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Regulation of Apoptosis by Modified Naringenin Derivatives in Human Colorectal Carcinoma RKO Cells

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Abstract Flavonoids are micronutrients that are widely detected in foods of plant origin and have been ascribed pharmacological properties. Several biological functions of flavonoids have been thus far identified, whereas there currently exists a lack of evidence to support the relationship between the structure-activity relationship and apoptosis-inducing activity. In an attempt to determine the importance of the OH group or substitution of the 5- or 7-carbon in the diphenylpropane skeleton of flavonoids, we selected 14 different flavonoids with different structures, particularly with regard to the 5- or 7-carbon, and found that naringenin treatment caused a slight decrease in the cell viability of the human colorectal carcinoma RKO cells. Next, in order to characterize the effects of specific substitutions of the 7-carbon of naringenin on apoptosis-regulatory activities, and in an attempt to develop anti-proliferative flavonoid derivatives that would be more effective against colon cancer, we originally synthesized several modified naringenin derivatives (MNDs) including 7-*O*-benzyl naringenin (KUF-1) and 7-*O*-(*m*-metoxybenzyl) naringenin (KUF-2). Treatment with KUF-1 or KUF-2 resulted in significant apoptosis-inducing effects concomitant with losses in mitochondrial membrane potential, caspase activation, intracellular ROS production, and sustained ERK activation. Our data show that KUF-1 or KUF-2 regulate the apoptosis of RKO cells via intracellular ROS production coupled with the concomitant activation of the ERK signaling pathway, thereby implying that hydroxylation or substitution at C7 is critical for the apoptosis-inducing activity of flavonoids. J. Cell. Biochem. 104: 259–273, 2008.

Key words: flavonoid; naringenin; modified naringenin derivatives; ERK; apoptosis; ROS

Colorectal cancer is one of the leading causes of cancer death among both men and women and the third most commonly diagnosed cancer and the second most common cause of cancer death in the western world [Jemal et al., 2003; Howe et al., 2006].

Apoptosis is a well-defined type of cell death, and can be readily distinguished from necrotic cell death. Several characteristics of apoptotic cell death, including DNA fragmentation, chromatin condensation, and apoptotic body formation have not been observed in necrotic cells [Hanahan and Weinberg, 2000; Li et al., 2001]. Previous studies have shown that the activation of the caspase cascade is involved in apoptosis [Planey et al., 2003]. Caspases which exist as inactive pro-caspases are proteolytically activated via multiple cleavages in cells undergoing apoptosis [Zhang et al., 2001]. The activated caspases cleave specific substrates, including PARP (poly(ADP-ribose) polymerase), an important apoptotic marker [Chen et al., 2002]. A variety of reports have also shown that the

Abbreviations used: MAPK, mitogen-activated protein kinase; PARP, poly(ADP-ribose) polymerase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; DAPI, 4′-6′-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; FACS, fluorescence activated cell sorting.

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mitogen-activated protein kinase (MAPK) pathways perform pivotal functions in apoptosis.

Flavonoids are plant pigments which have been detected in all parts of plants, including fruits, vegetables, nuts, leaves, flowers, and bark, and these compounds are major functional components of many herbal preparations used in traditional medical protocols [Lin et al., 1997; Chen et al., 2000, 2001; Ko et al., 2002]. They are low molecular weight polyphenolic compounds, which possess a basic 2-phenyl-benzo-gamma-pyrone structure harboring one or more hydroxyl groups. As the result of this basic chemical structure, one of the most obvious features of flavonoids is their ability to quench free radicals via the formation of resonance-stabilized phenoxyl radicals [Bors et al., 1997]. They have also been previously reported to perform a variety of biological functions, including apoptosis-inducing, free radical-scavenging, and anti-tumorigenic activities [Birt et al., 2001; Heijnen et al., 2002].

Flavonoids exert protective effects, which appear to be related to specific structural characteristics [Keinan-Boker et al., 2004]. Recent studies have shown that the differential effects of flavonoids are attributable to substituted functional groups [Constantinou et al., 1998; Kris-Etherton et al., 2002]. Furthermore, our previous reports have suggested that the OH group of the 5- or 7-carbon of the C6C3C6 skeleton is the principal determinant of the anti-oxidant and anti-apoptotic activities associated with flavonoids [Lee et al., 2005, 2007] Hence, investigations into the structurally related activities of flavonoids are important in terms of our understanding of their differential activities. The association between flavonoid intake and reduced disease risk was originally believed to be the consequence of the anti-oxidant effects of these compounds [Rice-Evans et al., 1996]. However, flavonoids are extensively metabolized in vivo, which attenuates their anti-oxidant capacity, and recent evidence appears to indicate that flavonoids and their metabolites exert other intracellular effects, including the direct modulation of cell signaling pathways, including the MAPK cascade [Schroeter et al., 2001; Spencer et al., 2001, 2003; Williams et al., 2004].

In this study, we describe the apoptotic cell death of human colorectal carcinoma RKO cells as the result of treatment with modified naringenin derivates (MNDs), including 7-*O*-benzyl naringenin (KUF-1), 7-*O*-(*m*-metoxybenzyl) naringenin (KUF-2), 7-*O*-(*p*-fluorobenzyl) naringenin (KUF-3), 7-*O*-(*m*-iodobenzyl) naringenin (KUF-4), 7-*O*-(2-naphtylmethyl) naringenin (KUF-5), 7-*O*-benzoxycarbonylmethyl naringenin (KUF-6), 7-*O*-(MeO-L-Leu-D-Pro-carbonylmethyl) naringenin (KUF-7), and 7-*O*-(MeO-Gly-D-Pro-carbonylmethyl) naringenin (KUF-8; Fig. 2A). The MNDs were originally generated via the substitution of the 7-carbon of the C3C6C3 skeleton of naringenin by specific benzyl or amino acid moieties. We evaluated the intracellular levels of ROS, caspase activation, and MAPK signal pathways in RKO cells treated with the MNDs and provide scientific evidence herein to demonstrate that hydroxylation or substitution at C7 is critical to the apoptosis-inducing activity of flavonoids, supporting the structurally related activities of flavonoids in apoptosis.

MATERIALS AND METHODS

Cell Culture and DNA Transfection

Human RKO colorectal carcinoma cell line [Beard et al., 1996; Goel et al., 2006] was cultured at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone) and 100 units/ml of penicillin/streptomycin. For DNA transfection, we plated the RKO cells at a density of 3×10^6 cells per 100 mm culture dish, incubated overnight, and then transfected the cells with the indicated expression vectors, using LipofectAMINE (Life Technologies).

Antibodies and Materials

The 2′,7′-dichloro-fluorescin diacetate (DCFH-DA) was acquired from Molecular Probes and the electrophoresis reagents and protein assay kit were purchased from Bio-Rad (Hercules). Antibodies against pro-caspase-3, pro-caspase-8, actin, JNK, p38 MAPK, phosphor-p38 MAPK, and cleaved poly(ADP-ribose) polymerase (PARP) were obtained from Santa Cruz, and the ERK1/2, phospho-JNK, and phospho-ERK (Thr202/Tyr204) antibodies were from Cell Signaling. Etoposide and PD98059 were acquired from BioMol. Natural flavonoids, including 4,7,4′-trihydroxy flavone (naringenin), 3-hydroxy flavone, and 7,8,4′-trihydroxy flavone were obtained from the INDOFINE Chemical Company (NJ).

Determination of Cell Viability

The RKO cells were plated at a density of 5×10^4 cells in 96-well plates, and cell viability was evaluated via a conventional MTT reduction assay. The cells were treated with MTT solution (final concentration, 0.25 mg/ml) for 2 h at 37°C. Dark blue formazan crystals forming in the intact cells were then dissolved with DMSO, and the absorbance was measured at 570 nm using an ELISA reader. The results were then expressed as percentages of MTT reduction, with the absorbance exhibited by the control cells being arbitrarily set as 100%.

General Procedure for the Preparation of Modified Naringenin Derivatives

For the preparation of 7-*O*-alkyl naringenin derivatives (KUF-1-6 and KUF-8), K_2CO_3 (1.0 equiv) was added to a solution of racemic naringenin (1.0 equiv) and alkyl bromide (1.0 equiv) in DMF at room temperature. After the resulting mixture was stirred at room temperature for 12 h, the mixture was dissolved in AcOEt and washed with 1N HCl and brine. The organic phase was dried over anhydrous $MgSO_4$, filtered, and concentrated. The crude material was purified by column chromatography (hexane-AcOEt 3:1) on silica gel to provide 7-*O*-alkyl naringenin in 65–50% yields. For the 7-*O*-(MeO-L-Leu-L-Pro-carbonylmethyl) naringenin derivative (KUF-7), K_2CO_3 (1.0 equiv) was added to a solution of naringenin (1.0 equiv), tetrabutylammonium iodide (1.0 equiv), and methyl *N*-(α-bromoacetyl)-prolinyl-leucinate (1.0 equiv) in methylene chloride at room temperature. After the resultant mixture was stirred for 24 h at room temperature, the reaction mixture was dissolved into AcOEt and washed with 1 N HCl and brine. The organic phase was dried over anhydrous $MgSO_4$, filtered, and concentrated. The crude material was purified via column chromatography (hexane-AcOEt 2:1) on silica gel to give 41% yield. The purities (>95%) of products were estimated by NMR. 1H NMR spectra were acquired on Bruker 400 (400 MHz 1H) spectrometer using chloroform-d or DMSO-d6 as the internal standard.

KUF-1: 1H-NMR (DMSO-d6, 400 MHz) 7.45–7.31 (m, 7H), 6.79 (d, J = 8.4 Hz, 2H), 6.17 (m, 2H), 5.48 (dd, J = 12.8, 2.8 Hz, 1H), 5.17 (s, 2H), 3.32 (dd, J = 13.1, 17.1 Hz, 1H), 2.72 (dd, J = 2.9, 17.1 Hz, 1H).

KUF-2: 1H-NMR (CDCl3, 400 MHz) 7.34–6.86 (m, 8H), 6.14 (m, 2H), 5.35 (dd, J = 13.0, 2.9 Hz, 1H), 5.04 (s, 2H), 3.82 (s, 3H), 3.08 (dd, J = 13.0, 17.2 Hz, 1H), 2.78 (dd, J = 2.9, 17.2 Hz, 1H).

KUF-3: 1H-NMR (CDCl3, 400 MHz) 7.35–7.30 (m, 6H), 6.89 (m, 2H), 6.03 (m, 2H), 5.34 (dd, J = 13.1, 2.9 Hz, 1H), 5.24 (s, 2H), 3.08 (dd, J = 13.1, 17.1 Hz, 1H), 2.78 (dd, J = 3.0, 17.1 Hz, 1H).

KUF-4: 1H-NMR (CDCl3, 400 MHz) 7.72–6.88 (m, 8H), 6.12–6.08 (m, 2H), 5.34 (dd, J = 12.8, 3.2 Hz, 1H), 4.96 (s, 2H), 3.06 (dd, J = 13.6, 18.0 Hz, 1H), 2.78 (dd, J = 2.8, 17.2 Hz, 1H).

KUF-5: 1H-NMR (CDCl3, 400 MHz) 7.73–6.70 (m, 11H), 6.14 (m, 2H), 5.32 (d, J = 13.0 Hz, 1H), 5.04 (s, 2H), 3.09 (dd, J = 13.0, 17.2 Hz, 1H), 2.79 (dd, J = 2.9, 17.2 Hz, 1H).

KUF-6: 1H-NMR (CDCl3, 400 MHz) 7.90–6.85 (m, 9H), 6.16 (m, 2H), 5.31 (m, 5H), 3.07 (dd, J = 13.0, 17.1 Hz, 1H), 2.76 (dd, J = 3.0, 17.1 Hz, 1H).

KUF-7: 1H-NMR (CDCl3, 400 MHz) 7.21 (d, J = 8.2 Hz, 2H), 6.83 (d, J = 8.2 Hz, 2H), 5.98 (m, 2H), 5.21 (m, 1H), 4.59 (m, 4H), 3.71 (s, 3H), 3.70–3.40 (m, 2H), 3.01 (m, 1H), 2.65 (m, 1H), 2.05 (m, 4H), 1.63 (m, 3H), 0.87 (m, 6H).

KUF-8: 1H-NMR (CDCl3, 400 MHz) 7.24 (d, J = 7.9 Hz, 2H), 6.85 (d, J = 7.9 Hz, 2H), 6.04 (m, 2H), 5.29 (m, 1H), 4.67 (m, 2H), 4.15 (m, 1H), 3.97 (m, 2H), 3.70 (s, 3H), 3.70–3.40 (m, 2H), 3.07 (m, 1H), 2.79 (m, 1H), 2.30–1.60 (m, 4H).

Nuclear DAPI Stain Analysis

We also conducted DAPI staining for the identification of apoptotic nuclei. RKO cells were collected at 2,000 rpm for 5 min, washed once in cold PBS, fixed in ice-cold methanol/acetic acid (1:1, v/v) for 5 min, then stained with 0.8 mg/ml of 4′,6-diamidino-2-phenolindole (DAPI) in darkness [Studzinski and Harrison, 1999]. Morphological changes in the apoptotic cells were visually assessed under a Zeiss Axiovert 200 microscope, at the fluorescence of the DAPI region (excitation, 351 nm; emission, 380 nm) [Cho et al., 2003; Ryoo et al., 2004].

Western Blot Analysis

Cells in 100 mm dishes were washed three times in ice-cold phospho-buffered saline

(PBS), scraped from the dishes, and then collected in extraction buffer (1% Triton X-100, 100 mM Tris–HCl, pH 7.5, 10 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM p-nitrophenyl phosphate, 1 mM PMSF). After the cells had been incubated on ice for 30 min, the lysates were centrifuged and the amount of proteins in the cleared lysates was quantified. An equal amount of proteins was then separated on 10–12% SDS–PAGE gel, and then transferred to nitrocellulose membranes (0.2 mm, Schleicher and Schuell). These membranes were blocked with 3–5% non-fat dry milk and 0.1% Tween-20 in Tris-buffered saline (TBS), and subsequently probed with primary antibody in TBS containing 3% non-fat dry milk and 0.1% Tween-20. The antibody-antigen complexes were then detected using goat anti-mouse IgG or goat anti-rabbit IgG peroxidase conjugates, followed by the use of an enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience).

Flow Cytometric Analysis

Cells were trypsinized and collected via 10 min of centrifugation at 1,000 rpm. The harvested cells were then washed in PBS, resuspended at a density of 1×10^6 cells/ml, fixed with 70% ethanol overnight at 4°C, and stained with PI solution (5 μg/ml propidium iodide, 0.1% Triton X-100, 0.1 mM EDTA, and 5 μg/ml RNase) for 20 min at 4°C. The stained DNA was then analyzed via flow cytometry (FACS Calibur; Beckton Dickinson).

Measurement of Mitochondrial Membrane Potential

The mitochondrial transmembrane potential was measured with DiOC6 [Le et al., 2006], a fluorochrome that is incorporated into cells depending upon the mitochondrial membrane potential [Kuo et al., 2007]. During apoptosis, mitochondrial-membrane potential is lost. Cells (1×10^6) were treated with MNDs for 24 h in a 60 mm dish. The changes in the $\Delta\psi m$ were determined by incubating PBS-washed 1×10^6 cells in 40 nM DiOC6 for 20 min at 37°C. After washing, the cells were resuspended in 1 ml PBS (pH 7.4) and were then analyzed using FACS CaliburTM flow cytometry. For the DiOC6-stained cells were excluded. At least 10,000 cells were analyzed for each sample.

Measurement of Intracellular ROS Levels

Intracellular ROS levels were assessed using the oxidant-sensitive fluorescent probe, DCFHDA, under inverted microscopy. Cells grown at 1×10^6 cells per 35 mm culture dish were maintained for 24 h in growth medium, then exposed to 5 mM DCFHDA for 30 min. The cells were washed in PBS, and a cover glass was placed atop the dish. DCF fluorescence (excitation, 488 nm; emission, 520 nm) was then imaged on an inverted fluorescence microscope. Intracellular peroxide production was also measured, using DCFHDA coupled with spectrofluorometry. Fluorescence was quantified with a Shimadzu RF5301 PC spectrofluorophotometer, set at an excitation of 504 nm and an emission of 524 nm [Royall and Ischiropoulos, 1993]. The cells were exposed to MNDs, for 12 h and then a 5 mM stock solution of DCFHDA was added to each culture dish 30 min prior to the assay. After incubation at 37°C, the cells were washed twice in ice-cold PBS, resuspended in 200 μl of PBS, and disrupted by three 10-s cycles of low-output sonication. The supernatants were then acquired after 10 min of centrifugation in a microcentrifuge and the crude extracts (500 μg of protein) were suspended in PBS, after which the fluorescence was recorded.

RESULTS

Effect of Flavonoids on the Cell Viability of Colorectal Carcinoma RKO Cells

Colorectal cancer is the third most common malignancy in the world, and accordingly is considered to be one of the primary public health concerns worldwide [Jemal et al., 2002]. In order to evaluate the effects of various flavonoids on the cell viability of human colorectal carcinoma RKO cells, we treated these cells with different quantities (10–30 μM) of several flavonoids. We previously suggested that the OH group or substitution of the 5- or 7-carbon of the C6C3C6 skeletons of flavonoids may represent an important determinant of the anti-oxidant and anti-apoptotic activities of flavonoids [Lee et al., 2005, 2007]. In order to determine the importance of the OH group or the substitution of the 5- or 7-carbons of flavonoids, we selected naringenin and 13 different flavones: naringenin (5,7,4′-trihydroxy flavanon) and three different flavones (5,7,3′,4′, 5′-pentahydroxy flavone, 5,7,3′-trihydroxy-4′-

methoxy flavone, and 5,7-dihydroxy-3′,4′, 5′-trimethoxy flavone) containing both the 5- and 7-hydroxyl or substitution group; three different flavones (7,8,4′-trihydroxy flavone, 4′-hydroxy-7-methoxy flavone, and 4′-hydroxy-5-methoxy flavone) having either 5- or 7-hydroxyl or substitution group; five different flavones (4′-hydroxy flavone, 3-hydroxy flavone, 3,2′-dihydroxy flavone, 3,3′-dihydroxy flavone, and 3,4′-dihydroxy flavone) devoid of 5- or 7-hydroxyl group including; two different flavones (6,4′-dihydroxy flavone and 4′-hydroxy-6-methoxy flavone) devoid of 5- or 7-hydroxyl group but containing 6-hydroxyl or substitution group (Fig. 1A). We then evaluated the cell viability of the RKO cells treated with different amounts of one of the 14 selected flavonoids via MTT assays. The MTT assay relies principally on the mitochondrial metabolic capacities of the viable cells and, hence, reflects the intracellular redox state. Two different flavones, 6,4′-dihydroxy flavone and 4′-hydroxy-6-methoxy flavone, which are devoid of 5- or 7-hydroxyl groups but harbor 6-hydroxyl or substitution groups, resulted in a dose-dependent increase in cell viability. Treatment with 5,7,3′-trihydroxy-4′-methoxy flavone, 3,2′-dihydroxy flavone also resulted in a dose-dependent increase in cell viability. Although the majority of the flavonoids employed in this study did not reduce the cell viability of the RKO cells, naringenin treatment resulted in a slight reduction in the cell viability of the RKO cells (Fig. 1B).

Effect of Several Synthetic Flavonoids on Cell Viability of RKO Cells

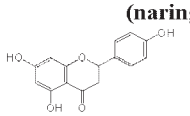
In order to determine the effects of the specific substitution of the 7-carbon of naringenin on apoptosis-regulatory activities, we originally synthesized several modified naringenin derivatives (MNDs), including 7-*O*-benzyl naringenin (KUF-1), 7-*O*-(*m*-metoxybenzyl) naringenin (KUF-2), 7-*O*-(*p*-fluorobenzyl) naringenin (KUF-3), 7-*O*-(*m*-iodobenzyl) naringenin (KUF-4), 7-*O*-(2-naphtylmethyl) naringenin (KUF-5), 7-*O*-benzoxycarbonylmethyl naringenin (KUF-6), 7-*O*-(MeO-L-Leu-D-Pro-carbonylmethyl) naringenin (KUF-7), and 7-*O*-(MeO-Gly-D-Pro-carbonylmethyl) naringenin (KUF-8; Fig. 2A). Initially, KUF-1 was synthesized to harbor an aromatic group in the C7 position of naringenin rather than the OH group. This naringenin derivative, KUF-1, appears to manifest a hydrogen bonding capacity differing from that of the original flavonoid, naringenin. Other MNDs, including KUF-2, KUF-3, KUF-4, KUF-5, and KUF-6, were also synthesized in an effort to determine the importance of hydrogen bonding capacity on the effects of the naringenin derivatives. Different quantities (10–30 μM) of naringenin derivatives were added to the RKO cell cultures, and their effects on the cell viability of RKO cells were assessed at several time points via MTT assays. Addition of the six-selected MNDs appeared to cause the cells to lose cell viability in a dose- and time-dependent manner, and this effect was more profound than was seen with naringenin (Fig. 2B,C). In particular, KUF-1 and KUF-2 evidenced the most dramatic effects on the cell viability of RKO cells, and their effects were more efficient than that of etoposide, a well-known apoptosis-inducing anti-cancer drug. For KUF-1 and KUF-2, the IC50 values for RKO cells were in the range of 20–30 μM, demonstrating a 10- to 15-fold greater growth inhibition than naringenin (Table I).

KUF-1- or KUF-2-Induced Apoptotic Cell Death of RKO Cells

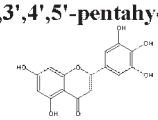
In order to determine whether the addition of MNDs induces apoptotic cell death, DAPI or PI staining of nuclear DNA was conducted in KUF-1, KUF-2, KUF-3, or KUF4-treated RKO cells. Initially, flow cytometric analyses of cell cycle distribution were conducted with the PI-stained RKO cells. Apoptosis was indicated by the accumulation of a sub-G1 population of MNDs-treated RKO cells. As the cells were exposed to KUF-1 or KUF-2, the percentage of apoptotic cells increased markedly (Fig. 3A). Nuclear morphology was also assessed via fluorescence microscopy using DAPI staining. Nuclear condensation and fragmentation became apparent upon exposure to MNDs (10 μM; Fig. 3B). In particular, KUF-1 and KUF-2 induced significant increases in apoptotic cell death in the RKO cell cultures. Changes in mitochondrial membrane potential ($\Delta\psi m$) were also analyzed in KUF-1- or KUF-2-treated RKO cells using the cationic lipophilic dye, DiOC6. Reductions in the degree of DiOC6 staining were considered reflective of the apoptosis-associated disruption of the mitochondrial inner transmembrane potential [Le et al., 2006]. KUF-1 or KUF-2 treatments induced progressive losses in mito-

A

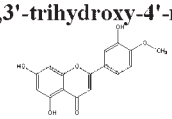
(1) : 5,7,4'-trihydroxy flavanon (naringenin)



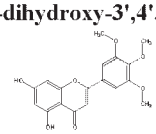
(2) : 5,7,3',4',5'-pentahydroxy flavone



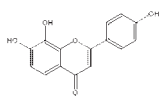
(3) : 5,7,3'-trihydroxy-4'-methoxy flavone



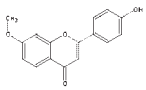
(4) : 5,7-dihydroxy-3',4',5'-trimethoxy flavone



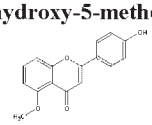
(5) : 7,8,4'-trihydroxy flavone



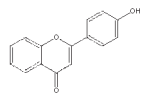
(6) : 4'-hydroxy-7-methoxy flavone



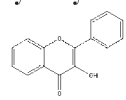
(7) : 4'-hydroxy-5-methoxy flavone



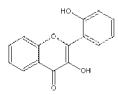
(8) : 4'-hydroxy flavone



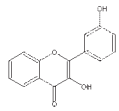
(9) : 3-hydroxy flavone



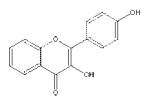
(10) : 3,2'-dihydroxy flavone



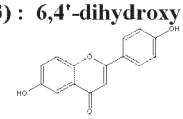
(11) : 3,3'-dihydroxy flavone



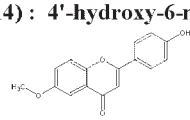
(12) : 3,4'-dihydroxy flavone



(13) : 6,4'-dihydroxy flavone



(14) : 4'-hydroxy-6-methoxy flavone



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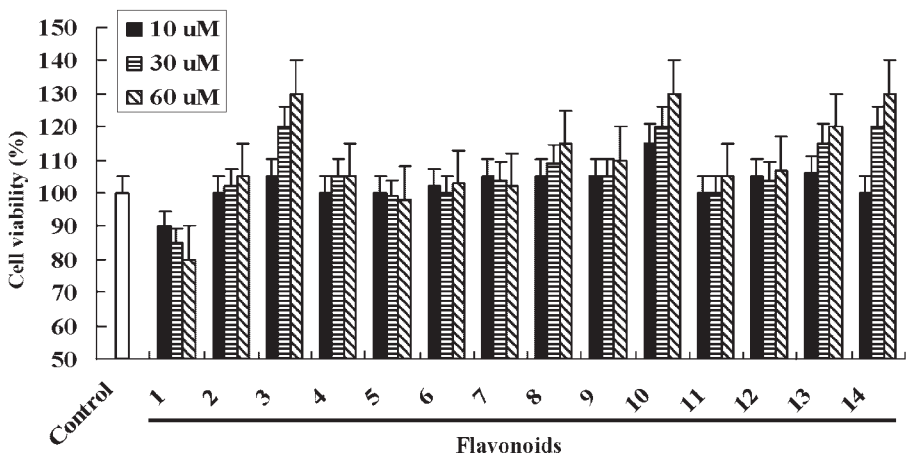


Fig. 1.

chondrial membrane potential and a concomitant increase in apoptotic cell death (Fig. 3C).

Stimulation of Caspase-3 and Caspase-8 Activities in KUF-1 or KUF-2-Induced Apoptosis

Previous reports showed that the induction of caspase activity is a pivotal event in apoptosis, and either caspase-3 or caspase-8 has been shown to be extensively involved in the apoptotic process. Caspase-3 recognizes the Asp-Giu-Val-Asp (DEVD) sequence, and cleaves a number of proteins, including PARP, one of the hallmarks of apoptosis. The addition of KUF-1 or KUF-2 induced the cleavage of PARP and the activation of caspase-3 and caspase-8 (Fig. 4A). In order to further determine whether the activation of caspase-8, as well as caspase-3 is necessary for MNDs-induced apoptosis, caspase inhibitors including the caspase-3-like protease inhibitor, Ac-DEVD-CHO, and the caspase-8-like protease inhibitor, Z-IETD, were employed to block intracellular protease activities. The addition of Ac-DEVD-CHO or Z-IETD evidenced an obvious preventive effect on MNDs-induced apoptosis (Fig. 4B and C). Moreover, we showed that activation of caspase-3 was mainly coming through activation of caspase-8, as the addition of Z-IETD almost completely inhibited the KUF-1- or KUF-2-induced activation of caspse-3 (Fig. 4D). These data show that the activation of caspase-8 and caspase-3-like activities is a downstream event in MNDs-induced apoptosis.

Generation of Intracellular ROS in the MNDs-Induced Apoptosis of RKO Cells

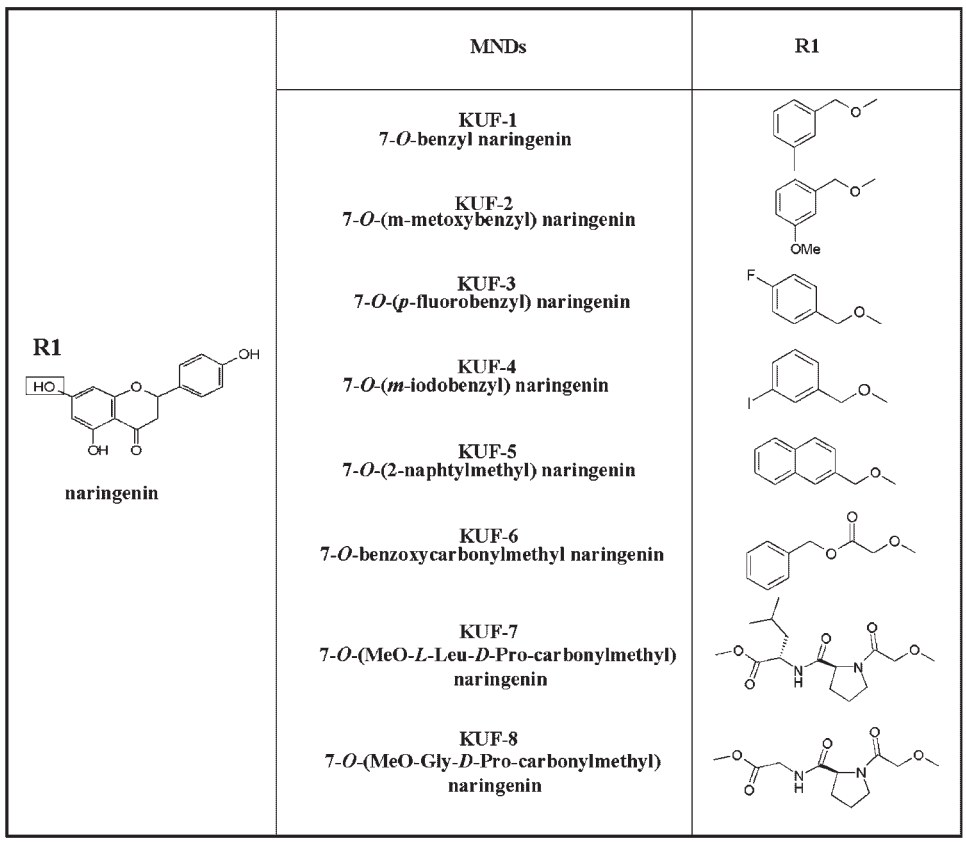
Several reports have indicated that a host of phenolic phytochemicals, including etoposide and flavonoids, can alter intracellular ROS levels. In order to further elucidate the molecular basis of the apoptotic effect of the MNDs, we attempted to determine whether the addition of MNDs induces intracellular ROS production. We measured the levels of intracellular ROS production using the oxidant-sensitive fluorescent dye, DCFH-DA. Apparent intracellular ROS generation was detected after treatment of the MNDs, and the increase of ROS production was almost completely antagonized by the addition of *N*-acetylcysteine, a well-known anti-oxidant and free radical scavenger (Fig. 5A,B). This result strongly indicates that the induction of intracellular ROS generation is involved in the MNDs-induced apoptosis of RKO cells.

The ERK Signaling Pathway Is Involved in the Apoptosis-Inducing Effects of KUF-1 or KUF-2

Several reports have shown that the MAPK signaling pathways perform important functions in the effects of chemotherapeutic agents [Lee et al., 2006]. In order to determine the role of the MAPK signaling pathways in the mechanism underlying MNDs-induced apoptosis, the expression and activation of ERK1/2, JNK/SAPK, and p38 MAPK were measured after the exposure of RKO cells to KUF-1 or KUF-2. The level of expression and activation of both p38 MAPK and JNK remained unaltered in response to KUF-1 or KUF-2 treatment, but KUF-1 and KUF-2 triggered significant upregulations of ERK1/2 phosphorylation (Fig. 6A). Moreover, pretreatment with PD98059, a MEK inhibitor, clearly antagonized both KUF-1 or KUF-2-induced PARP cleavage and caspase activation (Fig. 6B). In order to verify the involvement of ERK stimulation in the apoptotic effects associated with KUF-1 or KUF-2 treatment, we transfected the RKO cells with the kinase-inactive and dominant negative ERK2 mutant, ERK2-DN (K52R) [Lee et al., 2005]. ERK2-DN overexpression was determined to significantly override the apoptosis-inducing functions of KUF-1 or KUF-2 (Fig. 6C). When ERK2-DN was overexpressed, KUF-1- or KUF-2-induced caspase-3 activation and PARP cleavage were significantly attenuated, clearly

Fig. 1. Chemical structure of flavonoids used in this study and their effects on the cell viability of Human colon cancer RKO cells. **A**: The chemical structures of flavonoids 1–14: (1) naringenin; (2) 5,7,3′,4′,5′-pentahydroxy flavone; (3) 5,7, 3′-trihydroxy-4′-methoxy flavone; (4) 5,7-dihydroxy-3′,4′, 5′-trimethoxy flavone; (5) 7,8,4′-trihydroxy flavone; (6) 4′-hydroxy-7-methoxy flavone; (7) 4′-hydroxy-5-methoxy flavone; (8) 4′-hydroxy flavone; (9) 3-hydroxy flavone; (10) 3,2′-dihydroxy flavone; (11) 3,3′-dihydroxy flavone; (12) 3,4′-dihydroxy flavone; (13) 6,4′-dihydroxy flavone; (14) 4′-hydroxy-6-methoxy flavone. **B**: The RKO cells were treated with the indicated amounts of flavonoids, and cell viability was assessed via MTT assay. The RKO cells were exposed to different concentrations (10–30 μM) of each flavonoid for 48 h. As a control, the cells were treated with an equal amount of DMSO in the absence of flavonoids. Data were expressed as the means ± SE of values from three independent experiments.

A



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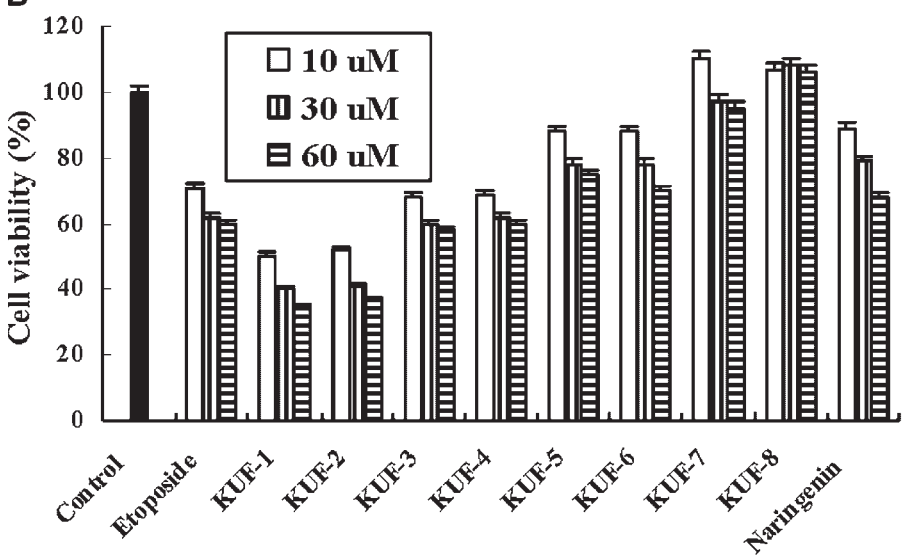

Fig. 2. Induction of cell death by modified naringenin derivatives (MNDs) in RKO cells. **A**: The chemical structures of MNDS. **B**: RKO cells were treated with different doses of MNDs for 24 h as indicated. Cells treated with a solution devoid of MNDs were used as controls (0). Cell viability was determined via MTT assay. **C**: Cells were incubated with the indicated MND (10 μM) for the indicated time periods. The data are expressed as the means ± SE of values from three independent experiments.

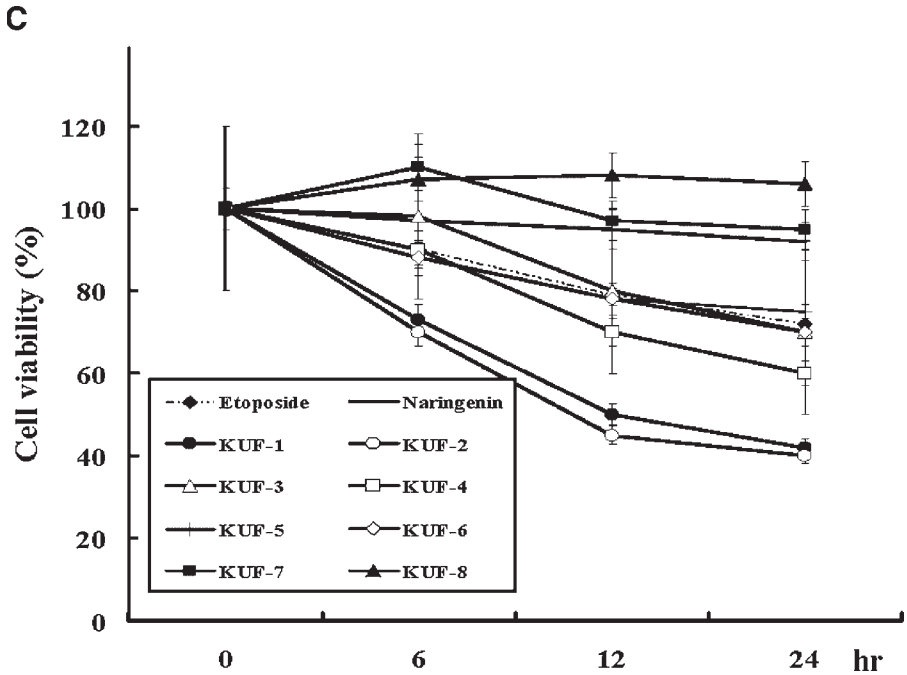

Fig. 2. (*Continued*)

indicating that the ERK signaling pathway is intimately involved in KUF-1- or KUF-2-induced apoptotic cell death in RKO cells.

DISCUSSION

Flavonoids evidence anti-tumor activity, and have been shown to perform a multitude of other functions, including the inactivation of carcinogens, induction of cell cycle arrest and apoptosis, inhibition of angiogenesis, reversal of multi-drug resistance, or a combination of these activities [Kanadaswami et al., 2005]. Flavonoids are polyphenolic compounds harboring 15 carbon atoms and two benzene rings joined by a linear three-carbon chain, constituting one of the most characteristic classes of compounds found in higher plants (Fig. 1A). Several thousand flavonoids are known to occur in nature, and they are both qualitatively and quantitatively one of the largest groups of natural products known [Depeint et al., 2002]. Various flavonoid subgroups are classified according to the substitution patterns of ring C, and both the oxidation state of the heterocyclic ring and the position of ring B are also important in their classification. The six major flavonoid subgroups are flavans-3-ol, flavone, flavonol, flavanone, and finally the isoflavonoids. The majority of these subgroups (flavanones, flavones, flavonols, and anthocyanins) harbor ring B at position 2 of the heterocyclic ring, but in the isoflavonoids, ring B occupies position 3. Moreover, flavonoids fall into two distinct groups, depending on the saturation status of the central heterocyclic ring. In cases in which the central heterocyclic ring is saturated, as in the flavanones and flavans, the flavonoids exhibit optical activity, due to the presence of one or more chiral centers. In cases in which the central heterocyclic ring is unsaturated, as in anthocyanidin, the flavones, and the flavonols, the molecule is achiral.

Several biological functions of flavonoids have been identified, but there currently exists a lack of evidence for any relationship between

TABLE I. Growth Inhibitory Activity of Six Selected MNDs in RKO Cells

	IC_{50} (μM)
Naringenin	>150
MNDs	
KUF-1	10
KUF-2	15
KUF-3	67
KUF-4	66
KUF-5	90
KUF-6	85

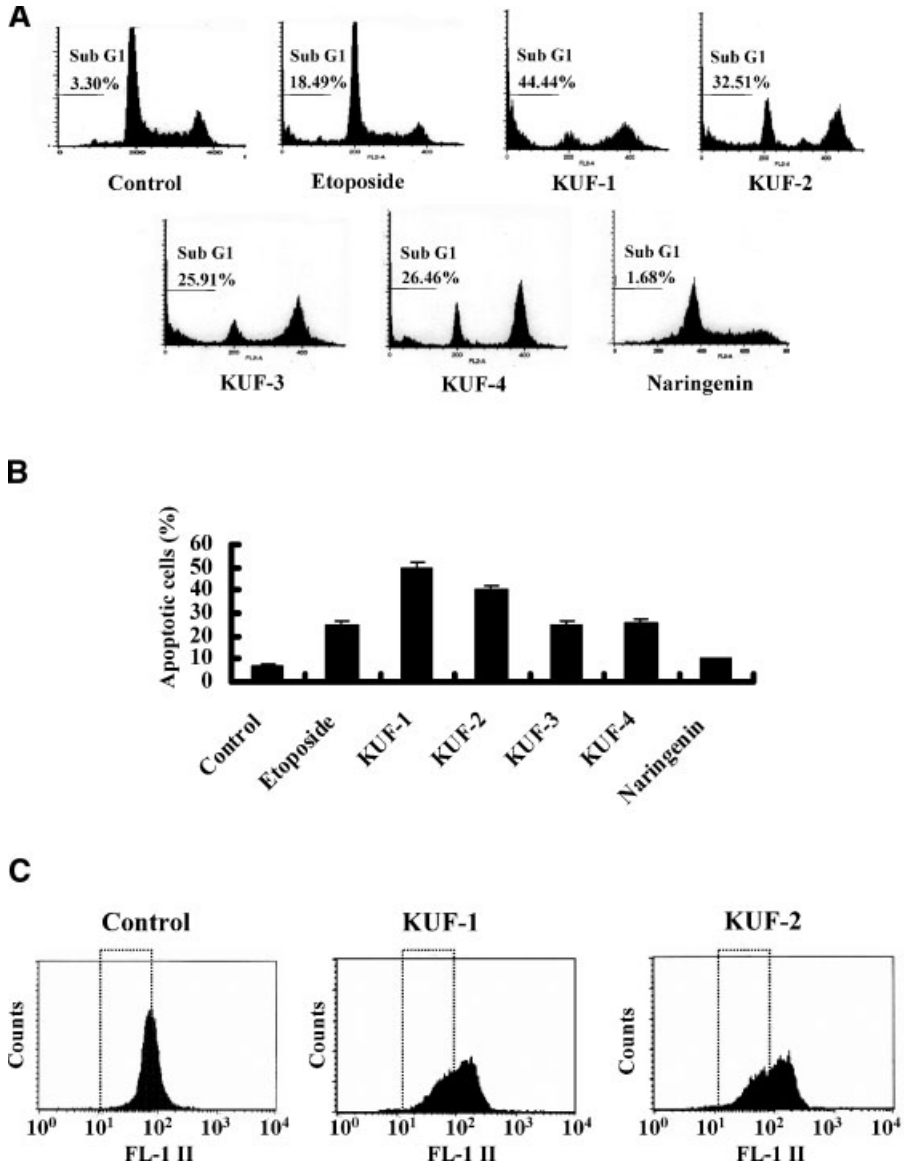

Fig. 3. Treatment of MNDs induced apoptosis in RKO cells. **A**: Cells (1×10^6 cells per ml) were cultured in the absence (control) or presence of 10 μM MND for 24 h. The cells were harvested and stained with propidium iodide and their DNA contents were analyzed by flow cytometry. The percentage of apoptotic cells was assessed by counting the cells with DNA contents below 2N. **B**: The cells were harvested, fixed with methanol/acetic acid (1:1, v/v) then loaded with 0.8 μg/ml DAPI for 5 min. Fluorescence images were acquired via fluorescence microscopy, and the cells evidencing apoptotic nuclear morphology were counted. Data are expressed as the means ± SE of values from three independent experiments. **C**: Cytofluorometric analysis of DiOC6-labeled RKO cells treated with KUF-1 or KUF-2 (10 μM). The cells were treated with the drug for 24 h prior to labeling with 25 nM DiOC6, and then the cells were detached and analyzed via flow cytometry. Histograms show all the acquired events.

the structure-activity relationship (SAR) and apoptosis-inducing activity. The role of hydroxylation of flavonoids in induction of apoptosis has been previously demonstrated [Kawaii et al., 1999a; Sergeev et al., 2006]. Previously, four structurally related flavonoids with or without rutinoside at 7-carbon were applied to investigate their apoptosis-inducing effects, providing direct evidence to suggest that the moiety at C7 is an important determinant for the induction of apoptosis by flavonoids [Chen et al., 2003]. We also suggested that substitutions on the 5- or/and 7-carbons in the diphenylpropane (C6C3C6) skeleton of the flavonoids might

significantly influence the apoptosis-regulating properties of these compounds [Lee et al., 2005]. In order to investigate the importance of the OH group or substitution of the 5- or 7-carbons of flavonoids, we selected 14 different flavonoids with different structures, particularly on the 5- or 7-carbon in the diphenylpropane skeleton (Fig. 1A). The majority of flavonoids employed in the present study effected no reductions in the cell viability of the cancer cells, but naringenin treatment caused a slight decrease in the cell viability of the RKO cells (Fig. 1B).

Although naringenin, a flavanone which is especially abundant in citrus fruits and in the tomato [Verhoeyen et al., 2002; Bugianesi et al., 2004], has been reported to evidence anti-proliferative effects in different cancer cells [Kawaii et al., 1999b; Frydoonfar et al., 2003; Virgili et al., 2004], naringenin evidenced only a slight anti-proliferative effect in human colon carcinoma RKO cells. In order to characterize the effects of specific substitutions of the 7-carbon of naringenin on apoptosis-regulatory activities, and in an attempt to develop anti-proliferative flavonoid derivatives that would be more effective against colon cancer, we originally synthesized several MNDs including 7-*O*-benzyl naringenin (KUF-1) and 7-*O*-(*m*-metoxybenzyl) naringenin (KUF-2; Fig. 2A). Treatment with the MNDs, particularly KUF-1 and KUF-2, caused the cells to lose cell viability in a dose- and time-dependent manner, and this effect was more prominent than was seen with naringenin treatment (Fig. 2B,C). KUF-1 or KUF-2 treatment evidenced the most dramatic apoptosis-inducing effects concomitant with progressive losses in mitochondrial membrane potential, activation of caspases, intracellular ROS production, and sustained ERK activation (Fig. 3). Our findings indicated that the activation of both caspase-3 and caspase-8-like activities are a downstream event in the KUF-1 or

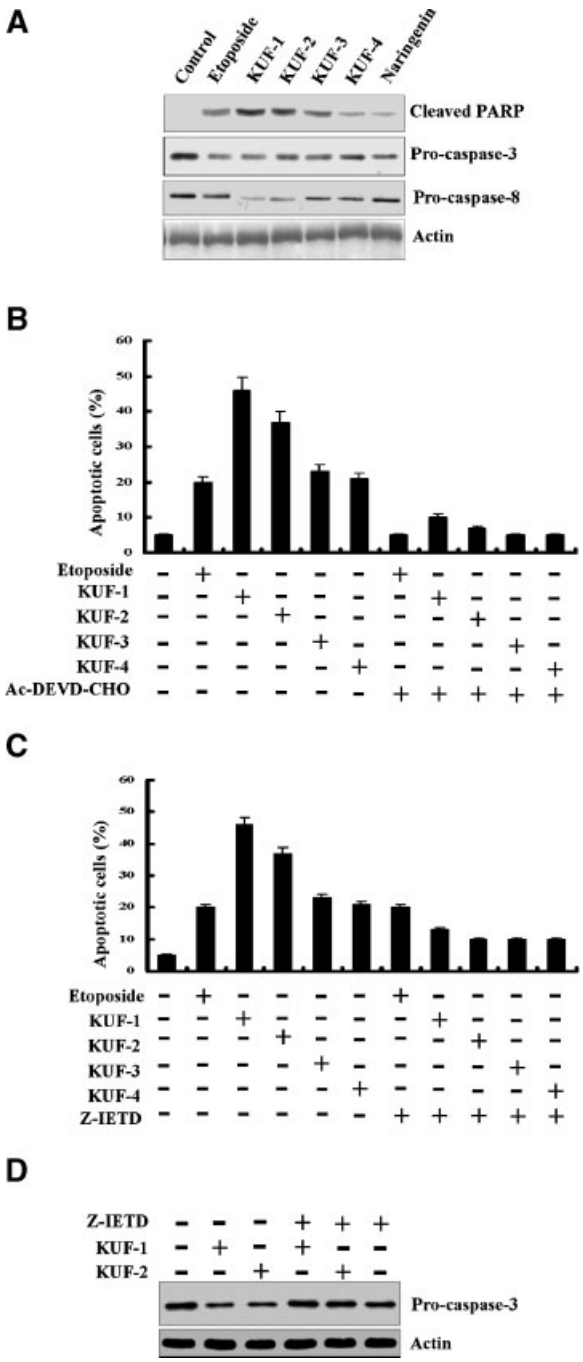

Fig. 4. Effect of specific caspase inhibitors on the MND-induced apoptosis. **A**: Cells were incubated for 24 h with MNDs (10 μM). The proteins were separated on 10% SDS–polyacrylamide gel, then transferred to nitrocellulose membranes. The cleavage of PARP and pro-caspase-8 and pro-caspase-3 was assessed via Western blotting. The blots were then reprobed with anti-actin antibody in order to confirm an equal amount of protein loading: **B**,**C**: After the pretreatment of the cells with a specific caspase inhibitor, such as Z-VAD-CHO (caspase-3, 10 μM) or Z-IETD (caspase-8, 10 μM) for 1 h, the cells were exposed to MND (10 μM) for 24 h. The cells were stained with DAPI and fluorescence images were obtained via fluorescence microscopy. Apoptotic cells evidencing nuclear condensation and fragmentation were then counted, and the percentage of cells undergoing apoptotic cell death was determined. The data are expressed as the means ± SE of values from three independent experiments. **D**: After the pretreatment of the cells with a specific caspase inhibitor, Z-IETD (caspase-8, 10 μM) for 1 h, the cells were exposed to MND (10 μM) for 24 h. Pro-caspase-3 was assessed via Western blotting.

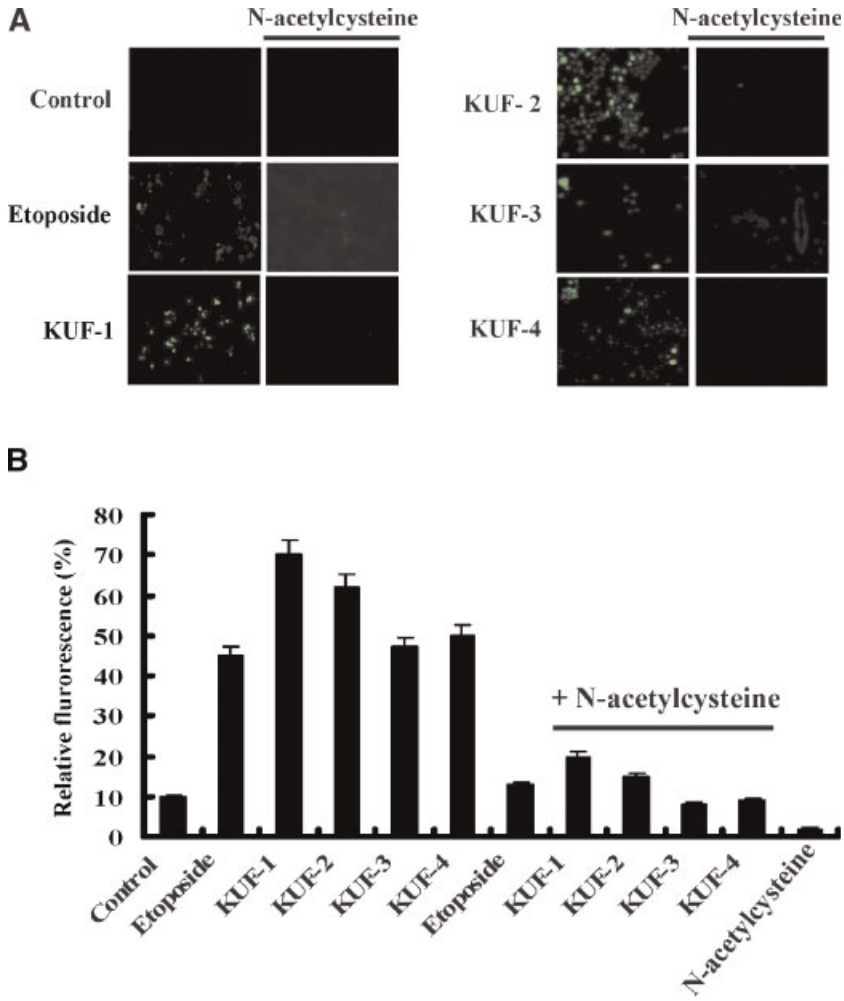

Fig. 5. Production of intracellular ROS in MND-induced apoptosis of RKO cells. **A**: Cells were incubated with specific MND (10 μM) for 12 h, and then intracellular ROS levels were evaluated via fluorescence microscopy with an oxidant-sensitive probe, DCFH-DA. For the indicated samples, cells were preincubated with *N*-acetylcysteine (NAC, 2 mM) for 1 h and then incubated with specific MND (10 μM) for 12 h. **B**: DCF fluorescence in the cells was quantified using a spectrofluorometer (excitation, 504 nm; emission, 524 nm). The results were expressed as the means ± SE of values obtained from three separate experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

KUF-2-induced apoptosis of RKO cells (Fig. 4). Moreover, the induction of intracellular ROS generation and the activation of the ERK signaling pathway were clearly shown to be intimately involved in KUF-1- or KUF-2-mediated apoptotic cell death in RKO cell cultures (Figs. 5 and 6). The anti-cancer effects of KUF-1 or KUF-2 were shown to be superior to that of etoposide, a well-known apoptosis-inducing anti-cancer drug.

Our results indicate that hydroxylation or substitution at C7 is crucial with regard to the apoptosis-inducing activity of flavonoids, a finding which represent a significant step forward in our understanding of the structure-activity relationship of flavonoids, and also shed new light on the molecular mechanisms underlying the differential effects of structurally different flavonoids. In conclusion, our study may provide helpful information, allowing us to further address the controversial pro-oxidant/anti-oxidant properties evidenced by various flavonoids and also augment our current understanding of the molecular mechanisms underlying the differential cellular functions associated with flavonoid treatment. The exact

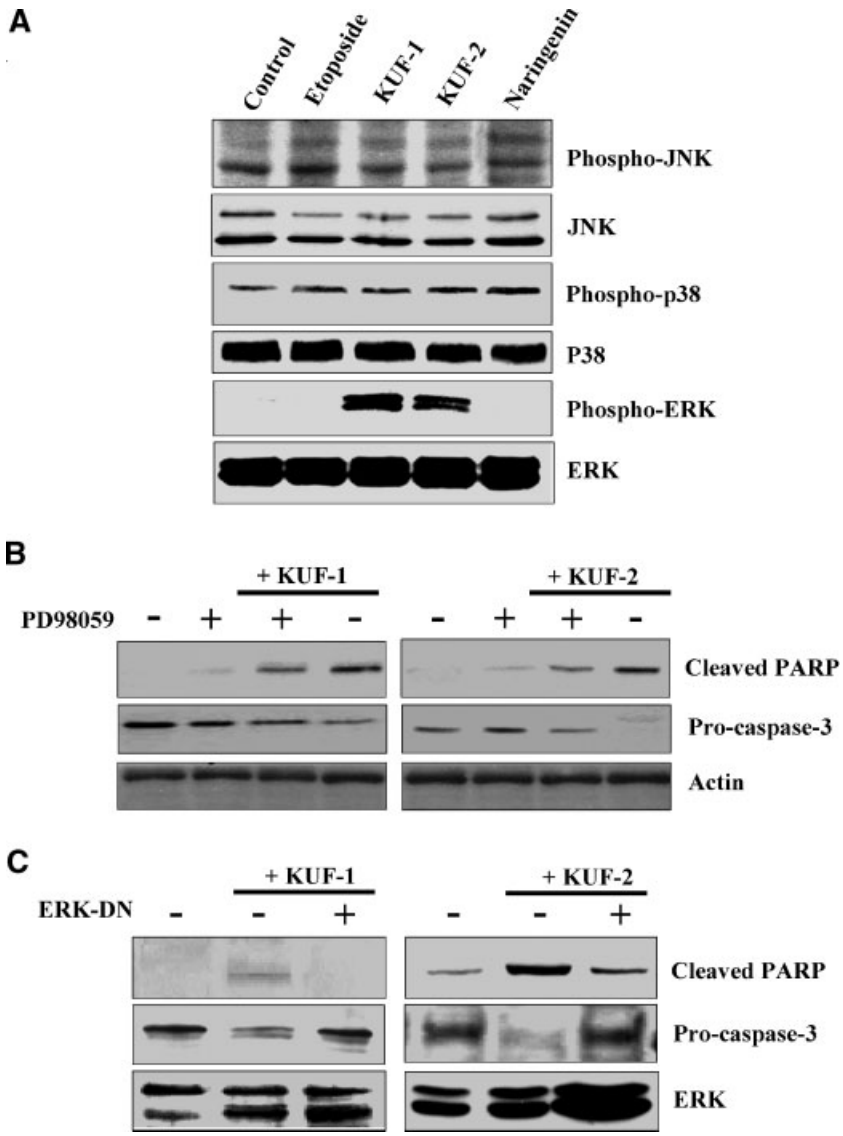

Fig. 6. Effect of MAP kinases in KUF-1- or KUF-2-induced apoptosis of RKO cells. **A**: Cells were exposed to 10 μM KUF-1 or KUF-2 for 24 h. The cells were lysed, and the cell lysates were resolved via SDS–PAGE. The phosphorylation status of the MAPKs was analyzed via Western blotting with anti-phospho-JNK, anti-phospho-ERK1/2, or anti-phospho-p38 MAPK antibody, as indicated. Equal protein loading was verified via the probing of the membranes with antibodies for the detection of the respective unphosphorylated proteins. **B**: RKO cells were pretreated with PD98059 (10 μM) for 1 h and then incubated with KUF-1 or KUF-2 (10 μM) for 24 h. Cell lysates were resolved via SDS–PAGE and analyzed by Western blotting with anti-caspase-3, anti-actin, or anti-PARP antibody. **C**: RKO cells were transfected with the dominant negative mutant form of ERK1 (ERK-DN). The transfected cells were treated for 24 h with KUF-1 or KUF-2 (10 μM) and the cell lysates were analyzed via SDS–PAGE and Western blotting using anti-ERK, anti-phospho-ERK, anti-PARP, or anti-caspase-3 antibody. The data are expressed as the means ± SE of values from three independent experiments.

mechanism underlying this structure-activity relationship may be elucidated in further studies.

ACKNOWLEDGMENTS

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