SPANDIDOS PUBLICATIONS

5-Fluorouracil combined with apigenin enhances anticancer activity through induction of apoptosis in human breast cancer MDA-MB-453 cells

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Abstract. We investigated the effects of combined treatment with 5-fluorouracil and apigenin on proliferation and apoptosis, as well as the underlying mechanism, in human breast cancer MDA-MB-453 cells. The MDA-MB-453 cells, which have been shown to overexpress ErbB2, were resistant to 5-fluorouracil; 5-fluorouracil exhibited a small dose-dependent anti-proliferative effect, with an IC_{50} of 90 μM. Interestingly, combined treatment with apigenin significantly decreased the resistance. Cellular proliferation was significantly inhibited in cells exposed to 5-fluorouracil at its IC_{50} and apigenin (5, 10, 50 and 100 μM), compared with proliferation in cells exposed to 5-fluorouracil alone. This inhibition in turn led to apoptosis, as evidenced by an increased number of apoptotic cells and the activation of caspase-3. To investigate the mechanism by which the combination of 5-fluorouracil and apigenin induces apoptosis, ErbB2 expression was analyzed. The level of ErbB2 was unchanged by 5-fluorouracil alone but was drastically reduced in cells treated with 5-fluorouracil plus apigenin. Moreover, compared with 5-fluorouracil alone, 5-fluorouracil in combination with apigenin at concentrations >10 μM exerted a pro-apoptotic effect via the inhibition of Akt expression. Taken together, our results suggest that 5-fluorouracil acts synergistically with apigenin inhibiting cell growth and inducing apoptosis via the down-regulation of ErbB2 expression and Akt signaling.

Introduction

Breast cancer is one of the most frequently diagnosed cancers in women, and its occurrence has been increasing in recent years. It is now the major cause of mortality and morbidity in Korean women, which may be attributable to changes from traditional to westernized lifestyles.

As a chemotherapy agent, 5-fluorouracil, an active metabolite of capecitabine, is widely used for the treatment of solid tumors such as breast cancers (1,2). However, the development of resistance is a major problem in the use of drugs such as 5-fluorouracil and limits the clinical utility of the drug. Attempts to solve this problem have taken various approaches, including the combined use of cancer drugs. In addition, it was recently shown that combined treatment with natural phytochemicals increased the efficiency and reduced the cytotoxicity of some cancer drugs.

Apigenin (4',5,7-trihydroxyflavone) is a member of the flavone subclass of flavonoids present in fruits and vegetables (3) and is considered to have various biological activities such as anti-inflammatory, anticancer and free-radical scavenging properties (4-7). Studies of human malignant cancer cell lines have shown that apigenin inhibits cancer cell growth via the promotion of cell cycle arrest and apoptosis (8,9). As a candidate anticancer agent, apigenin is of particular interest because it exhibits selective induction of cell cycle arrest and apoptosis in human prostate carcinoma cells without affecting normal cells (10,11). Moreover, apigenin is non-mutagenic and less cytotoxic than other flavonoids (12).

Therefore, in this study, we investigated the effects of 5-fluorouracil combined with apigenin on cellular proliferation and apoptosis using human breast cancer MDA-MB-453 cells, and we explored the underlying mechanism.

Materials and methods

Cell culture and apigenin treatment. Human breast cancer MDA-MB-453 cells were purchased from the KCLB (Korean Cell Line Bank, Korea). Cells was routinely maintained in RPMI-1640 (Gibco), supplemented with 10% FBS and antibiotics (50 U/ml of penicillin and 50 μg/ml streptomycin, Gibco) at 37˚C in a humidified atmosphere containing 5% CO_2. In cell proliferation analysis experiments, cells were treated with either 5-fluorouracil or apigenin alone, or vehicle alone for 24, 48 and 72 h. For apoptosis assay, cells were treated with 5-fluorouracil or apigenin either alone or combined, or vehicle alone for 72 h. Apigenin and 5-fluorouracil was purchased from Sigma and dissolved in DMSO (final concentration 0.1% in medium).

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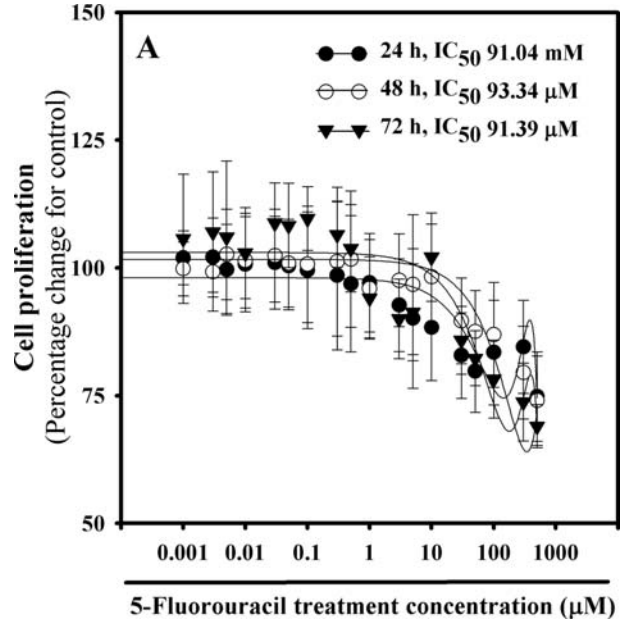

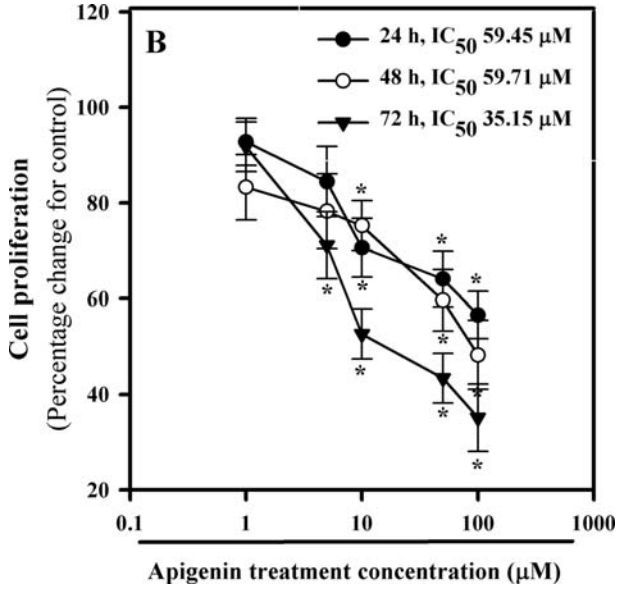

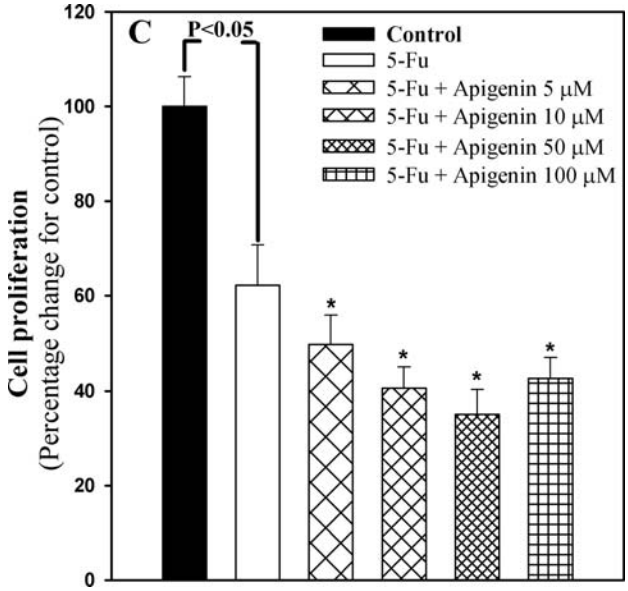

Figure 1. Effect of apigenin or 5-fluorouracil on cell proliferation of human breast cancer MDA-MB-453 cells. (A and B) Cells were exposed to either 5-fluorouracil (0.001-500 μM) or apigenin (1-100 μM) and incubated for 24, 48 and 72 h. (C) Cells were exposed to 5-fluorouracil at its IC_{50} concentration with apigenin (5, 10, 50 and 100 μM) for 72 h. All data are reported as the percentage change in comparison with the vehicle-only group, which were arbitrarily assigned 100% viability. *P<0.05, significantly different from the vehicle-only group (0.1% DMSO in medium, that is, apigenin concentration = 0).

Cell proliferation and cell death assay. Cell proliferation was determined using the MTT assay. Methyl thiazolyl tetrazolium (MTT) was added in cells exposed to either 5-fluorouracil or apigenin. Four hours later, DMSO was added to each well to dissolve the resulting formazan crystals and then absorbance was recorded at 490 nm in a microplate reader (SpectraMax Plus; Molecular Devices). The value of IC_{50} (i.e., the concentration of the extract required to inhibit cancer cell proliferation by 50% of the control level, which is each cells treated with only compound solvent) was estimated from the plot.

Cell apoptosis assay. Annexin V-FITC apoptosis kit (BD ApoAlert™, BD Biosciences Clontech, CA, USA) was used for apoptosis detection. Cells were trypsinized, washed twice in ice-cold PBS, and resuspended in 500 μl binding buffer (Sigma). Annexin V and propidium iodide solution were added to the cell preparations and incubated for 25 min in the dark. Binding buffer (400 μl) was then added to each tube and the samples were analyzed by a FACS Calibur instrument (BD Biosciences) equipped with CellQuest 3.3 software.

Immunoblotting assay. Cells were lysed in RIPA buffer (1% NP-40, 150 mM NaCl, 0.05% DOC, 1% SDS, 50 mM Tris, pH 7.5) containing protease inhibitor for 1 h at 4˚C. The supernatant was separated by centrifugation, and protein concentration was determined by Bradford protein assay kit II (Bio-Rad Laboratories, CA, USA). Proteins (25 μg/well) denatured with sample buffer were separated by 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (0.45 μm). The membranes were blocked with a 1% BSA solution for 3 h and washed twice with PBS containing 0.2% Tween-20, and incubated with the primary antibody overnight at 4˚C. Antibodies against cleaved caspase-3 and ß-actin were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., CA, USA) and used to probe the separate membranes. ErbB2, Akt, and phosphor-Akt were purchased from the Cell Signalling (Cell Signaling Technology, Inc., CA, USA), and caspase-3 and ß-actin were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc.). The next day, the immunoreaction was continued with the secondary goat anti-rabbit horseradish-peroxidase-conjugated antibody after washing for 2 h at room temperature. The specific protein bands were detected by Opti-4CN Substrate kit (Bio-Rad).

Apoptosis detection. For annexin-based FACS analysis, cells were trypsinized, washed twice in ice-cold PBS, and resuspended in 500 μl binding buffer (Sigma). Annexin V and propidium iodide solution were added to the cell preparations

ated for 25 min in the dark. Binding buffer (400 μl) added to each tube and the samples were analyzed by a FACS Calibur instrument equipped with CellQuest 3.3 software.

For caspase-3 activity analysis, cells were collected by trypsinization and lysed with lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin, 1 mg/ml leupeptin). Thereafter, the lysates were transferred to wells in a 96-well flat-bottom plate. A peptide with the caspase-3 target motif DEVD bound to the chromophore *p*-nitroanilide was added and incubated at 37˚C for 1 h. The intensity of the developed color was read at 405 nm in a microplate reader. In addition, cleaved caspase-3 expression was determined using immunoblotting assay as described above.

Statistical analyses. All data were expressed as percent compared with vehicle-treated control cells, which were arbitrarily assigned 100%. Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test (Sigma Stat, Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at $P<0.05$.

Results

Effect of 5-fluorouracil or apigenin on the cell proliferation of human breast cancer MDA-MB-453 cells. As presented in Fig. 1, the effects of 5-fluorouracil or apigenin on the cell proliferation of human breast cancer MDA-MB-453 cells were measured with the MTT assay. 5-Fluorouracil exhibited a small dose-dependent anti-proliferative effect, with an IC_{50} of 90 μM regardless of treatment time (Fig. 1A). In contrast, apigenin significantly inhibited cellular proliferation in a dose- and time-dependent manner ($P<0.05$, Fig. 1B); a significant anti-proliferative effect was observed in cells exposed to 5 μM apigenin for 72 h.

To determine the conditions necessary to produce a synergistic effect between the two compounds, cellular proliferation was investigated in cells exposed to 5-fluorouracil at its IC_{50} and apigenin at concentrations exceeding 5 μM (Fig. 1C). Combined treatment with 5-fluorouracil and 10 μM apigenin produced a first significant anti-proliferative effect. There was no significant difference in the anti-proliferative effect among the combined treatments with different concentrations of apigenin; all of the combined treatments produced a 56-68% reduction in proliferation, compared with that produced by 5-fluorouracil alone.

Effect of 5-fluorouracil or apigenin on ErbB2 and Akt expression of human breast cancer MDA-MB-453 cells. Compared with MCF-7 cells, MDA-MB-453 cells were shown to overexpress ErbB2 (Fig. 2A). Although the treatment of MDA-MB-453 cells with 5-fluorouracil alone slightly lowered ErbB2 expression compared with the expression in control cells, a significantly greater reduction was observed following combined treatment with 5-fluorouracil and 50 or 100 μM apigenin.

Under the conditions described above (i.e., MDA-MB-453 cells exposed to 5-fluorouracil at its IC_{50} combined with apigenin at 5, 10, 50 or 100 μM for 72 h), Akt expression and Akt phosphorylation were significantly decreased (Fig. 2B).

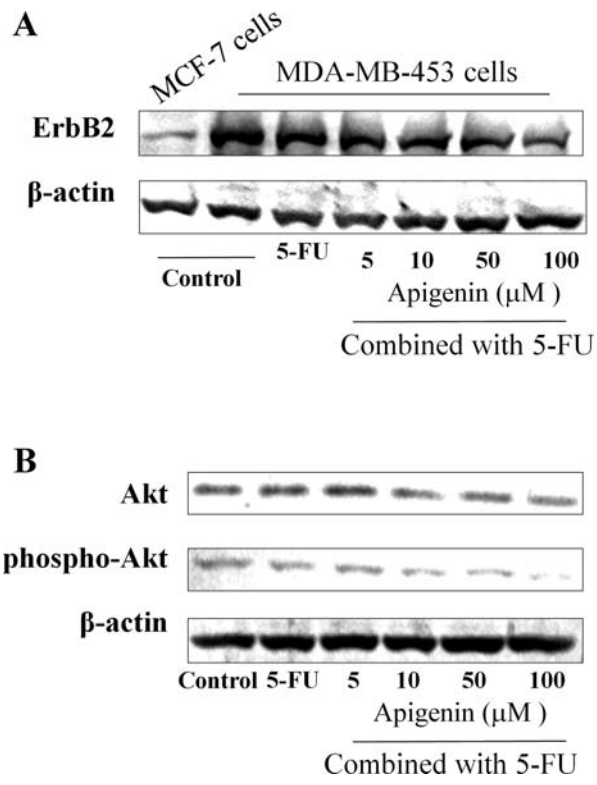

Figure 2. Effect of 5-fluorouracil combined with apigenin on ErbB2 (A) and Akt (B) expresssions of human breast cancer MDA-MB-453 cells. MDA-MB-453 cells were exposed to 5-fluorouracil at its IC_{50} concentration combined with apigenin (5, 10, 50 and 100 μM) for 72 h.

Effect of 5-fluorouracil combined with apigenin on the apoptosis induction of human breast cancer MDA-MB-453 cells. Treatment with 5-fluorouracil alone significantly increased the number of apoptotic cells, by 31.49% compared with control cells (Fig. 3A). In comparison, combined treatment with 5-fluorouracil and apigenin at concentrations >10 μM significantly increased the number of apoptotic cells by ~50% compared with the increase in apoptosis with 5-fluorouracil alone ($P<0.05$).

As shown in Fig. 3B, cleaved caspase-3 expression was clearly increased by treatment with 5-fluorouracil and apigenin. Consistent with this result, a significant increase in caspase-3 activity was observed in cells exposed to 5-fluorouracil and apigenin at concentrations exceeding 10 μM. Combined treatment with apigenin at 50 or 100 μM increased caspase-3 activity by 1.6- and 1.7-fold, respectively, compared with the activity in cells treated with 5-fluorouracil alone.

Discussion

Apigenin has been shown to efficiently inhibit proliferation in various breast cancer cell lines (13,14), which is in agreement with our results showing the dose- and time-dependent inhibition of human breast cancer MDA-MB-453 cell proliferation by apigenin. In contrast, the anti-proliferative effects of 5-fluorouracil were observed only at concen-

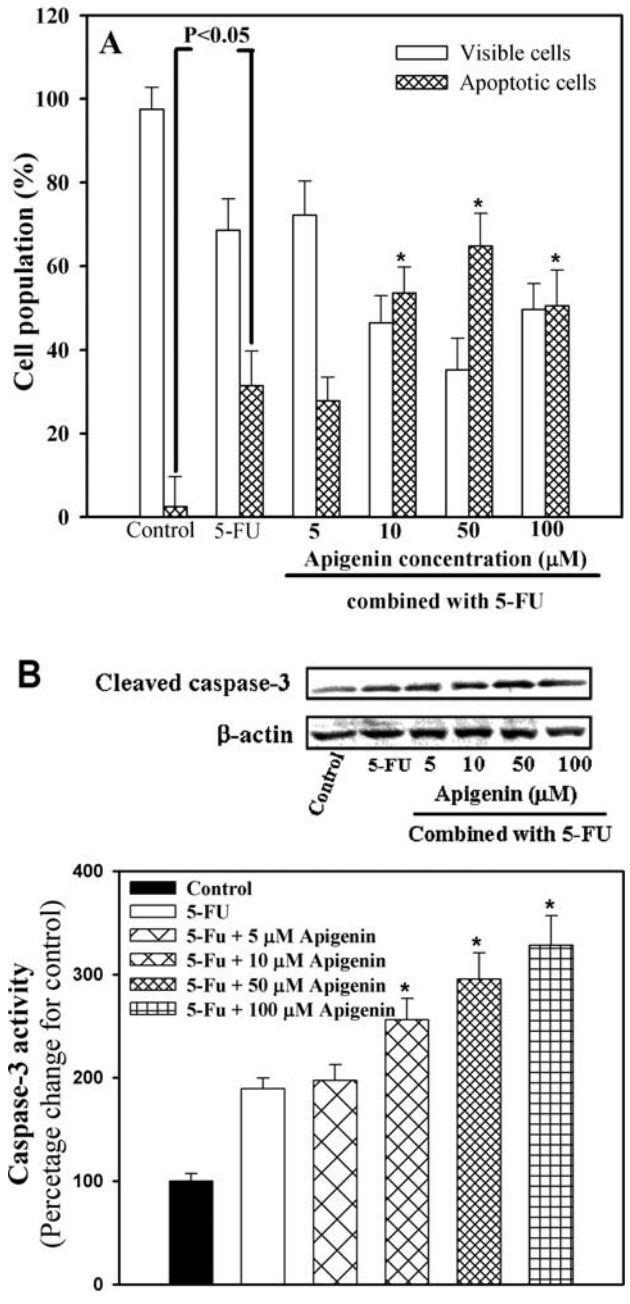

Figure 3. Effect of 5-fluorouracil combined with apigenin on apoptosis induction of human breast cancer MDA-MB-453 cells. Cells were exposed to 5-fluorouracil at its IC_{50} concentration combined with apigenin (5, 10, 50 and 100 μM) for 72 h. Apoptosis was determined using Annexin V-FITC apoptosis kit (A), caspase-3 expression and its activity (B). *P<0.05, significantly different from the vehicle-only group (0.1% DMSO in medium, that is, 5-fluorouracil or apigenin concentration = 0).

trations higher than those of apigenin (IC_{50}=35.15 μM). Previous studies have reported an IC_{50} of greater than 170 μM 5-fluorouracil in MDA-MB-453 cells (15). In the present study, treatment with 5-fluorouracil combined with apigenin at concentrations greater than 10 μM produced a significant anti-proliferative effect compared with that produced by treatment with 5-fluorouracil alone.

The reason for the poor response to 5-fluorouracil alone may be that the overexpression of ErbB2 renders human breast cancer cells more resistant to certain chemotherapeutic agents (15,16). It has been reported that *ErbB2* is distinct from other tumor-promoting genes in that it enhances the intrinsic metastatic potential of MDA-MB-435 cells without increasing their capacity for transformation (16). *ErbB2* (*HER-2/neu*) encodes a 185-kDa transmembrane glycoprotein (ErbB2) that belongs to the epidermal growth factor receptor family of type I receptor tyrosine kinases (ErbB family). ErbB2 overexpression has been reported in approximately 25-30% of human breast cancers (17,18).

Several mechanisms have been proposed to explain the inhibition of cancer cell growth by apigenin; these include the arrest of the cell cycle, the induction of apoptosis, and the modulation of signal transduction (19,20). It has been suggested that apigenin-induced apoptosis results from the depletion of ErbB2 following the dissociation of a complex containing ErbB2 and GRP94 (8,21). Aberrant ErbB2 receptor tyrosine kinase expression in breast tumor or cancer cell lines affects a range of signal transduction pathways that regulate basic cellular processes such as proliferation and survival (15,16,22).

In the present study, ErbB2 expression was higher in MDA-MB-453 cells than in MCF-7 cells. Exposure to 5-fluorouracil alone did not affect the expression of ErbB2 in MDA-MB-453 cells; however, combined treatment with 5-fluorouracil and apigenin at 50 or 100 μM strongly decreased ErbB2 expression. Structure-activity analyses (21) indicate that flavonoid induced down-regulation of ErbB2 in dependent with the presence of a B ring, a 3',4'-hydroxyl group, and a 2-phenyl group on the flavonoids structure. Notably, apigenin has these features and, in combination with 5-fluorouracil, appears to efficiently down-regulate ErbB2 expression.

The serine/threonine kinase protein kinase B or Akt (PKB/Akt), is a downstream target of phosphoinositide 3-kinase (PI3K), which is activated as a result of the ligand-dependent activation of receptor tyrosine kinases such as those of the ErbB receptor family. Akt plays critical regulatory roles in mammalian cell signaling, affecting such divergent cellular processes as apoptosis, cellular proliferation, differentiation, and metabolism (23,24). Akt is also activated in various cancer cells, and thus Akt signaling has become a target for cancer chemotherapy.

Flavonoids such as genistein and quercetin have been shown to play a role in Akt signaling (25,26), and the inhibition of Akt activity may facilitate the inhibition of proliferation and the induction of apoptosis in cancer cells. Several reports have suggested that apigenin exerts its anticancer effects by blocking the Akt pathway (25,27,28). In the present study, 5-fluorouracil combined with apigenin decreased both the expression and phosphorylation of Akt in an apigenin dose-dependent manner. Thus, the inhibition of the Akt pathway may be an important mechanism underlying the effects of 5-fluorouracil combined with apigenin in human breast cancer MDA-MB-453 cells.

To further scrutinize these results, we used fluorescence-activated cell sorting FACS to analyze apoptosis in MDA-MB-453 cells treated with 5-fluorouracil and apigenin, using the experimental conditions described above. Similar to the results of our cell proliferation experiments, combined treatment with 5-fluorouracil and apigenin increased the frequency

 sis, particularly at higher concentrations of apigenin (μM), compared with treatment with 5-fluorouracil alone.

Another biomarker of apoptosis, caspase-3 expression and activity, was also considered. Caspase-3 is a major death protease that catalyzes the cleavage of many key cellular proteins. In the present study, the activity of caspase-3 was significantly increased in cells exposed to 5-fluorouracil and apigenin at 50 or 100 μM. Thus, 5-fluorouracil and apigenin appear to have synergistic effects on the induction of apoptosis, at high concentrations of apigenin.

Based on the obtained results, 5-fluorouracil combined with apigenin exhibits anticancer activity via the inhibition of cellular proliferation and the induction of apoptosis. The present study is the first to show that treatment with 5-fluorouracil combined with apigenin stimulates the response of human breast cancer MDA-MB-453 cells to 5-fluorouracil. The significant effect of apigenin on the response to 5-fluorouracil appears to be mediated via the down-regulation of ErbB2 and Akt signaling. The present study suggests that these findings are encouraging for the clinical use of 5-fluorouracil in human breast cancers.

Acknowledgements

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