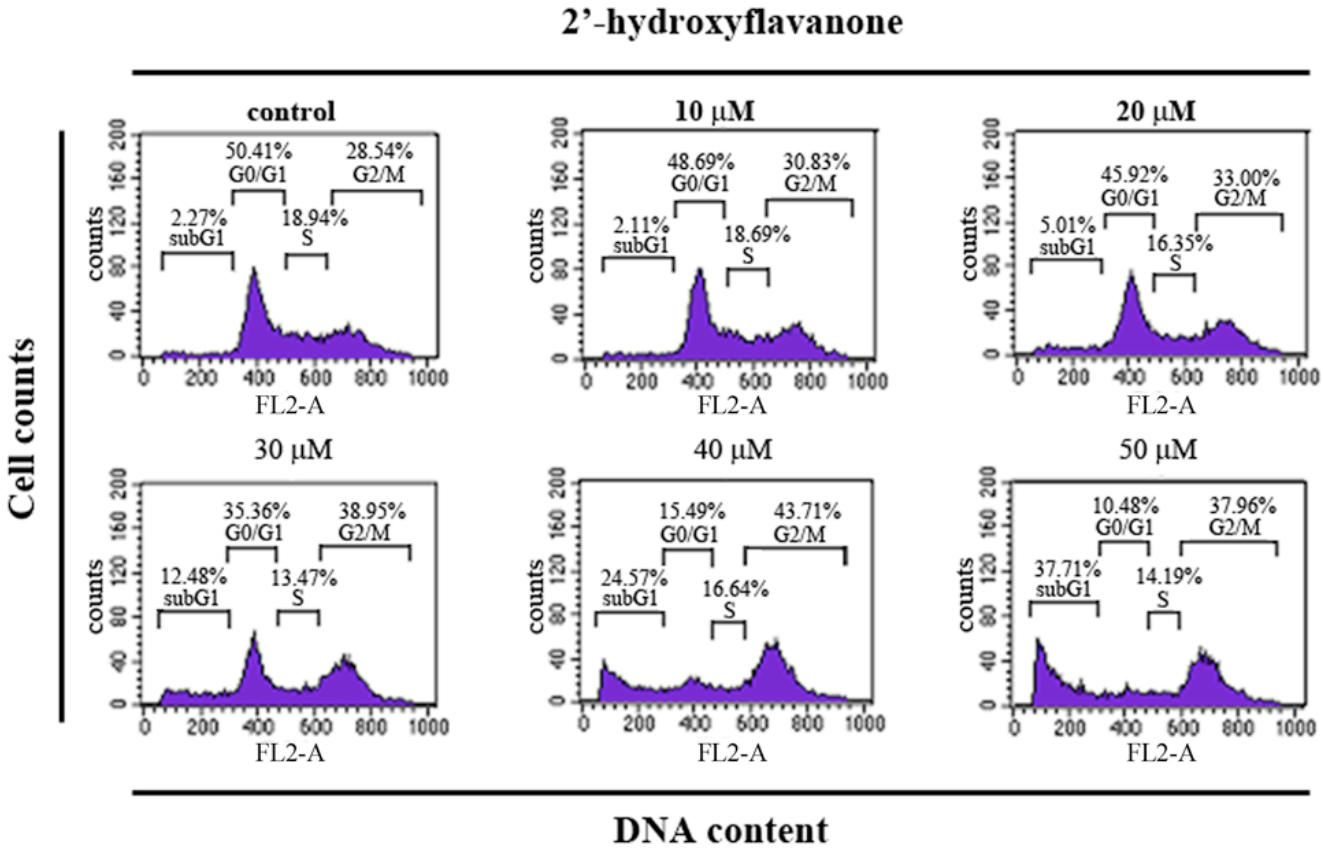

Figure 2. The effect of 2'-hydroxyflavanone on the cell cycle process of 143 B cells by flow cytometry. Cells were harvested after a 24 h treatment of 2'-hydroxyflavanone and then treated with PI for 30 min followed by a flow cytometric analysis for cell cycle distribution.

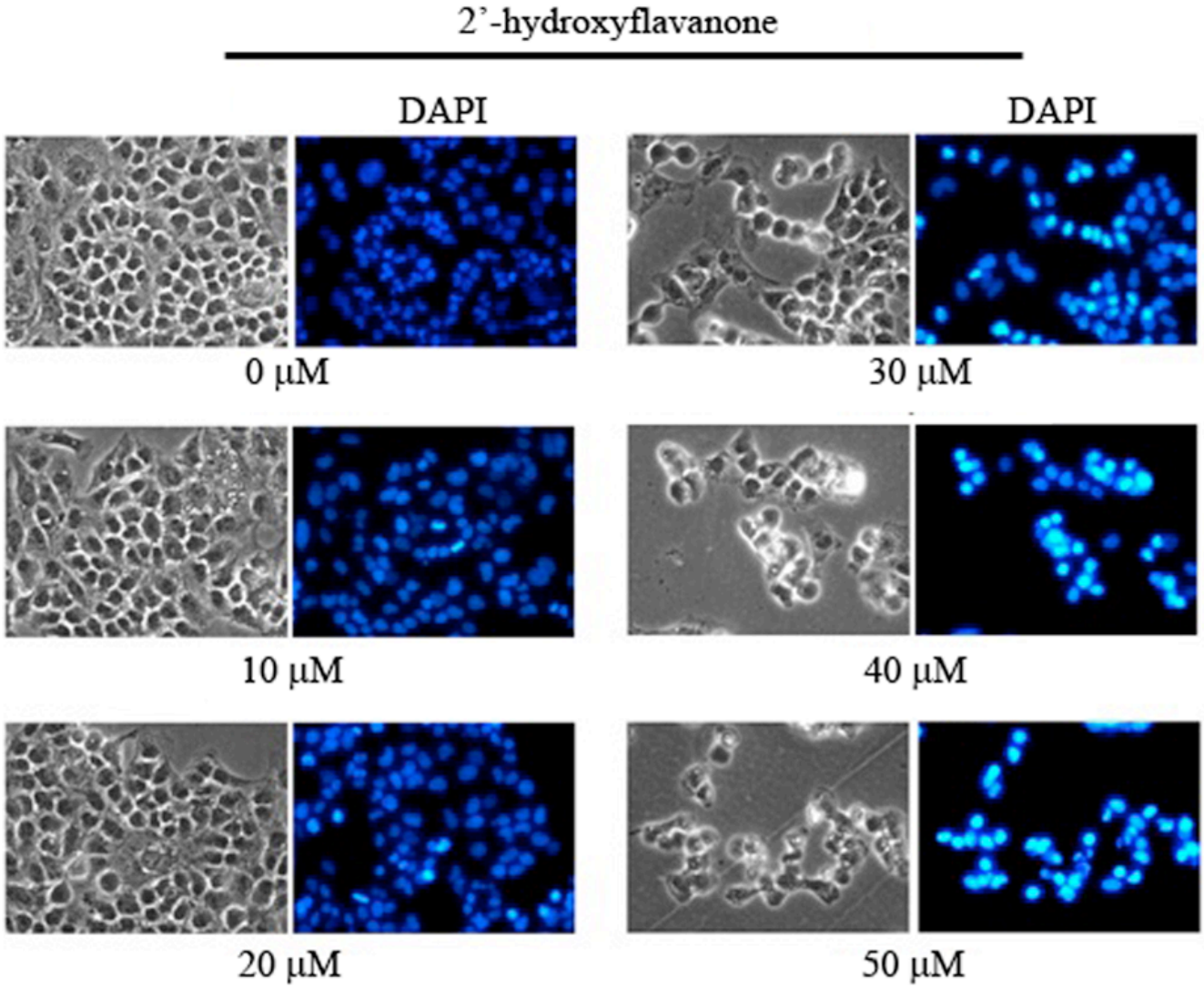

Figure 3. The apoptotic effect of 2'-hydroxyflavanone on chromatin aggregation of 143 B cells. Cells cultured with 2'-hydroxyflavanone were examined for apoptosis. For nuclear morphology of 143 B cells, cells were then stained with DAPI and observed under a UV-light microscope.

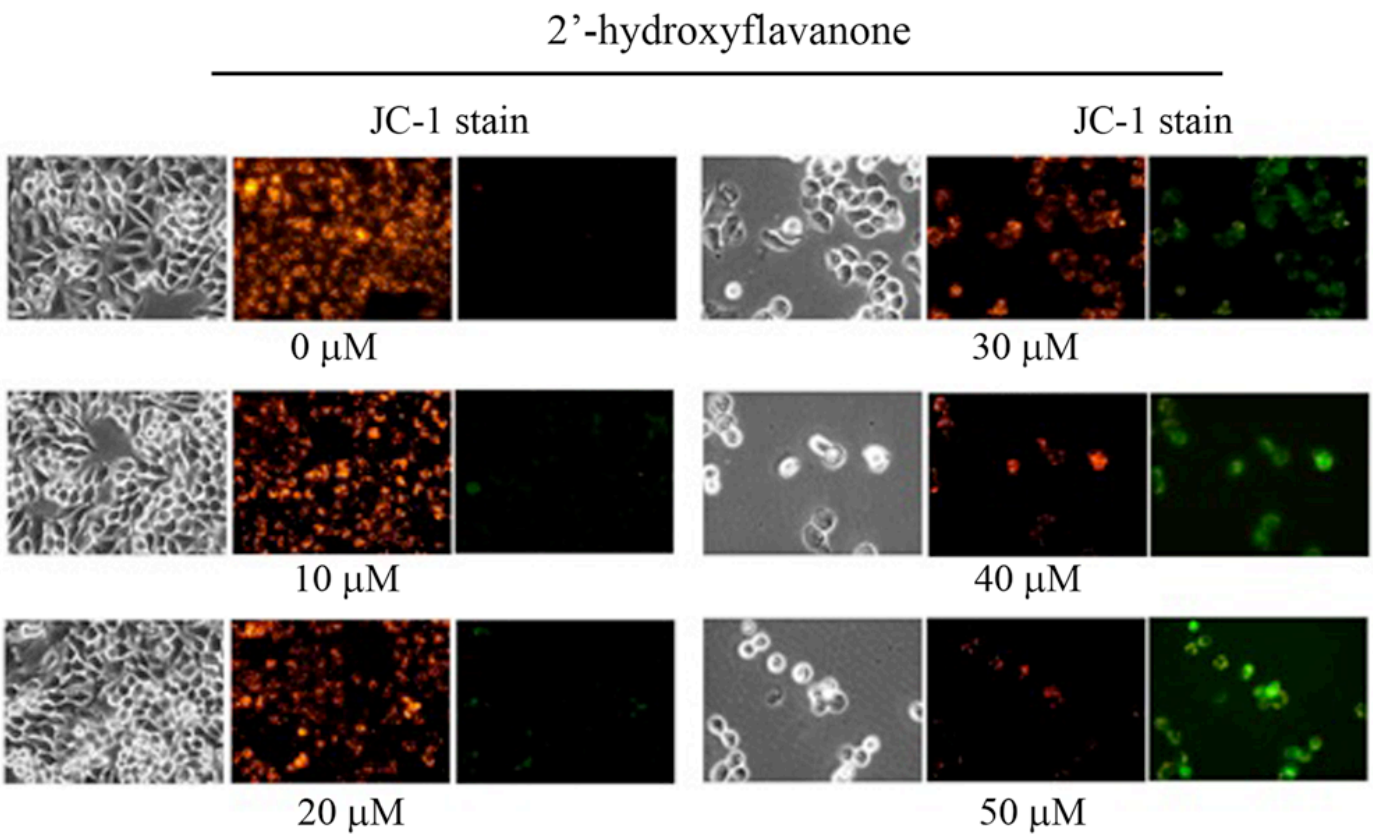

Figure 4. The apoptotic effect of 2’-hydroxyflavanone on the mitochondria potential of 143 B cells by JC-1 stai.

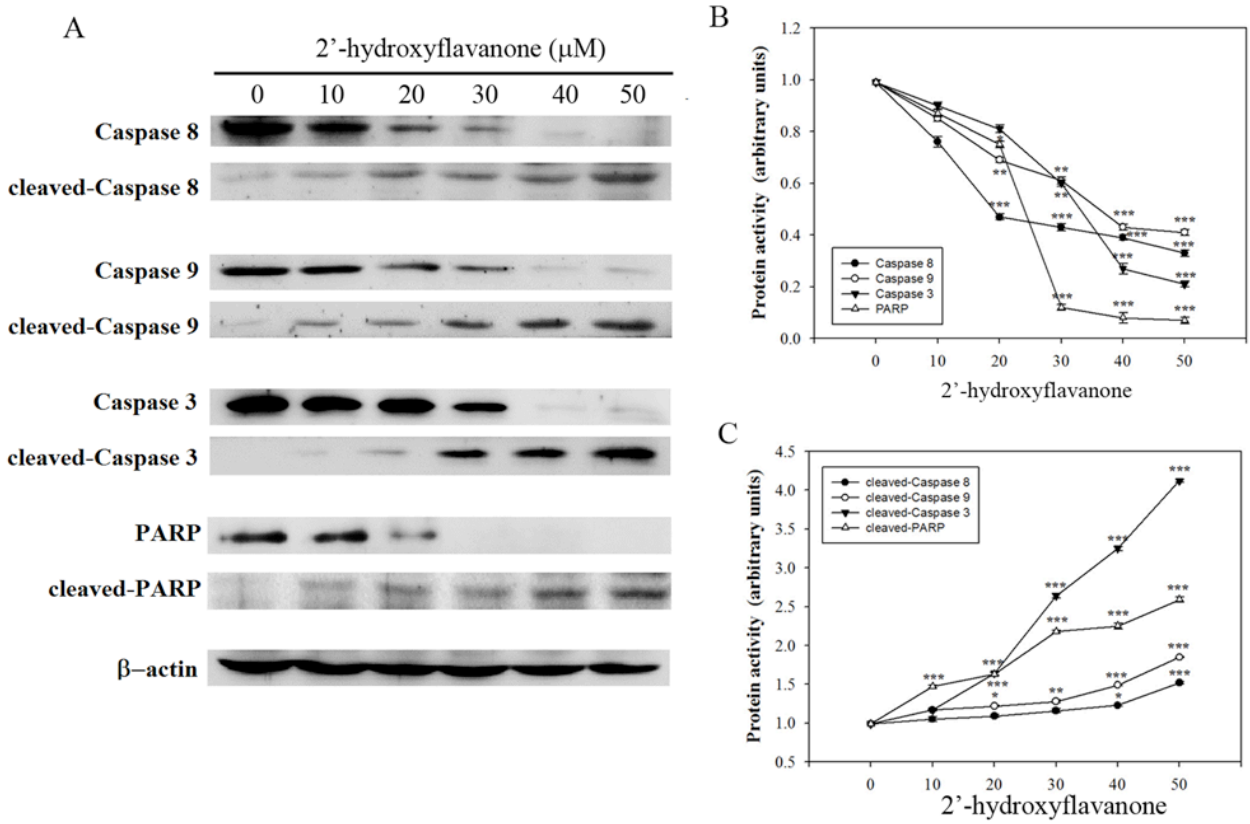

Figure 5. (A) The expression of caspase-8, caspase-9, caspase-3 and PARP by western blotting. (B) and (C) Quantification of three respective immunoblotting experiments is shown. Each datum represents mean ± standard deviation. Results were statistically evaluated by using one-way ANOVA with post hoc Dunnett's test (**, p <0.01; ***, p <0.001).

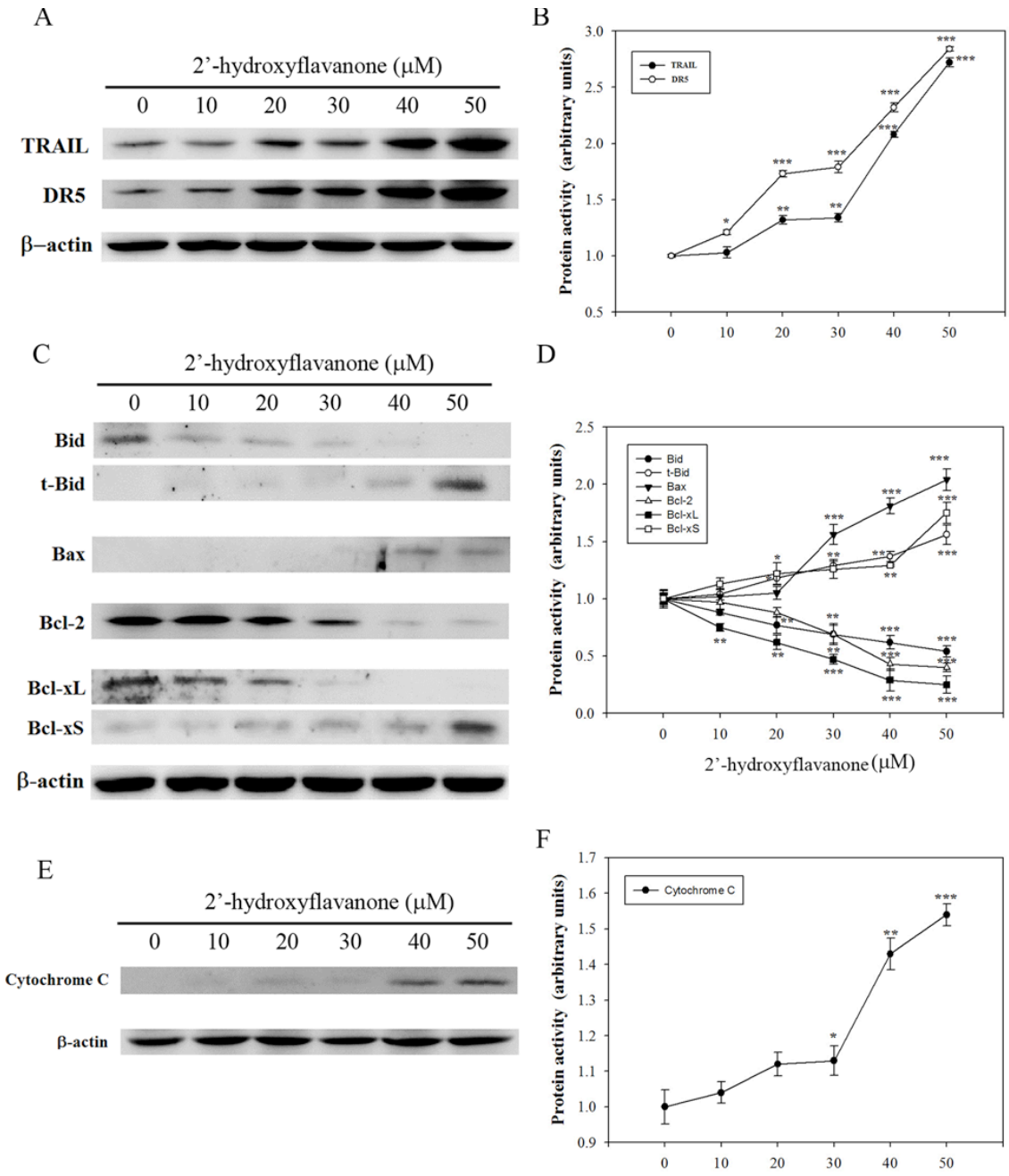

Figure 6. (A) The effect of 2'-hydroxyflavanone on the expressions of TRAIL and DR5 in 143 B cells. (B) Quantification of three respective immunoblotting experiments of TRAIL and DR5 is shown. (C) The effect of 2'-hydroxyflavanone on the expressions of Bcl-2 protein family in 143 B cells. (D) Quantification of three respective immunoblotting experiments of Bcl-2 protein family is shown. (E) The effect of 2'-hydroxyflavanone on the expression of cytochrome c in 143 B cells. (F) Quantification of three respective immunoblotting experiments of cytochrome c is shown. Results were statistically evaluated by using one-way ANOVA with post hoc Dunnett's test (*, p <0.005; **, p <0.01; ***, p <0.001).

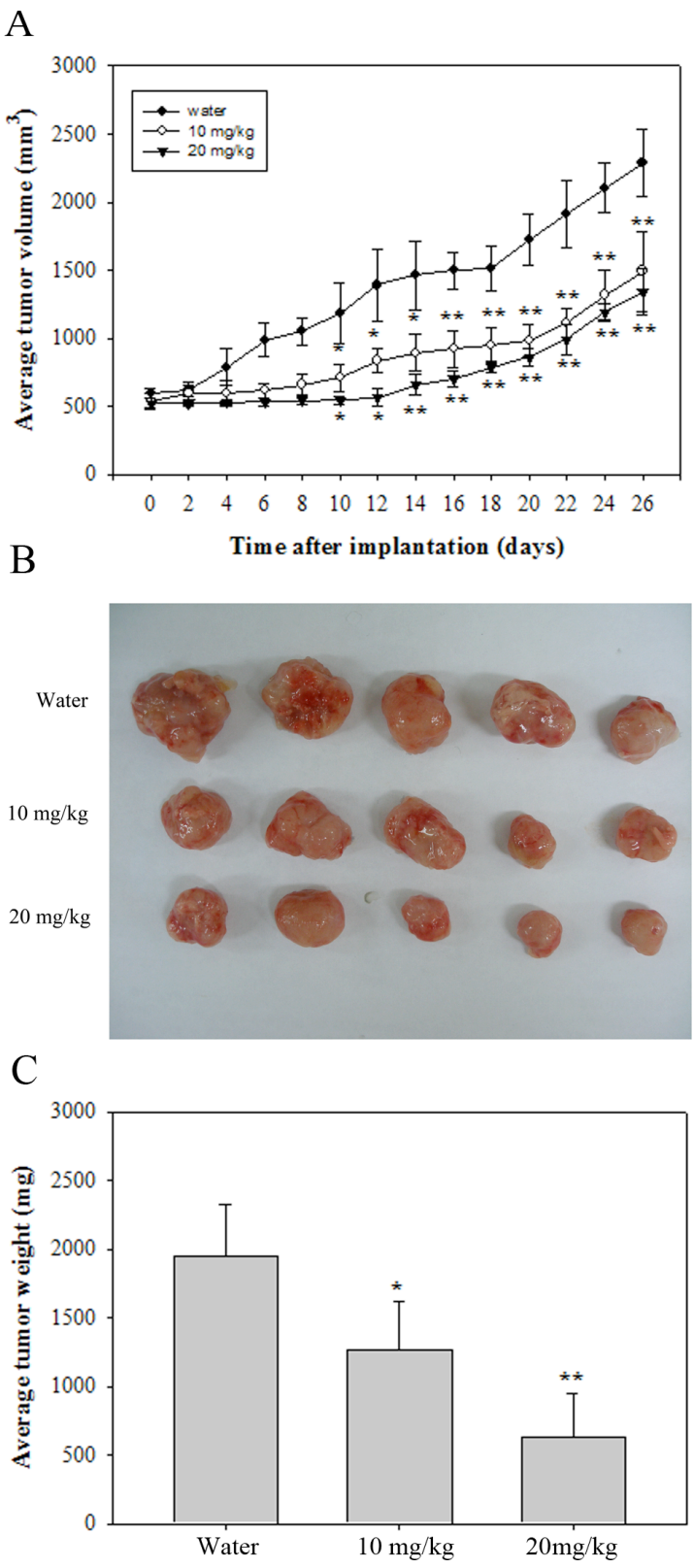

Figure 7. (A) The anti-tumor effect of 2'-hydroxyflavanone in the *in vivo* model. (B) The photographs of osteosarcoma tumor. (C) The effect of 2'-hydroxyflavanone on the tumor weight. Each datum represents mean ± standard deviation of at least three quantitative experiments. Results were statistically evaluated by using one-way ANOVA with post hoc Dunnett's test (**, p <0.01; ***, p <0.001).

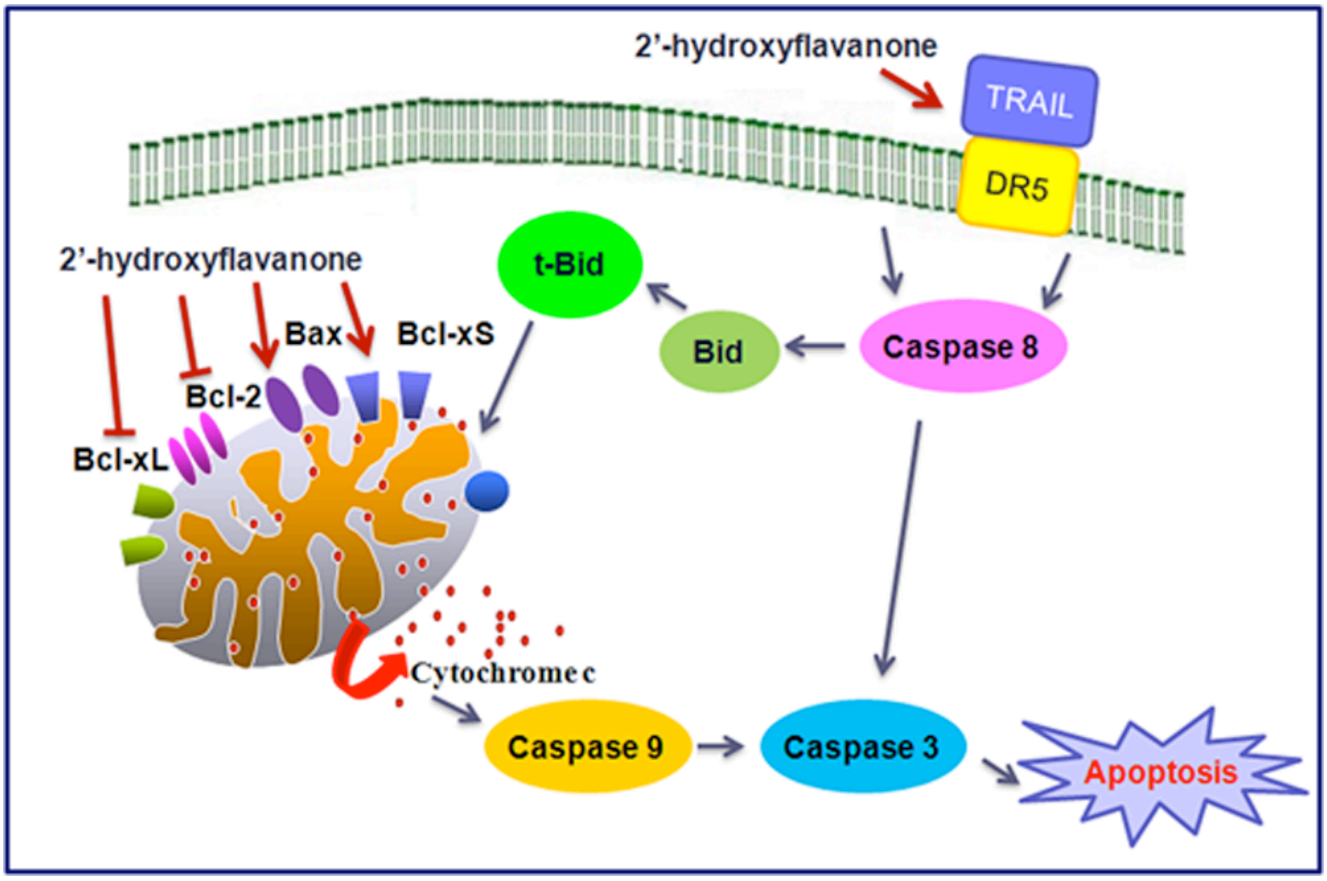

Figure 8. Model showing pathways that mediate 2'-hydroxyflavanone-induced apoptosis in 143 B cells.

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國科會補助計畫衍生研發成果推廣資料表

日期:2013/09/30

國科會補助計畫	計畫名稱: 探討結構相關之不同黃酮類化合物在骨癌細胞其細胞轉移、增生及細胞凋亡之相關性
	計畫主持人: 呂克修
	計畫編號: 101-2314-B-040-001- 學門領域: 骨科

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：呂克修	**計畫編號：**101-2314-B-040-001-
計畫名稱：探討結構相關之不同黃酮類化合物在骨癌細胞其細胞轉移、增生及細胞凋亡之相關性	

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	1	0	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%		
	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	1	1	100%	人次	
		博士生	1	1	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
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		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)

結果顯示腫瘤的生長會受到 2’-羥基黃烷酮的影響與抑制。這些結果顯示 2’-羥基黃烷酮會經由 TRAIL-DR5 路徑方式去活化細胞內的外在路徑與內在路徑，並且在 in vivo 實驗中對腫瘤的生長呈現有效的影響,因此 2’-羥基黃烷酮為一個在癌症化學預防發展上呈現有效生物活性的化合物。