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Full Length Research Paper

Induced-differentiation of two flavones and two flavonols on a human esophageal squamous cell carcinoma cell line (KYSE-510)

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The effects of two flavones (baicalein, chrysin) and two flavonols (galangin, kaempferol) on induction of differentiation in a human esophageal squamous cell carcinoma (KYSE-510) cell line were investigated in this study. Four compounds were found to be able to inhibit proliferation of KYSE-510 cells in a dose- and time-dependent manner. The inhibitory potency of them on the growth of KYSE-510 cells was in the order of galangin > chrysin > baicalein > kaempferol. The KYSE-510 cells treated with these compounds for 24 h showed differentiation characteristics including the differentiation-specific morphological changes, the augmentation of differentiation markers and the inhibition of human telomerase reverse transcriptase. The potency of these compounds on induction of differentiation was similar to that of them on growth inhibition. To elucidate the possible mechanisms by which these compounds modulate proliferation and differentiation in KYSE-510 cells, the alterations of three *genes* (p21^{waf1}, cyclin B1 and D1) related to proliferation and differentiation were investigated by real-time RT-PCR and Western blot. The results showed that the up-regulation of p21^{waf1} and down-regulation of cyclin B1 and D1 at the mRNA and protein levels in KYSE-510 cells treated with four compounds were observed, which implied that p21^{waf1}, cyclin B1 and D1 might be target *genes* of these compounds in inducing differentiation.

Key words: Flavones, flavonols, differentiation, esophageal cancer.

INTRODUCTION

Cancer of the esophagus which is one of the most lethal malignancies of all cancer (Tak and Naunheim, 2004) ranks as the ninth most common malignancy worldwide, occurs at a high frequency in Asia and South America (McCabe and Dlamini, 2005). This malignancy exists in two main forms with distinct etiological and pathological characteristics, squamous cell carcinoma (SCC) and adenocarcinoma (Stoner and Gupta, 2001). Esophageal squamous cell carcinoma accounts for more than 95% of esophageal malignancies (Kuwano et al., 2005). The development of human esophageal cancer is a multistep and progressive process. An early indicator of this process is an increased proliferation of esophageal epithelial cells morphologically including basal cell hyperplasia, dysplasia, and carcinoma *in situ* and advanced esophageal squamous cell carcinoma (Lehrbach et al., 2003). The two major risk factors are tobacco smoking and alcohol consumption (Kuwano et al., 2005). SCC of the esophagus is graded according to the degree of histological differentiation. The well-differentiated and moderately differentiated tumors display morphological features generally characteristic of normal epithelial differentiation (Grace et al., 1985).

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Abbreviations: BSA, Bovine serum albumin; **DMSO,** dimethyl sulfoxide; **EB,** ethidium bromide; **EDTA,** ethylenediaminetetraacetic acid; **FBS,** fetal bovine serum; **FITC,** fluorescein isothiocyanate; **5-FU,** 5-fluorouracil; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; **PBS,** phosphate- buffered saline; **PI,** propidium iodide; **PMSF,** phenylmethyl sulfonylfluoride; **RNase,** ribonuclease sodium dodesyl sulfate–polyacrylamide gel; **TBS,** tris buffered saline.

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Flavonoids are a group of more than 4000 polyphenolic compounds that occur naturally in foods of plant origin (Middleton et al., 2000; Ren et al., 2003). They are a large group of low molecular weight substances and according to the saturation level and opening of the central pyran ring, mainly into flavones, flavanols, isoflavones, flavonols, flavanones, and flavanonols (López-Lázaro, 2002; Ren et al., 2003; Wach et al., 2007). They present in practically all dietary plants, like fruits and vegetables. Additionally, flavonoids are also found in several medical plants, and herbal remedies containing flavonoids have been used in folk medicine around the world, especially in China (Ren et al., 2003), such as baicalein (isolated from the root of *Scutellaria baicalensis*, which is used as a traditional medicinal herb for centuries) (Yang et al., 2000) and galangin (present at high concentrations in propolis, a resinous material produced by the activities of honeybees and in India root, *Alpinia officinarum*, which is common spice and herbal medicine in Asia) (Heo et al., 2001; Bestwick and Milne, 2006). Flavonoids have a wide variety of biological effects and those of particular interest in relation to cancer prevention without affecting normal cells (López-Lázaro, 2002; Neuhouser, 2004a). A number of studies have suggested that consumption of vegetables, fruit, and beverages of plant containing flavonoids is consistently associated with a reduced risk of various cancers (Le Marchand et al., 2000; Neuhouser, 2004a, 2004b; Johnson, 2004; Bosetti et al., 2005, 2007), including esophageal cancer (Johnson, 2004; Rossi et al., 2007). Recent studies revealed that flavonoids exerted an extensive array of activities that might contribute to anti-carcinogenic effects including the mitigation of oxidative damage, antiproliferative effects, induction of apoptosis and inhibition of malignant transformation (Birt et al., 2001; Ren et al., 2003).

In addition to the anticancer properties mentioned previously, it is of interest that certain flavonoids is capable of causing poorly differentiated cancer cell lines to differentiate into cells exhibiting mature phenotypic characteristics (Middleton et al., 2000). For example, the flavones genistein, apigenin, luteolin, quercetin, and phloretin were found to induce differentiation of human acute myelogenous leukemia HL-60 cells into granulocytes and monocytes (Takahashi et al., 1998). Other studies also showed many citrus flavones and flavanones were active antiproliferative differentiation inducers in human leukaemia cells (Alexandrakis et al., 1999; Kawaii et al., 1999a, b; Kawii and Lansky 2004). Moreover, genistein were shown to arrest cell growth and induces neuroblastoma (NB) cell differentiation (Brown et al., 1998). Kaempferol was also shown to induce differentiation in partially differentiated KNC colon cancer cells (Nakamura et al., 2005). Although, differentiation therapy is best known for hematological malignancies, particularly leukemia (Aksentijevich and Flinn, 2003; Parmar and Tallman, 2003; Tolomeo et al., 2008), many studies only located in hematological malignancies, prostate, colon, and some other cancers. One of the most important pro-differentiation agents for cancer therapy is the retinoid (that is, vitamin A derivatives) (Tallman and Wiernik, 1992). Flavonoids are a group of differentiation-inducing compounds as well with a potentially low toxicity to normal cells than retinoids (Havsteen, 2002; López-Lázaro, 2002; Kawii and Lansky, 2004). In this study, we analyzed and compared differentiation-inducing effects of structurally related two flavones (baicalein and chrysin) and two flavonols (galangin and kaempferol) on human esophageal squamous cell line (KYSE-510), and elucidated possible molecular mechanisms responsible for their differentiation- inducing effects.

MATERIALS AND METHODS

Baicalein (> 98% of purity) and galangin (> 98% of purity) were purchased from Shanghai Yousi Biotechnology Co., Ltd. (Shanghai, China). Kaempferol (> 98% of purity) were purchased from Nanjing Qingze Medical Technology Co., Ltd. (Nanjing, Jiangsu, China). Chrysin (> 96% of purity), and MTT were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The following reagents were from Solarbio Science and Technology Co., Ltd. (Beijing, China): PI, EB, DMSO, RNase, BSA, sodium azide and materials for Western blot.

The mono- and poly-clonal antibodies (human reactive anti-$p21^{waf1}$ and β-actin) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).The anti-cyclin B1 and cyclin D1 were obtained from Lab Vision Co. (Fremont, CA, USA). The horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit antibody, fluorescien-conjugated affinipure goat anti-mouse IgG (H+L) antibody were from Zhongshan Gold Bridge Biotechnology Co., Ltd. (Beijing, China). The anti-cytokeratin 8 was obtained from Fuzhou Maixin Biotechnology Development Co., Ltd. (Fuzhou, Fujiang, China).

Cell culture

The KYSE-510 cell line was obtained from Tianjin Medical University Cancer and Hospital (TMUCIH, Tianjin, China). The cell line was established from a well-differentiated esophageal squamous cell carcinoma of a 67 year-old Japanese woman. KYSE-510 cells which express p53 mutation were cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G and 100 mg/ml streptomycin (all obtained from Solarbio) and kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

MTT assay

KYSE-510 cells (1×10^4/ well) were plated in 96-well plates and incubated for 24 h to allow the cells to attach, before treatment with two flavones (baicalein, chrysin) and two flavonols (galangin, kaempferol). Each compound was dissolved in dimethyl sulfoxide (DMSO) and made up with the medium so that the final concentration of the vehicle was not > 0.1% DMSO. The cells were treated with 10, 20, 40 and 80 µmol/L concentration of each

compound for 24, 48 and 72 h. Cells treated with 0.1% DMSO were served as a negative control. After incubation for specified time at 37°C in a humidified incubator, MTT (5 mg/ml in PBS) of 10 µl was added to each well and incubated for 4 h after which the plate was centrifuged at 1800 g for 5 min at 4°C. The MTT solution was removed from the wells by aspiration.

After careful removal of the medium, 100 µl of buffered DMSO was added to each well, and plates were shaken. The absorbance was recorded on a microplate reader (Bio Rad Laboratories, Hercules, CA, USA) at the wavelength of 570 nm. The effect of each compound on growth inhibition was assessed as percent cell viability (Zhang et al., 2009), where vehicle-treated cells were taken as 100% viable.

Immunofluorescence assay of differentiation marker

The cells were grown to about 70% confluence in glass bottom microwell dishes used for confocal laser scanning microscopy and were treated with each compound (80 µmol/L) for 24 h. The cultured cells were fixed in paraformaldehyde for 10 min, followed by incubation at 37°C for 30 min with specific mouse anti-cytokeratin 8 and then by incubation at 4°C for 1 h with fluorescence-conjugated goat anti-rabbit IgG (1:100 dilution).

Quantification of differentiation marker

The cells were grown to about 70% confluences in 6-well plates and treated with each compound (80 µmol/L) for 24 h. The cells were harvested by adding a solution of 1 mmol/L EDTA in PBS to remove the cells from the plate, and centrifuged at 1000 g at 4°C for 5 min, washed with 3 ml of PBS + 0.1% sodium azide and 1% BSA, count the cells using a minimum concentration of 1×10^6 cells/ml for each sample volumes of PBS+ 0.1% sodium azide and 1% BSA, proper dilution of anti-cytokeratin 8 (1:100 dilution) was added, and incubated on ice for 30 min. To each sample, 4 ml of PBS + sodium azide and BSA was added and mixed gently, centrifuged at 1000 g for 5 min, washed samples with 4 ml of PBS + sodium azide and BSA, later centrifuged samples at 1000 g for 5 min. Resuspended cells in 3 ml PBS + sodium azide, proper dilution of secondary antibody fluorescien-conjugated (1:100 dilution) was added and incubated on ice for 30 min.

To each sample, 3 ml of PBS + sodium azide and BSA was added and mixed gently, centrifuged at 1000 g for 5 min. Resuspended cells in 3 ml PBS + sodium azide and BSA to give a final concentration of $l \times 10^6$ to 5×10^6/ ml. The resuspended cells were analyzed by flow cytometry to determine the percentage of differentiated cells.

Telomerase activity assay

The cells were grown to about 70% confluence in 6-well plates and treated with each compound (80 µmol/L) for 24 h. Two micrograms of total RNA from each sample were subjected to reverse transcription using the SYBR® PrimeScript™ RT-PCR Kit (TaKaRa Biotechnology Co., Ltd., Dalian, Liaoning, China) according to the manufacturer's protocol.

Each real-time PCR was carried out in triplicate in a total of 20 µl reaction mixture (2 µl of cDNA, 10 µL of SYBR® Premix Ex Taq™, 0.4 µl of ROX Reference Dye II, 0.5 µL of each 10 mol/L forward and reverse primers, and 6.6 µl of H_2O) on ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA), Primers for hTERT cDNA amplification were added to a final concentration of 0.25 µmol/L, Primer for hTERT were listed in. Actin mRNA was used as an internal control. The amplification reactions were performed with an initial incubation step at 95°C for 10 s followed by 50 cycles of 95°C for 5 s, 60°C for 34 s. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and were normalized to β-actin expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

Real-time RT-PCR analysis

The cells were grown to about 70% confluence in 6-well plates and treated with each flavonoids (80 µmol/L) for 24 h. The extraction of RNA and real-time RT-PCR were manipulated as 2.6. Primers used for real-time PCR analysis were presented in. The PCR program was initiated by 10 s at 95°C before 40 thermal cycles, each of 5 s at 95°C and 34 s at 60°C. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and were normalized to β-actin expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

Western blot analysis

The cells (70% confluent) were treated with each compound (80 µmol/L) for 24 h. After which the media was aspirated, the cells were washed with cold PBS and ice-cold lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mmol/L PMSF (pH 7.4)] over ice for 30 min. The cells were scraped, and the lysate was collected in a microfuge tube. The lysate was cleared by centrifugation at 14000 g for 15 min at 4°C and the supernatant (total cell lysate) was used or immediately stored at -80°C. The protein concentration was determined by *Gene* Quan pro (Amarsham Biosciences, Uppsala, Sweden). Protein (50 µg) from each sample was resolved over 10 to 12% SDS-PAG and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% nonfat dry milk; 0.05% Tween 20; in 20 mmol/L TBS, pH 7.6) for 1 h at room temperature, incubated with appropriate rabbit monoclonal (Thermo Scientific, Fremont, CA, US) or mouse/rabbit polyclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, US) (1:2000 dilution) in blocking buffer from 2 h to overnight at 4°C, followed by incubation with goat anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate (Beijing Zhongshanjingqiao biotechnology Limited Company, Beijing, China) (1:2000 dilution) and detected by chemiluminescence and autoradiography using X-ray film (Applygen Technologies Inc., Beijing, China). Densitometric measurements of the bands in Western blot analysis were performed using Lab Works 4.5 image analysis software (UVP Bioimaging System, CA, US).

Statistical analyses

All data are expressed as means ±SE from at least three independent experiments. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) with Duncan's multiple range tests. Statistical significance was considered at $p < 0.05$. SPSS 13.0 software (SPSS Inc., Chicago, IL, US) and MS Excel 2003 (Microsoft Corporation, Redmond, WA, US) were used to analyze and report the data.

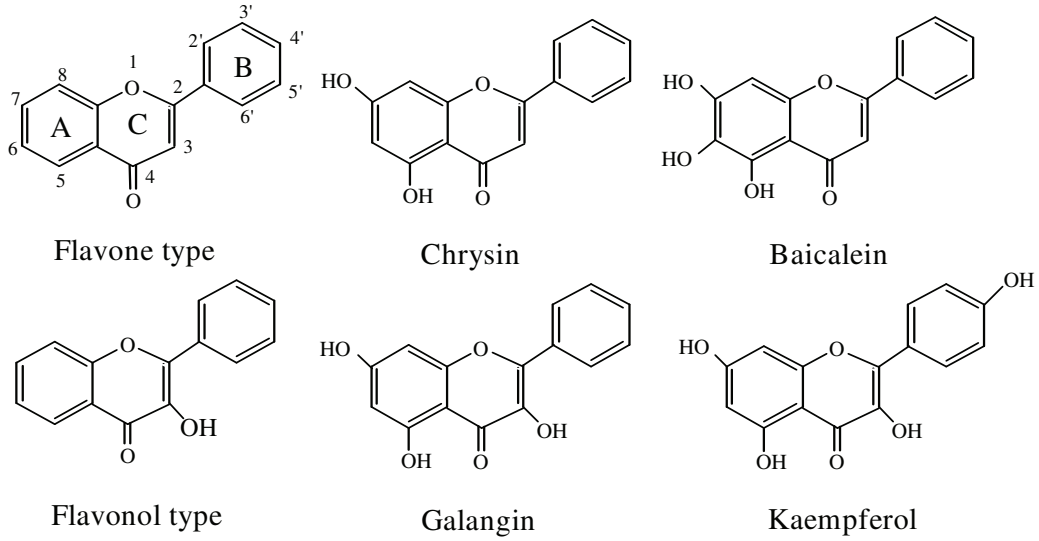

Figure 1. Chemical structures of two flavones and two flavonols selected in the presented study.

RESULTS

Growth inhibitory effects of two flavones and two flavonols on KYSE-510 cells

Two flavones (baicalein, chrysin) and two flavonols (galangin, kaempferol) with chemical structures as Figure 1 were selected in our work to reveal their induced-differentiation on a human esophageal squamous cell line (KYSE-510). The potential growth inhibitory effects of four compounds on a human esophageal squamous cell line (KYSE-510) was investigated first by MTT assay and shown in Figure 2. All compounds assayed were able to inhibit growth of KYSE-510 cells in a dose- and time-dependent manner (Figure 2a). There existed significant differences in growth inhibitory effects of these compounds on KYSE-510 cells. Galangin was the most active one with an estimated IC50 (50% inhibition of cell growth, 24 h) value of 50 μmol/L (Figure 2b). The potency of selected flavonoids on inhibition of KYSE-510 cells was in the order of: galangin (50 μmol/L) > chrysin (57 μmol/L) > baicalein (67 μmol/L) > kaempferol (139 μmol/L).

Morphological changes

Compared with the negative controls, KYSE-510 cells treated with four compounds showed dramatic morphological changes, as shown in Figure 3. With visual observation, the cells treated with four compounds briefly displayed an enlarged and flattened shape, an increased cytoplasmic-to-nuclear ratio, and a decreased cell density. These results were consistent with a differentiation phenotype.

Differentiation induction

To confirm the selected compounds having differentiation induction to KYSE-510 cells, confocal laser scanning microscopy and flow cytometry analysis were conducted to detect differentiation marker, cytokeratin 8, specific for esophageal squamous cell carcinoma. The cells were treated with each of four compounds (80 μmol/L) for 24 h. As shown in Figure 4, compared with vehicle-treated control, each treatment resulted in the augmentation of expression of differentiation marker cytokeratin 8 in KYSE-510 cells. The differentiation induced by these compounds was quantified by flow cytometry after labeling specific differentiation marker cytokeratin 8 of human esophageal squamous cells with FITC using indirect immuno- fluorescence. The analysis results revealed that four compounds (galangin, chrysin, baicalein, kaempferol) made an increase of the level of cytokeratin 8 in dose-dependent manner. The data also showed that potency of four compounds at 80 μmol/L on induction of differentiation was also similar to that of them on growth inhibition and morphological changes (Figure 5).

Human telomerase reverse transcriptase assay

The human telomerase reverse transcriptase (hTERT) is ingredient of telomerase and is often detectable in

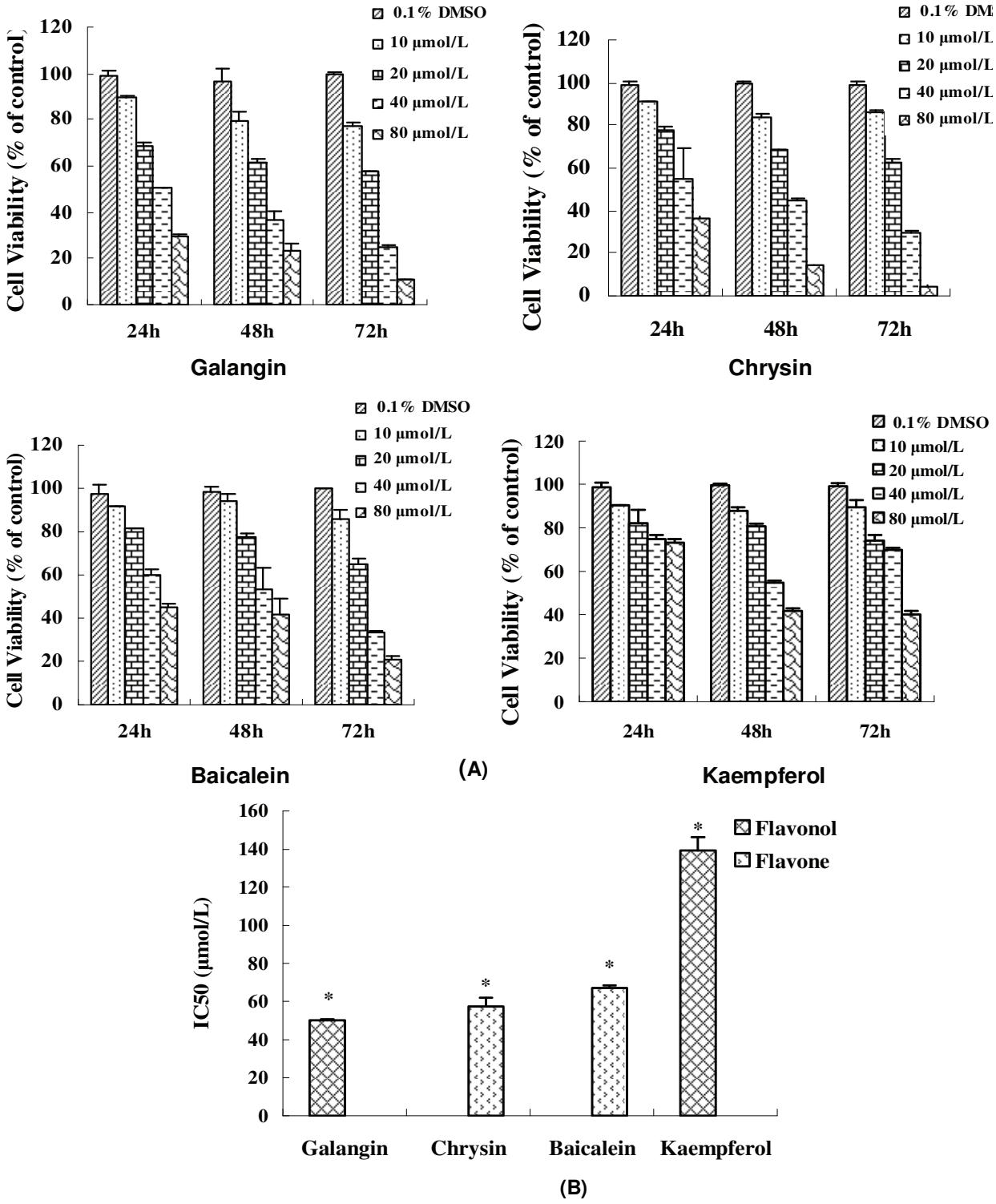

Figure 2. Inhibitory effect of two flavones and two flavonols on the growth of KYSE-510 cells (A) and their comparative effect on the growth of KYSE-510 cells (B). The cells were treated with specified concentrations of selected flavonoids for 24, 48 and 72 h, and growth inhibitory effects were determined by MTT assay. The values were represented as the percentage cell inhibition where vehicle-treated cells were regarded as 100%. The data represents the mean ± SE of three experiments each conducted in triplicate (A). The cells were treated with each compound at 80 μmol/L for 24 h. The values were represented as mean ± SE from three separate experiments each conducted in triplicate. The stars indicate that values were significantly different from each other (*$p \leq 0.05$) (B).

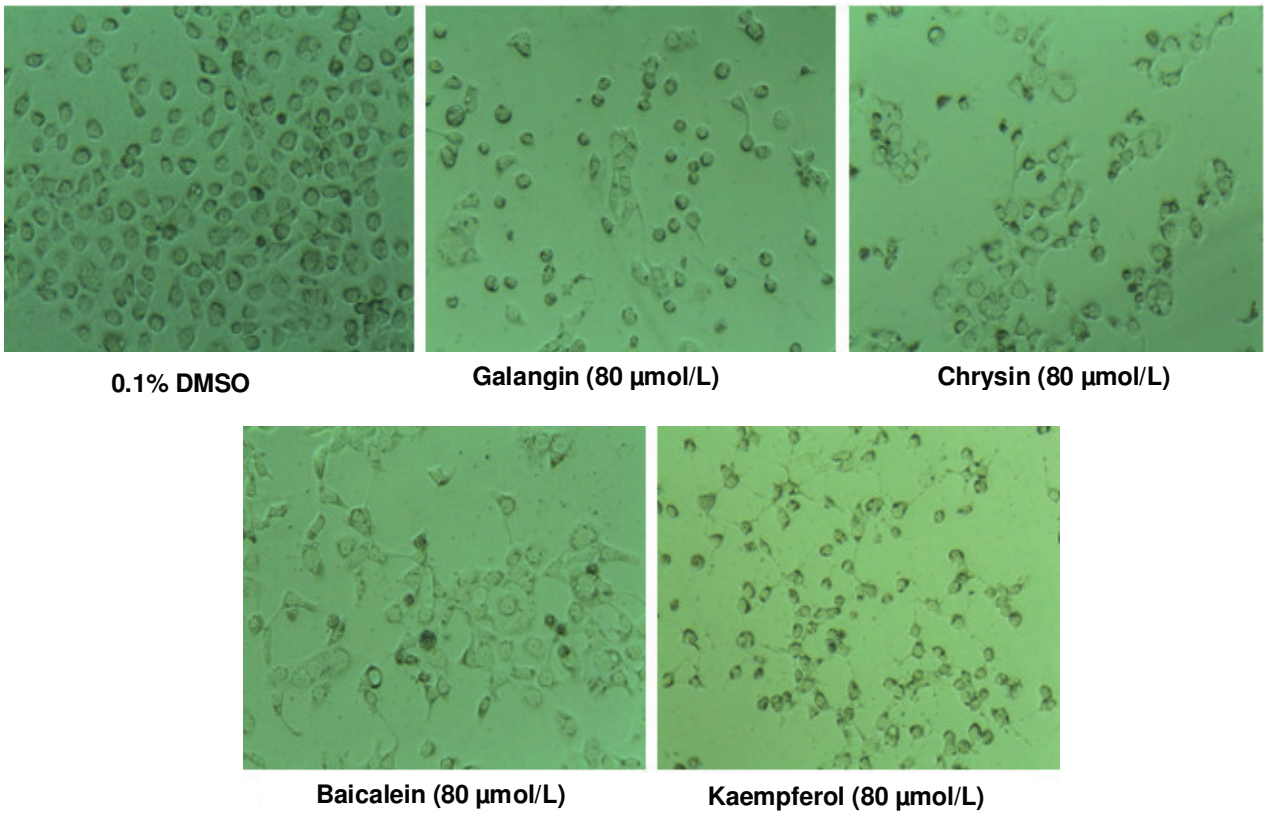

Figure 3. Effect of two flavones and two flavonols on the induction of differentiation in KYSE-510 cells. The cells were treated with each compound at 80 µmol/L for 24 h and showed differentiation phenotype, including an enlarged, flattened shape, an increased cytoplasmic-to-nuclear ratio, and decreased cell density.

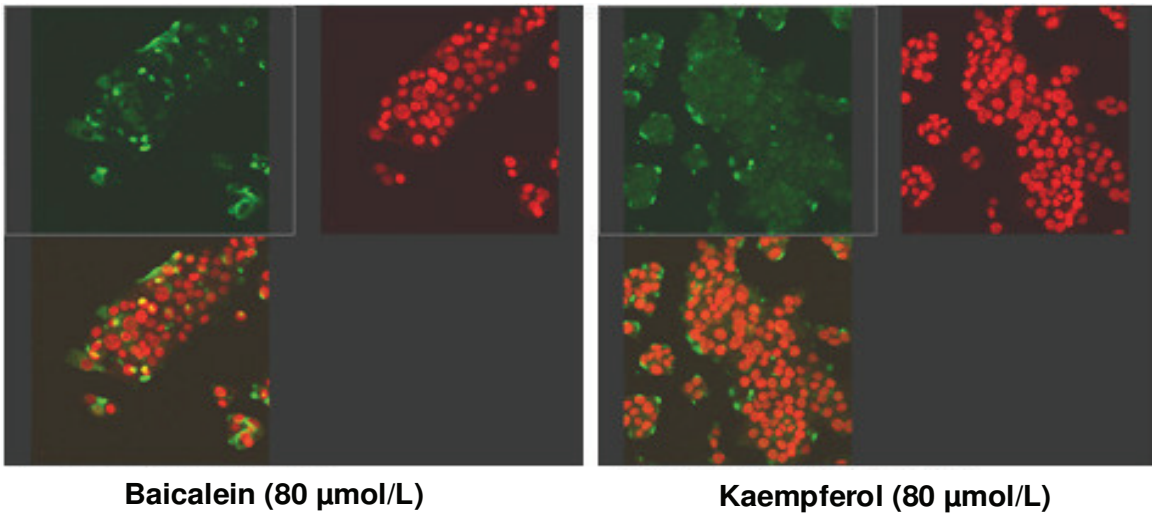

Figure 4. Effects of two flavones and two flavonols on induction of cytokeratin 8 expressions in KYSE-510 cells.The cells grown on glass bottom microwell dishes were treated with selected compound at 80 µmol/L for 24 h. Cytokeratin 8 positive cells were detected by fluorescein isothiocyanate (FITC)-labeled antibody (top-left) and nuclei reflecting cell density by PI (top-right). The result showed the augmentation of expression of differentiation marker cytokeratin 8 in KYSE-510 cells.

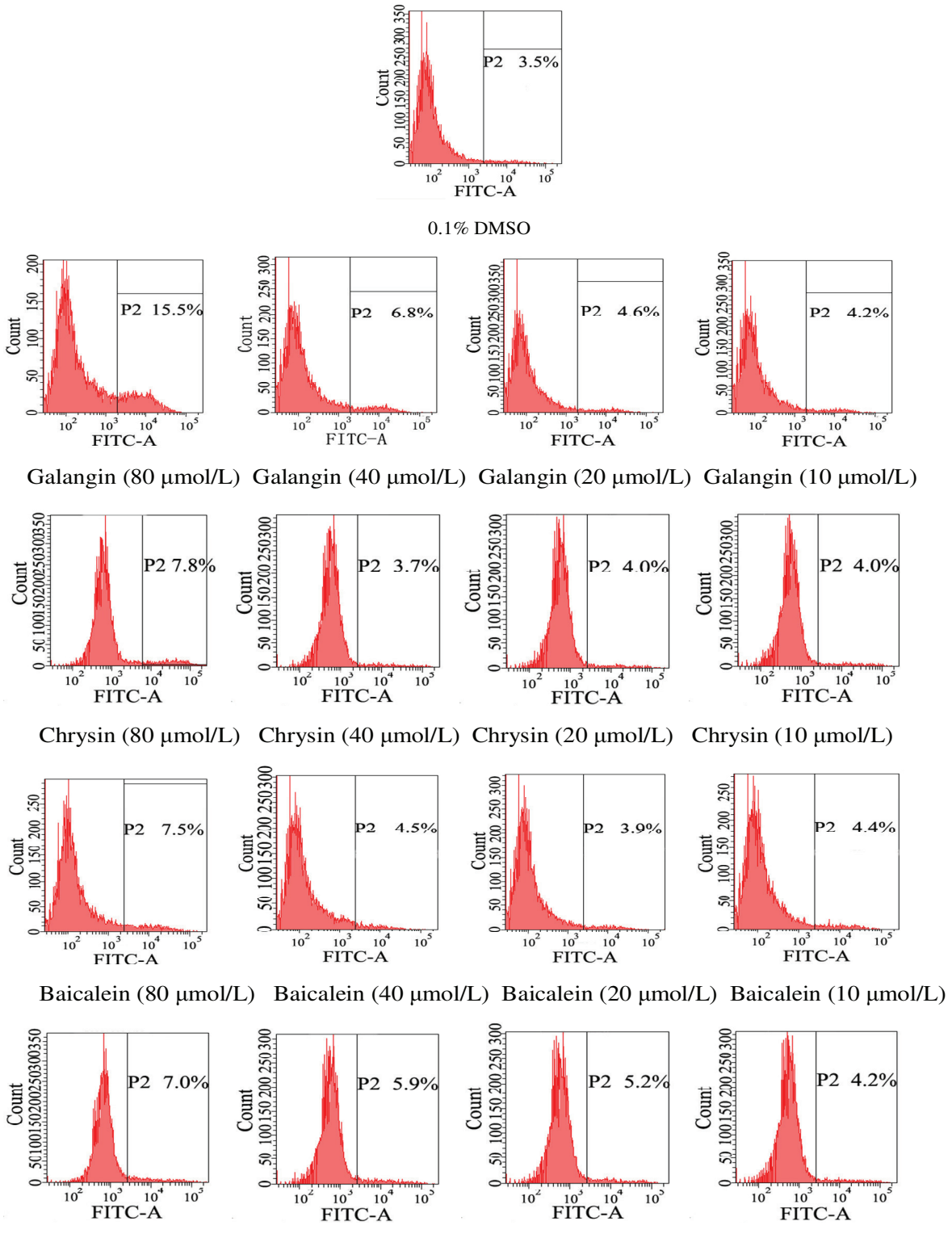

Figure 5. Effect of two flavones and two flavonols on induction of differentiation in KYSE-510 cells in a dose-dependent manner. The differentiation induced by 10, 20, 40 and 80 μmol/L concentration of each compound was quantified by flow cytometry with FITC. The analysis result revealed that the two flavones and two flavonols both made an increase of the level of cytokeratin 8 in a dose-dependent manner in gate P2.

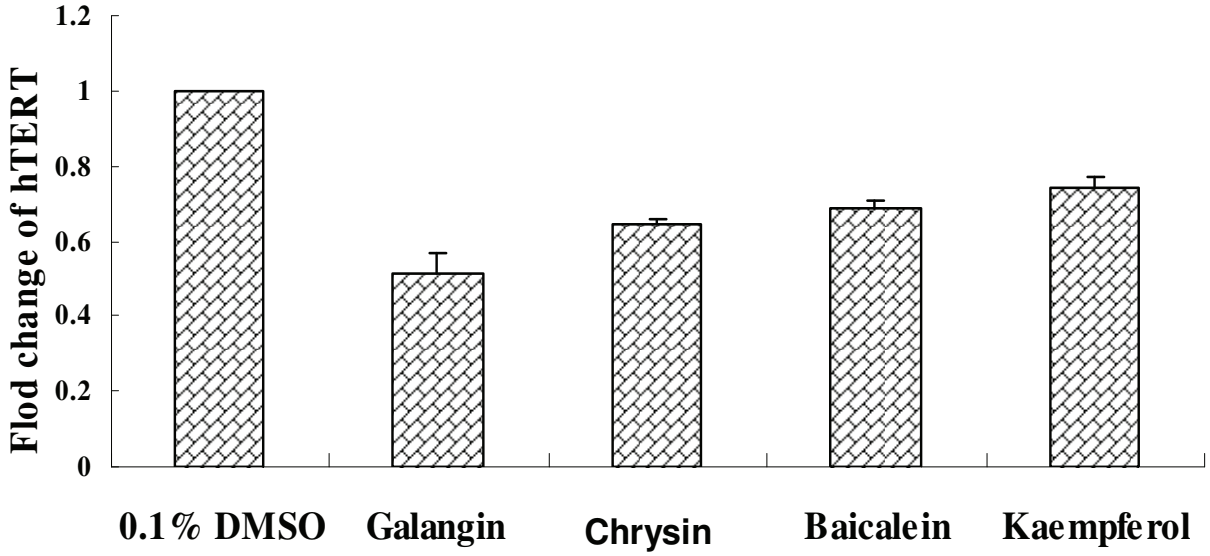

Figure 6. The effect of two flavones and two flavonols on the expression of human telomerase reverse transcriptase (hTERT) by Real-time RT-PCR analysis in KYSE-510 cells. The cells were treated with 80 μmol/L concentration of each compound for 24. Cells treated with 0.1% DMSO served as a negative control. The result indicated that each compound inhibited the expression of hTERT.

cancerous cells but not in normal somatic cells (Meyerson et al., 1997). The mRNA level of hTERT often correlates positively with telomerase activity and has thus been considered as the major determinant of telomerase activity (Counter et al., 1998; Kanaya et al., 1998; Kyo et al., 1999). Existing evidence indicates that down- regulation of hTERT may be somehow linked with cyto-differentiation (Bestilny et al., 1996; Savoysky et al., 1996; Xu et al., 1999).

In order to better understand the precise induction effect of differentiation of selected compounds on KYSE-510 cells, other markers of differentiation, mRNA expression of the human telomerase reverse transcriptase, were investigated in the cells exposed to these compounds. The alterations of hTERT were assayed by real-time RT-PCR assay. Each treatment showed a decrease expression of hTERT relative to the vehicle-treated control as shown in Figure 6, the potency was also similar to that of them on growth inhibition. The results indicated that selected compounds might inhibit KYSE-510 cells growth by inducing differentiation.

Real-time RT-PCR analysis

To verify the alterations of gene expression in KYSE-510 cells treated with four selected compounds at the mRNA level, real-time RT-PCR analysis was conducted for three genes ($p21^{waf1}$, cyclin B1 and cyclin D1) related to differentiation. As shown by the data in, treatments with each selected compound (galangin, chrysin, baicalein, kaempferol) resulted in the alteration of these gene expressions. The results indicated that selected compounds regulated the expression of some genes involved in cell growth, differentiation at the mRNA level. It was also suggested that the effects of flavones and flavonols on induction of cell differentiation in KYSE-510 cells might be depended on expression levels of these three *genes*.

Western blot analysis

In order to verify whether the alterations in expression of these three genes at the mRNA level ultimately would lead to the alterations at the protein level, a series of Western blot analysis were conducted. As shown in Figure 7, the protein level of cyclin B1 and cyclin D1 in KYSE-510 cells treated with selected compounds was down-regulated, and the protein levels of $p21^{waf1}$ were up-regulated. The potency of selected compounds on regulation of these three proteins was in agreement with the analysis data in real-time RT-PCR.

DISCUSSION

Growth inhibitory effects of two flavones and two flavonols on KYSE-510 cells

It is well known that flavonoids in the diet reduce the risk

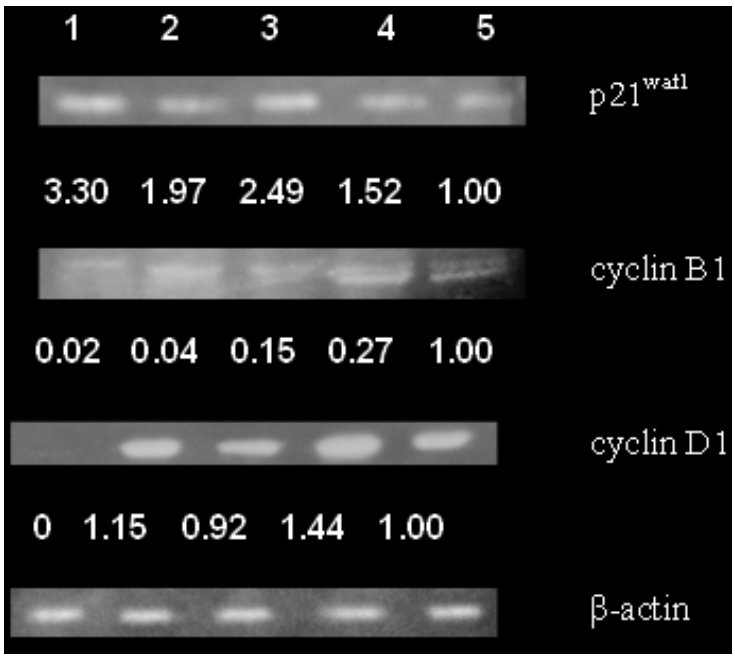

Figure 7. Effects of two flavones and two flavonols on the expression of proteins related to differentiation in KYSE-510. The cells were treated with vehicle (DMSO) only or selected compound at 80 μmol/L for 24 h and then harvested. Line 1, galangin; line 2, baicalein; line 3, chrysin; line 4, kaempferol; line 5, 0.1% DMSO. The values below the figure represented changes in protein expression of the bands normalized to β-actin regulated expression (fold change).

of cancer (Knekt et al., 1997; Garcia-Closas et al., 1999; Su and Arab, 2002; Neuhouser, 2004a, b). A number of studies have shown that flavonoids inhibit tumor cell growth of malignant cells (Zheng et al., 2005; Veeriah et al., 2006; Wang et al., 2000). Therefore, the biological effect of selected flavonoids on KYSE-510 cell growth was examined. The result showed that the two flavones and two flavonols inhibit cell proliferation of KYSE-510 in a dose- and time-dependent manne and the order of cytotoxic potency of these compounds was as galangin > chrysin > baicalein > kaempferol. This result was consistent with published results on other tumor cell lines (Wenzel et al., 2000; Pouget et al., 2001; Hsiao et al., 2007).

Effect of flavones and two flavonols on induction of differentiation on KYSE-510 cells

In stratified squamous epithelia, whether natural or cultured, cell division takes place in the basal layer. When cells leave this layer they undergo an orderly process of terminal differentiation that is characterized by specific molecular and morphological changes and no longer divide (Watt and Green, 1981; Crowe and Shuler, 1998). Histologically, cells become enlarged with abundant cytoplasm and express markers of terminal differentiation. Malignant transformation disrupts the normal process resulting in suprabasal cells retaining basal morphology and failing to express terminal differentiation markers (Crowe and Shuler, 1998; Crowe, 1998).

Studies have shown that flavonoids inhibit tumor cell growth, promote cell differentiation of malignant cells (Takahashi et al., 1998; Wenzel et al., 2000). Moreover, the activity of inducing differentiation of flavonoids can be confirmed by its action on many cancer cell lines, such as human breast squamous MCF-7 and MDA-MB-468 cell lines (Constantinou et al., 1998), human promyelocytic leukemia HL-60 cell line (Lee et al., 2001; Kawaii and Lansky, 2004), human myeloid K562 cell lines (Tolomeo et al., 2008), human melanoma TVM-A12, M14 and A-375 cell line (Serafino et al., 2004), human prostate carcinoma LNCaP cells (Zi and Agarwal, 1999) and other alimentary tract tumour cells (Gee and Johnson, 2001). However, Additional studies showed that some flavonoids were capable of suppressing cell differentiation or not promoting differentiation in keratinocyte. For example, curcumin inhibited proliferation and enhanced apoptosis but did not promote differentiation (Balasubramanian and Eckert, 2004) and apigenin suppressesed cell

differentiation and proliferation but did not enhance apoptosis (Balasubramanian et al., 2006). Taken together, their studies argue that chemopreventive agents of flavonoids have different mechanisms of action in differentiate cells.

In their previous study, three flavones and flavonols exerted cytotoxic effects on KYSE-510 (Zhang et al., 2009) and OE33 (Zhang et al., 2008) cells by causing G_2/M arrest and inducing apoptosis. In this process most malignant cells die via an apoptotic mechanism, whereas cells with differentiation potential will survive as a result of selection (Brown et al., 1998). Extending these studies, we here provide evidence that in addition to the anticancer properties mentioned earlier, flavonoids also have the ability to inhibit cancers through causing undifferentiated cancer cell lines to differentiate into cells exhibiting mature phenotypic characteristics. Therefore, the authors examined the biological effect of flavonoids on KYSE-510 cell differentiation.

Effect of flavones and flavonols on induction of morphological differentiation on KYSE-510 cells

This study had indicated that KYSE-510 cells treated with the four compounds showed dramatic morphological changes, including an enlarged, flattened shape, an increased cytoplasmic-to- nuclear ratio, and a decreased cell density, all of which were consistent with a differentiation phenotype, compared with the negative controls. In agreement with our results, some recent studies have also found that morphological changes during the differentiation of many cell lines, for example, neuroblastoma N_2A cells treated with genistein for 6 days exhibited morphological features of differentiation, as evidenced by the development of dendritic extensions (Brown et al., 1998), Human prostate carcinoma LNCaP cells treated with silibinin also resulted in a significant neuroendocrine differentiation of LNCaP cells, cells became elongated with prominent dendrite-like cyto-plasmic extensions where some of the dendrite-like extensions were connected to each other among neighboring cells, which were similar to that of neuroendocrine morphology (Zi and Agarwal, 1999). Meanwhile, cyanidin-3-O-β- glucopyranoside (C-3-G) treatment induced, in a dose- and time-dependent manner, melanoma cell differentiation characterized by a strong increase in dendrite outgrowth similarly to RA, which were a morphological feature of differentiated melanocytes and the first observable parameter of melanoma cell differentiation (Serafino et al., 2004). When human colon cancer KNC cells were cultivated for 10 days with kaempferol (20 mmol/L), dramatic morphological changes were also observed, including the appearance of neuron-like and triangular cells (Nakamura et al., 2005).

Effect of flavones and flavonols on inducing differentiation marker on KYSE 510 cells

Keratins are epithelia-specific intermediate filament proteins typically found in epithelial cells (Markey et al., 1991). According to their molecular weights and isoelectric points, they have been further subdivided into two subtypes, type I, which are acidic and have low molecular weights, and type II, which are basic or neutral and have high molecular weights. Keratin filaments are built from lateral and longitudinal interactions involving type I-II heterodimers (Morsi et al., 2006). Usually, only 2 - 6 keratins are expressed by an individual cell. Variability in expression of different keratins is related to species, cell type, state of cellular differentiation, and extracellular factors (Grace et al., 1985). A number of studies have demonstrated differentiation- dependent expression of keratins. Thus, they are being routinely used as markers of cell differentiation (Franke et al., 1982; Said et al., 1983; Burg-Kurland et al., 1986; Clausen et al., 1986; Swaf et al., 1990; Dale et al., 1990; Matsumoto et al., 2000). Keratin 8 is a type II keratin protein of 52 kD and a differentiation marker of squamous carcinoma cells (Grace et al., 1985) and their expressions are expected to be lower in SCC of esophagus, which are less differentiated. Data in this study showed that two flavones and flavonols restored a differentiated phenotype of KYSE-510 cells as judged by the increase of cytokeratin 8 by confocal laser scanning microscopy and increased expression in dose-dependent manner by flow cytometry (Figures 4 and 5). The potency of these flavonoids on induction of differentiation was in agreement with the results of cell growth analysis in our study (Figures 5 and 2b).

Cytokeratin is known as differentiation markers because of differentiation-dependent expression of keratins (Franke et al., 1982; Clausen et al., 1986; Thomas et al., 1987; Swaf et al., 1990; Moll, 1991, 1993; Stanwell et al., 1996). Variability in expression of different keratins is related to species, cell type, state of cellular differentiation, and extracellular factors (Grace et al., 1985). For example, in stratified epithelia, the cells of the basal layer are highly proliferating and express keratin 5/14. As they migrate into the upper layer, they become more differentiated. Cells of the uppermost layer are terminally differentiated cells that express keratin 4/13 or keratin 1/10 (Tamiji et al., 2005), depending upon the differentiation state of the tissue (Vaidya and Kanojia, 2007). In agreement with their study, some study showed the expressions of keratin 8 are expected to be lower in ESCC, which were less differentiated and might be a differentiation marker of esophagus squamous carcinoma cells (Grace et al., 1985). However, other study also reported keratin 13 and Annexin I might be a differentiation marker in normally differentiated squamous epithelial cells, and their expressions were expected to be lower in ESCC, which were less differentiated (Jetten et al., 1989). Meanwhile,

keratin 19 could be a marker for differentiation that decreases in less differentiated tumors (Morsi et al., 2006). It is postulated that variability in expression of different keratins is related to species, cell type, state of cellular differentiation, and extracellular factors.

Some of the cells treated with 10 and 20 μmol/L chrysin showed increased expression of cytokeratin 8 than cells treated with 40 μmol/L chrysin. In our treated cells with 40 μmol/L chrysin an initial differentiation response was followed by cell death. This is further supported by the tendency of some of the cells treated with the lower concentrations of chrysin to continue to show increased expression of cytokeratin 8 longer than cells treated with higher concentrations.

Effect of flavones and flavonols on expression of human telomerase reverse transcriptase

Telomerase is a ribonucleoprotein and has been shown to have reverse transcriptase activity and functions by adding DNA repeat sequences to the telomeric ends of chromosomes (Sanborn et al., 2000; Poole et al., 2001). In adult humans, telomerase activity in most normal tissues are low or no detectable but are found at high frequency across the whole spectrum of human cancers (Keith et al., 2002; Mokbel, 2003). The human telomerase complex consists of the telomerase reverse transcriptase, hTERT (the catalytic subunit), the RNA template, hTR, and a several telomerase associated proteins, including Hsp90, p23, and TEP-1 (Masutomi and Hahn, 2003; Mokbel, 2003). The RNA template is expressed in both normal and cancerous tissue, whereas hTERT is often detectable in cancerous cells but not in normal somatic cells (Meyerson et al., 1997). The mRNA level of hTERT often correlates positively with telomerase activity and has thus been considered as the major determinant of telomerase activity (Kraveka et al., 2003; Liu et al., 2004). The previous study demonstrated that telomerase activity down-regulation of hTERT in human cancer cells lines were correlated with the degree of differentiation, for example hepatocellular carcinoma (Kishimoto et al., 1998), human neuroblastoma SK-N-SH and SK-N-AS cell lines (Kraveka et al., 2003), human promyelocytic leukemia HL60 cell lines (Xu et al., 1999; Liu et al., 2004), human hepatoma-derived cell lines, PLC/PRF/5, HuH-7, and JHH-7 (Yasuda et al., 2002).

In order to better understand the precise effects of flavones and flavonols in inducing differentiation on KYSE-510 cells, the alterations of hTERT in KYSE-510 cells treated with flavones and flavonols were assayed. In this study, treatment of KYSE-510 cells with 80 μmol/L concentration of flavones and flavonols for 24 h resulted in the decrease of hTERT expression. The data showed that potency of these flavonoids on induction of differentiation was also similar to that of them on differentiation marker induction (Figures 6 and 7). The inhibition of hTERT during KYSE-510 cell differentiation demonstrates the effect of flavones and flavonols in inducing differentiation on KYSE-510 cells again. Many previous studies have also showed that a decrease in transcription of telomerase reverse transcriptase (hTERT) expression with the differentiation, such as hepatocellular carcinoma (Kishimoto et al., 1998), human neuroblastoma SK-N-SH and SK-N-AS cell lines (Kraveka et al., 2003), human promyelocytic leukemia HL60 cell lines (Xu et al.,1996, 1999; Liu et al., 2004), human hepatoma-derived cell lines, PLC/PRF/5, HuH-7, and JHH-7 (Yasuda et al., 2002).

Possible mechanism related to differentiation of KYSE-510 cells induced by two flavones and two flavonols

The development of a tissue requires the integration of cell cycle exit with morphological changes culminating in the specialized nature of a differentiated cell (Baldassarre et al., 2000; Zezula et al., 2001). Cell cycle progression in eukaryotes is controlled by a series of proteins named cyclins. At least eight cyclin *genes* have been identified in mammalian cells. They are classified into three groups: G_1 cyclins, an A-type cyclin, and two B-type cyclins (de Jong et al., 1999).

Cyclins D are required for progression through G_1. As cells enter G_1, the CDK4-and/or CDK6-cyclin D complex appears to be necessary for transition through early G_1 (Zi et al., 1998). The D-type cyclins can be induced by growth factors. Consistent with a role in proliferation, was identified as a potential oncogene which is overexpressed in a variety of different tumors, including breast (Gillett et al., 1994; Wani et al., 1997), esophageal (Doki et al., 1997) and colon (Hulit et al., 2004). Previous studies have showed that down-regulation of cyclin D1 concerned with differentiation (Spinella et al., 1999). Cyclin B is the major controlling cyclin in the G_2 phase of the cell cycle. Two B-type cyclins have been identified in mammalian cells, cyclin B1and cyclin B2 (Archer et al., 2005). Although, two types of cyclin B are now known to exist in mammals, most studies have focused on the expression and regulation of the first identified cyclin B. It has been shown that cyclin B1 over expression in several cancers, including esophageal carcinoma (Wang et al., 1997). The association of cyclin B1 repression and the induction of the differentiated phenotype had been described in human colon carcinoma-derived cell lines HT-29 (Archer et al., 2005). The cyclins act by binding to and stimulating the activities of a series of proteins named the cyclin dependent kinases (CDKs) to regulate the transition of the cell cycle. Whereas CDK activity is negatively regulated by the CDK-inhibitory proteins (CDKIs) which bind to the cyclin/cyclin-dependent kinase complex to inhibit cycle

progression, CDKI proteins are often absent or inactive in cancerous cells (Kamb et al., 1994; Graña and Reddy, 1995). At least seven CDKIs have been identified in mammalian cells, and on the basis of homology, they fall into the $p21^{waf1}$ and p16 families, each of which comprises several members. The p21 family includes $p21^{waf1}$ (also known as Cipl, WAF1, and so forth), Kipl/p27, and Kip2/p27 (Zi, et al., 1998; Crowe and Shuler, 1998). $P21^{waf1}$ is one of the most important cyclin-dependent kinase inhibitor (CDKI). Studies have report that up-regulation of p21 ^{waf1} are required for the appropriate differentiation of the oligodendrocytes (Zezula et al., 2001). $P21^{waf1}$ is not a transcriptional regulator itself, it is able to affect transcription indirectly through a number of mechanisms. One of the consequences of cdk inhibition by $p21^{waf1}$ might be to affect Rb differentiation-promoting functions separate from their cell-cycle inhibitory functions (Kaelin, 1997). Moreover, recent studies have revealed the existence of dual-function regulators that could participate in controlling both cell proliferation and differentiation. Certain cell-cycle regulators could directly regulate differentiation separate from their cell-cycle inhibitory functions (Zhu and Skoultchi, 2001). Thus, it was postulated that up-regulation of $p21^{waf1}$ and down-regulation of cyclin B1 and cyclin D1 might promote differentiation of KYSE-510 cells, independent of any role in regulating cell cycle exit.

Data in our analysis demonstrated up-regulation of $p21^{waf1}$and down-regulation of cyclin B1 and cyclin D1 at the mRNA level and protein levels (Figure 7) during inducing differentiation of KYSE-510 cells, which might be a possible molecular mechanism through which flavones and flavonols induce differentiation of cancer cells. In agreement with our results, some studies have shown an association between altered expressions of several cell-cycle related genes. There are several studies about the effect of $p21^{waf1}$ expression on differentiation. Zezula et al. (2001) reported that $p21^{waf1}$ were required for the establishment of differentiation of oligodendrocyte cells following growth arrest, independent of its ability to control exit from the cell cycle. By which of these mechanisms p21 might be promote KYSE-510 cells differentiation in our study, which is unlinked from its effects on the cell cycle. Other study also showed that the ERK signaling pathway mediated the up-regulation of the $p21^{waf1}$ expression that was induced by VES, which was required for monocytic differentiation of HL 60 cells (Lee et al., 2002). Meanwhile, Matsumoto et al. (2006) demonstrated that $p21^{waf1}$ expression was increased by 12-o-tetradecanoylphorbol 13-acetate (TPA) in other leukemia cell lines, including THP-1, U937, and KG-1, which differentiate into monocytes /macrophages TPA. However, in this case, $p21^{waf1}$ appeared to play a negative promoting role in differentiation (Devgan et al., 2006). Thus, the emerging consensus is that p21 can play an important regulatory function in differentiation that is not directly linked to its effects on the cell cycle and that can be either negative or positive depending on cell type and specific stages of differentiation.

Some studies had reported the association of down-regulation of cyclin B1 and induction of differentiation. Archer et al. (2005) had shown that cyclin B1 repression was linked to the differentiation process in colon cancer cells, not merely with growth arrest. The mechanism of cyclin B1 repression by butyrate requires prolonged histone hyperacetylation and was at least partly dependent on $p21^{waf1}$ expression. Meanwhile, the association of cyclin B1 repression and the induction of the differentiated phenotype in cells is similar to that described in other cells (Horiguchi-Yamada and Yamada, 1993; Jahn et al., 1994). Some previous studies had reported the association of decrease expression of cyclin D1 and induction of differentiation. Spinella et al. (1999) demonstrated that retinoids promoted ubiquitination and degradation of cyclin D1 during retinoid-induced differentiation of human embryonal carcinoma cells NT2/D1. However, some studies have also reported that increased levels of cyclin D1 during differentiation in cancer cells. Over expression of cyclin D1 has been reported in the TPA-treated HL60, THP-1, and KG-1 cells, which differentiates into monocytes/macrophages by TPA treatment (Matsumoto et al., 2006). Similarly, study reported that concerted over expression of the *genes* encoding cyclin D1 and $p21^{waf1}$ might also induce differentiation in squamous cell cancer (Jong et al., 1999). The results of these previous studies also corroborate the postulate that the expression changes of cyclin D1 by flavonoids is depended on both cell line and type of cancer. Based on this contradiction, it is postulated that the expression of cyclin D1 might be cell-type-specific. In our study, up-regulation of cyclin D1 in the cells treated with 80 μmol/L chrysin might be explained by this mechanism.

Conclusion

The differentiation-induced effects of two flavones (baicalein, chrysin) and two flavonols (galangin, kaempferol) on a human esophageal squamous cell carcinoma cell line (KYSE-510) were investigated in our work, and the molecular mechanism responsible for these effects were also preliminary studied. Our finding indicated that flavones and flavonols we selected in the presented study could induce differentiation in KYSE-510 cells. The potency of the four compounds on induction of differentiation was similar to that of them on growth inhibition, in an order of galangin > chrysin > baicalein > kaempferol. The alterations of three genes, $p21^{waf1}$, cyclin B1 and D1, which related to proliferation and differentiation of the cells, were investigated by real-time RT-PCR and Western blot. The evaluation results showed

that p21^{waf1}, cyclin B1 and D1 might be the target genes of four flavones and flavonols in inducing differentiation. These results might shed new light on some molecular mechanisms involved in flavonoid-induced differentiation on other cancer cells.

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