Published in final edited form as:
Cancer Detect Prev. 2007 ; 31(2): 110–118.

Inhibition of cell proliferation, induction of apoptosis, reactivation of *DLC1*, and modulation of other gene expression by dietary flavone in breast cancer cell lines:

Flavone-mediated alterations of genes that regulate tumor cell proliferation, cell cycle, and apoptosis contribute to chemopreventive and antitumoral effects of flavone in breast cancer

Veronika Ullmannova, PhD and **Nicholas C. Popescu, PhD**
Laboratory of Experimental Carcinogenesis, Center for Cancer Research, National Cancer Institute, 37 Convent Drive, MSC 4264, Bethesda, MD 20892

Abstract

Background—Dietary flavone was previously shown to increase the expression of deleted in liver cancer–1 gene (DLC-1) in HT-29 colon carcinoma cell line (Proteomics 2004;4:2455-64). DLC-1 that encodes a Rho GTPase-activating protein, functions as a tumor suppressor gene and is frequently inactivated or down-regulated in several common cancers. Restoration of DLC-1 expression suppresses in vitro tumor cells proliferation and tumorigenicity in vivo.

Methods—Here, the effect of flavone was examined in several DLC-1-deficient cell lines derived from different types human cancer using assays for cell proliferation, gene expression and transfer.

Results—We show that exposure to 15μM flavone increased *DLC1* expression in breast but not in liver or prostate carcinoma cells or a nonmalignant breast epithelial cell line. Flavone restored the expression of *DLC1* in the breast carcinoma cell lines MDA-MB-468, MDA-MB-361, and BT20 as well as in the colon carcinoma cell line HT-29 all of which are DLC-1-negative due to promoter hypermethylation. We further show that flavone inhibited cell proliferation, induced cell cycle arrest at G2-M, increased p21 Waf1 gene expression, and caused apoptosis. Microarray analysis of these aggressive and metastatic breast carcinoma cells revealed 29 flavone-responsive genes, among which the DNA damage–inducible *GADD* genes were up-regulated and the proto-oncogene *STMN1* and *IGFBP3* were down-regulated.

Conclusions—Flavone-mediated alterations of genes that regulate tumor cell proliferation, cell cycle, and apoptosis contribute to chemopreventive and antitumoral effects of flavone. Alone or in combination with demethylating agents, flavone may be an effective adjunct to chemotherapy in preventing breast cancer metastasis.

Author's key words

Flavone; breast cancer; colon cancer; DLC-1 gene; tumor suppressor genes; protooncogenes; DNA methylation; cell cycle; cell growth inhibition; apoptosis

Corresponding author: Nicholas C. Popescu, 37 Convent Drive, MSC 4264, Bethesda, MD 20892-4255. Tel.: 301-496-5688x240. Fax: 301-496-0734. e-mail: popescun@mail.nih.gov

1. Introduction

The *DLC1* (deleted in liver cancer–1) gene [1] encodes a Rho GTPase-activating protein and is expressed in most human tissues, but its expression is frequently down-regulated or silenced in various types of human cancer. Indeed, *DLC1* is emerging as a bona fide tumor suppressor gene, given that ectopic expression of DLC-1 in several common types human cancer cells that do not express the endogenous gene inhibits cell proliferation and induces caspase-3-mediated apoptosis in vitro as well as abolishes or reduces tumorigenicity in vivo [2,3,4,5,6,7,8]. Limited information is available on factors that regulate transcription of endogenous *DLC1*, however. Activation of peroxisome proliferator–activated receptor γ (PPARγ), which inhibits the growth of breast and prostate cancer cells as well as metastasis of lung tumor cells, was shown to increase expression of *DLC1* in association with inhibition of the invasiveness of lung cancer cells that overexpress PPARγ [9]. All-*trans* retinoic acid inhibits the proliferation of normal cells as well as that of various types of tumor cells, and culture of Wilms' tumor cells with all-*trans*-retinoic acid induced expression of *DLC1* [10]. Comprehensive gene expression profiling also revealed that doxorubicin, the most widely used drug for treatment of breast cancer, increased the expression of genes important in apoptosis, cell proliferation, cell cycle checkpoints, and suppression of metastasis, including that of *DLC1*, in breast cancer patients [11].

Progesterone confers protection against ovarian cancer. Gene expression profiling of human immortal nontumorigenic ovarian epithelial cells and ovarian cancer cells revealed that progesterone up-regulated the expression of several genes in the cancer cells [4]. Among these genes, four with known antitumorigenic function—those for ATF3, caveolin-1, NM23-H2, and DLC-1—were selected for post hoc functional analysis. Progesterone induced up-regulation of the expression of all four genes in the cancer cells but not in the nontumorigenic cells. Of these four genes, those for caveolin-1 and DLC-1 exhibited a broad spectrum of antitumor activity similar to that of bona fide tumor suppressor genes. Overexpression of DLC-1 in the ovarian cancer cells thus resulted in inhibition of cell growth, of colony formation in soft agar, and of cell migration as well as in the induction of apoptosis [4].

A protein and mRNA profiling study showed that flavone, a dietary constituent, increased the expression of DLC-1 in HT-29 human colon carcinoma cells [12], which constitutively express *DLC1* at only a low level as a result of promoter methylation [13]. We have now confirmed that flavone increases the amount of DLC-1 mRNA in HT-29 cells and have investigated whether this compound exerts a similar effect in other cell lines derived from several types of solid tumors deficient in DLC-1. We found that flavone reactivated *DLC1* expression in three breast carcinoma cell lines derived from aggressive or metastatic breast tumors. Given that DLC-1 exhibits marked antitumoral and antimetastatic activity in breast cancer cells, with *DLC1* being a candidate breast cancer susceptibility gene [2,6,14,15], we analyzed cellular and molecular alterations resulting from flavone exposure in the three breast carcinoma cell lines and a nontumorigenic breast epithelial cell line. Our results show that flavone targets additional genes important in regulation of cancer cell growth, repair of DNA damage, and apoptosis, effects that likely contribute to its antitumoral activity in breast cancer.

2. Materials and Methods

2.1. Cell culture

The breast carcinomas and immortalized breast epithelial cell lines MDA-MB-468, MDA-MB-361, BT20, MCF10F, colon carcinoma HT-29 cells, and prostate carcinoma 22RVi were obtained from American Type Culture Collection (Manassas, VA). The hepatocellular carcinoma (HCC) cell lines, Focus and 7703, were maintained in our laboratory repository. Cells were cultured under an atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's

medium–F12 supplemented with 10% fetal bovine serum and antibiotics (Biosource, Rockville, MD).

2.2. Assay of cell proliferation

Based on previous results with protein and mRNA profiling and other studies, in all experiments the concentration of 15μM flavone (2-phenyl-4*H*-1-benzopyran-4-one; Sigma, St. Louis, MO) was used [12,16]. For cell proliferation assay, cells were seeded in triplicate in 96-well plates (1×10^4 cells per well) and cultured with flavone for up to 4 days. Cell proliferation was evaluated with the colorimetric MTT reduction assay (Promega, Madison, WI) and measurement of absorbance at 490 nm with a microplate reader (Spektra Plus; PGC Scientific, Gaithersburg, MD).

2.3. Flow cytometry

Control or flavone-treated cells (1×10^6 per sample) were fixed in 70% ethanol, incubated for 5 minutes at 4°C with 0.1% Triton X-100 and RNase A (100 U/ml, Sigma), and then stained with propidium iodide (50μg/ml, Sigma). The cells were analyzed for cell cycle distribution and apoptosis by flow cytometry with a FACSort instrument (Becton Dickinson, Boston, MA) and FlowJo 6.4.1 software (Tree Star, Inc, Ashland, OR).

2.4. Assay of caspase-3 activity

The activity of caspase-3 was determined by using EnzChek Caspase-3 Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Fluorescence was measured with a Spektra Max Gemini instrument (PGC Scientific) at excitation and emission wavelengths of 480 and 520 nm, respectively. Serial dilutions of the R110 dye were used as a standard.

2.5. Quantitative RT-PCR analysis

Quantitative RT-PCR analysis of DLC-1 and $p21^{Waf1}$ mRNA was performed with primers using and a TaqMan probe as described previously [17,18]. Standard curves revealed similar reaction efficiencies for all PCR amplifications. PCR was performed with an ABI PRISM 7900 instrument, and the abundance of DLC-1 and $p21^{Waf1}$ mRNAs was normalized by that of GAPDH mRNA. Differences in gene expression induced by flavone treatment were evaluated by the $2^{-\Delta\Delta ct}$ method [19]; differences were considered significant when $2^{-\Delta\Delta ct}$ was ≥ 2 or ≤ 0.5.

2.6. Cell transfection

Breast cancer cell lines MDA-MB-468, MDA-MB-361, and BT20 at 70% confluence were transfected for 48 h with the adenoviral vector pAd/CMV/V5-GW (Invitrogen, Carlsbad, CA) containing full-length cDNAs for human DLC-1 or β-galactosidase (LacZ).

2.7. Immunoblot analysis

Control or flavone-treated cells (5×10^6) were lysed with the use of the M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) and Protease Inhibitor Cocktail (Sigma). Proteins were fractionated by SDS-polyacrylamide gel electrophoresis on a 4 to 20% gradient gel (Invitrogen) and then transferred to a nitrocellulose membrane (Invitrogen). After incubation with 5% skim milk (Bio-Rad, Hercules, CA) for 1 h, the membrane was incubated overnight with F-5 and DO-1 mouse monoclonal antibodies (1μg/ml) to $p21^{Waf1}$ and p53 (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. The membrane was reprobed with mouse monoclonal antibodies to GAPDH (MAB374; Chemicon, Tamacula, CA) at 0.5μg/ml for 1 h as a control for sample loading. Immune complexes were detected by incubation of the

membrane for 1 h with horseradish peroxidase–conjugated goat secondary antibodies (Santa Cruz Biotechnology) at a dilution of 1:20,000 and with the use of a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce). Blots were subjected to densitometric analysis with Gene Snap software (Syngene, Cambridge, UK).

2.8. cDNA microarray analysis

Microarrays (Operon Human Version 3.0) containing 34,580 probes were obtained from the NCI array facility (NCI ATC, Gaithesburg, MD). Total RNA was isolated from control or flavone-treated breast carcinoma cell lines with an RNeasy Mini Kit and was purified with a Qiaquick PCR Purification Kit (Qiagen). Samples of the purified total RNA (20μg) were subjected to RT with a SuperScript Indirect cDNA Labeling System (Invitrogen) and the resulting cDNA was labeled with Cy3 or Cy5 fluorescent dyes (Pharmacia, Piscataway, NJ). The labeled cDNA preparations were combined and allowed to hybridize overnight to the same microarray. Experiments were repeated with the same cDNA preparations labeled with the opposite combination of dyes. The ratio of the Cy3 and Cy5 signals was calculated for each spot on the array with GenePix 5.1 software (Axon Instruments, Sunnyvale, CA) and was further analyzed with the NCI mAdb array analysis tool.

3. Results

To confirm the previous finding by microarray analysis that flavone increased the amount of DLC-1 mRNA in HT-29 colon carcinoma cells [12], we examined the effect of flavone on DLC-1 mRNA by quantitative RT-PCR analysis. Exposure of HT-29 cells to 150μM flavone for 24 h induced a nearly five fold increase in the amount of DLC-1 mRNA (Fig. 1)

We next examined the effect of the same treatment in several other human cancer cell lines that, like HT-29 [13], express *DLC1* at a low level [6,8,17]. Among these cell lines, flavone induced up-regulation of DLC-1 mRNA in MDA-MB-468, MDA-MB-361, and BT20 breast carcinoma cell lines but not in the immortal nontumorigenic breast epithelial cell line MCF10F (Fig. 1) or in HCC cell lines (Focus, 7703) or prostate (22RVi) cancer cell lines (unpublished observations).

Given that ectopic expression of DLC-1 in various tumor cell types has been shown to perturb the cell cycle, inhibit cell growth, or induce apoptosis [4,6,7,8], we examined whether flavone exerts similar effects in breast carcinoma or nontumorigenic breast epithelial cell lines. Flavone treatment resulted in inhibition of the proliferation of MDA-MB-468, MDA-MB-361, BT20, and MCF10F cells that was apparent as early as 24 h and increased progressively for up to 4 days (Fig. 2).

This inhibitory effect on cell growth in malignant cell lines (MDA-MB-468, MDA-MB-361, BT20) was accompanied by changes in cell cycle progression and induction of apoptosis. Flavone treatment thus induced accumulation of cells with a sub-G_1 DNA content (apoptotic cells) as well as of cells arrested at the G_2-M transition of the cell cycle (Fig. 3).

These effects of flavone were not apparent with the nonmalignant cell line MCF10F, which instead manifested G_0-G_1 arrest in response to this agent. The induction of apoptosis in the malignant cell lines by flavone was confirmed by detection of increased caspase-3 activity in the flavone-treated cells (Fig. 4); again, such an effect was not observed in the nonmalignant cells.

We next examined the effect of flavone on expression of p21Waf1, an inhibitor of cell cycle progression, given that flavone-induced inhibition of the growth of colon tumor cells in vitro and in vivo is associated with both the induction of apoptosis and up-regulation of $p21^{Waf1}$

gene expression [16]. Furthermore, p21Waf1 is thought to be a downstream target of DLC-2, a member of the DLC family closely related to DLC-1 [20]. Flavone induced marked increases in the amounts of both $p21^{Waf}1$ mRNA and protein in all three breast cancer cell lines as well as in the nonmalignant breast epithelial cell line (Fig. 5, 6A and 6B).

Given that p53 binds directly to and increases the expression of p21Waf1 [21], we also examined the effect of flavone on the abundance of p53. Flavone increased the amount of p53 in MDA-MB-468 and BT20 cells, which harbor a mutant form of p53, but not in MDA-MB-361 cells, which are p53 negative, or in MCF10F cells, which express wild-type p53 (Fig. 6A and 6B).

To determine whether overexpression of p21 Waf1 was mediated by DLC-1 in the breast cancer cell lines, we transfected the cells with an adenoviral vector encoding DLC-1. Restoration of DLC-1 expression in these cells did not increase the expression of p21Waf1 (Fig. 7).

Finally, we used high-density oligonucleotide microarrays to identify genes differentially expressed in each of the three breast cancer cell lines before and after flavone treatment. Only genes that were expressed in all three cell lines and which showed at least a two fold change in expression level in response to flavone treatment were selected for further analysis. The output data set contained 29 genes whose expression was significantly up-or down-regulated by flavone in all three breast cancer cell lines (Table I). The Correlation Summary Report revealed that the flavone signatures in the estrogen receptor–negative cell lines MDA-MB-468 and BT20 were more similar to each other ($R = 0.92$) than to that in the estrogen receptor–positive cell line MDA-MB-361 ($R = 0.82$ and 0.83, respectively).

The flavone-responsive genes were functionally categorized according to the Gene Ontology classification. Genes were classified on the basis on their cellular localization and molecular function and were mapped to biological pathways with the use of the KEGG and BioCarta Pathways databases. The listed genes encoded intracellular products with the exception of those for the extracellular proteins stanniocalcin 2 (*STC2*) and insulin-like growth factor–binding protein 3 (*IGFBP3*). The Gene Ontology classification revealed the output dataset to contain genes whose products possess binding activity (15 proteins), act as signal transducers (12 proteins), exhibit catalytic activity (9 proteins), participate in the response to stress (7 proteins), contribute to metabolism (6 proteins), regulate cell growth or apoptosis (5 proteins), control transcription (3 proteins), function as chaperones (3 proteins), or are important in DNA repair (1 protein) or drug resistance (1 protein). Most of the flavone-responsive genes have already been implicated in the pathogenesis of malignant neoplasms of the breast (6 proteins) or lung (4 proteins), leukemia (3 proteins), melanoma (3 proteins), or prostate cancer, ovarian cancer, osteosarcoma, or psoriasis (2 proteins each).

The flavone-responsive genes known to be involved in breast cancer development include *GADD* genes, *STC2*, *STMN1* (stathmin 1), and *IGFBP3*. Among these genes, the DNA damage–inducible *GADD34*, *GADD153*, *GADD45A*, and *STC2* were significantly up-regulated in response to flavone treatment. Furthermore, flavone up-regulated the expression of *STC2* in both the estrogen receptor positive (MDA-MB-361) and estrogen receptor negative (MDA-MB-468, BT20) cell lines. The proto-oncogene *STMN1* and *IGFBP3* were significantly down-regulated by flavone treatment in all three cell lines.

4. Discussion

Both genetic and external factors are important in the development and prevention of breast cancer. Among such external factors, diet is thought to play an important role. A low incidence of breast cancer in Asia has been attributed in part to a high intake of flavonoids [22,23]. The incidence of cancer increases when Asian women move to the United States, where the average

diet is rich in fat and confers a strong significant protective effect during adolescence. Flavonoids and flavone exert a broad spectrum of biological effects by targeting genes involved in regulation of tumor cell proliferation, the cell cycle, and apoptosis, thus reducing the risk of cancer or inhibiting the growth of tumor cells [16,24,25,26,27]. Such effects were apparent in the present study. Flavone thus affected the expression of a variety of genes that regulate cell proliferation, the response to DNA damage, and apoptosis. Restoration of DLC-1 expression in breast tumor cells in which expression of endogenous *DLC1* is down-regulated or silenced has been shown to inhibit cell growth in vitro and to suppress the formation of tumors and metastases in athymic nude mice [2,6]. The effects of flavonoids on the expression of proto-oncogenes and tumor suppressor genes in vitro or in vivo have been examined in only a relatively small number of studies with human or rat mammary cells. The induction of apoptosis in mammary gland cells from young adult female rats by the soy isoflavone genistein was accompanied by an increase in the expression of the tumor suppressor gene *PTEN* [28]. A diet rich in two other soy isoflavones increased the expression of *BRCA1* and *BRCA2* in the mammary glands of ovariectomized rats [29]. In immortalized human breast epithelial cells, genistein inhibited cell proliferation by down-regulating expression of the *MET* proto-oncogene and up-regulating that of the tumor suppressor gene *EGR1* as well as that of the immediate-early response genes *FOS* and *JUN* [30]. The inhibition by genistein of the growth of human breast tumor cells derived from an invasive carcinoma was associated with a marked increase in *BRCA2* expression [31]. Collectively, these observations indicate that up-regulation of the expression of tumor suppressor genes by flavone or flavonoids is a contributing factor to their antitumoral activity.

In addition to the increase in the expression of *DLC1* in all three breast carcinoma cell lines flavone inhibited tumor cell proliferation, arrested the cells in G_2-M of the cell cycle, induced up-regulation of $p21^{Waf1}$ and caspase-3–mediated apoptosis was detected. A recent study suggested that DLC-2, which is closely related to DLC-1, functions in a Rho-independent manner and that $p21^{Waf1}$ is a downstream effector of Rho-DLC-2 signaling. Introduction of a DLC-2 vector into breast cancer cells already transfected with $p21^{Waf1}$ cDNA did not further inhibit cell proliferation [20]. In a study with HCC cells, it has been concluded that DLC-2 inhibition of cell growth occurs via a pathway that does not involve $p21^{Waf1}$ [32]. Our present results with breast cancer cells suggest that $p21^{Waf1}$ is not a downstream effector of DLC-1 and that flavone stimulation of $p21^{Waf1}$ expression is independent of that of DLC-1 expression.

Although ectopic expression of DLC-1 in HCC cells was previously shown to induce accumulation of hypodiploid (apoptotic) cells, arrest of the cell cycle at G_2-M was not observed [8]. The flavone-induced G_2-M arrest apparent in breast cancer cells in the present study was likely mediated by $p21^{Waf1}$, which induces such arrest in a variety of cell types [33,34,35, 36]. In contrast to its effects in malignant cells, flavone induced G_0-G_1 arrest without apoptosis in nonmalignant breast epithelial cells, suggesting that nonmalignant cells might be able to regain their proliferative capacity.

Microarray analysis revealed that flavone induced deregulation of several genes already implicated in breast cancer. The *GADD* family of genes is implicated in the induction of apoptosis [37]. The observed up-regulation of *GADD34*, *GADD153*, and *GADD45A* as well as that of *DLC1*, which also induces apoptosis, might be responsible for the induction of programmed cell death in flavone-treated breast tumor cells. Although a contribution of $p21^{Waf1}$ cannot be ruled out, the induction of apoptosis does not appear to be a consequence of increased $p21^{Waf1}$ expression in most cell types examined [38]. STC2 possesses growth-inhibitory activity and its expression correlates with that of the estrogen receptor in breast tumors [39,40]. Flavone-induced up-regulation of *STC2* was apparent in both estrogen receptor–positive and –negative breast cancer cell lines in the present study.

Among the genes whose expression was down-regulated by flavone treatment were *IGFBP3*, which encodes a positive regulator of cell proliferation and whose overexpression predicts an unfavorable prognosis in breast cancer [41], and *STMN1*, an important proto-oncogene in breast cancer progression [42,43]. *STMN1* is overexpressed in breast tumors harboring mutant p53 and is responsible for resistance to microtubule-based anticancer treatment [42], suggesting that down-regulation of *STMN1* expression by flavone may reduce breast tumor cell resistance to such treatment. Silencing of *STMN1* expression by RNA interference in breast cancer cell lines harboring p53 mutations was found to inhibit cell proliferation, restore cell cycle regulation, and induce apoptosis [43]. Targeting of *STMN1* in breast cancers with p53 mutations, which are frequently more aggressive and refractory to therapy, may thus represent a potential new therapeutic approach. In this context and given that more than half of all human cancers lack p53 function [44,45], our observations on the effects of flavone in breast cancer cells with p53 mutations may also be relevant and have therapeutic implications. Our data indicate that flavone-induced up-regulation of the expression of $p21^{Waf1}$ is independent of the expression of its binding partner p53, given that increased expression of $p21^{Waf1}$ was detected in tumor cells in which the p53 gene is mutated. Flavone also induced apoptosis in these cells. The flavonoid apigenin was also shown to induce apoptosis in HT-29 colon carcinoma cells [in which the p53 gene is also mutated [46].

Flavone and flavonoids have been shown to reactivate the expression of genes silenced by methylation, including tumor suppressor genes. (–)Epigallocatechin-3-gallate, the major polyphenol in green tea, was shown to inhibit DNA methyltransferase activity and to reverse the methylation of several genes including that of a tumor suppressor gene in cancer cell lines [47]. Genistein and related soy isoflavones were also shown to reactivate the gene for the tumor suppressor p16INK4a as well as those for retinoic acid receptor β, O6-methylguanine methyltransferase, and mut L homolog 1 in colon, esophageal, and prostate cancer cell lines [48]. Our results now suggest that flavone manifests a similar activity in breast cancer cells. Flavone thus restored the expression of *DLC1* in breast and colon carcinoma cell lines all of which manifest silencing of the endogenous gene as a result of promoter hypermethylation. Promoter hypermethylation is a major mechanism of *DLC1* down-regulation and inactivation in many other types of solid tumors and hematological malignancies [13,17,49], and reactivation of *DLC1* expression by dietary constituents thus warrants further investigation.

Various dietary compounds have been shown to have potential therapeutic benefits [48,50, 51,52]. Our present results obtained with cell lines derived from aggressive or metastatic breast tumors suggest the possibility that administration of flavone, alone or in combination with DNA methyltransferase and histone deacetylase inhibitors, may be an effective adjunct to chemotherapy.

Acknowledgements

This work was supported by Intramural Research Program of the NIH, National Cancer Institute. Bethesda, Maryland, USA

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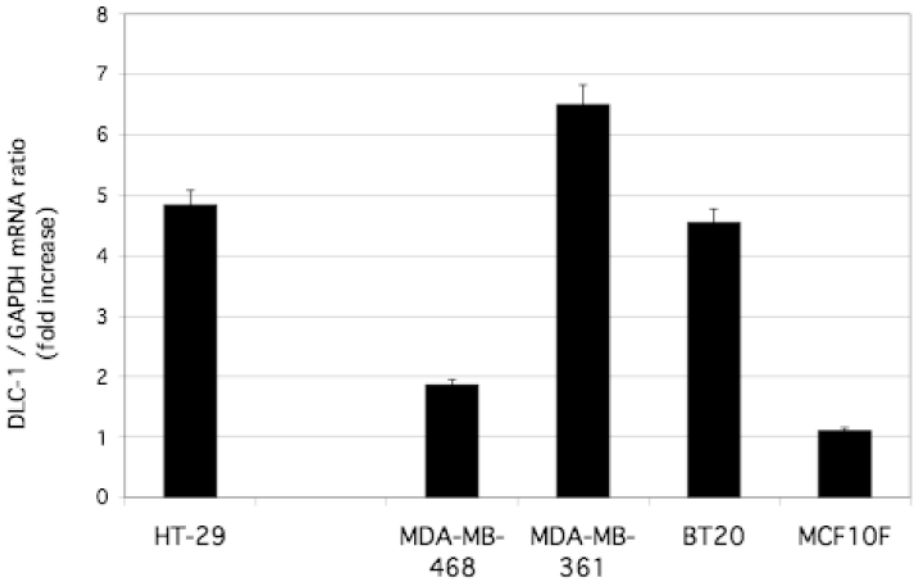

Figure 1.

Effects of flavone on DLC1 expression in HT-29 colon carcinoma cells, breast carcinoma cells (MDA-MB-468, MDA-MB-361, BT20), and a normal breast epithelial cell line (MCF10F). Cells were incubated in the absence or presence of 150μM flavone for 24 h, after which total RNA was isolated and subjected to quantitative RT-PCR analysis of DLC-1 mRNA. The level of normalized DLC1 expression for flavone-treated cells is shown relative to that in nontreated cells (see materials and methods). Data are the means ± SEM from independent experiments.

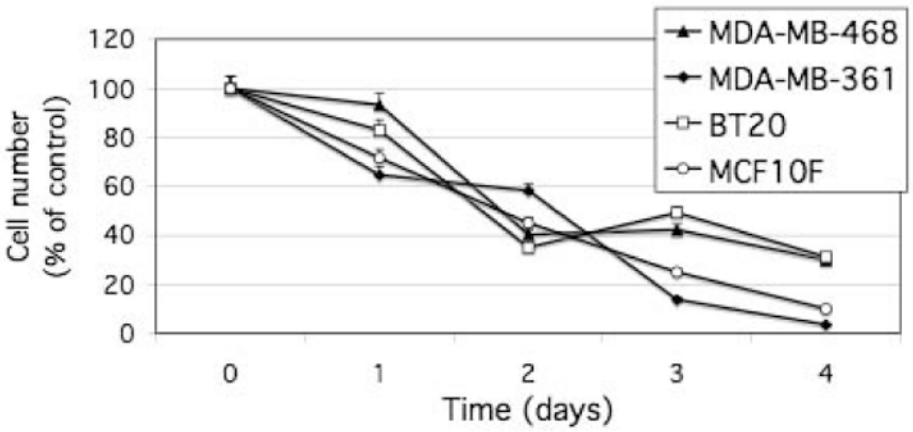

Figure 2.

Inhibition of cell proliferation by flavone in breast carcinoma cell lines and a normal breast epithelial cell line. Cells were incubated in the absence or presence of 150μM flavone for the indicated times, after which cell number was determined by the MTT assay. Cell number in flavone-treated cultures was expressed as a percentage of that in control cultures. Data are means ± SD of triplicates from a representative experiment.

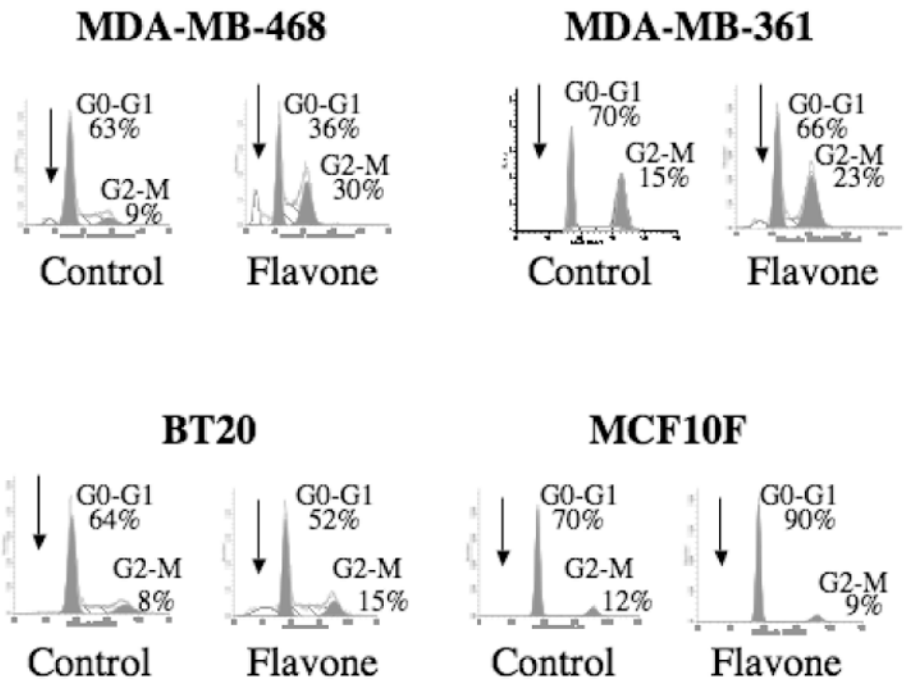

Figure 3.

Effect of flavone on cell cycle distribution in breast carcinoma cell lines and a nonmalignant breast epithelial cell line. Cells were incubated in the absence (control) or presence of 15μM flavone for 24 h, after which DNA content was determined by flow cytometry. Arrows indicate the sub-G_1 population, and the percentages of cells in G_0-G_1 or G_2-M are shown.

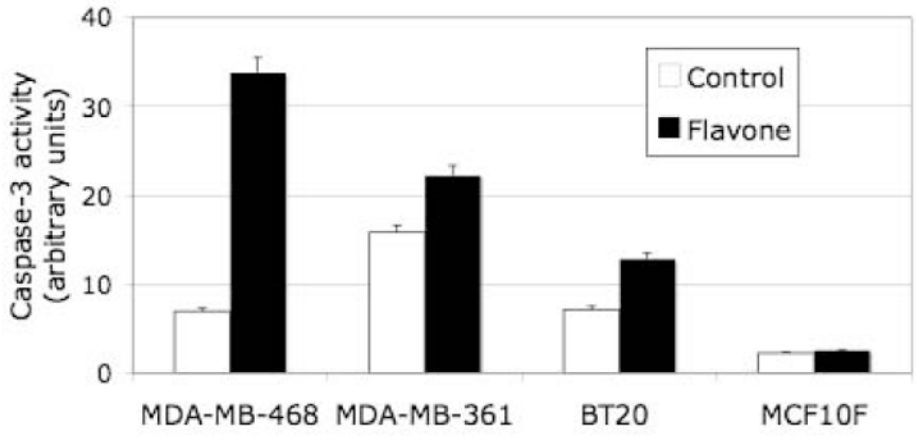

Figure 4.

Effect of flavone on caspase-3 activity in breast carcinoma cell and nonmalignant breast epithelial cells. Cells were incubated in the absence or presence of 15μM flavone for 24 h, after which the activity of caspase-3 was determined. Data are expressed in arbitrary units and are means ± SD of triplicates from a representative experiment.

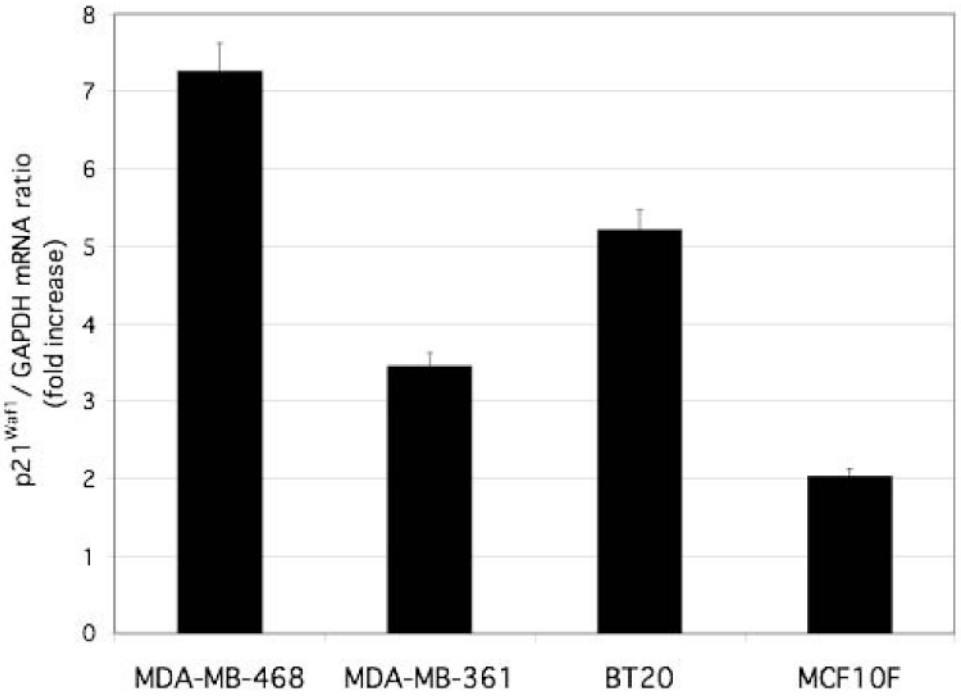

Figure 5.
Effect of flavone on p21Waf1 gene expression in breast carcinoma cells and nonmalignant breast epithelial cells. Cells were incubated in the absence or presence of 150μM flavone for 24 h, after which total RNA was isolated and subjected to quantitative RT-PCR analysis of p21Waf1 mRNA. Data were normalized by the amount of GAPDH mRNA, and the effect of flavone was evaluated by the $2^{-\Delta\Delta ct}$ method and considered significant when $2^{-\Delta\Delta ct}$ was ≥2 or ≤0.5. The abundance of p21Waf1 mRNA in flavone-treated cells is shown relative to that in nontreated cells. Data are the means ± SEM from independent experiments.

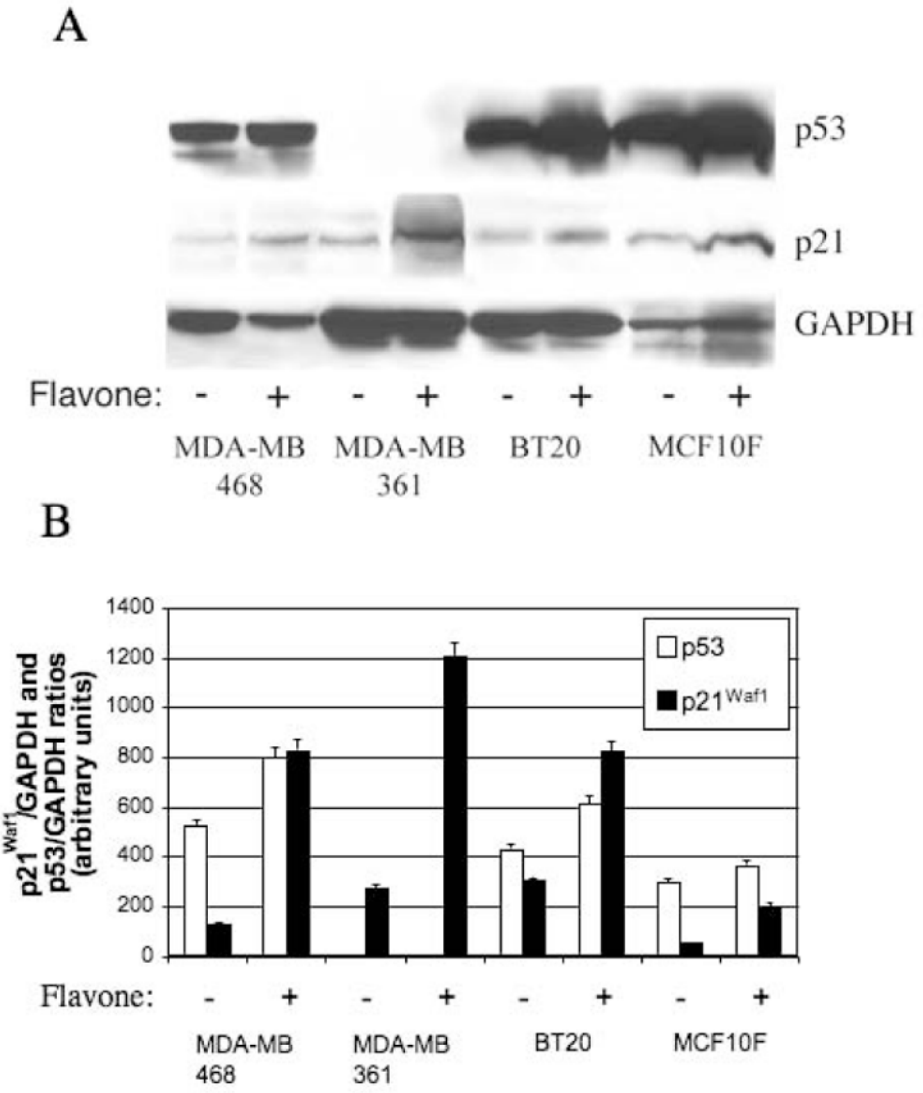

Figure 6.

Effects of flavone on p21Waf1 and p53 abundance in breast cancer cell lines) and a normal breast epithelial cell line. (**A**) Cells were incubated in the absence or presence of 15μM flavone for 24 h, after which cell lysates were subjected to immunoblot analysis with antibodies to p53, to p21Waf1, or to GAPDH. (**B**) Blots similar to that in (**A**) were subjected to densitometric analysis, and the amounts of p21Waf1 and p53 were normalized by that of GAPDH.

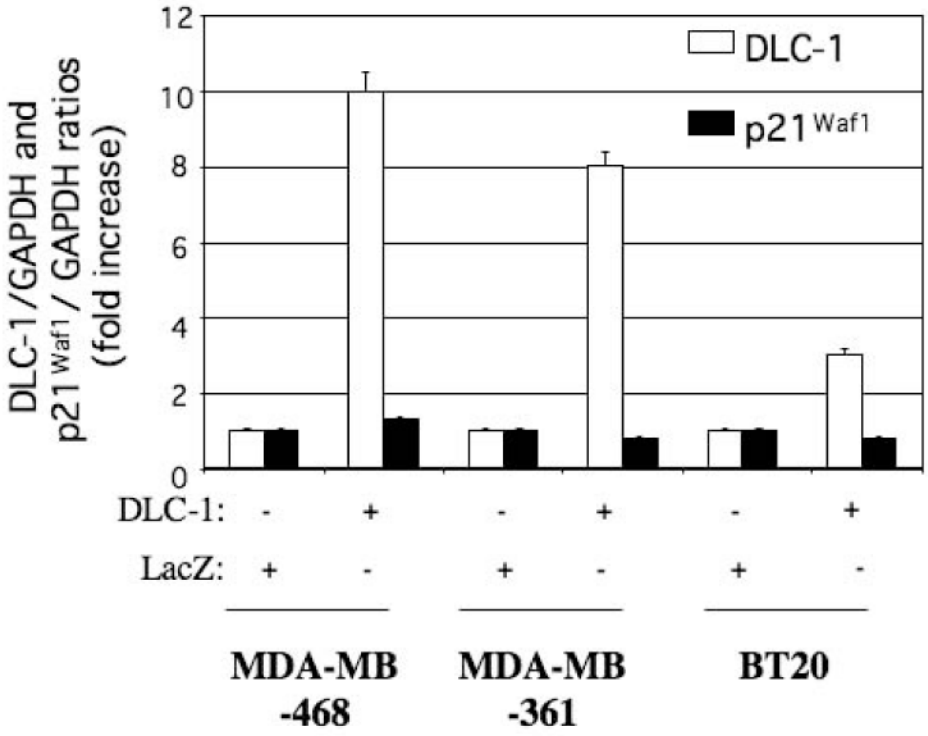

Figure 7.

Effect of ectopic DLC-1 expression on p21Waf1 abundance in the breast cancer cell lines MDA-MB-468, MDA-MB-361, and BT20. Cells were transfected with expression vectors for DLC-1 or LacZ (control), and the abundance of DLC-1 and p21Waf1 mRNAs was subsequently determined by quantitative RT-PCR analysis. Data were normalized by the amount of GAPDH mRNA, and the normalized data were expressed relative to the corresponding value for cells transfected with the control vector. Differences between cells transfected with the DLC-1 vector and those transfected with the control vector were evaluated by the $2^{-\Delta\Delta ct}$ method and considered significant when $2^{-\Delta\Delta ct}$ was ≥ 2 or ≤ 0.5. Data are the means ± SEM from independent experiments.

Table I

List of flavone-responsive genes in breast cancer cell lines MDA-MB-468, MDA-MB-361 and BT20 revealed by micro-array analysis. Gene involvement in the cellular biology was determined by KEEG/ BioCarta pathway and Gene Ontology reports. Their relationship to human disease was obtained from NCI Gene Report and online Gene Cards database.

Gene	UniGene ID	Description	Biological Pathway and Function	Disease relationship
Table 1: Up-regulated genes				
DDIT3 (GADD153)	Hs.505777	DNA-damage-inducible transcript 3	B, CC/AP, p38 MAPK SP, TR	Cell injury, colon carcinoma, liposarcoma myxoid, malignant neoplasm musculoskeletal, melanoma, myeloid leukemia, osteosarcoma
SRXN1	Hs.516830	Sulfiredoxin 1 homolog (S. c.)	B, CA, response to oxidative stress	
SQSTM1	Hs.437277	Sequestosome 1	Response to stress, RANK SP	Paget disease of bone
HMOX1	Hs.517581	Heme oxygenase (decycling) 1	B, CA, IL-10 anti-inflammatory SP, porphyrin and chlorophyll metabolism, regulation of I-kappa B /NF-kappa B SP, response to oxidative stress	Atherosclerosis, cell injury, cerebral vasospasm, inflammation, ischemia, jaundice neonatal, malaria cerebral, shock
PPP1R15A (GADD34)	Hs.76556	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	CC/AP, response to DNA damage stimulus	Herpes simplex, malignant neoplasm
SLC20A1	Hs.187946	Solute carrier family 20 (phosphate transporter), member 1	Regulation of I-kappa B /NF-kappa B SP	
AKR1C3	Hs.78183	Aldo-keto reductase family 1, member C3	CA, prostaglandin and leukotriene metabolism	
STC2	Hs.233160	Stanniocalcin 2	B, cell-cell signaling, response to nutrient	Malignant neoplasm of breast
OSIL		Ubiquitin-binding protein p62		
HERPUD1	Hs.146393	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	Destruction of misfolded proteins by the ER-associated protein degradation system, unfolded protein response	
PRNP	Hs.472010	Prion protein (p27-30)	B, response to oxidative stress	Creutzfeldt-Jakob disease, fatal familial insomnia, Gerstmann-Straussler disease, Huntington disease-like 1, kuru
RABGAP1	Hs.271341	RAB GTPase activating protein 1		
GADD45A	Hs.80409	Growth arrest and DNA-damage-inducible	CC/AP, hypoxia, p53 SP, response to stress	Ataxia telangiectasia, lung carcinoma, leukemia, malignant neoplasm of breast, melanoma, ovarian epithelial carcinoma
PBEF1	Hs.489615	Pre-B-cell colony enhancing factor 1	B, CA, nicotinate and nicotinamide metabolism, positive regulation of cell proliferation, ST	Systemic infection
TMEM38B	Hs.411925	Transmembrane protein 38B		
NEU1	Hs.520037	Sialidase 1	CA, glycosphingolipid metabolism, N-Glycan degradation	Influenza, malignant neoplasm of breast and metastasis, sialidosis
SEC63	Hs.529957	SEC63-like (S. c.)	B, ST	Autosomal dominant polycystic liver disease (pcld)
DKFZP434 F0318	Hs.23388	Hypothetical protein		
DNAJB9	Hs.6790	DNAJ (Hsp40) homolog, subfamily B, member 9	B, chaperon regulator	
Table 1: Down-regulated genes				
RAD51L3	Hs.125244	RAD51-like 3 (S. c.)	B, CA, recombination repair of DNA	Malignant neoplasm of breast
RARRES3	Hs.17466	Retinoic acid receptor responder	Tumor suppressor, growth regulator	Malignant neoplasm, psoriasis
STMN1	Hs.209983	Stathmin 1/oncoprotein 18	B, drug resistance, MAPK SP	Acute erythroblastic leukemia, lymphoma, lung carcinoma, osteosarcoma, plasmacytoma
FBXO4	Hs.165575	F-box protein 4	CA, ubiquitination	
SUB1	Hs.229641	SUB1 homolog (S. c.)	B, TR	
S100A2	Hs.516484	S100 calcium binding protein A2	B, hypothetical tumor suppressor	Basal cell carcinoma, malignant neoplasm of mouth, melanoma, non-small cell lung carcinoma, papillary carcinoma, psoriasis, squamous carcinoma

Gene	UniGene ID	Description	Biological Pathway and Function	Disease relationship
IGFBP3	Hs.450230	Insulin-like growth factor binding protein 3	B, CC/AP, enzyme regulator, food intake and Energy homeostasis, hypoxia, p53 SP	Acromegaly, hypoglycemia, malignant neoplasm of breast, pituitary dwarfism ii, prostate carcinoma
E2F8	Hs.523526	E2F transcription factor 8	B, TR	
AMOT	Hs.528051	Angiomotin	B, regulation of vascular permeability and angiogenesis	
TUBB	Hs.533059	Beta tubulin	B, CA, gap junction, ST	Cerebral amyloid angiopathy familial, ciliary motility disorders, non-small cell lung carcinoma, ovarian epithelial carcinoma, polyneuropathy, prostate carcinoma,

B= binding activity, CA= catalytic activity, CC/AP= cell cycle/apoptosis, SP = signaling pathway, ST= signal transducer, TR= transcription regulator activity