Molecular targets for apigenin-induced cell cycle arrest and apoptosis in prostate cancer cell xenograft

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Abstract

Apigenin (4′,5,7-trihydroxyflavone) is a promising chemopreventive agent abundantly present in fruits and vegetables that has been shown to promote cell cycle arrest and apoptosis in various malignant cell lines. To determine whether pharmacologic intervention with apigenin has a direct growth inhibitory effect on human prostate tumors implanted in athymic nude mice, we examined cell cycle regulatory molecules as precise molecular targets of apigenin action. Apigenin feeding by gavage to these mice at doses of 20 and 50 μg/mouse/d in 0.2 mL of a vehicle containing 0.5% methyl cellulose and 0.025% Tween 20 resulted in significant decreases in tumor volume and mass of androgen-sensitive 22Rv1 and androgen-insensitive PC-3-implanted cells. Oral intake of apigenin resulted in dose-dependent (*a*) increase in the protein expression of WAF1/p21, KIP1/p27, INK4a/p16, and INK4c/p18; (*b*) down-modulation of the protein expression of cyclins D1, D2, and E; and cyclin-dependent kinases (cdk), cdk2, cdk4, and cdk6; (*c*) decrease in retinoblastoma phosphorylation at serine 780; (*d*) increase in the binding of cyclin D1 toward WAF1/p21 and KIP1/p27; and (*e*) decrease in the binding of cyclin E toward cdk2 in both types of tumors. In addition, apigenin feeding resulted in stabilization of p53 by phosphorylation at serine 15 in 22Rv1 tumors, which seems to exhibit p53-dependent growth inhibitory responses. Apigenin intake by these mice also resulted in induction of apoptosis, which positively correlated with serum and tumor apigenin levels. Taken together, this is the first systematic *in vivo* study showing the involvement of cell cycle regulatory proteins as potential molecular targets of apigenin. [Mol Cancer Ther 2006;5(4):843–52]

Introduction

One of the most desirable goals in cancer chemoprevention is the identification of natural agents with demonstrable efficacy against defined molecular targets. Cancer chemoprevention is a means of cancer control in which a malignancy is prevented or reversed by pharmacologic intervention with naturally occurring and/or synthetic agents (1, 2). Apigenin (4′,5,7-trihydroxyflavone) is a naturally occurring nontoxic, nonmutagenic plant flavonoid commonly present in fruits and vegetables with proven anti-inflammatory and anticarcinogenic effects in various animal tumor model systems (3–5). Apigenin has been shown to suppress angiogenesis in melanoma and carcinoma of the breast, skin, and colon (6–10). The effects of apigenin seem to be primarily mediated through suppression of the expression of hypoxia-inducible factor 1-α, cyclooxygenase-2, nitric oxide synthase-2, vascular endothelial growth factor, and lipoxygenase (4, 11, 12). Apigenin has shown potential to inhibit growth in several human cancer cells, including breast, colon, skin, thyroid, leukemia, and prostate (8–10, 13–15). These cell inhibitory effects are mediated via cell cycle arrest and induction of apoptosis. The molecular targets of apigenin-mediated cell growth inhibition and apoptosis are through activation of caspases, inhibition of fatty acid synthase, topoisomerase inhibition, nuclear factor-κB inhibition, and modulation in Bax and Bcl-2 ratio (15–18).

An event that frequently accompanies human cancer development is deregulation of the cell cycle machinery. Normal eukaryotic cells progress through the cell cycle in a regulated manner because of a cascade of biochemical events that coordinates the transition of cells from one phase to another (19). A series of events must be completed before entry of cells into S phase. These events include elevations in D-type cyclins (D1, D2, and D3) and cyclin E levels in early and late G_1 phase of the cell cycle. D-type cyclins form complexes with cyclin-dependent kinase (cdk) 2, cdk4, or cdk6 and promote cell cycle progression by phosphorylation of the tumor suppressor protein retinoblastoma (Rb), which is required for entry into S phase (19, 20). G_1-phase cdks are positively regulated by cyclins and cdks and negatively regulated by cdk inhibitors such as WAF1/p21, KIP1/p27, and the INK4 family proteins (21). In addition to Rb, another tumor suppressor gene, *p53*, is also required for maintenance of G_1 checkpoint controls. Wild-type p53 is a transcription factor whose half-life increases after DNA damage (19–21). As a result, p53

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transcriptionally activates the WAF1/p21 promoter, resulting in increase in WAF1/p21 protein levels (21, 22). WAF1/p21 also forms complexes with G_1 cyclin-cdks and inhibits their kinase activity (23). This mechanism allows cells to stop and repair DNA defects before DNA replication occurs in the S phase. An ideal chemopreventive agent may have direct effects on the cell cycle regulatory molecules.

Apigenin is a potent inhibitor of several protein tyrosine kinases, including epidermal growth factor receptor and src tyrosine kinase (24). Apigenin has also been shown to inhibit activation of phosphatidylinositol 3-kinase, protein kinase B/Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2, casein kinase-2, and other upstream kinases involved in the development and progression of cancer (24–27). Despite growing interest in apigenin as a chemopreventive agent, the mechanisms by which it induces cell cycle arrest and apoptosis remain incompletely understood. Several groups, including our laboratory, have investigated the molecular mechanisms of apigenin-mediated cell cycle arrest in cell culture systems (9, 10, 14, 18). It has been shown that apigenin can inhibit cyclin and cdks *in vitro* and can up-regulate the endogenous cdk inhibitors (i.e., WAF1/p21 and KIP1/p27), and that this up-regulation is associated with loss in G_1 cdk activity, Rb dephosphorylation, and p53 stabilization (18). However, the exact relevance of these molecules during pharmacologic intervention with apigenin has not been elucidated. We studied the efficacy of p.o. intake of apigenin on the growth of human prostate cancer cells *in vivo* as xenografts in immunodeficient mice to evaluate whether the cell cycle regulatory proteins that are critical for driving prostate cancer cells through G_1 checkpoints may be potential molecular targets of apigenin action.

Materials and Methods

Materials

Androgen-sensitive human prostate carcinoma 22Rv1 and androgen-insensitive PC-3 cells were obtained from American Type Culture Collection (Manassas, VA). Apigenin (>95% purity) was obtained from A.G. Scientific, Inc. (San Diego, CA). M30-Apoptosense ELISA kit was purchased from Alexis Biochemicals (San Diego, CA). Fetal bovine serum was obtained from Life Technologies (Gaithersburg, MD). Antibodies recognizing full-length poly(ADP)ribose polymerase and its cleaved product, phospho-p53 (Ser^{15}) and anti-p-Rb (Ser^{780}), were purchased from Cell Signaling Technology (Fremont, CA). Monoclonal antibodies for WAF1/p21, KIP1/p27, INK4a/p16, INK4c/p18, cyclin D1, cyclin D2, cyclin E, cdk2, cdk4, cdk6, and p53 recognizing both mutant and wild-type protein were obtained from Lab Vision Corporation (Fremont, CA).

Cell Culture and Treatments

Human prostate carcinoma 22Rv1 and PC-3 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, and 1% penicillin and streptomycin. Cells were maintained at 37°C in a humidified CO_2 incubator. Log-phase growing cells (60% confluent) were treated with apigenin (10, 20, 40, and 80 μmol/L) dissolved in DMSO (maximum concentration, 0.1%) in complete culture medium for 24 hours. The cells were trypsinized thereafter, washed twice with cold PBS, and centrifuged. The pellet was resuspended in 50 μL cold PBS and 450 μL cold methanol for 1 hour at 4°C. The cells were centrifuged at 1,100 rpm for 5 minutes, the pellet was washed twice with cold PBS, suspended in 500 μL PBS, and incubated with 5 μL RNase (20 μg/mL final concentration) for 30 minutes. The cells were chilled over ice for 10 minutes and stained with propidium iodide (50 μg/mL final concentration) for 1 hour and analyzed by flow cytometry.

Animals

Approximately 8-week-old male athymic nude mice (weighing 28 ± 3 g) were obtained from Ireland Cancer Center and housed at the Association for Assessment and Accreditation of Laboratory Animal Care–accredited athymic nude mice facility of Case Western Reserve University. The animals were fed with the autoclaved Teklad 8760 high-protein diet and tap water *ad libitum* throughout the study.

Tumor Implantation

Approximately 1 million 22Rv1 and PC-3 cells suspended in 0.05 mL medium and mixed with 0.05 mL Matrigel were s.c. injected in the left and right flank of each mouse to initiate tumor growth. After implantation, the animals were kept under supervision for growth of tumor.

Apigenin Feeding

Apigenin (10 mg) was suspended in 1 mL vehicle material (0.5% methyl cellulose and 0.025% Tween 20) by sonication for 30 seconds at 4°C and further diluted for appropriate concentration. Apigenin, 20 and 50 μg/mouse/d (w/v), was administered by gavage in 0.2 mL of a vehicle consisting of 0.5% methyl cellulose and 0.025% Tween 20, daily for 8 weeks throughout the study.

Experimental Design

To determine the effect of apigenin on prostate tumor growth, 22Rv1 tumors were grown s.c. in athymic nude mice as previously described (28). Two weeks after cell inoculation, animals were divided into three equal groups of six mice each. The first group received only 0.2 mL vehicle material by gavage daily and served as a control group. The second and third groups of animals received 20 and 50 μg/mouse/d doses of apigenin in vehicle for 8 weeks, respectively. Similar treatment protocol was followed for PC-3 tumors. In all the experiments, animals were monitored daily and their body weights were recorded weekly throughout the studies. Once the tumor xenografts started growing, their sizes were measured twice weekly in two dimensions with calipers. At the termination of the experiment, tumors were excised and wet weights of tumors were recorded.

Apoptosis by ELISA

Apoptosis was assessed in tumor lysates by M30-Apoptosense ELISA kit (Alexis Biochemicals) according to the protocol of the manufacturer and color developed was read at 450 nm against the blank, and values were plotted against standards provided and expressed as units per liter.

Western Blotting

For Western blotting, appropriate amounts of cell lysates (25 μg protein) were resolved over 4% to 20% Tris-glycine polyacrylamide gel and then transferred onto the nitrocellulose membrane. The blots were blocked using 5% nonfat dry milk and probed using appropriate primary antibodies in blocking buffer overnight at 4°C. The membrane was then incubated with appropriate secondary antibody horseradish peroxidase conjugate (Amersham Life Sciences, Inc., Arlington Heights, IL) followed by detection using chemiluminescence ECL kit (Amersham Life Sciences). For equal loading of protein, the membrane was stripped and reprobed with anti-β-actin antibody. Densitometric measurements of the bands in Western blot analysis were done using digitalized scientific software program UN-SCAN-IT purchased from Silk Scientific Corporation (Orem, UT).

Immunoprecipitation

Cell lysate (200 μg) was immunoprecipitated with 2 μg appropriate primary antibody and was incubated at 4°C for 3 hours. Protein A/G agarose beads (20 μL) were added and incubated overnight at 4°C. Immunoprecipitated proteins were washed four times with lysis buffer, electrophoresed by SDS-PAGE, and analyzed by Western blotting as previously described (29).

High-Performance Liquid Chromatography Analysis

Serum samples (0.2 mL) and tissue homogenate from the various experimental groups were deproteinized by adding 0.4 mL methanol, vortex-mixed for 60 seconds, and centrifuged at 2,200 × *g* for 15 minutes at 4°C. The supernatant (0.6 mL) was collected into the tube and evaporated to dryness by vacuum freeze drying. The residue was dissolved in 200 μL methanol and chromatographically analyzed by high-performance liquid chromatography system. Analytic reversed-phase high-performance liquid chromatography was done for apigenin estimation on a Waters 600 system, Amphotech, Ltd. (Beverly, MA), connected to a Waters UV detector set at 349 nm using a Vydac 250 mm, 5 μm C18 columns. The mobile phase consisting of 2% formic acid-acetonitrile-methanol (40:35:25) was run at a flow rate of 1 mL/min. Apigenin concentrations in serum samples were calculated based on the sum of the area of each diastereomer peak and compared with the standard curve.

Statistical Analysis

Changes in tumor volume and body weight during the course of the experiments were visualized by scatter plot. Differences in tumor volume (mm^3) and body weight at the termination of the experiment among three treatment groups were examined using ANOVA followed by Turkey multiple comparison procedure. Additionally, 22Rv1 and PC-3 tumor volumes were compared using unpaired *t* test. All tests were two-tailed and *P* values <0.05 were considered to be statistically significant.

Results

Apigenin Causes Cell Cycle Arrest at G_0-G_1 Phase in Prostate Carcinoma Cells

In the first experiment, we examined the effect of apigenin on the cell cycle perturbations. Compared with the vehicle-treated controls, apigenin treatment resulted in an appreciable arrest of 22Rv1 and PC-3 cells in G_0-G_1 phase of cell cycle after 24 hours of treatment. Apigenin treatment caused an arrest of 59.4% cells in G_0-G_1 phase of the cell cycle at 10 μmol/L (P = 0.024), which further increased to 61.3% at 20 μmol/L (P = 0.008), 62.1% at 40 μmol/L (P = 0.004), and 65.0% at the highest dose of 80 μmol/L (P = 0.003) in 22Rv1 cells. Essentially similar observations were recorded with PC-3 cells where following apigenin treatment of cells at 10, 20, 40, and 80 μmol/L doses resulted in 51.4% (P = 0.005), 57.4% (P = 0.0002), 60.8% (P = 0.0003), and 62.6% (P = <0.0001) arrest, respectively (Table 1). This increase in G_0-G_1 cell population was accompanied with a concomitant decrease of cell number in S-phase and G_2-M phase in both cell

Table 1. Effect of apigenin on cell cycle distribution in 22Rv1 and PC-3 cells

Treatments	G_0-G_1 phase	G_2-M phase	S phase	Sub-G_1 phase
22Rv1 cells				
Vehicle only	54.7 ± 2.4 (—)	15.8 ± 1.8 (—)	29.5 ± 1.6 (—)	2.0 ± 0.8 (—)
10 μmol/L apigenin	59.4 ± 2.1 (0.024)	15.9 ± 1.4 (NS)	24.7 ± 1.3 (0.005)	7.9 ± 1.1 (0.0005)
20 μmol/L apigenin	61.3 ± 1.8 (0.008)	16.3 ± 1.6 (NS)	22.4 ± 1.2 (0.002)	9.5 ± 1.3 (0.0002)
40 μmol/L apigenin	62.1 ± 2.0 (0.004)	16.2 ± 1.5 (NS)	21.7 ± 1.5 (0.001)	11.3 ± 1.2 (0.0001)
80 μmol/L apigenin	65.0 ± 1.7 (0.003)	17.8 ± 1.2 (NS)	17.2 ± 1.4 (0.0001)	32.3 ± 1.6 (<0.0001)
PC-3 cells				
Vehicle only	47.6 ± 1.4 (—)	30.2 ± 1.4 (—)	22.2 ± 1.1 (—)	0.4 ± 0.2 (—)
10 μmol/L apigenin	51.4 ± 1.5 (0.005)	24.5 ± 1.2 (0.008)	24.1 ± 1.5 (NS)	1.2 ± 0.5 (0.0034)
20 μmol/L apigenin	57.4 ± 1.2 (0.0002)	24.6 ± 1.3 (0.008)	18.0 ± 1.5 (0.03)	5.2 ± 0.8 (0.007)
40 μmol/L apigenin	60.8 ± 1.8 (0.0003)	22.5 ± 1.6 (0.003)	16.7 ± 1.2 (0.004)	10.1 ± 1.0 (0.002)
80 μmol/L apigenin	62.6 ± 1.3 (<0.0001)	17.2 ± 1.4 (0.0005)	20.2 ± 1.7 (NS)	18.4 ± 2.2 (0.0008)

NOTE: The cells were treated with vehicle alone or with indicated doses of apigenin for 24 hours, stained with propidium iodide, and analyzed by flow cytometry. Percentage of cells in sub-G_1, G_0-G_1, S, and G_2-M phases were calculated using cell-fit computer software. Each value represents the mean ± SD from three independent experiments. Values are represented as percentage of cell population. Values in parens represent *P* values.
Abbreviation: NS, not significant.

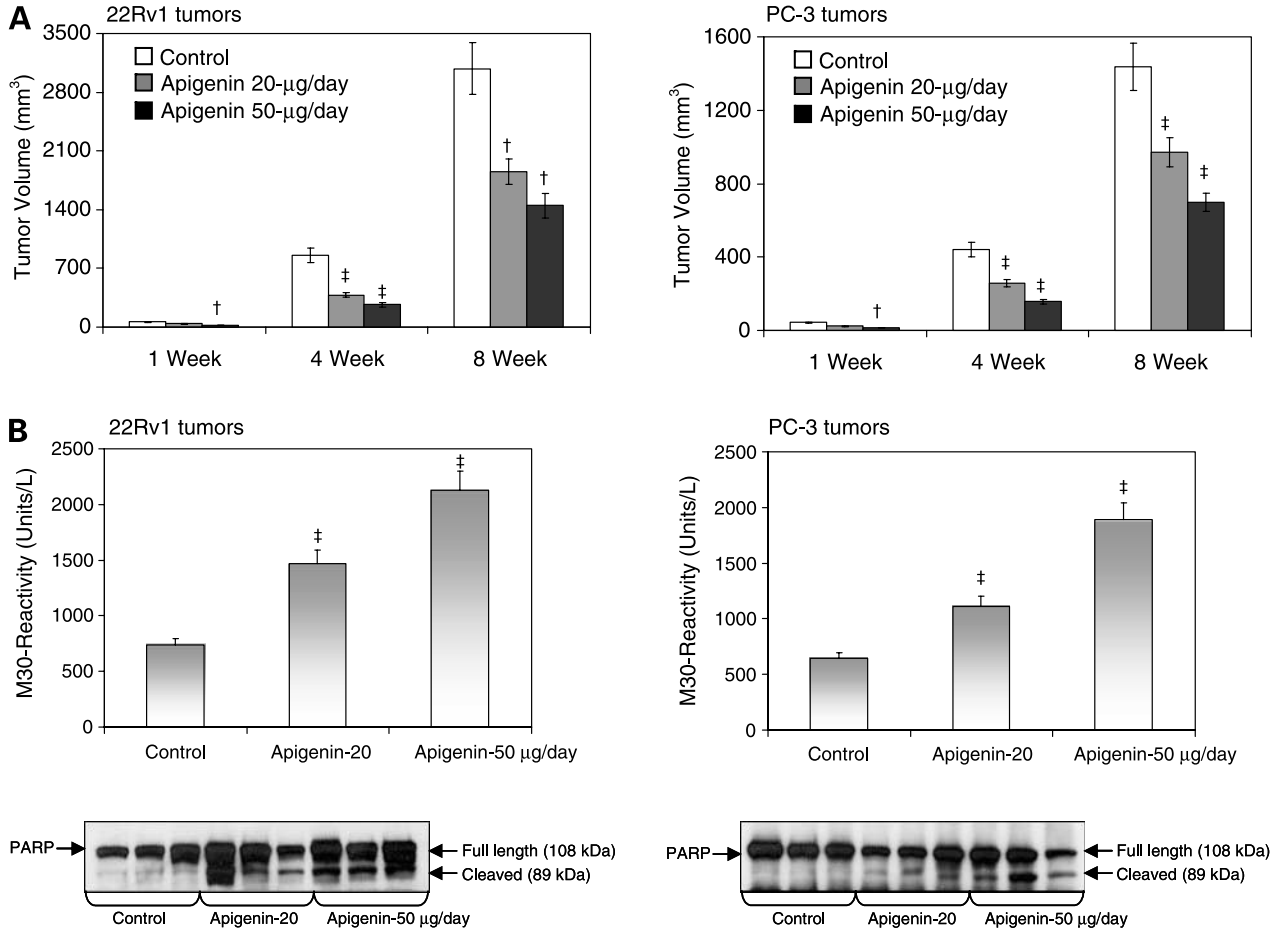

Figure 1. Effect of p.o. intake of apigenin on tumor growth and apoptosis in athymic nude mice implanted with 22Rv1 and PC-3 tumors. **A,** ~1 million cells were injected in both the flanks of each mouse to initiate ectopic prostate tumor growth as described in Materials and Methods. Mice were fed *ad libitum* with Teklad 8760 autoclaved high-protein diet. Apigenin was provided with 0.5% methyl cellulose and 0.025% Tween 20 as vehicle, to these animals p.o. on a daily basis 2 wks after cell inoculation. Group I, control received 0.2 mL vehicle only; group II, 20 μg/mouse apigenin in 0.2 mL vehicle; group III, 50 μg/mouse apigenin in 0.2 mL vehicle, daily for 8 wks. Once the tumor xenograft started growing, their sizes were measured twice weekly in two dimensions throughout the study. Tumor volume (mm^3) is represented as mean of 8 to 10 tumors in each group. Results show tumor volume from control and treated groups after 1, 4, and 8 wks of apigenin feeding. Tumors were excised, weighed, and measured at the termination of the study. *Columns,* mean of 8 to 10 tumors from each group; *bars,* SE. **B,** tumors were harvested for extraction of total protein to determine the extent of apoptosis by M-30 apoptosense ELISA assay and Western blotting by cleavage of poly(ADP)ribose polymerase in various groups as described in Materials and Methods. *Columns,* mean of six to eight tumor samples in each treatment; *bars,* SE. †, $P < 0.05$; ‡, $P < 0.001$ at the termination of the experiment.

lines. Further, treatment of 22Rv1 and PC-3 cells with apigenin resulted in accumulation of cells in sub-G_1 phase of the cell cycle. Treatment of 22Rv1 cells with apigenin resulted in 7.9% cells in sub-G_1 phase at 10 μmol/L ($P = 0.0005$), which further increased to 9.5% at 20 μmol/L ($P = 0.0002$), 11.3% at 40 μmol/L ($P = 0.0001$), and 32.3% at the highest dose of 80 μmol/L ($P < 0.0001$), respectively. Similarly, PC-3 cells exhibited sub-G_1 phase accumulation of 1.2% at 10 μmol/L ($P = 0.0034$), 5.2% at 20 μmol/L ($P = 0.007$), 10.1% at 40 μmol/L ($P = 0.002$), and 18.4% at 80 μmol/L ($P = 0.0008$) doses of apigenin treatment, which may be indicative of apoptosis (Table 1). The extent of sub-G_1 accumulation was more pronounced in 22Rv1 cells than in PC-3 cells. Taken together, these data suggest that essentially similar mechanism of apigenin-mediated cell cycle arrest and apoptosis occurs in both androgen-sensitive 22Rv1 and androgen-insensitive PC-3 cells and cell cycle regulatory molecules operative at G_1-checkpoint, which may be essential targets of apigenin.

Apigenin Intake Inhibits the Growth of Prostate Tumor Xenograft in Nude Mice

To study the effect of apigenin feeding on prostate tumor development, we used *in vivo* human prostate tumor xenograft derived from 22Rv1 and PC-3 cancer cells in nude mice (Fig. 1A). In vehicle-treated controls, progressive tumor growth was observed during 8 weeks of study period and tumor mass became visible and measurable at day 9 of 22Rv1 cells implantation and day 13 of PC-3 cell implantation, respectively. Compared

with control, 20 and 50 μg/mouse/d apigenin treatment to nude mice beginning 2 weeks after tumor implantation, tumor volumes were reduced by 39% and 53% ($P < 0.01$ and 0.002), and wet weights of tumor by 31% and 42% ($P < 0.05$), respectively, at the end of the studies. Almost similar effects were noted in PC-3 tumors where 20 and 50 μg/mouse/d apigenin treatment for 8 weeks resulted in 32% and 51% inhibition in tumor growth, and wet weight tumors by 28% and 40% compared with vehicle-treated controls (Fig. 1A). Overall, apigenin elicited a marked dose-dependent inhibitory effect on tumor xenograft that remained constant up to termination of the experiment.

We also determined the body weight gain and diet consumption profiles of control and apigenin-fed animals. These variables are reflective of the gross toxicity of a test agent. In apigenin-fed mice, body weight gain and diet consumption profiles were almost comparable with the vehicle-treated control group. A modest difference remained between the control and apigenin-treated groups, which was attributed to the reduced tumor volume and weight (data not shown).

Oral Intake of Apigenin Induces Apoptosis in Tumor Xenograft in Nude Mice

Previous studies have shown that apigenin has potential to induce apoptosis in cancer cells *in vitro*; therefore, we evaluated the effect of apigenin feeding on induction of apoptosis in tumor xenografts. As shown in Fig. 1B, p.o. intake of apigenin at doses of 20 and 50 μg/mouse/d for 8 weeks after tumor implantation resulted in marked induction of apoptosis in both 22Rv1 and PC-3 tumor xenografts as shown by M-30 apoptosome reactivity. Compared with vehicle-treated control, 1.9- and 3.0-fold increases ($P < 0.0001$) in induction of apoptosis was observed in 22Rv1 tumors. In PC-3 tumors, 1.7- and 2.9-fold increase ($P < 0.0001$) in the induction of apoptosis was observed after 20 and 50 μg/mouse/d of apigenin feeding. Similar results were noted in tumor xenografts where apigenin feeding led to significant dose-dependent increases in the cleaved product of poly(ADP)ribose polymerase in both types of tumor xenograft (Fig. 1B).

Apigenin-Mediated Tumor Growth Inhibition and Apoptosis Correlates with Mouse Serum and Tumor Apigenin Levels

Next, we determined the levels of apigenin in serum and evaluated whether these levels correlate with tumor growth inhibition and induction of apoptosis. Assessment of the levels of apigenin in the serum of apigenin-fed mice was done by a standard high-performance liquid chromatography profile as previously reported (28). Animals in the vehicle-treated control exhibited nondetectable levels of apigenin in serum and tumor tissue. As shown in Fig. 2, apigenin feeding at 20 and 50 μg/mouse/d for 8 weeks to 22Rv1 tumor-implanted mice resulted in 0.78 ± 0.02 and 1.06 ± 0.04 μmol/L apigenin in serum and 0.30 ± 0.02 and 0.62 ± 0.04 μmol/g tumor ($P < 0.0001$), respectively. In PC-3 tumors, apigenin intake at 20 and 50 μg/mouse/d resulted in 0.80 ± 0.02 and 1.1 ± 0.03 μmol/L apigenin in serum and 0.32 ± 0.01 and 0.66 ± 0.03 μmol/g tumor ($P < 0.0001$), respectively (Fig. 2). The increase in apigenin concentration in the serum and tumors of apigenin-fed mice positively correlated with inhibition of tumor growth and induction of apoptosis.

Apigenin Intake Increases Protein Levels of Cdk Inhibitors in Tumor Xenograft in Nude Mice

Next, we evaluated the effect of dietary intake of apigenin on the protein levels of various cdk inhibitors operative at the G_1 checkpoint. These cdk inhibitors include the CIP/KIP and INK4 family of proteins. As shown in Fig. 3A, daily p.o. intake of apigenin at doses of 20 and 50 μg/mouse/d administered to 22Rv1-implanted nude mice exhibited dose-dependent increases in the protein expression of WAF1/p21, KIP1/p27, INK4a/p16, and INK4c/p18, compared with vehicle-treated controls. Similar

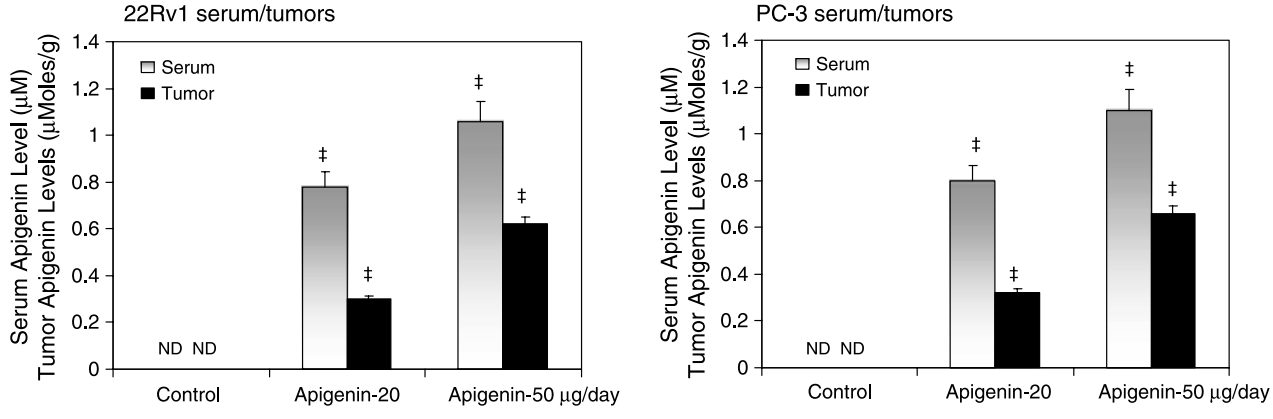

Figure 2. Pharmacologically attainable apigenin concentration in serum and tumor of athymic nude mice implanted with 22Rv1 and PC-3 tumors. Apigenin levels in serum and tumors were detected by high-performance liquid chromatography analysis as described in Materials and Methods. *Columns,* five to six serum and tumor samples from various groups; *bars,* SE. ‡, $P < 0.001$; *ND,* nondetectable.

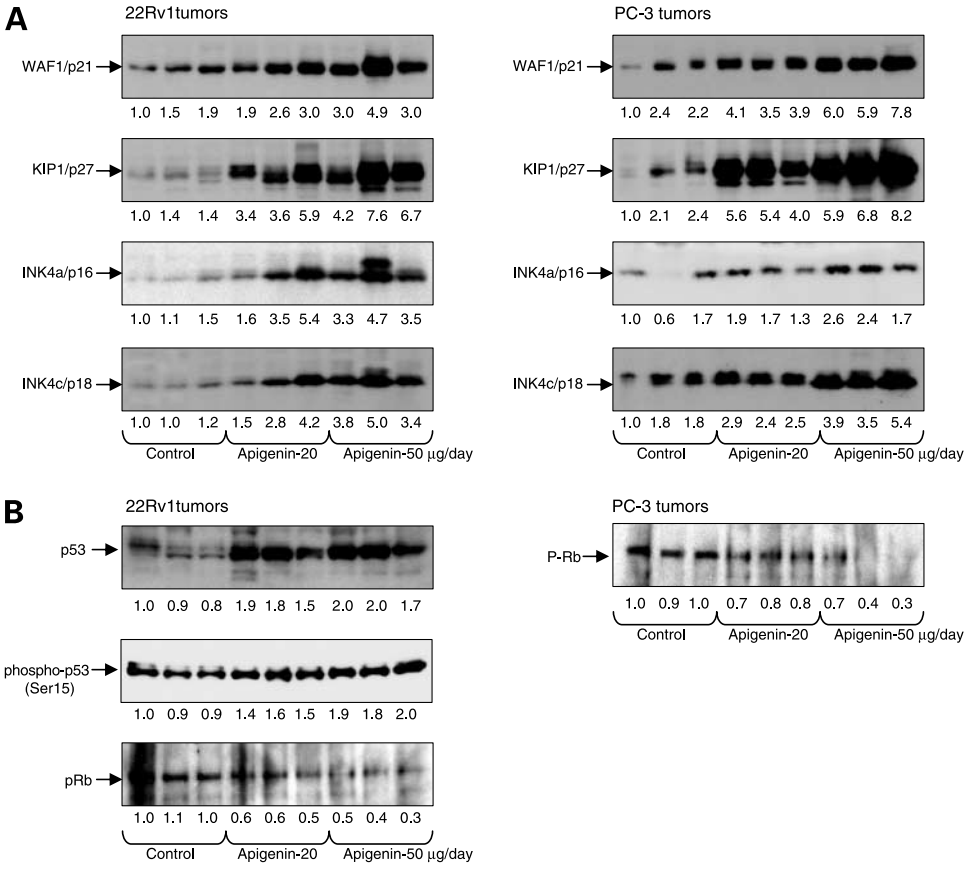

Figure 3. Effect of p.o. intake of apigenin on protein expression of (**A**) cdk inhibitors: WAF1/p21, KIP1/p27, INK4a/p16, and INK4c/p18; and (**B**) p53 and its phosphorylation at serine 15, phosphorylation of Rb at serine 780 in athymic nude mice implanted with 22Rv1 and PC-3 tumors. Tumors were harvested for extraction of total protein to determine the levels of these proteins by Western blotting and chemiluminescent detection. *Numbers below the blots,* fold modulation in the protein expression normalized to β-actin. The details are described in Materials and Methods. The blots are from three different tumors from various treated groups as indicated.

results were observed in PC-3 tumors, in which there were dose-dependent increases in the protein expression of WAF1/p21, KIP1/p27, INK4a/p16, and INK4c/p18, compared with vehicle-treated controls (Fig. 3A).

Oral Intake of Apigenin Increases p53 Expression and Decreases Rb Phosphorylation in Tumor Xenograft of Nude Mice

Tumor suppressor genes *p53* and *Rb* are required for maintenance of proper checkpoint controls in G_1 phase of cell cycle; therefore, we evaluated the effect of apigenin intake on the levels of these proteins. As shown in Fig. 3B, daily p.o. intake of apigenin at 20 and 50 μg/mouse/d administered to nude mice resulted in a dose-dependent increase in the expression of wild-type p53 protein, in 22Rv1-implanted tumors, which harbor functional p53. Wild-type p53 is a short-lived protein and its induction in response to cellular stress is followed by posttranslational modifications, such as phosphorylation and acetylation of critical serine residues. To examine the mechanism by which apigenin mediate increase in p53 levels, we determined the phosphorylation status of p53 at most common site of serine 15 residue, which has been shown to increase the half-life of this protein. Apigenin feeding resulted in dose-dependent increase in phosphorylation of p53 at serine 15 residue in 22Rv1-implanted tumors. In addition, apigenin feeding to nude mice implanted with 22Rv1 tumors resulted in a dose-dependent decrease in Rb phosphorylation at serine 780. In contrast, PC-3-implanted tumors, which are p53 null cells, did not exhibit any alteration in p53 expression (data not shown). However, a dose-dependent decrease at serine 780 phosphorylation of Rb was observed in apigenin-fed tumors compared with vehicle-treated control (Fig. 3B).

Apigenin Intake Decreases Protein Expression of Cyclins and Cdks in Tumor Xenograft of Nude Mice

Next, we assessed the protein expression of the cyclins and cdks, which are operative in the G_1 phase, to determine whether their levels are altered after apigenin feeding. As shown in Fig. 4A and B, p.o. intake of apigenin at doses of 20 and 50 μg/mouse/d administered to 22Rv1-implanted nude mice for 8 weeks resulted in dose-dependent decreases in the protein expression of cyclins D1, D2, and E, which positively correlated with the decreased expression of cdk2, cdk4, and cdk6, compared with vehicle-treated controls. Similar results were noted in mice with PC-3 tumors; apigenin intake resulted in dose-dependent decreases in the protein expression of cyclins D1, D2, and E with corresponding decreases in the levels of cdk2, cdk4, and cdk6 proteins after 8 weeks of apigenin feeding (Fig. 4A and B).

Oral Intake of Apigenin Modulates Binding of Cyclins, Cdks, and Cdk Inhibitor of Tumor Xenograft in Nude Mice

Normal progression of cells through the cell cycle requires a balance between each component of the cdk

inhibitor–cyclin-cdk complex. We therefore investigated the effect of apigenin feeding on the binding between WAF1/p21-cyclin D1, KIP1/p27-cyclin D1, and cdk2-cyclin E. In this study, one of the two proteins was immunoprecipitated using an appropriate antibody and the effect on the binding was assessed by probing the immunoblot using antibody directed against the other protein. As shown in Fig. 5, p.o. intake of apigenin at 20 and 50 μg/mouse/d administered to nude mice for 8 weeks implanted with 22Rv1 tumors resulted in a dose-dependent increase in the binding of cyclin D1 toward WAF1/p21 and KIP1/p27. Apigenin intake also resulted in a dose-dependent decrease in the binding of cyclin E toward cdk2 in 22Rv1 tumors, compared with vehicle-treated controls. Similar results were noted in PC-3-implanted tumors in which apigenin intake for 8 weeks resulted in dose-dependent increase in the binding of cyclin D1 toward WAF1/p21 and KIP1/p27 along with decrease in the binding of cyclin E toward cdk2, respectively (Fig. 5).

Discussion

The use of naturally occurring dietary agents is becoming increasingly appreciated as an effective means of managing many types of cancer in an approach known as cancer chemoprevention (1, 2). Among these natural dietary agents, flavonoids, a constituent of different classes of food and beverages, have been intensely studied in recent years. Apigenin is a common dietary flavonoid widely distributed in many fruits and vegetables, including parsley, onions, oranges, tea, chamomile, wheat sprouts, and some seasonings (10, 11). Apigenin has shown considerable promise as a chemopreventive agent because it produces a wide variety of effects in cell culture systems and in several animal tumor model systems (3–18). Recent studies suggest that apigenin may also possess therapeutic effects (11, 28, 29). Apigenin is a particularly attractive agent due to its limited toxicity, wide availability, and its differential effects on normal versus cancer cells (30). Importantly, at physiologically attainable concentrations, apigenin induces cancer cells to undergo apoptosis, without a similar effect on normal cells (30). Previous studies in our laboratory and elsewhere have shown that apigenin promotes cell cycle arrest and apoptosis in several types of malignant cells (9, 10, 29, 30). The precise molecular mechanisms by which apigenin promotes these effects in preclinical models of cancer have not been elucidated. Deciphering the molecular mechanisms by which apigenin imparts its antiproliferative effects may be important because it may lead to the development of new strategies for the management of cancer. We provide the first evidence that apigenin feeding to mice with prostate xenograft tumors reduces tumor mass, possibly by cell cycle arrest and induction of apoptosis.

In cancer, the cell cycle is frequently deregulated, contributing to tumorigenesis. Defects in cell cycle control occur through multiple mechanisms, which include (*a*) amplification or overexpression of D-type cyclins; (*b*) loss,

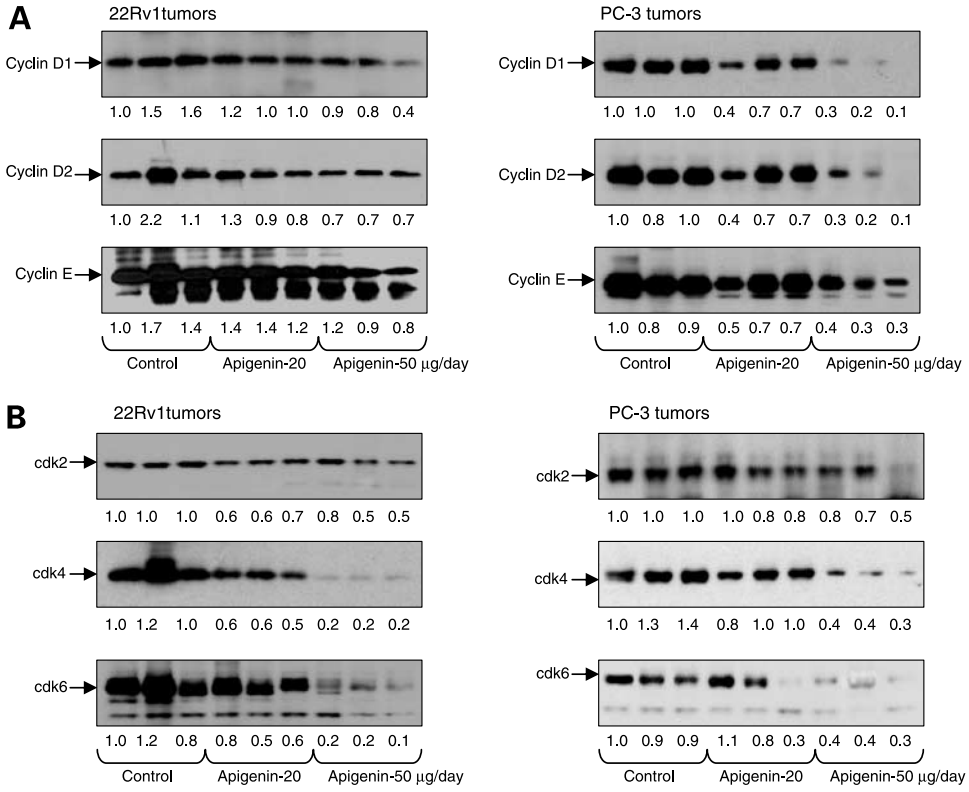

Figure 4. Effect of p.o. intake of apigenin on protein expression of (**A**) cyclins D1, D2, and E; and (**B**) cdk2, cdk4, and cdk6 in athymic nude mice implanted with 22Rv1 and PC-3 tumors. Tumors were harvested for extraction of total protein to determine the levels of these proteins by Western blotting and chemiluminescent detection. *Numbers below the blots,* fold modulation in the protein expression normalized to β-actin. The details are described in Materials and Methods. The blots are from three different tumors from various treated groups as indicated.

mutation, or methylation of the cdk4/6 inhibitor INK4a/p16; (*c*) constitutive activation of cdk4 via mutation; (*d*) viral inactivation of WAF1/p21; (*e*) low expression of KIP1/p27; and (*f*) loss or mutation of the tumor suppressor genes *Rb* and/or *p53* (ref. 31 and references therein). These oncogenic events confer a greater susceptibility for progression through the cell cycle. In eukaryotes, the cell cycle is governed by the activities of the cdks and their regulatory cyclin partners (19, 20). The cdk-cyclin complex is activated at various checkpoints after specific intervals during the cell cycle and can be regulated by a number of exogenous factors. The cdks (cdk2, cdk4, and cdk6) involved in the G_1 progression are activated, in part, by the binding of D-type cyclins in early G_1 and cyclin E in late G_1 phase. Cyclin D complexes with cdk4 and cdk6 to generate an active kinase complex that phosphorylates a variety of cytoplasmic substrates, the most well characterized being the Rb tumor suppressor protein (20). Cyclin E forms a complex with cdk2 to further phosphorylate Rb with the release of general transcription factor E2F-1, which then stimulates the transcription of genes involved in DNA replication (20, 21). Our studies show that apigenin treatment cause cell cycle arrest at G_0-G_1 phase along with sub-G_1 accumulation in both 22Rv1 and PC-3 cells. Further studies have shown that apigenin feeding to nude mice tumor xenograft resulted in significant decreases in the protein levels of cdks (cdk2, cdk4, and cdk6) and cyclins (D1, D2, and E) operative at the G_1 checkpoint, along with a decrease in Rb phosphorylation, leading to cell cycle arrest and subsequent apoptosis that is reflected by reduced tumor growth.

Cell cycle progression may be further regulated by activation or inhibition of phosphorylation of cdk-cyclin complex by small proteins known as cyclin kinase inhibitors. The cdk inhibitors include the CIP/KIP and INK family of proteins (21–23). Several cdk inhibitors have been shown to have a role in the regulation of G_1-S progression (22, 30). The cdk inhibitors function by binding to cyclin D-cdk complexes to inhibit their kinase activity. Our studies indicate dose-dependent induction of WAF1/p21 in apigenin-fed tumor xenografts. Many studies have shown that certain exogenous stimuli may result in a p53-dependent and p53-independent induction of WAF1/p21, which, in turn, may trigger a series of events ultimately resulting in cell cycle arrest and/or apoptosis (32, 33). Our data showing an induction of WAF1/p21 by apigenin seems to be transcriptionally up-regulated in p53-dependent fashion via posttranscriptional phosphorylation of p53 at serine 15 in 22Rv1 tumors (harboring wild-type p53) and p53-independent up-regulation in PC-3 tumors (with null p53). Similar studies in cell culture have shown that apigenin activates the WAF1/p21 promoter dependent on p53-binding elements (34). In addition, apigenin has been shown to stimulate expression of WAF1/p21 mRNA and protein in a p53-independent manner (35). Therefore, apigenin seems to be capable of exerting its effect on cancer cells with or without p53 mutations. Further studies are required to elucidate the precise molecular targets of apigenin, in particular with regard to p53 activation and stabilization.

A number of reports in the literature have shown that cdk inhibitor KIP1/p27 functions as an integral break during cell cycle progression (36). Similarly, the INK4 family members, especially INK4a/p16 and INK4c/p18, have also been implicated in the regulation of cell cycle progression at the G_1-to-S transition via inhibiting cdk4 and/or cdk6 (37). Our studies have shown that apigenin may impart growth inhibition in both androgen-sensitive 22Rv1 and androgen-insensitive PC-3 tumors, via cell cycle deregulation involving all major cdk inhibitors of G_1 phase.

Cell cycle regulatory molecules (cdk, cyclins, and cdk inhibitors) are believed to be critical regulatory elements that control the progression of cells in early and late G_1 phases of the cell cycle (20). Many studies have shown that cells treated in culture respond by modulation of cdk activity but did not show direct binding to suspected kinase targets (refs. 25, 38, 39 and references therein). Although apigenin has been shown to modulate protein levels of

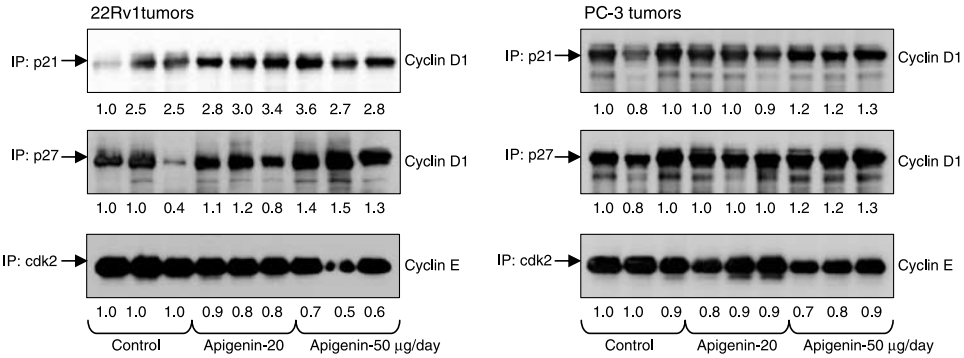

Figure 5. Effect of p.o. intake of apigenin on interbinding between WAF1/p21, KIP1/p27, and cdk2 with cyclins in athymic nude mice implanted with 22Rv1 and PC-3 tumors. Tumor lysates were prepared, 200 μg protein was immunoprecipitated and subjected to SDS-PAGE, and the binding with the other protein was determined by Western blotting directed against the other protein. *Numbers below the blots,* fold modulation in the protein expression normalized to β-actin. The details are described in Materials and Methods. The blots are from three different tumors from various treated groups as indicated.

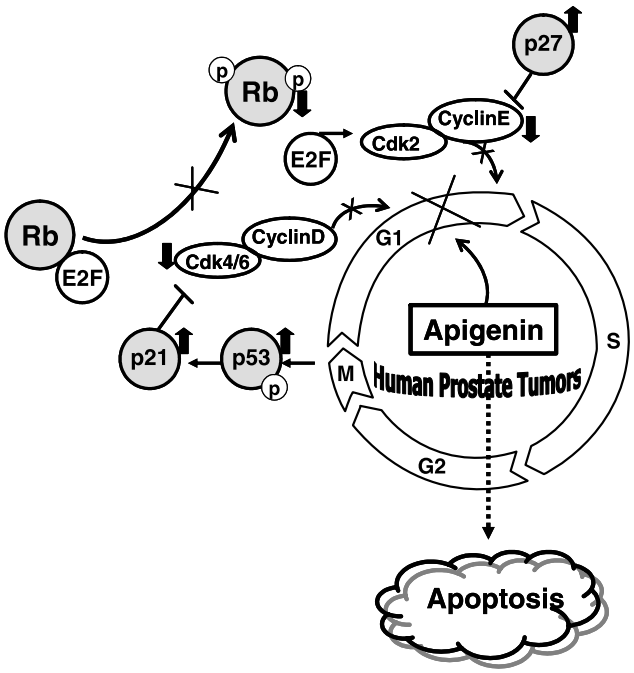

Figure 6. Schematic representation for apigenin-mediated cell cycle deregulation and apoptosis in athymic nude mice implanted with human prostate cancer cells. Increases (↑) and decreases (↓) in protein expression of various cell cycle regulatory molecules operative at G_1 checkpoint.

various cdk molecules in cell culture, the precise molecular targets of apigenin during pharmacologic intervention are presently unknown. Our data, showing a decrease in the protein levels of cyclins (D1, D2, and E) and the cdks (cdk2, cdk4, and cdk6) after apigenin feeding in both tumor types, are in agreement with the observation that the cdks and cyclins operate in association with each other by forming complexes that may bind to and are inhibited by cdk inhibitors. These series of events act in concert with the effects of p53: The increase in the levels of WAF1/p21 and KIP1/p27 and apigenin-mediated decrease in phosphorylation of pRb likely leads to growth arrest and apoptosis in tumor xenografts. The schematic representation is shown in Fig. 6.

Epidemiologic data shows that diets rich in flavonoids are associated with decreased incidence of certain cancers, suggesting that ingested flavonoids play an important role in delaying or inhibiting cancer progression (40). Consumption of traditional Asian diet rich in plant flavonoids profoundly decreases the risk of some diseases in Asian countries compared with Western nations (41). The potential contribution of flavonoids to the cancer-preventive effects of Asian diets depends on the daily intake and uptake of these agents. A recent study on rats after a single p.o. dose of apigenin has shown a high-elimination half time (~90 hours), suggesting that frequent consumption may result in accumulation of flavonoids in plasma and tissues (42). Studies have further shown that upon absorption, the flavonoids are metabolized by methylation or by conjugation with gluconate or sulfate via dual recycling involving both enteric and enterohepatic pathways (43, 44). Our *in vivo* mouse studies have shown that continuous apigenin feeding decreased tumor mass and increased apoptosis in tumor xenografts, suggesting apigenin uptake that remains biologically active after ingestion. Importantly, the dose-dependent inhibition of tumor volume and apoptosis positively correlates with higher serum and tumor apigenin levels.

The average intake of flavonols and flavones range from 6 mg/d in Finland to 64 mg/d in Japan with intermediate intakes in the United States (13 mg/d), Italy (27 mg/d), and the Netherlands (33 mg/d). These estimates are based on the analyses of five flavonoids, including apigenin, in composite food samples for population in the seven countries study (45). The concentrations of apigenin used in our studies are pharmacologically attainable in humans in terms of total flavones/flavonols consumption. The dose of 20 μg/mouse/d relates to the median apigenin intake (40-50 mg/d) and the higher dose of 50 μg/mouse/d apigenin is comparable with an intake of ~120 mg/d of apigenin by an adult (46). Taken together, these studies support the notion that apigenin may impart a broad spectrum of biological and chemopreventive effects by affecting a number of molecular targets that influence cell cycle progression and apoptosis.

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References

1. Tsao AS, Kim ES, Hong WK. Chemoprevention of cancer. CA Cancer J Clin 2004;54:150 – 80.

2. Sporn MB, Suh N. Chemoprevention: an essential approach to controlling cancer. Nat Rev Cancer 2002;2:537 – 43.

3. Birt DF, Walker B, Tibbels MG, Bresnick E. Anti-mutagenesis and anti-promotion by apigenin, robinetin and indole-3-carbinol. Carcinogenesis 1986;7:959 – 63.

4. Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF, Lin JK. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 1999;20:1945 – 52.

5. Birt DF, Mitchell D, Gold B, Pour P, Pinch HC. Inhibition of ultraviolet light induced skin carcinogenesis in SKH-1 mice by apigenin, a plant flavonoid. Anticancer Res 1997;17:85 – 91.

6. Liu LZ, Fang J, Zhou Q, Hu X, Shi X, Jiang BH. Apigenin inhibits expression of vascular endothelial growth factor and angiogenesis in human lung cancer cells: implication of chemoprevention of lung cancer. Mol Pharmacol 2005;68:635 – 43.

7. Caltagirone S, Rossi C, Poggi A, et al. Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. Int J Cancer 2000;87:595 – 600.

8. Wei H, Tye L, Bresnick E, Birt DF. Inhibitory effect of apigenin, a plant flavonoid, on epidermal ornithine decarboxylase and skin tumor promotion in mice. Cancer Res 1990;50:499 – 502.

9. Yin F, Giuliano AE, Law RE, Van Herle AJ. Apigenin inhibits growth and induces G_2-M arrest by modulating cyclin-CDK regulators and ERK MAP kinase activation in breast carcinoma cells. Anticancer Res 2001;21: 413 – 20.

10. Wang W, Heideman L, Chung CS, Pelling JC, Koehler KJ, Birt DF. Cell-cycle arrest at G_2-M and growth inhibition by apigenin in human colon carcinoma cell lines. Mol Carcinog 2000;28:102 – 10.

11. Fang J, Xia C, Cao Z, Zheng JZ, Reed E, Jiang BH. Apigenin inhibits VEGF and HIF-1 expression via PI3K/AKT/p70S6K1 and HDM2/p53 pathways. FASEB J 2005;19:342 – 53.

12. O'Prey J, Brown J, Fleming J, Harrison PR. Effects of dietary flavonoids on major signal transduction pathways in human epithelial cells. Biochem Pharmacol 2003;66:2075 – 88.

13. Yin F, Giuliano AE, Van Herle AJ. Signal pathways involved in apigenin inhibition of growth and induction of apoptosis of human anaplastic thyroid cancer cells (ARO). Anticancer Res 1999;19:4297 – 303.

14. Knowles LM, Zigrossi DA, Tauber RA, Hightower C, Milner JA. Flavonoids suppress androgen-independent human prostate tumor proliferation. Nutr Cancer 2000;38:116 – 22.

15. Wang IK, Lin-Shiau SY, Lin JK. Induction of apoptosis by apigenin and related flavonoids through cytochrome *c* release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. Eur J Cancer 1999; 35:1517 – 25.

16. Constantinou A, Mehta R, Runyan C, Rao K, Vaughan A, Moon R. Flavonoids as DNA topoisomerase antagonists and poisons: structure-activity relationships. J Nat Prod 1995;58:217 – 25.

17. Brusselmans K, Vrolix R, Verhoeven G, Swinnen JV. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J Biol Chem 2005;280:5636 – 45.

18. Gupta S, Afaq F, Mukhtar H. Involvement of nuclear factor-κB, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. Oncogene 2002;21:3727 – 38.

19. Malumbres M, Barbacid M. To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer 2001;1:222 – 31.

20. Massague J. G_1 cell-cycle control and cancer. Nature 2004;432: 298 – 306.

21. Senderowicz AM. Cyclin-dependent kinases as new targets for the prevention and treatment of cancer. Hematol Oncol Clin North Am 2002; 16:1229 – 53.

22. Facchinetti MM, De Siervi A, Toskos D, Senderowicz AM. UCN-01-induced cell cycle arrest requires the transcriptional induction of p21 (waf1/cip1) by activation of mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway. Cancer Res 2004;64:3629 – 37.

23. Yoo YD, Choi JY, Lee SJ, et al. TGF-β-induced cell-cycle arrest through the p21 (WAF1/CIP1)-G_1 cyclin/Cdks-p130 pathway in gastric-carcinoma cells. Int J Cancer 1999;83:512 – 7.

24. Geahlen RL, Koonchanok NM, McLaughlin JL, Pratt DE. Inhibition of protein-tyrosine kinase activity by flavanoids and related compounds. J Nat Prod 1989;52:982 – 6.

25. Way TD, Kao MC, Lin JK. Apigenin induces apoptosis through proteasomal degradation of HER2/neu in HER2/neu-overexpressing breast cancer cells via the phosphatidylinositol 3-kinase/Akt-dependent pathway. J Biol Chem 2004;279:4479 – 89.

26. Llorens F, Miro FA, Casanas A, et al. Unbalanced activation of ERK1/2 and MEK1/2 in apigenin-induced HeLa cell death. Exp Cell Res 2004;299: 15 – 26.

27. Hessenauer A, Montenarh M, Gotz C. Inhibition of CK2 activity provokes different responses in hormone-sensitive and hormone-refractory prostate cancer cells. Int J Oncol 2003;22:1263 – 70.

28. Shukla S, Mishra A, Fu P, MacLennan GT, Resnick MI, Gupta S. Upregulation of insulin-like growth factor binding protein-3 by apigenin leads to growth inhibition and apoptosis of 22Rv1 xenograft in athymic nude mice. FASEB J 2005;19:2042 – 204.

29. Shukla S, Gupta S. Suppression of constitutive and tumor necrosis factor α-induced nuclear factor (NF)-κB activation and induction of apoptosis by apigenin in human prostate carcinoma PC-3 cells: correlation with down-regulation of NF-κB-responsive genes. Clin Cancer Res 2004; 10:3169 – 78.

30. Gupta S, Afaq F, Mukhtar H. Selective growth-inhibitory, cell-cycle deregulatory and apoptotic response of apigenin in normal versus human prostate carcinoma cells. Biochem Biophys Res Commun 2001;287: 914 – 20.

31. Whittaker SR, Walton MI, Garrett MD, Workman P. The cyclin-dependent kinase inhibitor CYC202 (R-roscovitine) inhibits retinoblastoma protein phosphorylation, causes loss of cyclin D1, and activates the mitogen-activated protein kinase pathway. Cancer Res 2004;64:262 – 72.

32. Colman MS, Afshari CA, Barrett JC. Regulation of p53 stability and activity in response to genotoxic stress. Mutat Res 2000;462:179 – 88.

33. Amundson SA, Myers TG, Fornace AJ, Jr. Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. Oncogene 1998;17:3287 – 99.

34. Zheng PW, Chiang LC, Lin CC. Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. Life Sci 2005; 76:1367 – 79.

35. Takagaki N, Sowa Y, Oki T, Nakanishi R, Yogosawa S, Sakai T. Apigenin induces cell cycle arrest and p21/WAF1 expression in a p53-independent pathway. Int J Oncol 2005;26:185 – 9.

36. Belletti B, Nicoloso MS, Schiappacassi M, et al. p27 (kip1) functional regulation in human cancer: a potential target for therapeutic designs. Curr Med Chem 2005;12:1589 – 605.

37. Roussel MF. The INK4 family of cell cycle inhibitors in cancer. Oncogene 1999;18:5311 – 7.

38. Ferriola PC, Cody V, Middleton E, Jr. Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. Biochem Pharmacol 1989;38:1617 – 24.

39. Torkin R, Lavoie JF, Kaplan DR, Yeger H. Induction of caspase-dependent, p53-mediated apoptosis by apigenin in human neuroblastoma. Mol Cancer Ther 2005;4:1 – 11.

40. Neuhouser ML. Dietary flavonoids and cancer risk: evidence from human population studies. Nutr Cancer 2004;50:1 – 7.

41. Knekt P, Kumpulainen J, Jarvinen RF, et al. Flavonoid intake and risk of chronic diseases. Am J Clin Nutr 2002;76:560 – 8.

42. Gradolatto A, Basly JP, Berges R, et al. Pharmacokinetics and metabolism of apigenin in female and male rats after a single oral administration. Drug Metab Dispos 2005;33:49 – 54.

43. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr 2002;22:19 – 34.

44. Chen J, Lin H, Hu M. Metabolism of flavonoids via enteric recycling: role of intestinal disposition. J Pharmacol Exp Ther 2003;304:1228 – 35.

45. Hertog MG, Kromhout D, Aravanis C, et al. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch Intern Med 1995;155:381 – 6.

46. Hollman PC, Katan MB. Dietary flavonoids: intake, health effects and bioavailability. Food Chem Toxicol 1999;37:937 – 42.