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Molecules and Cells

(*E*)-1-(3,4-Dihydroxyphenethyl)-3-Styrylurea Inhibits Proliferation of MCF-7 Cells through G_1 Cell Cycle Arrest and Mitochondria-Mediated Apoptosis

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Growing interest in the beneficial effects of antioxidants has inspired the synthesis of new phenolic acid phenethyl ureas (PAPUs) with enhanced antioxidant potential. We have previously shown the capacity of one PAPU compound, (*E*)-1-(3,4-dihydroxyphenethyl)-3-styrylurea (PAPU1), to induce caspase-dependent apoptosis in melanoma cells. In the present study, we examined the anti-proliferative effects of PAPU compounds on MCF-7 human breast cancer cells and determined the molecular mechanisms involved. Treatment with PAPU compounds inhibited predominantly proliferation in these cells, where the PAPU1 was the most efficient form. Flow cytometric analysis showed that PAPU1 blocked cell cycle progression in the G_0/G_1 phase, and reduced the proportion of cells in G_2/M phase. This was related to the inhibition of cell cycle regulatory factors, including cyclin D/E and cyclin-dependent kinase (CDK) 2/4, through induction of $p21^{Cip1}$. PAPU1 also induced the mitochondrial-mediated and caspase-dependent apoptosis in MCF-7 cells. This was evidenced by cellular changes in the levels of Bcl-2 and Bax, loss of the mitochondrial membrane potential, release of cytochrome *c* into the cytosol, and caspase-9 activation. Collectively, our results suggest that G_1 cell cycle regulatory proteins and mitochondrial pathways are the crucial targets of PAPU1 in the chemoprevention of breast cancer cells.

INTRODUCTION

Naturally occurring phenolic compounds show diverse anti-carcinogenic and anti-inflammatory activities that may originate in part from their antioxidant properties (Fang et al., 2008; Korkina, 2007; Rahman et al., 2006). The phenolic acids caffeic-, ferulic-, and *p*-coumaric acid, for example, display antioxidant (Hudson et al., 2000; Masuda et al., 2008; Srinivasan et al., 2007), anti-inflammatory (Song et al., 2008), anti-melanogenic (Mathew and Abraham, 2004), and other cancer chemopreventive activities (Hudson et al., 2000).

The close correlation of the antioxidant potential of phenolic compounds with anticancer activity has guided our efforts to develop phenolic acid antioxidants with greater potency than the original phenolic acids. We previously synthesized various phenolic acid phenethyl ureas (PAPUs) from the phenolic acids containing one aromatic hydroxyl group by a Curtius rearrangement. The compounds synthesized were: (*E*)-1-(3,4-dihydroxyphenethyl)-3-styrylurea (PAPU1), (*E*)-1-(3,4-dihydroxystyryl)-3-phenethylurea (PAPU2), (*E*)-1-(3,4-dihydroxystyryl)-3-(4-hydroxyphenethyl)urea (PAPU3), (*E*)-1-(3,4-dihydrophenethyl)-(3,4-dihydroxystyryl)urea (PAPU4), (*E*)-1-(4-methoxyphenethyl)-3-(4-methoxystyryl)urea (PAPU5), (*E*)-1-(3,4-dihydroxyphenethyl)-3-(4-hydroxystyryl)urea (PAPU6). We recently found that the synthetic PAPU compounds have a pro-apoptotic potential in melanoma cells, where PAPU1 was one of the major effective forms (Kim et al., 2009). However, the antitumor potential of PAPU1 varied between cell types, in that in melanoma and NIH3T3 cells, PAPU1 induced apoptosis sensitively, whereas in MCF-7 cells it appeared to inhibit proliferation rather than a direct cytotoxicity at the same condition examined.

Although the anti-proliferative activity of phenolic antioxidants does involve apoptosis induction (Miranda et al., 1999), the mechanisms involved might be differed depending on the type of cancer cells and the tested compounds. Daidzein may induce apoptosis through cell cycle arrest at the G_0/G_1 interface or in G_2 phase (Choi and Kim, 2008). In contrast, some phenolic antioxidants induce apoptosis directly, without altering the cell cycle distribution (Kim et al., 2009; Su et al., 2000). Thus we postulated that PAPU compounds exert their anti-cancer effects differently according to the type of cells, and in MCF-7 cells they induce cell cycle arrest as the primary effects.

In this study, we investigated the anti-proliferative activity of six synthetic PAPU compounds on MCF-7 human breast cancer cells. We also examined signaling pathways the compounds invoke to inhibit the cell cycle and induce apoptosis.

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MATERIALS AND METHODS

Chemicals and plastics

Unless otherwise specified, all of the chemicals, culture plastics, and antibodies were purchased from the Sigma Chemical Co. (USA), Becton-Dickinson (Franklin Lakes, USA), and Santa Cruz Biotechnology Inc. (USA), respectively. PAPU compounds were dissolved in absolute ethanol prior to use and the final ethanol concentration did not exceed 0.5% (v/v) in any experiment.

Cell cultures and PAPU treatment

MCF-7 human breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, USA) and antibiotics (100 IU/ml penicillin G and 100 μg/ml streptomycin). Cells were resuspended at 10^6 cells/ml in medium, and either spread onto 35-mm culture dishes in 2 ml aliquots or distributed in 100 μl aliquots to 96-well flat-bottomed plates. Just prior to the PAPU treatment, the medium in all of the cultures was replaced with fresh medium (DMEM supplemented with 1% FBS), and the replacement did not induce cell cycle arrest during the experimental periods. At various times after PAPU sample treatment (0 to 100 μM), the cells were processed for further analyses.

Cell proliferation assay

Cell proliferation was measured by direct counting. Initially, the cells were seeded into 12-well culture plates at 2×10^4 cells/ml and cultured in DMEM containing 10% FBS at 37°C for 24 h. When cells reached up to 70% confluence, the medium was replaced with DMEM containing 1% FBS and various concentrations (0-100 μM) of PAPU compounds. At various times (0-72 h) of treatment, the cells per well were collected after treatment with trypsin-EDTA and then counted using a hemocytometer.

Measurement of cell viability and cytotoxicity

To evaluate the viability of cells exposed to PAPU compounds, we used 3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) (Mosmann, 1983). Absorbance was measured at 560 nm using the SpectraCount™ ELISA reader (Packard Instrument Co., USA) and the results were represented as a MTT reducing activity. Cytotoxicity was also measured using a trypan blue exclusion assay. The cells were stained with 0.4% trypan blue, and one hundred cells for each treatment were counted.

Measurement of DNA synthesis

One μCi of [*methyl*-^{3}H] Thymidine deoxyribose (TdR) (Amersham Pharmacia Biotech Inc., USA) was added to each well for the last 24 h of cultures co-incubated with PAPU compounds. Cells were collected from the plates with a cell harvester (Inotech Inc., Switzerland), and the tritium incorporated into total DNA was measured using a liquid scintillation counter (Packard Instrument Co.).

Flow cytometric analysis

The effects of PAPU on DNA fragmentation and cell cycle distribution were determined by flow cytometric analysis after PI staining. PAPU-treated cells (2×10^6 cells/sample) were first fixed with 80% ethanol at 4°C for 24 h, then incubated overnight at 4°C with 1 ml of a PI staining mixture containing 25 μg/ml PI. After staining, 10^4 cells from each sample were analyzed using the FACS Calibur® system (Becton Dickinson, USA). The cell cycle progression was determined using the WinMDI 2.9 program.

Agarose gel electrophoresis

Cells were incubated with a lysis buffer [1% nonidet P (NP)-40 and 1% sodium dodecyl sulfate (SDS) in 50 mM Tris, pH 8.0] at 65°C for 1 h. DNA was extracted with phenol/chloroform/isoamyl alcohol and formation of DNA ladders was analyzed using 1-2% agarose gel electrophoresis followed by ethidium bromide staining.

Western blot analysis

Cells were lysed in a lysis buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). Equal amounts (20 μg) of protein from each sample were analyzed by 12% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, UK). The blots were treated with PBS containing 5% skim milk for 1 h, probed with primary antibodies at room temperature for 2 h, and then incubated with secondary antibodies for 1 h. The blots were then developed with enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech) and used to expose X-ray film (Eastman-Kodak, USA).

Small interfering RNA transfection

Gene silencing of p21 was performed using siRNA for human p21 and a control siRNA from Santa Cruz Biotechnology. Transient transfection was carried out using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's instructions. Cellular levels of the proteins specific for the siRNA transfection were checked by immunoblotting as described above, and all experiments were performed 24 h after transfection.

Immunoprecipitation

Equal amounts (100 μg/sample) of protein were incubated at 4°C with a $p21^{Cip1}$-specific antibody and protein A/G plus agarose in an immunoprecipitation buffer (0.5% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF). The immunoprecipitate specific for $p21^{Cip1}$ was analyzed by 12% SDS-PAGE, blotted onto polyvinylidene difluoride (PVDF) membranes, and probed with primary antibodies specific for cell cycle regulatory proteins and Bcl-2 family proteins.

Cell fractionation

Cells were washed with PBS and resuspended for 20 min in an ice-cold lysis buffer (250 mM sucrose, 20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM each of EGTA, EDTA, DTT and PMSF, and 10 μg/ml each of leupeptin, aprotinin and pepstatin A). The cells were centrifuged at $750 \times g$ for 10 min at 4°C, and the supernatants were further centrifuged at $10,000 \times g$ for 25 min at 4°C to prepare the cytosolic fraction. The pellets were resuspended in the lysis buffer and used as the mitochondrial fraction after centrifugation at $10,000 \times g$ for 25 min.

Measurement of mitochondrial membrane potential (MMP)

Cells were stained with 50 nM 3,3′-dihexyloxa-carbocyanine iodide ($DiOC_6$; Molecular Probes, USA) for 20 min at 37°C. The fluorescence related to MMP was measured using a FACS Calibur® system (Becton Dickinson), and the level of MMP was determined using the WinMDI 2.9 program.

Measurement of caspase activity

Cell lysates (100 μg) prepared from PAPU-exposed cells were incubated with 100 μM of the synthetic substrate Ac-IETD-AMC

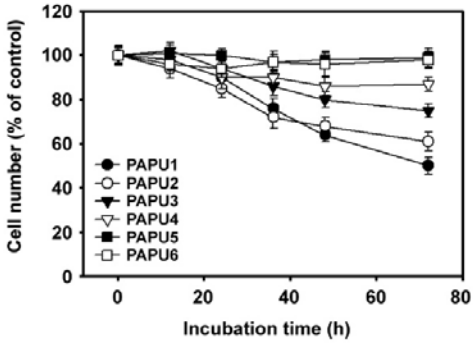

Fig. 1. Effects of PAPU compounds on cell growth. MCF-7 cells were treated with 100 μM of PAPU compounds (PAPU1-PAPU6) for various times (0-72 h), and the cells were then counted using a hemocytometer.

(for caspase-8 activity) or Ac-LEHD-AMC (for caspase-9 activity) at 37°C for 2 h in 1 ml reaction buffer (100 mM Hepes, 10% sucrose, 0.1% Chaps, and 10 mM dithiothreitol, pH 7.5). Fluorescence intensities were measured using a microplate spectrophotometer (Bio-Tek Instruments, Inc., USA).

Statistical analyses

Unless otherwise specified, all of the data were expressed as a mean ± standard deviation (SD) of triplicate experiments. One-way analysis of variance (ANOVA; SPSS version 17.0 software) followed by Scheffe's teat was used for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

RESULTS

PAPU1 reduces more sensitively DNA synthesis by the cells than viability of the cells

Treatment of the cells with 100 μM of the PAPU compounds caused a time-dependent decrease in cell number relative to control cultures. The compounds exhibited anti-proliferative efficiency in the following order: PAPU1 > PAPU2 > PAPU3 > PAPU4 (Fig. 1). However, PAPU5 and PAPU6 did not prevent proliferation of the cells at the same experimental conditions. PAPU compounds also suppressed proliferation of the cells in a dose-dependent manner, where PAPU1 was the most potent compound (data not shown). This result was in part consistent with our previous data showing that PAPU1 was one of the most potent drugs in inhibiting proliferation and inducing apoptosis in melanoma cells. Thus we selected PAPU1 in the next experiments and further examined the possible mechanisms by which PAPU1 inhibits the proliferation of MCF-7 cells.

The addition of PAPU1 did not decrease MTT reducing activity within 24 h after exposure, even at the highest concentration tested (100 μM) (Fig. 2, top panel). Exposure to 100 μM PAPU1 for 48 h decreased viability slightly, and increased the proportion of trypan blue-positive cells (Fig. 2, middle panel). In contrast, PAPU1 (100 μM) significantly reduced TdR incorporation even at 24 h after exposure (Fig. 2, bottom panel), and by 72 h, reduced it to 22% of the control level, although only 23% of cells stained with trypan blue. Under these same conditions, PAPU1 did not cause cytotoxic effects in primary cultured fibroblasts (data not shown).

PAPU1 induces G_1 cell cycle arrest with the concomitant suppression of G_1 phase regulatory proteins in MCF-7 cells

We subsequently analyzed cell cycle progression in PAPU1-exposed cells using flow cytometry after propidium iodide (PI) staining. Treatment with PAPU1 for 48 h resulted in the accumulation of cells in the G_0/G_1 phase, with a concomitant depletion of cells in G_2/M phase (Fig. 3A). In the presence of 50 and 100 μM PAPU1, the proportion of cells in G_0/G_1 phase increased gradually from 54%, the control value, to 67 ± 2.9% and 71 ± 3.1%, respectively (Fig. 3B). The proportion of cells in G_2/M phase after 100 μM PAPU1 treatment was reduced to approximately 8% as compared to the untreated control value (27.1 ± 0.8%).

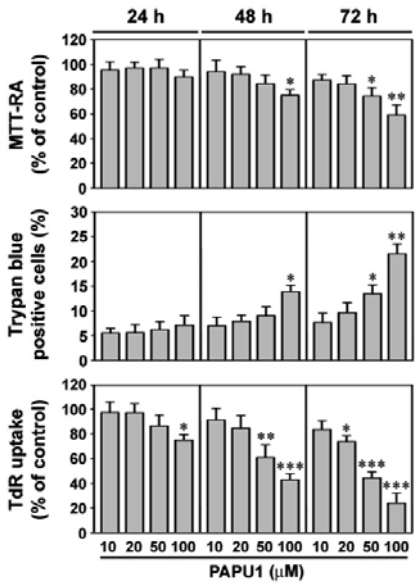

Fig. 2. Effects of PAPU1 on MTT reducing activity, cytotoxicity, and DNA synthesis in MCF-7 cells. The cells were treated with PAPU1 at the indicated concentrations for 0-72 h, then processed for MTT assay (top panel), trypan blue staining (middle panel), and TdR incorporation (bottom panel). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. the untreated control values.

Following exposure to 100 μM PAPU1 for 24 h, levels of the G_1-checkpoint-related proteins, cyclin-dependent kinase-4 (CDK4) and cyclin D, decreased slightly in the MCF-7 cells (Fig. 3C). In addition to CDK4 and cyclin D, the expression of CDK2 and cyclin E declined markedly in the PAPU1-treated cells at 48 h of treatment (Figs. 3C and 3D). PAPU1 also reduced the level of proliferating cell nuclear antigen (PCNA) in a time-dependent manner (Fig. 3E). However, levels of CDK1, cyclin A and pRb were not affected after PAPU1 treatment in a significant level (Figs. 3C-3E).

Up-regulation of $p21^{Cip1}$ was related to the PAPU1-mediated G1 arrest in MCF-7 cells

To identify upstream effectors in the PAPU1-induced G_1 phase arrest, we determined the expression patterns of p53 and the CDK inhibitors (CDKIs), $p21^{Cip1}$ and $p27^{Kip1}$, by western blot analysis. Although p53 and $p21^{Cip1}$ proteins were scarcely detectable in control cells, they increased significantly in cells exposed to 100 μM PAPU1, and the increase in $p21^{Cip1}$ was most prominent at 48 h of treatment (Fig. 4A). In contrast, treatment of cells with PAPU1 did not increase the expression of $p27^{kip1}$ in a significant level.

The expression of $p21^{Cip1}$ may proceed by either a p53-dependent or p53-independent mechanism (El-Deiry, 1994; Huang et al., 1998). To distinguish these mechanisms in PAPU1-

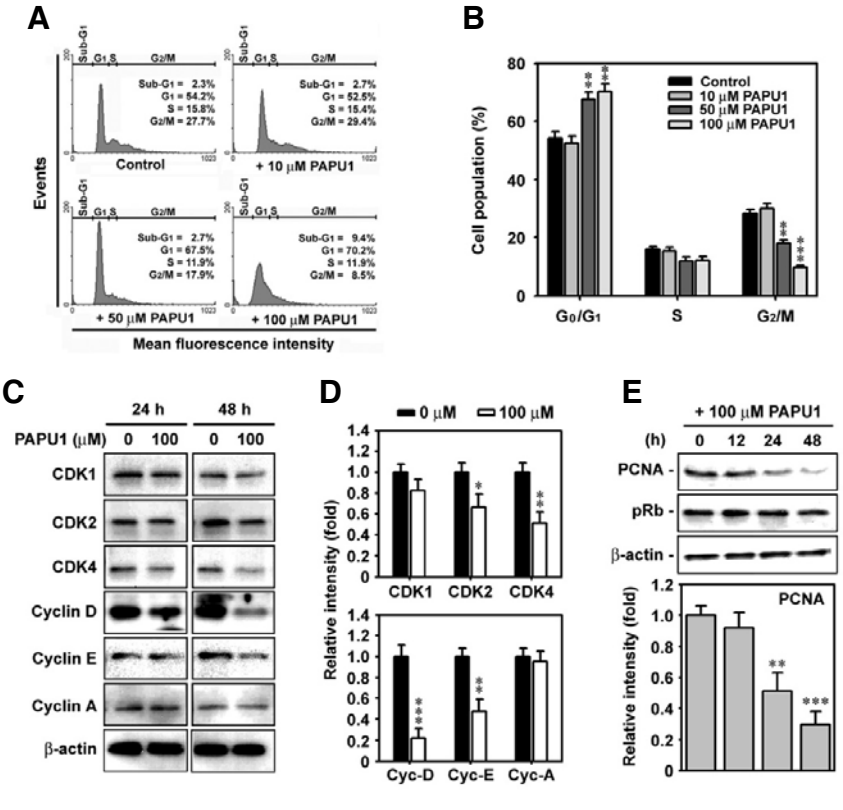

Fig. 3. Cell cycle analysis and the expression of cell cycle regulatory proteins in PAPU1-treated MCF-7 cells. (A) Cells were exposed to increasing concentrations (0-100 μM) of PAPU1 for 48 h, and DNA contents were analyzed using a flow cytometer after PI staining. The figures show a representative staining profile for 10,000 cells per experiment. (B) The percentages of cells in each cell cycle phase were calculated with WinMDI 2.9. (C) MCF-7 cells were also exposed to 100 μM PAPU1 for 24 and 48 h, and then protein lysates were analyzed by Western blotting using primary antibodies specific to CDKs and cyclins. (D) The data from triplicate experiments were quantified by densitometry after normalizing the bands to β-actin. (E) In addition, the levels of PCNA and pRb in these cells exposed to 100 μM PAPU1 for the indicated times were analyzed by immunoblotting. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs. the untreated control values.

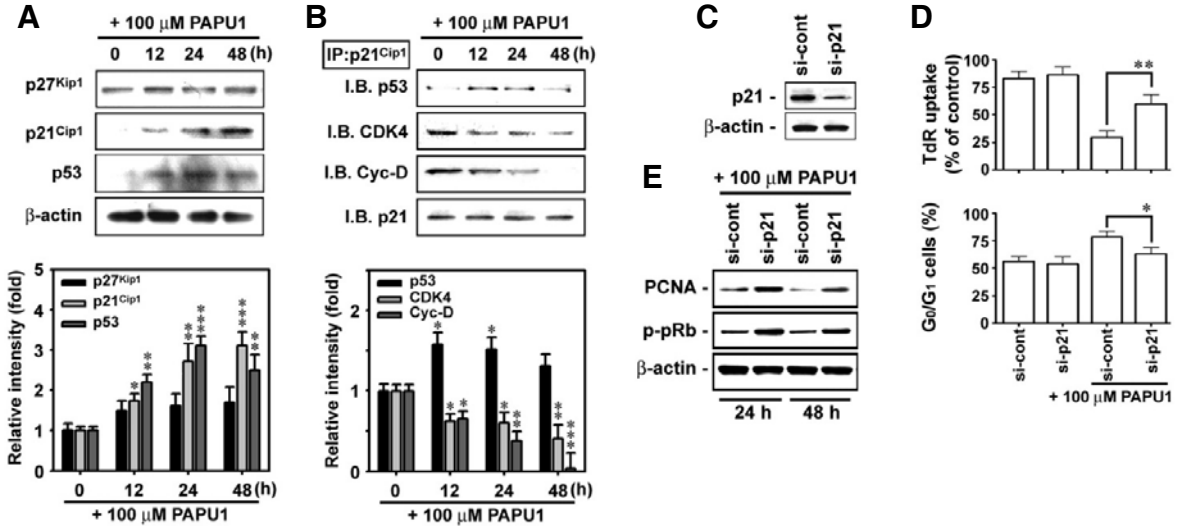

Fig. 4. Effect of PAPU1 on the expression of cyclin-dependent kinase inhibitors in MCF-7 cells. (A) Cells were exposed to 100 μM PAPU1 for the indicated times (0-48 h), and protein lysates were analyzed by immunoblotting with primary antibodies specific to $p21^{Cip1}$, $p27^{Kip1}$, and p53. β-Actin was used as control marker, and the data from triplicate experiments were quantified by densitometry. (B) Protein lysates were immunoprecipitated with $p21^{Cip1}$ antibody. Western blots were prepared and probed with antibodies to p53, CDK4, and cyclin D to detect these proteins on the blots. The data from three independent experiments were also quantified by densitometry. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs. the untreated control values. (C) Cells were transfected with either $p21^{Cip1}$-specific siRNA or control siRNA (si-cont), and at 24 h of transfection, the level of $p21^{Cip1}$ was determined by Western blot assay. (D) Transfected cells were treated with 100 μM PAPU1 and after 24 h-co-incubation, they were processed for the analyses of DNA synthesis (upper panel) and cell cycle progression (below panel). $*p < 0.05$ and $**p < 0.01$ indicate significant differences between the experiments. (E) The si-p21 transfected cells were also incubated with 100 μM PAPU1 for the indicated times and then processed for the Western blot analysis.

treated cells, we prepared Western blots with $p21^{Cip1}$-specific immunoprecipitates. By 12 h after PAPU1 exposure, the level of $p21^{Cip1}$-associated p53 had increased, and it remained at this same level for up to 24 h (Fig. 4B). In contrast, the levels of

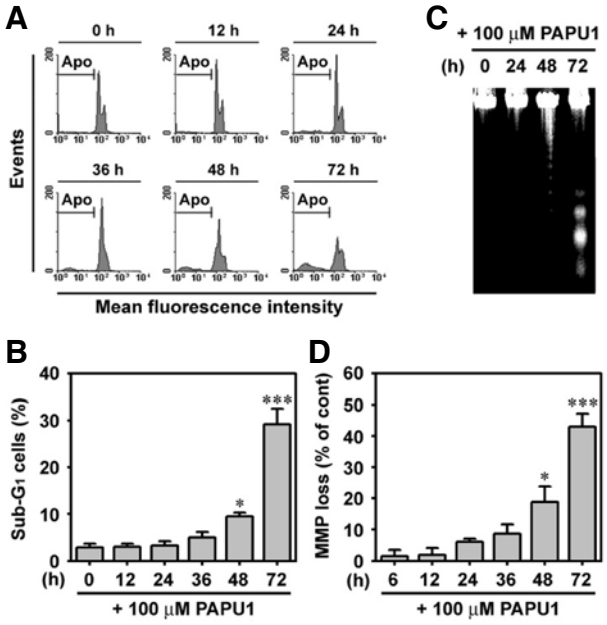

Fig. 5. Prolonged exposure to PAPU1 results in the DNA fragmentation in MCF-7 cells. Cells were exposed to 100 μM PAPU1 for various times (0-72 h), then processed for cell cycle analysis after PI staining (A), for DNA laddering after agarose gel electrophoresis (C), and for MMP determination after $DiOC_6$ staining (D). In the experiments of (B) and (D), the percentage of cells was calculated using WinMDI 2.9 program, and the data represent the mean ± SD of experiments performed in triplicate. *$p < 0.05$ and ***$p < 0.001$ vs. the untreated control cells.

$p21^{Cip1}$-associated CDK4 and cyclin D were dramatically decreased after PAPU1 treatment in a time-dependent manner. This is similar to the case that the immunoprecipitates were analyzed using anti-cyclin E or CDK2 antibody (data not shown). Blockage of p21 expression by it specific siRNA inhibited significantly the PAPU1-mediated growth inhibition as well as prevented the accumulation of MCF-7 cells into the G_0/G_1 phase (Figs. 4C and 4D). Furthermore, si-p21 transfection transparently attenuated the PAPU1-induced suppression of PCNA and p-pRb protein levels in the cells (Fig. 4E). These results indicated that $p21^{Cip1}$ played critical role in the G_0/G_1 arrest of cell cycle progression in PAPU1-treated cells, where p53 is also in part involved.

PAPU1 induces the loss of MMP and DNA fragmentation in MCF-7 cells

The cell population in the sub-G_1 phase of cell cycle progression, which means the apoptotic cells accompanied by a DNA fragmentation within cells, was also increased in MCF-7 cells after PAPU1 treatment for a long time (more than 36 h) (Figs. 5A and 5B). This was supported by the result from agarose gel electrophoresis, which showed the appearance of DNA ladders after 48 h and its dramatic increase at 72 h after PAPU1 treatment (Fig. 5C). In accordance with the results from PI staining and DNA fragmentation analyses, prolonged incubation with PAPU1 reduced MMP in the cells, with an approximate reduction of 43% in cells exposed to 100 μM PAPU1 for 72 h (Fig. 5D).

Regulation of Bcl-2 family proteins and caspase activation by PAPU1

Exposure of MCF-7 cells to PAPU1 resulted in a dramatic decrease in Bcl-2 protein level after 48 h of treatment, and a slight decrease in Bcl-X_L protein expression was observed after 72 of PAPU1 treatment (Fig. 6A). A significant increase in the level of Bax protein was observed after PAPU1 treatment. Cytosolic accumulation of cytochrome *c* from the mitochondria was also observed in a dose- (Fig. 6B, upper panel) and time-dependent manner (Fig. 6B, below panel) after PAPU1 treatment.

There was no change in the level of caspase-8 activity after PAPU1 treatment (Fig. 6C). However, the activity of caspase-9 was significantly increased in proportion to PAPU1 concentration, and the pancaspase inhibitor, z-VAD fmk (20 μM) completely inhibited caspase-9 activation (Fig. 6D). The inhibitor also diminished the PAPU1-induced increase in the cells stained positively with trypan blue and in the migration of cells into the sub-G_1 phase (Fig. 6E). The inhibitor specific for caspase-8 did not attenuate the PAPU1-caused toxicity (data not shown).

Down-regulation of p21 attenuates PAPU1-mediated apoptotic death

The role of p21 on PAPU1-mediated apoptosis was also investigated. As shown in Fig. 7, treatment of MCF-7 cells with 100 μM PAPU1 induced an apparent accumulation of cells in the sub-G_1 phase, whereas this was attenuated by blockage of p21 expression through siRNA transfection. This result was supported by the observation that PAPU1-mediated increase in the number of trypan blue positive cells was significantly inhibited by si-p21 transfection (Fig. 7B, below panel).

DISCUSSION

Naturally occurring phenolic acids draw increasing interest as potential chemopreventive and therapeutic agents (Hudson et al., 2000; Kadoma et al., 2008; Mathew and Abraham, 2004; Shahidi et al., 2007). Some phenolic acids may be absorbed from the intestine by active transport, and by that route, attenuate oxidative stress (Konishi et al., 2004; 2005). We showed previously that PAPU1, a new synthetic phenethyl urea, displays stronger antitumor potential than the phenolic acids (Kim et al., 2009). Here we demonstrated that PAPU1 leads to G_1 phase arrest by up-regulating $p21^{Cip1}$, and induces apoptosis through mitochondrion- and caspase-dependent pathways.

An agent with antitumor activity may inhibit cell proliferation by either a cytostatic or directly cytotoxic effect. That PAPU1 inhibited thymidine incorporation more potently than it induced cell death points to a cytostatic mechanism for the antiproliferative effect it exerts in MCF-7 cells. We also found that the PAPU1-mediated growth inhibition results from cell cycle

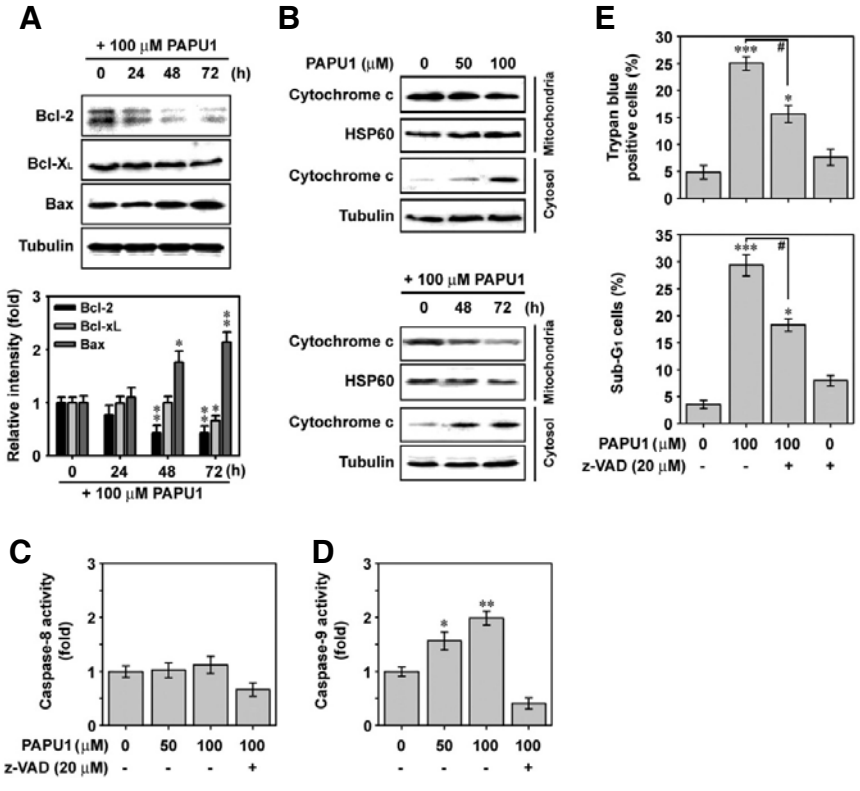

Fig. 6. Effects of PAPU1 on the expression of Bcl-2 family proteins and on the caspase activation in MCF-7 cells. (A) Protein lysates from cells exposed to 100 μM PAPU1 for the indicated times were analyzed by Western blotting using the antibodies specific for Bcl-2, Bcl-X_L and Bax. α-Tubulin was used as control marker, and the data from triplicate experiments were quantified by densitometry. (B) These cells were also treated with various concentrations (0-100 μM) of PAPU1 for 72 h (upper panel) or with 100 μM PAPU1 for 0, 48, and 72 h (below panel), and distribution of cytochrome *c* in the mitochondrial and cytosolic fractions was analyzed by Western blotting. In addition, cells were exposed to the indicated concentrations of PAPU1 for 72 h in the presence or absence of 20 μM z-VAD-fmk. The activities of caspase-8 (C) and caspase-9 (D) were determined by incubation of 100 μg of total protein with the substrates, Ac-IETD and Ac-LEHD-AMC. (E) These cells were also stained with trypan blue before counting dead cells with a hemocytometer (upper panel) or were stained with PI before flow cytometric analysis (below panel). *p < 0.05, **p < 0.01 and ***p < 0.001 vs the untreated control cells. #p < 0.05 vs the experiments.

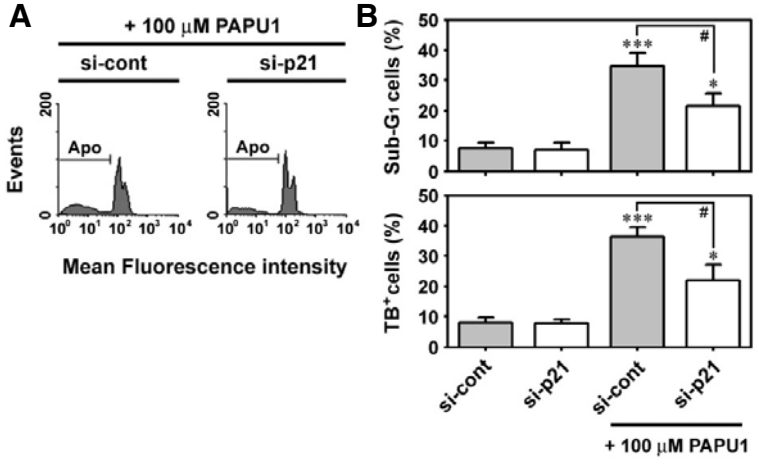

Fig. 7. Positive role of p21 on PAPU1-mediated apoptosis in MCF-7 cells. The si-control and si-p21 transfected cells were incubated with and without 100 μM PAPU1 for 72 h, and DNA contents were analyzed using a flow cytometer after PI staining (A). The figures show a representative staining profile for 10,000 cells per experiment. (B) The percentages of cells in each cell cycle phase were calculated with WinMDI 2.9. *p < 0.05 and ***p < 0.001 vs the untreated si-control values. #p < 0.05 vs the experiments.

arrest in the G_1 phase.

Signals from diverse metabolic pathways converge to control cell cycle progression (Sherr, 1996). Key factors in growth inhibition include the cyclins, CDKs, and CKIs (Braun-Dullaeus et al., 2004; Sherr, 2000). Our finding that PAPU1 arrests the cell cycle in G_0/G_1 phase led us to assume that this compound acts primarily in the early stages of cell cycle progression to inhibit proliferation. CDK2 and CDK4 act during the transition from G_0/G_1 to S phase, through formation of complexes with cyclin E and cyclin D (Jirawatnotai et al., 2004; Martin et al., 2005). These complexes phosphorylate many proteins, and importantly, mediate the hyper-phosphorylation of pRb and PCNA expression (Dzau et al., 2002). In this study, PAPU1 inhibited the CDKs and cyclins involved in transition from G_0/G_1 to S phase. PAPU1 strongly suppressed the levels of CDK2, CDK4, cyclin D, and cyclin E, without corresponding effects on CDK1 and cyclin A. These findings imply that PAPU1 induces G_0/G_1 arrest primarily by inhibiting the activation of G_1 phase-related proteins, which leads in turn to the decrease of PCNA (and growth suppression) in breast cancer cells. Further, the present results showing the blockage of PCNA expression and pRb phosphorylation by down-regulating p21 protein level clearly suggest the critical roles of p21 in the arrest of cell cycle progression.

Two classes of CKIs may inhibit the kinase activity of CDK-cyclin complexes (Sherr and Roberts, 1999). Members of the

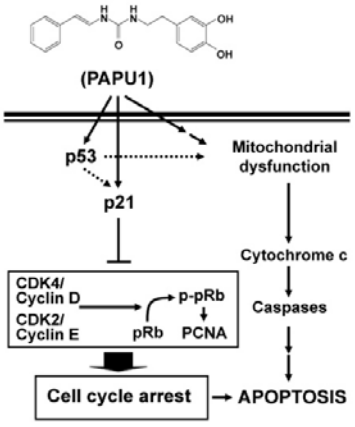

Fig. 8. Proposed signaling pathways involved in the cell cycle arrest and apoptosis induced by PAPU1 in MCF-7 human breast cancer cells.

INK4 family ($p16^{INK4a}$ and $p15^{INK4b}$) inhibit only CDK4 and CDK6 (Orgeta et al., 2002), while members of the Cip family ($p21^{Cip1}$ and $p27^{Kip1}$) inhibit all CDKs (Conqueret, 2003). Many anticancer compounds express their anti-proliferative potential by up-regulating $p21^{Cip1}$ and/or $p27^{Kip1}$ expression. Daidzein, for example, arrests the cell cycle at the G_1 phase in MCF-7 cells by up-regulating $p21^{Cip1}$ and $p57^{Kip2}$ (Choi and Kim, 2008). Pentagalloylglucose, a major component of Chinese medicine, induces G_1 phase arrest in MCF-7 cells through down-regulation of CDK4 and CDK2 and up-regulation of $p21^{Cip1}$ and $p27^{Kip1}$ (Chen et al., 2003). Similar to these reports, we found that PAPU1 induces the G_1 phase arrest in MCF-7 cells through up-regulation of $p21^{Cip1}$ in partial association with p53 expression. Moreover, the present findings reveal that the PAPU1-mediated increase in the sub-G_1 cells and cytotoxicity was diminished by inhibiting p21 protein level. There are considerable findings showing the roles of CDKIs on cell cycle arrest and apoptosis induced by a chemopreventive compound. For example, treatment of MCF-7 cells with arsenic trioxide increased the mRNA and protein levels of p21 and p27, and knocking down the CDKIs inhibited significantly cell cycle arrest and apoptosis caused by the agent (Wang et al., 2010). These findings led us to suggest that PAPU-mediated p21 up-regulation and the subsequent arrest of cells in the G_1 phase were at least partially involved in the PAPU-1-induced apoptosis of MCF-7 cells.

Many lines of evidence demonstrated that p53 could affect cell cycle arrest and cell death depending on the expression; over-expression of p53 may figure prominently in the regulation of apoptosis (Lowe et al., 1993; Muller et al., 1998), whereas a mild expression of the protein can lead to G_1 cell cycle arrest (Chen et al., 2000). Our current data showed that p53 expression was at least in part involved in the PAPU1-induced G_1 arrest in MCF-7 cells. Notwithstanding, we did not exclude a possibility that PAPU1-stimulated p53 induction was also associated with apoptosis induction. This is because that changes in Bax and Bcl-2 levels correlate closely with imbalances in mitochondrial homeostasis, which can be affected by p53 expression. It was reported that hydrogen peroxide induces both growth arrest and apoptosis in normal human fibroblasts, and p53 induction is required for these activities (Muller et al., 1998). Similarly, tanshinone, a traditional herbal medicine, induced apoptosis in colon cancer cells through both a mitochondrial pathway and p21-mediated G_1 phase arrest, and these actions involve both p53 and Bax expression (Su et al., 2008). In order to clarify the involvement of p53 in G_1 arrest and/or apoptosis in PAPU1-treated MCF-7 cells, more detailed experiments using p53 knock down cells will be needed.

There are two pathways of caspase activation; one is the involvement of cytochrome *c*-related activation of caspases such as caspase-9 and caspase-3, which correlates mitochondrial stress, and other is the activation of caspase-8 accompanied by binding to the Fas-activated death domain (FADD). Our present data verify that the mitochondria-mediated caspase activation is mainly involved in the PAPU1-mediated apoptosis. This can be proven by the suppression in Bcl-2 and Bcl-X_L with the concomitant increase in Bax protein expression, the cytosolic accumulation of cytochrome *c*, and the activation of caspase-9, but not of caspase-8, in PAPU1-exposed MCF-7 cells. The result showing that z-VAD-fmk inhibited PAPU1-mediated cell death further conformed the involvement of caspase cascades. Further, the involvement of caspase cascade and mitochondrial-mediated death signaling in MCF-7 cells by acacetin is well documented (Shim et al., 2007).

In summary, this study could lead to a schematic mechanism in PAPU1-induced growth inhibition and apoptosis as described in Fig. 8. As shown in the figure, PAPU1 inhibited the proliferation of MCF-7 cells through cell cycle arrest in the G_0/G_1 phase, in close association with the up-regulation of $p21^{Cip1}$ and down-regulation of cyclins and CDKs involved in the G_1 cell cycle. The mitochondrial dysfunction accompanied by a decrease in Bcl-2 and Bcl-X_L and increase in Bax protein expression, loss of MMP, and cytoplasmic release of cytochrome *c*, and the subsequent activation of caspase cascades plays critical role in PAPU1-induced apoptosis in MCF-7 cells. In addition, p21-mediated growth inhibition was also associated with the PAPU1-mediated apoptosis in the cells. Collectively, these results reveal that G_1 cell cycle regulatory proteins and mitochondrial pathways are the critical targets responsible for the anti-proliferative and apoptosis inducing effects of PAPU1 in breast cancer cells.

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REFERENCES

Braun-Dullaeus, R.C., Mann, M.J., Sedding, D.G., Sherwood, S.W., Von der leyen, H.E., and Dzau, V.J. (2004). Cell cycle-dependent regulation of smooth muscle cell activation. Arterioscler. Thromb. Vasc. Biol. *24*, 845-850.

Chen, Q.M., Liu, J., and Merrett, J.B. (2000). Apoptosis or senescence-like growth arrest: influence of cell-cycle position, p53, p21 and bax in H_2O_2 response of normal human fibroblasts. Biochem. J. *37*, 543-551..

Chen, W.J., Chang, C.Y., and Lin, J.K. (2003). Induction of G1 phase arrest in MCF human breast cancer cells by pentagalloylglucose through the down-regulation of CDK4 and CDK2 activities and up-regulation of the CDK4 inhibitors $p27^{Kip}$ and $p21^{Cip}$. Biochem. Pharmacol. *65*, 1777-1785.

Cho, E.J., and Kim, G.H. (2008). Daidzein causes cell cycle arrest at the G1 and G2/M phases in human breast cancer MCF-7 and MDA-MB-453 cells. Phytomedicine *15*, 683-690.

Conqueret, O. (2003). New roles for p21 and p27 cell-cycle inhibitors: A function of each cell compartment? Trends Cell. Biol. *13*, 65-70.

Dzau, V.J., Braun-Dullaeus, R.C., and Sedding, D.G. (2002). Vascular proliferation and atherosclerosis: new perspectives and

therapeutic strategies. Nat. Med. *8*, 1249-1256.
El-Deiry, W.S. (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. *54*, 1169-1174.
Fang, S.C., Hsu, C.L., and Yen, G.C. (2008). Anti-inflammatory effects of phenolic compounds isolated from the fruits of *Artocarpus heterophyllus*. J. Agric. Food Chem. *56*, 4463-4468.
Huang, C., Ma, W., Goranson, A., and Dong, Z. (1999). Resveratrol suppresses cell transformation and induces apoptosis through a p53-independent pathway. Carcinogenesis *20*, 237-242.
Hudson, E.A., Dinh, P.A., Kokubun, T., Simmonds, M.S., and Gescher, A. (2000). Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. Cancer Epidemiol. Biomarkers Prev. *9*, 1163-1170.
Jirawatnotai, S., Aziyu, A., Osmundson, E.C., Moons, D.S., Zou, X., Kineman, R.D., and Kiyokawa, H. (2004). Cdk4 is indispensable for postnatal proliferation of the anterior pituitary. J. Biol. Chem. *279*, 51100-51106.
Kadoma, Y., and Fujisawa, S. (2008). A comparative study of the radical-scavenging activity of the phenolcarboxylic acids caffeic acid, *p*-coumaric acid, chlorogenic acid and ferulic acid, with or without 2-mercaptoethanol, a thiol, using the induction period method. Molecules *13*, 2488-2499.
Kim, J.H., Jang, Y.O., Kim, B.T., Hwang, K.J., and Lee J.C. (2009). Induction of caspase-dependent apoptosis in melanoma cells by a synthetic compound, (E)-1-(3,4-dihydroxyphenethyl)-3-styrylurea. BMB Rep. *42*, 806-811.
Konishi, Y., Hitomi, Y., and Yoshioka, E. (2004). Intestinal absorption of *p*-coumaric and gallic acids in rats after oral administration. J. Agric. Food Chem. *52*, 2527-2532.
Konishi, Y., Hitomi, Y., Yoshida, M., and Yoshioka, E. (2005). Absorption and bioavailability of artepillin C in rats after oral administration. J. Agric. Food Chem. *53*, 9928-9933.
Korkina, L.G. (2007). Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. Cell. Mol. Biol. *53*, 15-25.
Lowe, S.W., Ruley, H.E., Jacks, T., and Housman, D.E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957-967.
Martin, A., Odajima, J., Hunt, S.L., Dubus, P., Ortega, S., Malumbres, M., and Barbacid, M. (2005). Cdk2 is dispensable for cell cycle inhibition and tumor suppression mediated by p27 (Kip1) and p21(Cip1). Cancer Cell *7*, 591-598.
Masuda, T., Yamada, K., Akiyama, J., Someya, T., Odaka, Y., Takeda, Y., Tori, M., Nakashima, K., Maekawa, T., and Sone, Y. (2008). Antioxidation mechanism studies of caffeic acid: identification of antioxidation products of methyl caffeate from lipid oxidation. J. Agric. Food Chem. *56*, 5947-5952.
Mathew, S., and Abraham, T.E. (2004). Ferulic acid: an antioxidant found naturally in plant cell walls and feruloyl esterases involved in its release and their applications. Crit. Rev. Biotechnol. *24*, 59-83.
Miranda, C.L., Stevens, J.F., Helmrich, A., Henderson, M.C., Rodriguez, R.J., Yang, Y.H., Deinzer, M.L., Barnes, D.W., and Buhler, D.R. (1999). Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (*Humulus lupulus*) in human cancer cell lines. Food Chem. Toxicol. *37*, 271-285.
Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods *65*, 55-63.
Muller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber, M., Friedman, S.L., Galle, P.R., Stremmel, W., Oren, M., et al. (1998). p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. J. Exp. Med. *188*, 2033-2045.
Orgeta, S., Malumbers, M., and Barbacid, M. (2002). Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochim. Biophys. Acta *1602*, 73-87.
Rahman, I., Biswas, S.K., and Kirkham, P.A. (2006). Regulation of inflammation and redox signaling by dietary polyphenols. Biochem. Pharmacol. *72*, 1439-1452.
Shahidi, F., Alasalvar, C., and Liyana-Pathirana, C.M. (2007). Antioxidant phytochemicals in hazelnut kernel (*Corylus avellana* L.) and hazelnut byproducts. J. Agric. Food Chem. *55*, 1212-1220.
Sherr, C.J. (1996). Cancer cell cycles. Science *274*, 1672-1677.
Sherr, C.J. (2000). The Pezcoller lecture: cancer cell cycles revisited. Cancer Res. *60*, 3689-3695.
Sherr, C.J., and Roberts, J.M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. *13*, 1501-1512.
Shim, H.Y., Park, J.H., Paik, H.D., Nah, S.Y., Kim, D.S.H.L., and Han, Y.S. (2007). Acacetin-induced apoptosis of human breast cancer MCF-7 cells involves caspase cascade, mitochondrial-mediated death signaling and SAPK/JNK1/2-c-Jun activation. Mol. Cells *24*, 95-104.
Song, J.J., Cho, J.G., Hwang, S.J., Cho, C.G., Park, S.W., and Chae, S.W. (2008). Inhibitory effect of caffeic acid phenethyl ester (CAPE) on LPS-induced inflammation of human middle ear epithelial cells. Acta Otolaryngol. *128*, 1303-1307.
Srinivasan, M., Sudheer, A.R., and Menon, V.P. (2007). Ferulic Acid: therapeutic potential through its antioxidant property. J. Clin. Biochem. Nutr. *40*, 92-100.
Su, S.J., Yeh, T.M., Lei, H.Y., and Chow, H.N. (2000). The potential of soybean foods as a chemoprevention approach for human urinary tract cancer. Clin. Cancer Res. *6*, 230-236.
Su, C.C., Chen, G.W., and Lin, J.G. (2008). Growth inhibition and apoptosis induction by tanshinone I in human colon cancer Colo 205 cells. Int. J. Mol. Med. *22*, 613-618.
Wang, X., Gao, P., Long, M., Lin, F., Wei, J.X., Ren, J.H., Yan, L., He, T., Han, Y., and Zhang, H.Z. (2010). Essential role of cell cycle regulatory genes p21 and p27 expression in inhibition of breast cancer cells by arsenic trioxide. Med. Oncol. (in Press).