Flavones inhibit breast cancer proliferation through the Akt/FOXO3a signaling pathway

Chia-Hung Lin, Ching-Yao Chang, Kuan-Rong Lee, Hui-Ju Lin, Ter-Hsin Chen and Lei Wan

Abstract

Background: Flavones found in plants display various biological activities, including anti-allergic, anti-viral, anti-inflammatory, anti-oxidation, and anti-tumor effects. In this study, we investigated the anti-tumor effects of flavone, apigenin and luteolin on human breast cancer cells.

Methods: The anti-cancer activity of flavone, apigenin and luteolin was investigated using the MTS assay. Apoptosis was analyzed by Hoechst 33342 staining, flow cytometry and western blot. Cell migration was determined using the culture inserts and xCELLigence real-time cell analyzer instrument equipped with a CIM-plate 16. Real-time quantitative PCR and western blot were used to determine the signaling pathway elicited by flavone, apigenin and luteolin.

Results: Flavone, apigenin and luteolin showed potent inhibitory effects on the proliferation of Hs578T, MDA-MB-231 and MCF-7 breast cancer cells in a concentration and time-dependent manner. The ability of flavone, apigenin and luteolin to inhibit the growth of breast cancer cells through apoptosis was confirmed by Hoechst33342 staining and the induction of sub-G1 phase of the cell cycle. Flavone, apigenin and luteolin induced forkhead box O3 (FOXO3a) expression by inhibiting Phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB)/Akt. This subsequently elevated the expression of FOXO3a target genes, including the Cyclin-dependent kinase inhibitors p21 (p21) and p27 (p27), which increased the levels of activated poly(ADP) polymerase (PARP) and cytochrome c.

Conclusion: Taken together, these data demonstrated that flavone, apigenin and luteolin induced cell cycle arrest and apoptosis in breast cancer cells through inhibiting PI3K/Akt activation and increasing FOXO3a activation, which suggest that flavone, apigenin and luteolin will be the potential leads for the preventing and treating of breast cancer.

Keywords: Breast cancer, Flavones, FOXO3a, Akt, p27, Apoptosis

Background

Breast cancer is one of the most common types of cancer affecting women in western countries, and in recent years, the number of deaths caused by breast cancer has been increasing in Taiwan. Despite the new promising breakthrough in therapeutics, the annual breast cancer mortality rate continues to increase, and one million new cases are diagnosed every year [1]. Numerous risk factors for breast cancer etiology have been identified, including genetic, gender, age, alcohol consumption, smoking and obesity [2–5].

Breast cancer develops as a consequence of cellular changes that increase the rate of cell division and metastasis, decrease the rate of apoptosis, or both. These changes often involve dysregulation of key signal transduction pathways within the cell that transmit extracellular signals to transcription factors, resulting in changes in gene expression. Previous studies have shown that increased protein kinase B (PKB)/Akt activity can promote breast cancer cell survival and therapeutic resistance [6, 7]. Forkhead box O3 (FOXO3a), a downstream target of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, belongs to a family of transcription factors that are characterized by a distinct forkhead DNA-binding domain [8]. Activation of PI3K/Akt signaling causes phosphorylation of FOXO3a, thereby inhibiting its activity and translocating it out of the nucleus, where it becomes subject to proteasomal degradation in the cytosol [9]. The cytoplasmic expression level of FOXO3a is correlated with Akt phosphorylation.

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and is associated with poor prognosis in breast cancer [10]. Nuclear localization of FOXO3a promotes the expression of multiple target genes such as p21\(^{kip1}\) (p21), p27\(^{kip1}\) (p27), and cyclin D, which results in cell cycle arrest to inhibit the growth of cancer cells [9, 11, 12]. Cell cycle arrest in the G1, G2-M, and S phases can lead to apoptosis [13, 14]. FOXO transcription factors are also involved in the cellular stress response, and they regulate cell cycle progression and apoptosis [15]. In particular, FOXO3a plays a vital role in the initiation of cell cycle arrest, in addition to its involvement in DNA damage-mediated apoptosis [16]. Moreover, FOXO3a is an important tumor suppressor that is underexpressed in many breast cancers. Several anti-cancer drugs have been shown to increase the expression of FOXO3a, which suggest it is a tangible therapeutic target for breast cancer therapy [17].

Surgical resection, radiation therapy, and chemotherapy are among the main treatment options for breast cancer patient. In addition, there is growing need to discover new chemopreventive agents that are effective in preventing/treating breast cancer. Polyphenols are compounds found in food plants and Chinese herbs, and they can be divided into various classes on the basis of their molecular structure. Flavonoids constitute a class of polyphenols that can be further divided into the following six subclasses: flavones, flavanones, flavanols, flavonols, isoflavones, and anthocyanidins [18, 19]. A recent study showed that flavonoids exhibit various beneficial biological properties such as anti-inflammatory [20, 21], anti-viral [22, 23], anti-allergic [24], anti-oxidant, [25, 26] and anti-tumor activities [27–31]. Researchers have also discovered that flavones inhibit tumor growth by promoting apoptosis in cancer cells [27, 28, 32]. Compounds in the flavone subclass, including flavone, apigenin, and luteolin, are present in fruits and vegetables and are considered to be potent dietary phytochemicals that are effective in cancer chemoprevention [33, 34]. Although different mechanisms and signaling pathways have been proposed as targets of flavone, apigenin, and luteolin, these compounds were studied individually and occasionally by using different model cancer cells. However, whether these structurally similar compounds could induce common pathways among different types of cancer cells to potentiate their chemoprevention activity remains to be elucidated. The objectives of the present study were to investigate the anti-proliferative role of flavone, apigenin, and luteolin in MCF-7, Hs578T, and MDA-MB-231 human breast cancer cells, and to elucidate the common molecular pathways in these cells.

**Methods**

**Cell culture**

The human Breast cancer cell lines Hs578T, MDA-MB-231 and MCF-7 were purchased from American Type Culture Collection. Cells were cultured in Dulbecco’s modified Eagle medium (Invitrogen), supplemented with 10 % fetal bovine serum (Invitrogen) and 1 % penicillin-streptomycin solution (Invitrogen) at 37 °C in a 5 % CO\(_2\) incubator. No approvals were required for this study, which complied with all relevant regulations.

**Cell viability assay**

Cell viability was determined using the MTS/PMS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt)/phenazine methosulfate) assay. Hs578T (2 × 10\(^5\) cells/well), MDA-MB-231 (2 × 10\(^5\) cells/well) and MCF-7 (4 × 10\(^5\) cells/well) cells were seeded in 96-well plates (2 × 10\(^5\) cells/well) and treated with IC\(_{50}\) different concentrations of flavone, apigenin, and luteolin for 21 d. Thereafter, the cells were fixed with methanol for 15 min, followed by incubation with 0.5 % crystal violet for 30 min and rinsing with deionized distilled water.

**Hoechst 33342 staining for detection of cell apoptosis**

Apoposis was analyzed by Hoechst 33342 staining. The MCF-7, Hs578T, and MDA-MB-231 cells were seeded in 6-well plates (2 × 10\(^5\) cells/well) and treated with IC\(_{50}\) concentrations (Table 1) of flavone, apigenin and luteolin for 24 h. Cells were stained with 40 mg/mL Hoechst 33342 (Sigma). Nuclear morphology was assessed using the cell membrane penetration DNA dye Hoechst 33342. Then, the cells were visualized under a fluorescence microscope with a blue filter.

**Cell cycle analysis**

For cell cycle analysis, cells were seeded in a 10-cm dish (5 × 10\(^5\) cells) and allowed to adhere overnight. Cells were then treated with IC\(_{50}\) concentrations (Table 1) of flavone, apigenin and luteolin for 24 h. Cells were harvested and fixed in ice-cold 70 % ethanol overnight at −20 °C. Cells were washed with ice-cold PBS and subsequently suspended in staining buffer (20 μg/mL propidium iodide; 0.1 % Tween 20; 0.2 mg/mL RNase A in PBS), and incubated at room temperature for 30 min. Using flow cytometry (FACSort instrument and analysis software; ModFit LT), the cells were analyzed with regard to cell cycle distribution and apoptosis.

**Colony formation assay**

Cells were plated in a 25 T flask at a density of 2 × 10\(^5\) cells and treated with IC\(_{50}\) concentrations (Table 1) of flavone, apigenin and luteolin for 21 d. Thereafter, the cells were then fixed with methanol for 15 min, followed by incubation with 0.5 % crystal violet for 30 min and rinsing with deionized distilled water.
Wound healing assay
Cell migration was determined using the culture inserts (ibidi). Cells were then trypsinized, counted, plated into each well of the culture-inserts (3.5 × 10⁴ cells per well), and incubated at 37 °C in a humidified atmosphere with 5 % CO₂. Cells were allowed to attach for 12 h, and then the culture inserts were gently removed. MCF-7 breast cancer cells were treated with flavone (88 μM), apigenin (30 μM), and luteolin (43 μM) for 24 h. Images were processed and analyzed using Image Plus software.

Analysis of cell migration and invasion
To monitor cell migration/invasion in real time, we used the xCELLigence Real-Time Cell Analyzer (RTCA) DP Instrument equipped with a CIM-plate 16 (Roche, Indianapolis, IN), which is a 16-well system in which each well is composed of upper and lower chambers separated by an 8-μm microporous membrane. Migration/invasion was measured as the relative impedance change (cell index) across microelectronic sensors integrated into the bottom side of the membrane. For cell migration experiments, cells (7.5 × 10⁴ /well) were added in duplicates to the upper chambers. MCF-7 breast cancer cells were treated with flavone (88 μM), apigenin (30 μM) and luteolin (43 μM). Migration/invasion was monitored every hour for 9 h. For quantification, the cell index at the indicated time points was averaged from three independent measurements.

RNA extraction, reverse transcription (RT), and real-time PCR
Total RNA from Hs578T, MDA-MB-231 and MCF-7 cells was extracted using the RNaseasy Mini Kit (Qiagen, Valencia, CA). RNA (4 μg) was reverse-transcribed using
the Superscript First Strand synthesis system for conversion to cDNA (Invitrogen, Carlsbad, CA). Primers and probes for amplification and detection were selected from the Universal Probes Library (Roche, UK). The primer sequences used were as follows: FOXO3a (forward: acctaaagctacttacagtccc, reverse: gactgtgctagctggtc), p21 (forward: ccctagagggcaagtacctg, reverse: atctgaagttccatcgctcac), and p27 (forward: ccttagagggcaagtacctg, reverse: agtagaactgcggcaacgtg). Amplification was performed in a LightCycler480 system (Roche, UK) beginning with an initial heating at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 1 s. Gene expression levels were determined using glyceraldehyde 3-phosphate dehydrogenase as a control.

Western blot
Whole cell lysates were obtained by direct lysis of the cells using an ice-cold Mammalian Protein Extraction Reagent (M-PER, Pierce). Nuclear and cytoplasmic fractionations were performed using the Nuclear and Cytoplasmic Extraction Kit (Pierce). Protein (20 μg) was resolved by 10 % SDS-PAGE and electro-transferred onto a polyvinylidene difluoride membrane. Western blotting was performed according to standard methods, using anti-cleaved-PARP, anti-p53, anti-cytochrome c, anti-Akt, anti-phosphorylated-Akt (ser473), anti-FOXO3a, anti-p21, anti-p27 and anti-β-actin antibodies (Cell Signaling Technology). The membranes were developed using an enhanced chemiluminescence detection system, horseradish peroxidase substrate (Millipore) and an ImageQuant LAS-4000 Chemiluminescence and Fluorescence Imaging System (FujiFilm).

Statistical analysis
For each study group, data were derived from at least three independent experiments. Statistical analysis was performed using a Student’s t-test or Chi-Square test to compare differences in values between the control and experimental groups. The results are presented as the mean ± SD. P < 0.05 indicated a statistically significant difference.

Results
Effects of flavone, apigenin and luteolin on cell viability and apoptosis of breast cancer cells
To determine the effect of the flavone, apigenin, and luteolin on MCF-7, Hs578T, MDA-MB-231 cancer cells and on a non-tumorigenic MCF-10A cell, a MTS/ PMS assay was performed. The IC50 for flavone, apigenin and luteolin on these cells are listed in Table 1. Flavone, apigenin and luteolin showed a concentration-dependent effect on the cell viability of MCF-7, Hs578T, and MDA-MB-231 cells (Fig. 1a, b, and c). Cell viabilities were significantly decreased treating with 12.5–100 μM flavone, apigenin, and luteolin for 72 h. Long-term effects of flavone, apigenin, and luteolin on breast cancer cells, were further assessed by colony formation assay. After 21 d of treatment with flavone, apigenin, and luteolin the colony numbers were suppressed by 2- to 3-fold (Fig. 1d, e).

To delineate the mechanisms of action of flavone, apigenin and luteolin in breast cancer cells. Cell cycle analyses were performed using flow cytometry (Fig. 2a, b, and c). Flavone induced arrest at G1 phase in Hs578T and MDA-MB-231 cells, and at the G2-M phase in MCF-7 cells. Treatment with apigenin and luteolin induced arrest at G2-M and S phases in Hs578T and MDA-MB-231 cells and at S phase in MCF-7 cells. Thus, although all three compounds inhibited the growth of Hs578T, MDA-MB-231, and MCF-7 cells, their regulatory effects on the cell cycle differed.

We further examined the cyclin B, and cyclin D1 markers for G1, G2-M, and S phase arrest in MCF-7 cells. Consistent with the observation that the flavone, apigenin, and luteolin arrested MCF-7 cell cycle at the G2-M or S phase, the expression levels of cyclin B and cyclin D1 were reduced after treatment with these compounds for 24 and 48 h (Fig. 2d).

The induction of apoptosis by flavone, apigenin, and luteolin was detected by Hoechst 33342 staining (Fig. 2e, f). Treatment with flavone, apigenin and luteolin increased the number of apoptotic cells in MCF-7, Hs578T, and MDA-MB-231 breast cancer cells. In addition, western blot analysis in MCF-7 cells revealed that the expression of p53 increased and that PARP was cleaved to its intermediate forms (Fig. 2g). The activation of PARP indicated an induction of the intrinsic apoptosis pathway by the flavone, apigenin and luteolin. Treatment with these compounds also increased the release of cytochrome c into the cytosol of MCF-7 cells (Fig. 2g).

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For each study group, data were derived from at least three independent experiments. Statistical analysis was performed using a Student’s t-test or Chi-Square test to compare differences in values between the control and experimental groups. The results are presented as the mean ± SD. P < 0.05 indicated a statistically significant difference.

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Flavone, apigenin and luteolin inhibited cell motility
To examine cell proliferation and migration, scratch wound migration assays were conducted. Flavone, apigenin and luteolin effectively reduced the migration of MCF-7 cells into the wounded area (Fig. 3a, b). To obtain further details, cell migration was measured in real time, and treatment with flavone, apigenin, and luteolin decreased the migration ability of MCF-7 cell (Fig. 3c). These results confirm that flavone, apigenin, and luteolin directly inhibit MCF-7 cell migration, ruling out the influence of proliferation on cell motility.
Flavone, apigenin, and luteolin activate FOXO3a, which is associated with a change in the signal transduction pathway

We further determined the effect of flavone, apigenin, and luteolin on the expression of FOXO3a, a transcription factor and tumor suppressor, in the three cancer cell types. Treatment of Hs578T, MDA-MB-231, and MCF-7 cells with flavone, apigenin, and luteolin for 24 h led to an increase in the expression RNA levels of FOXO3a (Fig. 4a). To investigate whether flavone, apigenin and luteolin affect the FOXO3a expression in breast cancer cells, we performed western blot analyses on the nuclear and cytoplasmic fractions of MCF-7 cells treated with the IC50 concentrations of flavone, apigenin, and luteolin for 48 h. We found that these compounds increased the expression of FOXO3a in all the cells (Fig. 4b).

FOXO3a is downstream target of Akt. Akt kinase regulates breast cancer proliferation and survival [35]. Inhibiting Akt phosphorylation modulates the activities of FOXO3a and subsequently affects cell proliferation, apoptosis, and differentiation [36]. We therefore examined the roles of flavone, apigenin, and luteolin in Akt signaling. Akt was predominantly phosphorylated...
in control cells, whereas Akt phosphorylation in cells treated with flavone, apigenin, and luteolin for 48 h showed a marked decrease which was consistent with the decreased expression levels of FOXO3a protein (Fig. 4c, d).

We found that all three flavones suppressed Akt phosphorylation and increased FOXO3a expression. Akt inhibits of p21 and p27 promoter activity through reduction of FOXO3a expression [36–38]. Previous studies have suggested that anti-cancer drugs upregulated p21 and p27, and this effect may play an important role in drug-induced cell cycle arrest in human cancer. Therefore, we examined the expression of the proteins p21 and p27, which are known targets of FOXO3a in MCF-7 cells (Fig. 5). The results indicated the flavone, apigenin, and luteolin induced upregulation of FOXO3a, which subsequently induced the expression of p21 and p27. To confirm this finding, parallel cell cultures were treated with flavone, apigenin, and luteolin, and found an increase in p21 and p27 mRNA levels (Fig. 5a). This finding suggests that the increased expression levels of p21 and p27 observed in the western blot analyses (Fig. 5b–d) resulted from an increase in transcription. These results are consistent with our observation of alterations in PI3K/Akt, FOXO3a, p21, and p27 expression levels after treatment with flavone,
apigenin, or luteolin, suggesting that the flavone compound-mediated inhibition of cell proliferation and apoptosis were mediated at least part by regulation of the PI3K/Akt/FOXO3a/p27 signaling pathway.

Discussion

Despite recent advances in medicinal chemistry, intrinsic and acquired resistance to chemotherapy treatments and the possibility of relapse present drawbacks in the treatment of breast cancer [39]. Because of the clear risks posed by chemotherapy, researchers worldwide have started searching for natural products that have better anti-carcinogenic activity without side effects. In addition, we believe that long chemotherapy treatment weakens the immunological defense system of the body and leaves patients susceptible to other infections and diseases. Radiation therapy is another treatment to combat cancer but also shows several potentially harmful side effects including weakened immune system and the potential to induce carcinogenesis itself. Therefore, there is an urgent need to develop chemoprevention approaches for the prevention of cancer. Chemoprevention is an important function area of oncology that focuses on the prevention of cancer using natural or synthetic agents. Recently, natural plant extracts and compounds have received widespread attention because of their potential beneficial effects on human health. Natural compounds provide a wide array of potential drug candidates for cancer therapy with various roles and targets [40, 41]. Accumulating evidence indicates that curcumin [15], quercetin [42], emodin [43], resveratrol [14] and wogonin [44] of natural origin induce apoptosis and inhibition of cell proliferation in multiple tumor cell lines including A549 lung cancer cells, hepatoma HepG2 cells, MCF-7 breast cancer cells and LNCaP prostate carcinoma cells. Flavonoids are the most common polyphenolic compounds, as they are ubiquitously present in foods of plant origin [45].

Many practitioners have used flavone compounds to treat a wide variety of ailments, including cancer [21, 23, 24, 46, 47]. Accumulating evidence indicates flavone compounds have been shown to have anti-cancer effects on cancer cell lines.
and anti-proliferative activities in in vitro and in vivo [48, 49]. In this study, we examined the mechanisms by which flavone, apigenin, and luteolin induced cytotoxicity in breast cancer cells. We have shown that flavone, apigenin and luteolin induce cell cycle arrest and apoptosis in breast cancer cells. The induction of cell apoptosis occurs in response to various stresses, including activation of p53 [50], which leads to its nuclear translocation and activation of targets such as cyclin D1 and p21 that regulate the cell cycle and trigger apoptosis [51, 52]. Our results indicate that the cell cycle arrest of MCF-7 cells treated with three compounds may be associated with the inhibition of cyclin B and cyclin D1-mediated cell cycle-arrest responses. Therefore, flavone, apigenin and luteolin may inhibit breast cancer cells proliferation via cell cycle arrest and apoptosis.

The PI3K-Akt signaling pathway plays a vital role in tamoxifen and cytotoxic chemotherapeutic drug resistance...
enhanced Akt activity has been shown to elevate resistance to tamoxifen and cytotoxic drugs by promoting cell proliferation and survival [54]. Many cancers acquired drug resistance by PI3K/Akt pathway activation, which has been observed during the administration of paclitaxel in breast cancer [55]. Our results showed that flavone, apigenin and luteolin treatment substantially suppressed PI3K/Akt phosphorylation at Ser473 in MCF-7 cells.

Members of the forkhead class O (FOXO) family of transcription factors are crucial for regulating various physiological processes, including proliferation, metabolism, cell differentiation, cell cycle arrest, DNA repair and apoptosis [56]. FOXO3a are important targets of PI3K/Akt signaling pathway [11]. The Akt mediated phosphorylation of FOXO3a is known to transport FOXO3a out of nucleus and retain FOXO3a in the cytoplasm [57]. FOXO3a has also been shown to regulate cell cycle arrest and apoptosis through the activation of transcriptional targets such as p27 and p21 [36]. The nuclear localization of FOXO3a and its subsequent transcriptional activity were known to be a prognosis marker for breast cancers [9]. Our results demonstrated that treatment of breast cancer cells with flavone, apigenin, and luteolin for 12 h led to an inhibiting Akt activation and increasing the expression levels of FOXO3a, which subsequently increase the expression levels of p27 and p21 to inhibit the proliferation of breast cancer cells.
Metastasis is complex processes and accounts for the death of most cancer patients. In wound healing assay, we found that treatment of the flavone, apigenin and luteolin suppressed MCF-7 cells migration. Inhibiting Akt signaling reduced the migration and invasion of gastric cancer cells [58], which may be due to up-regulation FOXO3a. In renal cancer cells, FOXO3a has been identified as a key factor in metastasis. Over-expression of FOXO3a in renal cancer cells could inhibit tumor metastasis [59]. Flavone, apigenin and luteolin inhibit breast cancer cells migration was through inhibiting Akt activation and increasing FOXO3a expression.

Epidemiologic and clinical studies suggest that higher intake of plant flavonoids can prevent cancer through their interaction with various genes and enzymes [60]. Our study demonstrated that these three compounds suppressed cell proliferation in human breast cancer cells, in part, by acting on the Akt/FOXO3a/p27 signaling pathway. Flavone, apigenin, and luteolin also inhibited the proliferation of Hs578T, MDA-MB-231 and MCF-7 cells by promoting cell cycle arrest, cell apoptosis and inhibiting cell migration and invasion. These effects were associated with FOXO3a activation. These compounds may thus reduce the risk of carcinogenesis by affecting the Akt/FOXO3a/p27 signaling pathway and serve as chemopreventive agents (Fig. 6).

Conclusions
Collectively, our study findings suggests that flavone, apigenin, and luteolin have chemopreventive properties against breast cancer, and these compounds are useful as potential preventive or therapeutic agent in the management of human breast cancer.

Abbreviations
FOXO3a: Forkhead box O3; FOXO: Forkhead box class; Akt: Protein kinase B; PI3K: Phosphatidylinositol-3-kinase; CKI: Cyclin-dependent kinase inhibitor; PARP: (poly(ADP) polymerase; p53: Tumor protein 53; FBS: Fetal bovine serum; RT-PCR: Reverse transcription-PCR; IC50: Half maximal inhibitory concentration; M-PER: Mammalian protein extraction reagent; PVP: Polyvinylidene difluoride; ECL: Enhanced chemiluminescence; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
CHL performed laboratory work, all experiments and data analysis, as well as wrote the manuscript. HJL, CYC and THC conceived and designed the study. CHL prepared the manuscript with LW and allocated funding for the work. LW and KRL critically revised the manuscript and provided scientific direction. All authors read and approved the final manuscript.

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