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Requirement for Ras/Raf/ERK pathway in naringin-induced G_1-cell-cycle arrest via p21WAF1 expression

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Naringin, an active flavonoid found in citrus fruit extracts, has pharmacological utility. The present study identified a novel mechanism of the anticancer effects of naringin in urinary bladder cancer cells. Naringin treatment resulted in significant dose-dependent growth inhibition together with G_1-phase cell-cycle arrest at a dose of 100 μM (the half maximal inhibitory concentration) in 5637 cells. In addition, naringin treatment strongly induced p21WAF1 expression, independent of the p53 pathway, and down-regulated expression of cyclins and cyclin dependent kinases (CDKs). Moreover, treatment with naringin induced phosphorylation of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase and c-Jun N-terminal kinase. Among the pathways examined, only PD98059, an ERK-specific inhibitor, blocked naringin-dependent p21WAF1 expression. Consistently, blockade of ERK function reversed naringin-mediated inhibition of cell proliferation and decreased cell-cycle proteins. Furthermore, naringin treatment increased both Ras and Raf activation. Transfection of cells with dominant-negative Ras (RasN17) and Raf (RafS621A) mutant genes suppressed naringin-induced ERK activity and p21WAF1 expression. Finally, the naringin-induced reduction in cell proliferation and cell-cycle proteins also was abolished in the presence of RasN17 and RafS621A mutant genes. These data demonstrate that the Ras/Raf/ERK pathway participates in p21WAF1 induction, subsequently leading to a decrease in the levels of cyclin D1/CDK4 and cyclin E–CDK2 complexes and naringin-dependent inhibition of cell growth. Overall, these unexpected findings concerning the molecular mechanisms of naringin in 5637 cancer cells provide a theoretical basis for the therapeutic use of flavonoids to treat malignancies.

Introduction

Bladder cancer is the fifth most common cancer in men and eighth most common in women. Bladder cancer has a very high incidence of recurrence—at 70% or greater (1). Bladder cancer comprises a wide range of tumors, including transitional cell carcinoma (2,3). Transitional cell carcinoma is classified histopathologically into three types: superficial tumors (papillary tumors), tumors confined to the bladder wall (pT1 and pTa tumors) and invasive tumors (stages T2–T4). Patients with superficial bladder cancer are at risk for recurrence of the disease and/or progression to invasive diseases, which is potentially lethal (4). For these people, effective preventive measures are needed.

Abbreviations: CDK, cyclin dependent kinase; ERK, extracellular signal-regulated kinase; EV, empty vector; GTP, guanosine triphosphate; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, small interfering RNA.

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The p21WAF1 is the primary known mediator of checkpoint-induced arrest during the G_1 phase of the cell cycle. The p21WAF1 is a target for diverse signals, which cause cell growth arrest and promote differentiation. Introduction of p21WAF1 protein into human untransformed and malignant cells induces cell-cycle arrest (5–7). The kinase activities of the cyclin–cyclin dependent kinase (CDK) complexes are negatively regulated by CDK inhibitors, such as p21WAF1 (8,9), which blocks cell-cycle progression by binding and inactivating the cyclin–CDK complex system (6,7). A mutation in p21WAF1 that specifically abrogated binding to CDKs was identified in cells isolated from a primary breast tumor (10), suggesting that p21WAF1 has tumor-suppressive activity.

The mitogen-activated protein kinase (MAPK) family of enzymes is composed of related serine/threonine protein kinases that integrate diverse signals, thereby directing cellular responses to proliferative cues or stressful stimuli (11–13). Three major mammalian MAPK subfamilies have been described: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs, also called stress-activated protein kinase) and p38 MAPKs. In general, JNK and p38 MAPKs are only weakly activated by growth factors, but are highly activated in response to a variety of stress signals, including tumor necrosis factor, ionizing and short-wavelength ultraviolet irradiation (UVC) and hyperosmotic stress. Activation of JNK and p38 MAPK is most frequently associated with induction of apoptosis (14,15). In contrast, ERK activation is typically associated with cell survival, proliferation and differentiation in response to activation by mitogens and cell survival factors (15–17). However, activation of ERKs has recently been found to contribute to cell death in neuronal cells, human medullary thyroid cancer cells and keratinocytes *in vitro* (18–20). Overactivity of the ERK-mediated pathway promotes oncogenesis in a variety of cell types (21,22). Furthermore, the ERK pathway has been implicated in p21WAF1 upregulation and in cell-cycle arrest mediated by pharmacological agents in colon cancer cells (23).

Flavonoids are naturally occurring polyphenols that are present in a wide variety of fruits and vegetables. Naringin (the glycoside of the flavonone, naringenin), a bioflavonoid found in grapefruit and other citrus fruits, has antioxidant (24) and antiatherogenic effects (8). Naringin also has proven to have antiviral activity (9) and to reduce the level of cytochrome P450 1A2 protein (25). Moreover, recent studies have shown that increased dietary consumption of naringin is associated with reduced risk of certain cancers, such as breast and lung cancers (26,27). Although many studies have analyzed the effects of naringin on tumor growth inhibition in several cell lines (26–28), the relevant pathway integrating cell-cycle regulation and signaling pathways involved in growth inhibition in cancer cells remains to be identified.

The present study sought to examine the roles of the MAPK-signaling pathways in regulating naringin-induced inhibition of cell growth in 5637 bladder cancer cells. Although ERK, JNK and p38 MAPK all were activated in response to naringin treatment, only ERK activity appeared to be involved in regulating cell growth. Using a variety of strategies to manipulate ERK activity, the Ras/Raf/ERK pathway was identified as a novel mechanism of naringin-induced cell growth inhibition that was mediated via p21WAF1-induced cell-cycle arrest.

Materials and methods

Materials

Naringin was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Polyclonal antibodies to cyclin E, CDK2 and CDK4 were obtained from Santa Cruz (Santa Cruz Biotechnology, CA). Polyclonal antibodies to cyclin D1, p21WAF1, p53, p27, ERK, Raf, phospho-Raf, phospho-ERK, p38 MAPK, phospho-p38 MAPK, JNK and phospho-JNK were obtained from New England Biolabs (Ipswich, MA). PD98059, SP600125 and SB203580 were obtained from Calbiochem (San Diego, CA). Anti-Ras antibody was obtained

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from Transduction Laboratories (San Jose, CA). The pCMV vector encoding dominant-negative Ras (RasN17) and dominant-negative Raf (RafS621A) were from Clontech (Mountain View, CA). Small interfering RNA (siRNA) oligonucleotides targeting p53 were designed and synthesized as described previously (29). p53 validated siRNA was purchased from Ambion (Austin, TX).

Cell cultures

The human bladder carcinoma cell lines (5637 and T24) and human primary cells (fibroblast) were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (4.5 g glucose/l) supplemented with 10% fetal calf serum, L-glutamine and antibiotics (Biological Industries, Beit Haemek, Israel) at 37°C in a 5% CO_2 humidified incubator.

Cell viability assay

Subconfluent, exponentially growing 5637 cells in 24-well plates were incubated with naringin for various lengths of time. Cell viability was determined using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium to the formazan product by mitochondrial dehydrogenase (30). The formazan product is quantified by measuring absorbance at 490 nm.

[^{3}H]thymidine incorporation

The 5637 cells, grown to near confluence in 24-well tissue culture plates, were made quiescent and treated with naringin, as indicated. The [^{3}H]thymidine incorporation experiment was performed as described previously (31).

Cell-cycle analysis (fluorescence activated cell sorter)

Cells were harvested, fixed in 70% ethanol and stored at −20°C. Cells then were washed twice with ice-cold phosphate-buffered saline and incubated with RNase and the DNA intercalating dye, propidium iodide. Cell-cycle phase analysis was performed using a Becton Dickinson Facstar flow cytometer equipped with Becton Dickinson cell fit software.

Immunoprecipitation, immunoblotting and immune complex kinase assays

Growth-arrested cells were treated with naringin in the presence of 10% fetal bovine serum for various durations at 37°C. Cell lysates were prepared, and immunoprecipitation, immunoblotting and immune complex kinase assays were performed as described previously (31).

Creation of p21WAF1 promoter reporter constructs

The human p21WAF1 promoter construct, WWW-luc (p21WAF1P), was a gift from Dr Bert Vogelstein (32). Preparation of p21WAF1PΔ2.3 has been described previously by Datto *et al.* (33).

Transient transfection

Each plasmid was transfected into cells using the Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. The luciferase activity was tested using a luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Firefly luciferase activities were standardized for β-galactosidase activity.

Affinity precipitation of the active form of Ras (Ras-guanosine triphosphate)

Cells seeded in 100 mm dishes at subconfluency (5×10^4 cells/cm^2) were grown and treated with naringin for various durations. After washing with ice-cold phosphate-buffered saline once, the cells were lysed by addition of 500 μl lysis buffer (25 mM HEPES, 10 mM ethylenediaminetetraacetic acid, 1% Igepal CA630, complete protease inhibitor cocktail from Roche Diagnostics (Palo Alto, CA), 1 mM sodium orthovanadate and 10% glycerol). The lysate was clarified by centrifugation for 15 min at 14 000*g* and the protein concentration of the lysate was determined by using the bicinchoninc acid assay (Pierce, Rockford, IL). Equal amounts of cell lysates (500 μg) were subjected to affinity precipitation for Ras-guanosine triphosphate (GTP) using 10 μl of an agarose suspension conjugated with glutathione S-transferase (GST) fusion protein, which corresponded to the human Ras-binding domain of c-Raf (Upstate Biotechnology, Lake Placid, NY). After a 1 h incubation at 4°C, the agarose was washed three times with lysis buffer and boiled with 30 μl sodium dodecyl sulfate sample buffer. The product was resolved by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by immunoblotting with anti-Ras antibody (34).

Transfection of siRNA

All siRNAs were diluted to 50 μM stock concentration and used as a 1000× stock. Transfection of siRNA was done in six-well tissue culture plates with cells at 20% confluency. siRNA was introduced into the cells using lipofectamine 2000 transfection reagent according to the manufacturer's protocols (Invitrogen, CA). After 24 h incubation with the 100 μM naringin, cells were then harvested for immunoblotting to determine the effect on cell-cycle protein.

Statistical analysis

When appropriate, data were expressed as means ± SEs. Data were analyzed by factorial analysis of variance and Fisher's least significant difference test where appropriate. Statistical significance was set at $P < 0.05$.

Results

Naringin reduces the proliferation of human urinary bladder cancer cells

To investigate the inhibitory effect of naringin on proliferation of bladder cancer cells, 5637 cells were grown in medium containing 10% fetal bovine serum in the absence or presence of various concentrations of naringin (0–150 μM) for 24 h. The results of the MTT assay (Figure 1A) demonstrated that naringin significantly inhibited cell viability in a concentration-dependent manner. In addition, the effect of naringin on cell proliferation was determined by [^{3}H]thymidine incorporation after treatment with various concentrations of naringin for 24 h. Naringin had a concentration-dependent inhibitory effect on 5637 cell growth (Figure 1B). Vehicle (ethanol) had no effect on the basal levels of cell viability and thymidine incorporation (data not shown).

Naringin-induced G_1-phase cell-cycle arrest

Flow cytometric analysis was performed to determine whether naringin-induced cell growth inhibition was due to cell-cycle arrest at a specific point in the cell cycle. Upon treatment with 100 μM naringin (the half maximal inhibitory concentration as assessed by MTT assay and [^{3}H]thymidine incorporation into cells), a flow cytometric analysis demonstrated that naringin treatment (100 μM) resulted in accumulation of significant numbers of cells in the G_1 phase of the cell cycle, indicative of cell-cycle arrest (Figure 2C–G). Next, the effect of naringin on cell-cycle regulatory molecules, operative in the G_1 phase of the cell cycle, was examined. To determine if naringin-induced growth inhibition of 5637 cells was due to decreased activation of the cell-cycle machinery, expression of cell-cycle regulatory molecules was examined using immunoblot and kinase assays. Treatment of cells with naringin for 24 h resulted in a dose-dependent decrease in the expression of cyclin D1 and cyclin E, as well as CDK2 and CDK4 (Figure 2A). The kinase activities associated with the CDKs drive cell-cycle progression through the transition checkpoints because they activate cyclins—the essential components of cyclin–CDK complexes. Therefore, kinase activities associated with CDK2 and CDK4 were assessed in naringin-treated cells. CDK complexes were immunoprecipitated using specific anti-CDKs antibodies, and the levels of CDK-associated kinase activities were measured using retinoblastoma (Rb) protein or histone H1 as the substrate. The kinase activities of the CDK2 and CDK4 immunoprecipitates were markedly inhibited after treating 5637 cells with naringin (Figure 2B).

Naringin-induced cell-cycle arrest is associated with upregulation of p21WAF1

The effect of naringin on the induction of p21WAF1, which regulates progression of the cell cycle at the G_1–S-phase transition checkpoint, was examined using immunoblotting (6,7). Immunoblot analysis revealed that treatment of 5637 cells with naringin resulted in significant dose-dependent induction of p21WAF1 compared with untreated cells (Figure 2C). However, naringin had no effect on induction of p27. Moreover, under similar experimental conditions, expression of the p53 tumor suppressor protein was unaffected, suggesting that it is unlikely that p27 and p53 are involved in the cell-cycle arrest induced by naringin (Figure 2C).

Naringin treatment strongly induced p21WAF1 expression in 5637 cells. Because induction of p21WAF1 has been shown to increase interaction between p21WAF1 and CDKs, leading to a decrease in kinase activity (6,7), the effects of naringin on interactions between p21WAF1 and CDKs were examined. To assess the effect of naringin on this protein–protein interaction, cell extracts were subjected to immunoprecipitation using anti-CDK2 or -CDK4 antibodies. After

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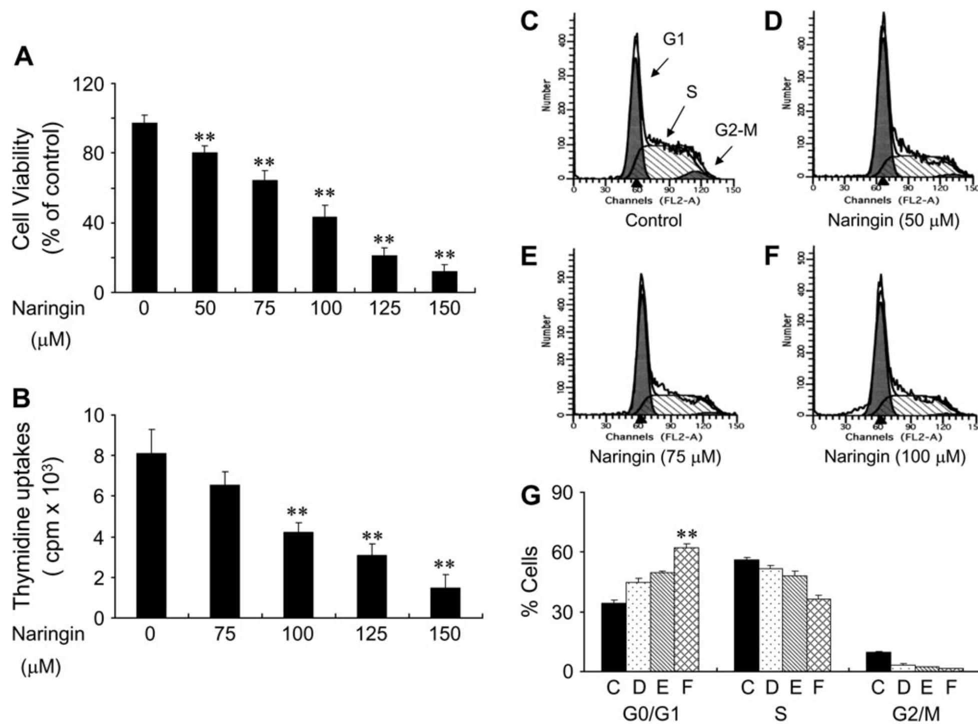

Fig. 1. Naringin suppressed cell proliferation via G_1-cell-cycle arrest in 5637 cells. (**A**) Subconfluent, exponentially growing cells were incubated with naringin for 24 h at indicated concentrations in 10% Dulbecco's modified Eagle's medium. Cell viability was determined by using a modification of the MTT assay. (**B**) Measurement of DNA replication by thymidine uptake as a marker of cell proliferation. Cells were grown to near confluence in 24-well tissue culture plates and treated with naringin as indicated. Cells were incubated for an additional 24 h and labeled with [methyl-^{3}H]thymidine at 1 μCi/ml during the last 12 h of this time period. Results are presented as means ± SEs from three triplicate experiments. $^{**}P < 0.01$ compared with no naringin treatment. Naringin induces G_1-cell-cycle arrest in 5637 cells. Cells were treated with 0 (**C**), 50 (**D**), 75 (**E**) and 100 μM naringin (**F**). Cells were subjected to flow cytometric analysis to determine the effect of naringin on cell-cycle distribution. (**G**) The percentage of cells in each population is shown as the mean ± SE from three triplicate experiments. $^{**}P < 0.01$ compared with no naringin treatment.

sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotting, membranes were probed with anti-p21WAF1. In naringin-treated cells, the association of CDK2 with p21WAF1 was maintained at high levels (Figure 2D). Levels of p21WAF1–CDK4 complexes also were increased in 5637 cells 24 h after naringin treatment (Figure 2D). These results suggest that the increased interaction between induced p21WAF1 and CDKs plays an important regulatory role in inhibiting CDK kinase activity, leading to G_1 arrest after naringin treatment in human bladder 5637 cancer cells. Moreover, p21WAF1 promoter activity was increased as a result of naringin treatment (Figure 2E). Collectively, these results demonstrate that naringin induces expression of p21WAF1 protein and enhances p21WAF1 promoter activity.

p21WAF1 is a direct downstream target of p53 and is activated by p53 binding to the two p53-binding sites that reside in the p21WAF1P promoter (Figure 2E) (35,36). Naringin induction relative to basal activity was not altered in the deletion mutant, p21WAF1PΔ2.3, which is devoid of p53-binding sites (Figure 2E). This result indicates that naringin is capable of inducing p21WAF1, independent of the p53 pathway. p21WAF1 promoter activity was increased in 5637 cells following naringin treatment (Figure 2E). In addition, the luciferase activity of the deletion mutant, p21WAF1PΔ2.3, remained unchanged compared with p21WAF1P (Figure 2E). Moreover, ablation of p53 production using siRNA also had no apparent effect on naringin-induced upregulation of p21 expression (Figure 2F), suggesting that naringin induces p21WAF1 induction in VSMC via a p53-independent pathway.

Effects of naringin on ERK, JNK and p38 MAPK activation in 5637 cells

To determine whether naringin affects MAPK activation, time course experiments, measuring ERK1/2, JNK and p38 MAPK activation in response to naringin, were performed in 5637 cells. Cells were exposed to naringin for various incubation times. Figure 3A shows an immunoblot of naringin-treated cells using antibodies specific for phosphorylated ERK1/2, JNK and p38 MAPK. The results of these experiments indicated that ERK1/2, JNK and p38 MAPK were significantly activated by naringin. Naringin increased the amount of phosphorylated ERK1/2, JNK and p38 MAPK at 12 h, suggesting that the naringin inhibits cell growth via activation of the ERK1/2, JNK and p38 MAPK pathways.

The effects of specific kinase inhibitors on MAPK activation then were analyzed. PD98059 is known to selectively block the activity of MAP kinase kinase, which activates ERK1/2 kinases. SB203580 is a specific inhibitor of p38 MAPK, and SP600125 inhibits JNK activity. Naringin-induced phosphorylation of the MAPKs (ERK1/2, p38 MAPK and JNK) was inhibited by PD98059, SB203580 and SP600125 (Figure 3B). These results suggest that naringin induced activation of ERK1/2, p38 MAPK and JNK in 5637 cells.

Next, we examined the effect of naringin on cell viability and growth inhibition using another bladder cancer cell line T24 and normal fibroblast cells. As shown in Figure 3C and D, treatment with naringin (50–150 μM) had no effect on cell growth in culture of normal fibroblast cells. However, under similar experimental conditions, naringin significantly inhibited the cell viability and cell proliferation in T24 cells (Figure 3C and D). In addition, naringin treatment strongly induced p21WAF1 expression and phosphorylation of ERK (Figure 3E–G). These results suggest that naringin has a general inhibitory effect in bladder cancer cells.

Naringin-induced p21WAF1 is blocked by PD98059, a specific inhibitor of ERK

Because MAPK activity was induced by naringin, the role of p21WAF1 induction in MAPK activity was investigated. To elucidate

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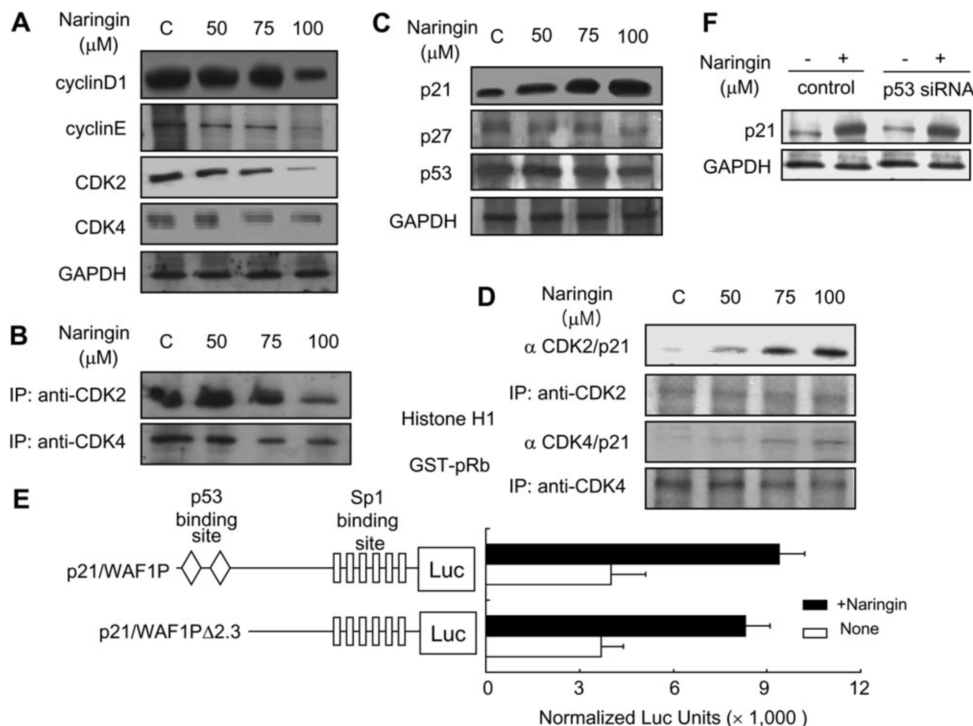

Fig. 2. Naringin-induced cell-cycle arrest is associated with upregulation of p21WAF1 in 5637 cells via a p53-independent pathway. (**A** and **C**) The 5637 cells were treated with naringin at the indicated concentrations and immunoblot analysis was performed with antibodies specific for cyclin D1, cyclin E, CDK2, CDK4, p21WAF1, p27 and p53. The results from representative experiments were normalized to glyceraldehyde-3-phosphate dehydrogenase expression. (**B**) Cells were treated with naringin at the indicated concentrations for 24 h and were then harvested. Total cell lysates were immunoprecipitated with anti-CDK2 and anti-CDK4 antibodies. The kinase reaction was performed using histone H1 (for CDK2) or GST-Rb (for CDK4) as substrate. (**D**) Equal amounts of cell lysates were subjected to immunoprecipitation with anti-CDK2 and anti-CDK4 antibodies. The immunoprecipitates were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the samples were transferred to a nitrocellulose membrane, followed by immunoblot analysis with an anti-p21WAF1 antibody. The results from representative experiments were normalized to immunoprecipitated CDK2 and CDK4 expression. (**E**) Stimulation of p21WAF1 promoter activity by naringin treatment in 5637 cells. Luciferase activity was determined in cell lysates as described under Materials and Methods. The indicated values are the means of three triplicate experiments. (**F**) The cells were transfected with p53 siRNA (right panel) or control siRNA (left panel) and treated with 100 μM naringin for 24 h. Harvested cells were immunoblotted for p21WAF1.

the signal cascade triggered by MAPK, the dependence of p21WAF1 induction on the MAPK-signaling pathway was investigated. Thus, 5637 cells were pretreated for 40 min with or without kinase inhibitors, such as PD98059, SB203580 and SP600125, followed by exposure to 100 μM naringin. As shown in Figure 4A, the naringin-induced increase in p21WAF1 expression was reduced to the level of the control by PD98059 treatment. SP600125 had a slight reversed effect on naringin-mediated p21WAF1 expression. However, SB203580 had no apparent effect on naringin-induced p21WAF1 levels. In addition, CDK2 and CDK4 protein levels also were reversed after PD98059 treatment for 24 h (Figure 4B). These results strongly suggest that the ERK-signaling pathway is required for the regulation of p21WAF1-mediated G_1-phase cell-cycle arrest in response to naringin.

Naringin-induced inhibition of cell growth is reversed by ERK inhibition

To investigate the effect of ERK activation on naringin-induced cell growth inhibition, cells were pretreated with PD98059, followed by the MTT and [^{3}H]thymidine incorporation assays (Figure 4C and D). The 5637 cells were pretreated for 40 min with or without 40 μM PD98059, followed by treatment with 100 μM naringin in the presence of 10% serum. As shown in Figure 4C and D, the inhibition of cell viability and [^{3}H]thymidine incorporation induced by naringin treatment were reversed by pretreatment with PD98059. However, SB203580 and SP600125 had no effect on naringin-induced cell growth inhibition (data not shown), suggesting that the ERK-signaling pathway is involved in naringin-induced inhibition of growth in 5637 cells.

Effects of a dominant-negative RasN17 mutant gene on naringin-mediated ERK, p21WAF1, growth inhibition and decreased CDKs levels

The results described above led to an examination of whether or not Ras, an activator of the ERK1/2-signaling pathway, is involved in naringin-induced growth inhibition. First, the ability of naringin to induce activation of Ras in 5637 cells was examined. To achieve this objective, cellular levels of the active form of Ras (Ras-GTP) were analyzed via specific binding to GST-conjugated c-Raf immobilized on agarose. The bound Ras-GTP was then assayed by immunoblotting with an anti-Ras antibody. As shown in Figure 5A, naringin is capable of inducing activation of Ras in 5637 cells, whereas the expression of Ras remained unchanged after treatment with naringin.

To elucidate whether Ras activation is required for naringin-induced ERK phosphorylation, 5637 cells were transfected with a dominant-negative Ras (RasN17) or an empty vector (EV) and then treated with naringin. As shown in Figure 5B, naringin treatment of 5637 cells, or of cells transfected with the EV, induced ERK1/2 activity. Transfection with RasN17 resulted in near complete suppression of ERK1/2 activity, suggesting that naringin may activate ERK through a Ras-dependent pathway in 5637 cells.

The involvement of Ras in p21WAF1 expression and naringin-induced inhibition of cell growth was further investigated by transfecting a dominant-negative RasN17 mutant gene into 5637 cells. As shown in Figure 5C, D and E, naringin treatment of 5637 cells, or of cells transfected with the EV, induced p21WAF1 expression and decreased [^{3}H]thymidine incorporation and CDK2 and CDK4 expression. Transfection with the RasN17 gene blocked naringin-induced

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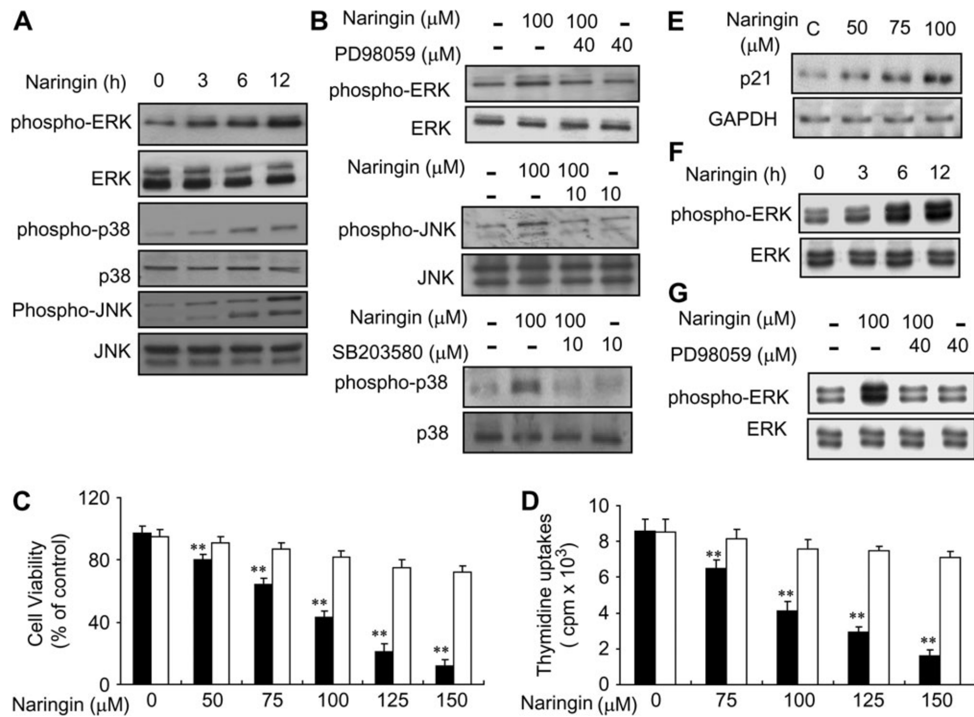

Fig. 3. Naringin induced ERK1/2, JNK and p38 MAPK phosphorylation. (**A**) Cells were harvested, lysed and the phosphorylation levels of ERK1/2, JNK and p38 MAPK were detected by immunoblot analysis using antibodies phospho-specific for ERK1/2, JNK and p38 MAPK. (**B**) The 5637 cells were pretreated for 40 min with PD98059 (40 μM), SB203580 (10 μM) and SP600125 (10 μM) before cells were treated with naringin (100 μM) at 12 h. (**C** and **D**) Effect of cell viability and growth inhibition in T24 and normal fibroblast cells using MTT assay and thymidine uptake experiments. (**E–G**) Naringin induced p21WAF1 expression and phosphorylation of ERK1/2 in T24 cells. $^{**}P < 0.01$ compared with no naringin treatment.

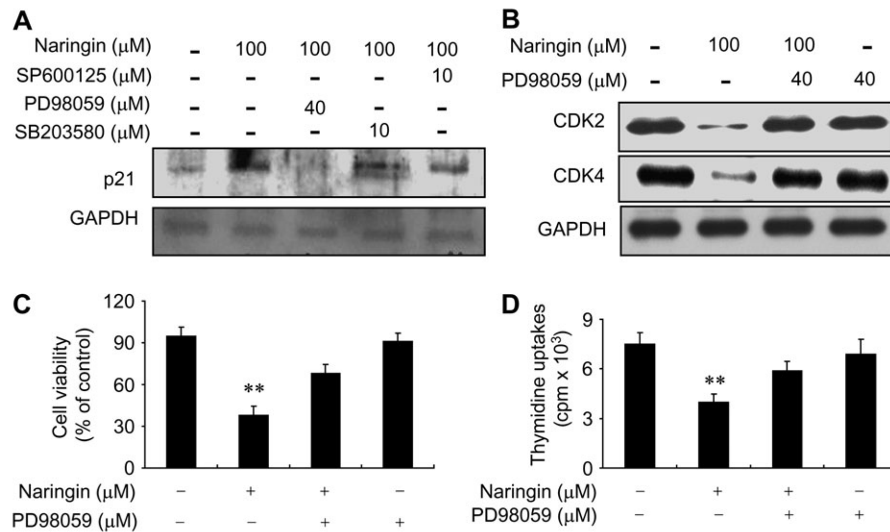

Fig. 4. MAP kinase kinase 1/2 inhibitor reversed p21WAF1 expression, decreased growth inhibition and G_1-phase cell-cycle-associated proteins by naringin. (**A** and **B**) Cells were plated in plates and were preincubated for 40 min in the absence or presence of PD98059 (40 μM), SB203580 (10 μM) and SP600125 (10 μM). Cells were then treated with 100 μM naringin, followed by immunoblot analysis performed with antibodies specific for p21WAF1, CDK2 and CDK4. The results from representative experiments were normalized to glyceraldehyde-3-phosphate dehydrogenase expression. (**C** and **D**) Cells were pretreated for 40 min with 40 μM PD98059 before cells were treated with 100 μM naringin at 24 h. MTT assay and thymidine uptake experiments were determined as described under Materials and Methods. Indicated values are means of triplicate wells. $^{**}P < 0.01$ compared with no naringin treatment.

p21WAF1 expression and prevented the naringin-induced decrease in [^{3}H]thymidine incorporation and in CDK2 and CDK4 expression. These results show that naringin regulates p21WAF1 expression, growth inhibition and decreases CDKs in 5637 cells by activating a Ras-dependent pathway. Collectively, these results suggest that the Ras/ERK-signaling pathway must be involved in p21WAF1-mediated G_1-phase cell-cycle arrest and growth inhibition in 5637 cells in response to naringin treatment.

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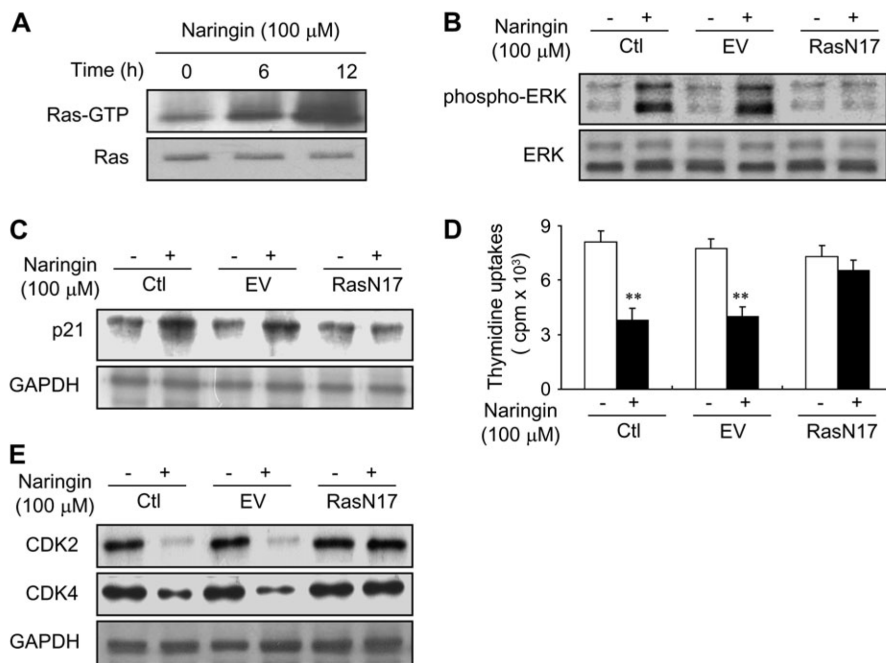

Fig. 5. Effects of a dominant-negative RasN17 mutant gene in the naringin-mediated cell growth inhibition (**A**) Time course for naringin-induced Ras activation. The 5637 cells were stimulated by naringin for 6 or 12 h and lysed. Cellular content of Ras active form (Ras-GTP) was analyzed by immunoblotting as described in Materials and Methods. (**B**, **C** and **E**) Immunoblot of ERK1/2, p21WAF1, CDK2 and CDK4 in cells after 12 h treatment with naringin. Cells were transfected with an EV or with RasN17. Cellular content of phosphorylated ERK1/2, p21WAF1, CDK2 and CDK4 was analyzed by immunoblotting as described in the Materials and Methods section. (**D**) Thymidine uptake experiments were determined as described under Materials and Methods. Results are presented as means ± SEs from three triplicate experiments. $^{**}P < 0.05$ compared with no naringin treatment in 5637 cells transfected with the RasN17 mutant gene.

The RafS621A mutant gene reversed the effects of naringin treatment on growth inhibition, CDKs levels, ERK activation and p21WAF1

The requirement for the activation of the Ras, Raf and ERK pathways, which are associated with cell proliferation, has been demonstrated for various cell functions in response to various stimuli (37–39). The activation of Raf, which is a downstream molecule of Ras, also was observed in naringin-treated 5637 cells. Figure 6A shows an immunoblot analysis of 5637 cells treated with naringin using antibodies specific for phosphorylated Raf. The results of this experiment indicate that Raf is significantly activated by naringin. Subsequently, experiments were carried out to demonstrate direct involvement of Raf activation in naringin-induced ERK and p21WAF1 expression in 5637 cells. The 5637 cells were transiently transfected with an EV or RafS621A mutant gene and stimulated with naringin. As shown in Figure 6B and C, naringin treatment of 5637 cells or of cells transfected with EV induces ERK activity and p21WAF1 expression (Figure 6B and C). This activation of ERK and p21WAF1 was decreased in cells transfected with the RafS621A mutant gene. In addition, the naringin-induced decrease in [^{3}H]thymidine incorporation and in CDKs expression (Figure 6D and E) was not observed in RafS621A mutant gene transfectants. These results show that naringin regulates ERK activation, p21WAF1 expression, growth inhibition and decreased CDKs levels in 5637 cells by activating a Raf-dependent pathway. Collectively, these results suggest that the Ras/Raf/ERK-signaling pathway must be involved in naringin-induced growth inhibition via activation of p21WAF1-mediated cell-cycle arrest in 5637 cells.

Discussion

Recently, the use of naturally occurring compounds in the development of antitumor agents has become a critical topic in the scientific and industrial communities. Citrus fruits contain various flavonoids, and among these naturally occurring compounds, naringin has been pharmacologically evaluated as a potential anticancer agent (26–28). However, the exact molecular mechanisms underlying the cellular effects resulting from naringin treatment have yet to be fully explained. To the best of our knowledge, no previous studies have focused on flavonoid-induced cell growth inhibition due to p21WAF1-mediated G_1-phase cell-cycle arrest via the Ras/Raf/ERK-signaling pathway in cancer cells.

Treatment of 5637 cells with naringin (50–150 μM) resulted in decreased cell viability and thymidine uptake, but not in normal fibroblast cells. A number of studies have investigated the effects of naringin on cancer cell responses. Consistent with the results of the present study, several studies reported that naringin inhibited cell growth in different cancer cell lines (26–28). Moreover, the results of the present study indicate that naringin caused G_1-phase cell-cycle arrest together with a decrease in cyclin D1/CDK4 and cyclin E/CDK2, which are involved in cell-cycle progression from the G_1- to S-phase. The data demonstrate that a significant upregulation in p21WAF1 occurred during the G_1-phase arrest in 5637 cells treated with naringin. Interestingly, however, naringin had no effect on the expression of p27 and p53 as determined by immunoblot analysis, promoter assay and siRNA experiment, suggesting that naringin-induced accumulation of p21WAF1, independent of the p53 pathway, may also be responsible for the G_1-phase arrest in 5637 cells. To our knowledge, this is the first systematic study examining the involvement of each component of the cyclin dependent kinase inhibitor–cyclin–CDK machinery during naringin-induced cell-cycle arrest.

The importance of MAPK-signaling pathways in regulating inhibition of cell growth during conditions of stress has been widely investigated (18–20). The effect of naringin on early signal transduction pathways was examined using ERK, p38 MAPK and JNK. Naringin treatment resulted in upregulation of ERK, JNK and p38 MAPK phosphorylation. Previous studies have demonstrated that the MAPK pathway is involved in cell growth inhibition (15–20,30) and/or the regulation of the cell cycle (23,30,40,41). Because naringin treatment induced p21WAF1, the role of MAPK in the regulation of p21WAF1 expression was investigated. To examine the relationship between the

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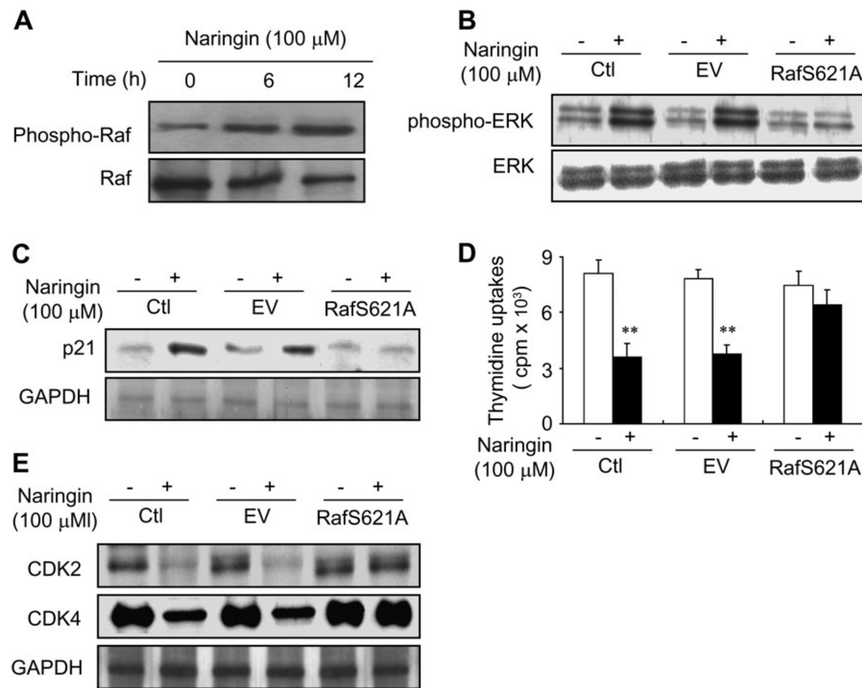

Fig. 6. Effects of the dominant-negative RafS621A mutant gene on naringin-induced cell growth inhibition. (**A**) The levels of phosphorylated Raf protein were increased in 5637 cells after 12 h treatment with naringin (100 μM). (**B**, **C** and **E**) Immunoblot of ERK1/2, p21WAF1, CDK2 and CDK4 in cells 12 h after treatment with naringin. Cells were transfected with an EV or with the RafS621A mutant gene. Cellular content of phosphorylated ERK1/2, p21WAF1, CDK2 and CDK4 was analyzed by immunoblotting as described in the Materials and Methods section. (**D**) Thymidine uptake experiments were performed as described in Materials and Methods. Results are presented as means ± SEs from three triplicate experiments. $^{**}P < 0.05$ compared with no naringin treatment in 5637 cells transfected with the RafS621A mutant gene.

MAPK-signaling pathway and the regulation of p21WAF1, pharmacological inhibitors (PD98059, SB203580 and SP600125) were employed. Of considerable interest in this study was the marked decrease in naringin-induced expression of p21WAF1 only by PD98059 treatment. Under the same experimental conditions, SB203580 and SP600125 treatment had no effect on naringin-induced p21WAF1 expression (Figure 4A). These results suggest that activation of these two kinases is not required for naringin-induced p21WAF1 expression, although naringin activates both p38 kinase and JNK2. The ERK-signaling pathway appears to involve multiple signal transduction pathways, which are used to accomplish a variety of functions (18–20,23). Many studies have supported the general view that activation of the ERK pathway delivers a survival signal that counteracts the proapoptotic effects of JNK and p38 MAPK activation (11–15). However, recent reports have maintained that sustained activation of ERK also is involved in apoptosis (18–20). Robust ERK stimulation has been reported to suppress cell-cycle progression, via induction of cell-cycle inhibitor proteins, including p21WAF1 and p27KIP (42). The results of the present study suggest that ERK signaling is involved in naringin-induced inhibition of cell growth due to induction of p21WAF1 in 5637 cells. This study identified the first example of an interaction between activation of the ERK-signaling pathway and regulation of p21WAF1 in cells treated with flavonoids.

The effects of naringin on cell proliferation and on the cell-cycle-associated proteins, CDK2 and CDK4, were confirmed by inhibition of ERK. Consistent with the observations that naringin treatment inhibited cell growth and modulated cell-cycle-associated proteins, blockade of ERK with PD98059 rescued cell proliferation and cell-cycle-associated proteins. These data provide evidence that ERK is a key mediator of naringin-induced inhibition of cell growth.

In general, the ERK cascade is activated by mitogenic stimuli and is believed to mediate both cell proliferation and survival (15–17). The activation of ERK by extracellular growth signals is mediated via activation of a small G-protein, Ras, or its downstream effector, Raf, which is the target molecule of activated Ras. Ultimately, ERK activation enhances cell proliferation (37–39,43,44). In contrast, sustained expression of activated Ras or Raf can elicit cell growth arrest in many cell types *in vitro* (45,46). Ras/Raf-mediated growth arrest is accompanied by induction of CDKI, such as p16INK4a, p21WAF1, p27KIP1 and tumor suppressor p53, and by downregulation of phosphorylation of Rb or of the E2F family (20,45,46). However, the mechanism by which Ras or Raf activation, in response to flavonoid induction, promotes cell growth arrest remains to be elucidated. Accordingly, the involvement of a Ras/Raf-mediated pathway in naringin-induced inhibition of 5637 cell growth was examined because both Ras and Raf were activated by naringin. In this study, to determine whether Ras/Raf is required for naringin-induced activation of ERK, 5637 cells were transfected with dominant-negative Ras (RasN17) and dominant-negative Raf (RafS621A). Naringin-induced ERK activation was suppressed by transfection with the dominant-negative mutants of RasN17 and RafS621A in cells, suggesting that Ras/Raf plays a key role in naringin-induced activation of the ERK-signaling cascade in 5637 cells.

To gain further insight into the mechanism of naringin-mediated cell growth inhibition in 5637 cells, the influence of Ras/Raf activation on the ERK1/2 kinase cascades that control cell growth and cell-cycle machinery proteins was examined. Transfection of cells with RasN17 and RafS621A mutant genes abolished the naringin-induced inhibition of cell growth, together with upregulation of p21WAF1 levels and inhibition of CDK2 and CDK4 expression. The results obtained for the RasN17 and RafS621A overexpression experiments now provide evidence that the Ras/Raf-signaling pathway is involved in naringin-induced inhibition of cell growth via p21WAF1-mediated cell-cycle regulation in 5637 cells. Thus, a Ras/Raf-dependent ERK-signaling pathway may be responsible for naringin-induced inhibition of cell proliferation.

The present study provides important new insight into the molecular mechanisms of the effects of naringin in 5637 cancer cells. First,

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naringin reduces cell viability and cell proliferation. In addition, naringin arrests the cell cycle at the G_1 phase in 5637 cells and the naringin-induced cell-cycle arrest can be attributed to the inhibition of cyclin D1/CDK4 and cyclin E–CDK2 complexes by the increased expression of p21WAF1, independent of p53 protein levels. Finally, the naringin-induced inhibition of cell growth appears to be linked to the activation of Ras/Raf/ERK through p21WAF1-mediated G_1-phase cell-cycle arrest. The findings of the present study may, in part, explain the therapeutic effects of naringin for treatment of urinary bladder cancer.

The results of the present study are a significant step forward in understanding the activity of flavonoids, and it also sheds new light on the molecular mechanisms underlying the unexpected effects of the flavonoid, naringin. Although recent data from our laboratory have demonstrated the importance of the ERK-signaling pathway in some natural products (unpublished data), additional studies are required to clearly elucidate the relationship between the ERK pathway and the activity of other flavonoids.

Others have found that suppression of the ERK pathway is important in flavonoid-induced inhibition of cell growth. Recent studies have suggested that flavonoid inhibition of ERK activation represents a primary approach for treatment of human malignancies. Although this may indeed be true, the results of the present study indicate that this approach should not be generalized to all types of cancer. In contrast, the findings of the present study, which support a role for the Ras/Raf/ERK-signaling pathway in enhancing naringin-induced cell growth inhibition, suggest that strategies designed to activate this signal cascade may enhance the therapeutic effectiveness of the flavonoid, naringin.

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