

Synergistic apoptotic effect of celecoxib and luteolin on breast cancer cells

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Abstract. Breast cancer is heterogeneous and often hormone-dependent. There are many breast cancer treatment options, including endocrine therapy, chemotherapy, radiotherapy and targeted therapy. Unfortunately, not all patients respond to first-line treatments, and others will eventually relapse despite an initial response. Therapeutic options for these patients are limited. In the past decade, several studies have demonstrated the antitumor effect of celecoxib and luteolin in breast cancer as single treatment. The effect of combination treatment of celecoxib and luteolin in human breast cancer cells has not been well characterized. The present study examined the synergistic effect of celecoxib and luteolin on the human breast cancer cell lines MCF-7 and MDA-MB-231. We analyzed cell proliferation, cell death, apoptosis and changes in protein expression by performing cell survival assays, apoptosis assays and western blotting. The combination treatment significantly decreased cancer cell viability, and it had a greater efficiency in killing tumor cells after 72 h of treatment, compared to treatment with either agent alone or the control in a concentration- and time-dependent manner ($P=0.01$). The combination treatment demonstrated a greater than additive increase in breast cancer cell apoptosis ($P=0.01$). Decreased levels of Akt phosphorylation (pAkt) were noted after celecoxib and luteolin combination treatment. The combination of celecoxib and luteolin provided superior inhibition of breast cancer cell growth than either celecoxib or luteolin treatment alone. These results suggest that celecoxib and luteolin combination may be a new possible treatment option for breast cancer.

Introduction

Breast cancer is a serious concern in many countries. Despite public education, cancer prevention and screening, increase in

early diagnosis and advances in cancer management, 200,000 women develop breast cancer and more than 40,000 women die of breast cancer in the United States annually (1,2). Since breast cancer is typically a hormone-dependent cancer, targeted endocrine therapy has led to a significant improvement in the outcomes for women with estrogen receptor (ER)-positive breast cancer. However, not all patients respond to first-line endocrine treatment, and other patients will eventually relapse despite an initial response to the treatment (3). Therapeutic options for these patients are limited. Additionally, patients with hormone receptor-negative breast tumors that are not human epidermal growth factor receptor 2 (HER2)-positive [(i.e., triple-negative breast cancer (TNBC)] account for ~15% of breast cancer patients and these TNBC patients have not benefitted from generally well-tolerated, anti-HER2 drugs or targeted endocrine treatment strategies (4-6). Although considerable progress has been achieved through the development of new drugs and treatment strategies, current therapy is unable to elicit a clinical response in these patients (7).

In the last two decades, there have been major efforts to identify the signaling mechanisms responsible for these patients, and new therapeutic targets are being explored against cancer signaling pathways. Cyclooxygenase-2 (COX-2) is overexpressed in approximately 70% of *in situ* cases and 60% of invasive breast cancer, and elevated COX-2 expression in invasive breast cancer leads to increased tumor recurrence and decreased patient survival (8,9). COX-2-specific inhibitors have been shown to promote growth arrest and induce apoptosis in various cancers, including breast cancer, by down-regulating the prosurvival signaling pathway, protein kinase B (PKB)/Akt (10,11). The Western New York Exposures and Breast Cancer Study results showed a significant reduction in breast cancer risk associated with recent and adult lifetime non-steroidal anti-inflammatory drug use (odds ratio 0.73, 95% confidence interval: 0.51-1.03) (12). Luteolin, 3', 4', 5, 7-tetrahydroxyflavone, is a common flavonoid that exists in many types of plants including fruits, vegetables, and medicinal herbs and acts as antioxidants, estrogenic regulators, and antimicrobial agents (13). Studies have been demonstrated that the anticancer property of luteolin is associated with inducing apoptosis, which involves redox regulation, DNA damage, protein kinase inhibition to cancel cell proliferation, and suppression of metastasis and angiogenesis (13,14).

The objective of this study was to examine the effect of combination treatment of celecoxib and luteolin in human breast

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cancer cells and to subsequently determine the mechanism of action.

Materials and methods

Cell lines. Estrogen receptor-positive MCF-7 human breast cancer cells and estrogen receptor-negative MDA-MB-231 human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with heat inactivated 10% fetal bovine serum with 300 mg/l L-glutamine, 25 mM HEPES and 25 mM NaHCO₃, 100 µg/ml streptomycin, and 100 U/ml penicillin. The cells were grown in a humidified incubator at 37°C and 5% CO₂ atmosphere.

Cell survival assays. The effect of celecoxib (Celebrex[®], Pfizer Inc., St. Louis, MO, USA) and luteolin (Sigma Chemical Co.) on human breast cancer cell growth was determined by cell counting following XTT (Roche, Mannheim, Germany) assays. The XTT assay was performed as recommended by the manufacturer. One thousand cells were seeded in triplicates in 6-well plates with or without celecoxib and/or luteolin and assayed 72 h later. After incubation, cells treated with celecoxib were fixed in dimethyl sulfoxide (DMSO; Sigma Chemical Co.) and cells treated with luteolin were fixed in isopropyl Alcohol (Duksan pure chemicals, Kyungkido, Korea). Following fixation, the cells were treated with the XTT cell proliferation kit (Roche). The 570-nm absorbance was read using an automated spectrophotometric miniplate reader EL808 (Ultramicroplate reader; Bio-Tek Instruments, Inc., Winooski, VT, USA). The values were normalized and plotted as the percentage change compared to control cells (mean ± standard error of the mean).

Apoptosis assays. Apoptotic MCF-7 and MDA-MB-231 cells were identified using the 5-bromo-2-deoxyuridine/terminal deoxynucleotidyl transferase (TdT)-mediated 2'-deoxyuridine 5'-triphosphate (dUTP)-biotin nick end labeling assay (TUNEL) and Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. TUNEL assay (Apoptosis detection kit s7100; Chemicon international Inc, Temecula, USA) was performed according to the manufacturer's protocol. Briefly, total of 1x10⁵ cells/ml were incubated with celecoxib and/or luteolin for 72 h. Then, the cells were washed and incubated with staining solution (10 µl of TdT reaction buffer, 0.75 µl of TdT enzyme, and 8 µl of FITC-dUTP) overnight. The following day, cells were rinsed and resuspended in 1 ml PI/RNase solution. After incubation in the dark for 30 min at room temperature, a manual cell count was performed to obtain the percentage of apoptotic cells.

Annexin V-FITC and PI staining was performed using the detection kit according to the manufacturer's protocol (BD Pharmingen, CA, USA). Total of 1x10⁵ cells/ml were incubated with celecoxib and/or luteolin for 72 h. The cells were washed with cold phosphate-buffered saline and suspended in 100 ml buffer (10 mM HEPES, 10 mM NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl₂). After 5 ml of Annexin V-FITC and 5 ml of PI were added, the cells were incubated for 15 min at room temperature in the dark. After this incubation, 400 ml of binding buffer solution was added, and the cells were analyzed via

flow cytometry. The quadrant containing Annexin V-FITC-positive/PI-negative cells represents early apoptotic cells, and the quadrant containing Annexin V-FITC-positive and PI-positive cells represents cells undergoing the end stage of apoptosis, necrotic cells or dead cells.

Western blotting. The cells were lysed, and the protein concentration was determined with the Bio-Rad colorimetric Assay (Bio-Rad Laboratories, Hercules, CA, USA). Lysates were resolved by western blot analysis on 10% sodium dodecylsulfate gels. The lanes were loaded with 50 µg of protein and electrophoresed for 2 h at 90 V. Proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk and incubated with antibodies directed against β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt (Cell Signaling, Beverly, MA, USA), STAT 3 (Cell Signaling) and pAkt (Cell Signaling) overnight at 4°C. Membranes were washed and incubated with secondary antibody for 1 h at room temperature. Membranes were then developed, and protein signals were detected using enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK). Membranes were incubated with antibody against β-actin (Santa Cruz Biotechnology) to assess equal protein loading, and results were subjected to densitometry analysis.

Statistical analysis. Statistical analyses were performed between control and treatment groups and among the different experimental groups. Continuous data are presented as the means and standard error deviations with median values. Comparisons of means were carried out using One way ANOVA. Differences with a value of P<0.05 were considered to be statistically significant.

Results

Effect of celecoxib and luteolin on breast cancer cells in vitro. Breast cancer cells were treated with DMSO, isopropyl alcohol, celecoxib, luteolin, or a celecoxib + luteolin combination treatment. DMSO was used as a control for celecoxib and isopropyl alcohol was used as a control for luteolin. Cell viability was assessed using XTT assays. MCF-7 cells were treated with celecoxib and/or luteolin at various concentrations (0, 10, 25, 50, 75, 100 µM). As shown in Fig. 1A based on the XTT assay results, the viability of the MCF-7 cells was inhibited by treatment with either celecoxib or luteolin after 72 h of treatment in a concentration-dependent manner (celecoxib: P=0.03, luteolin: P=0.04). At a concentration of 50 µM, celecoxib decreased viability to 25.9% compared to controls, and 100 µM celecoxib reduced viability to 73.8% compared to controls. At a concentration of 25 µM, luteolin decreased viability to 34.2% compared to controls, and at 50 µM luteolin reduced viability to 76.3% compared to controls. MDA-MB-231 cells were treated with celecoxib and/or luteolin at various concentration (0, 10, 25, 50, 75, 100 µM). MDA-MB-231 cell viability was inhibited by 72 h of celecoxib treatment in a concentration-dependent manner (Fig. 1B, P=0.01). However, luteolin alone did not inhibit MDA-MB-231 cell viability (P=0.78). At a concentration of 50 µM, celecoxib decreased cell viability to 27.7% compared to controls, and at 100 µM, it reduced cell viability 81.6% compared to controls. At a concentration of

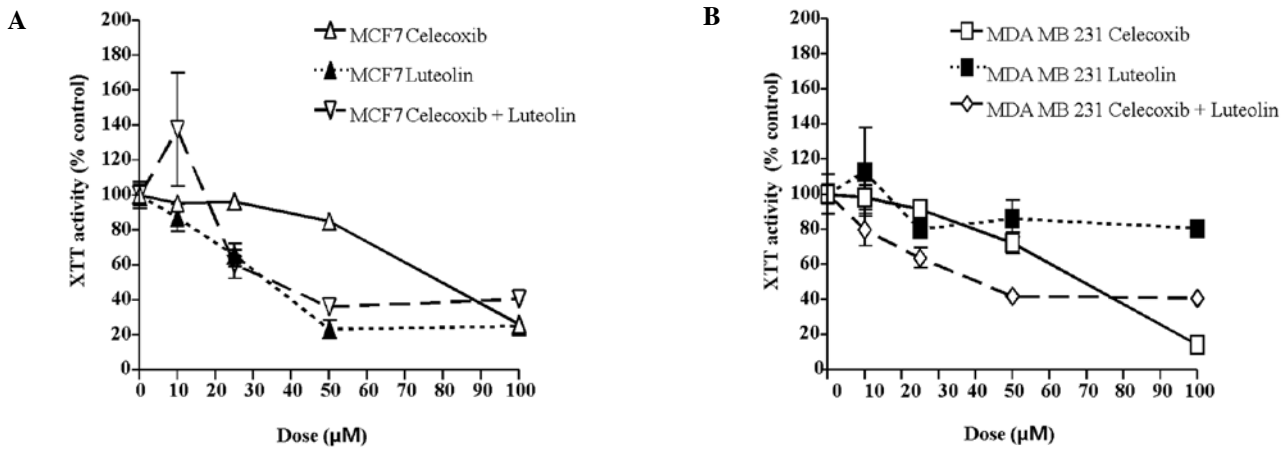


Figure 1. Cell viability of MCF-7 (A) and MDA-MB-231 (B) breast cancer cells. Cells were treated with celecoxib or luteolin or both as indicated for after 72 h. Cell death was determined using the trypan blue exclusion method (*P<0.05).

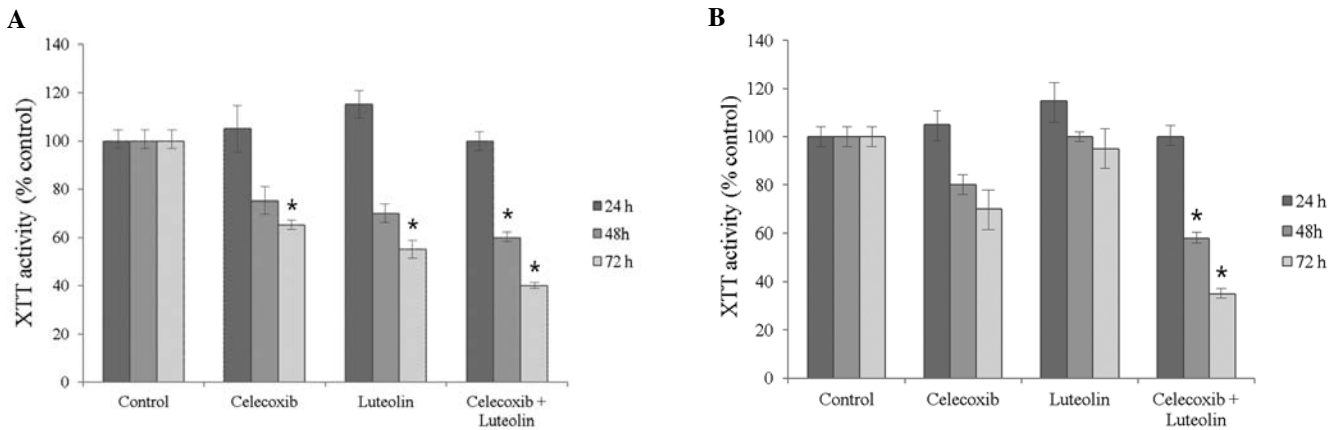


Figure 2. Cell viability of the breast cancer cell lines MCF-7 (A), MDA-MB-231 (B) treated with celecoxib (75 µM) or luteolin (50 µM) or both for 24, 48 and 72 h. Viability was determined using XTT assays (*P<0.05).

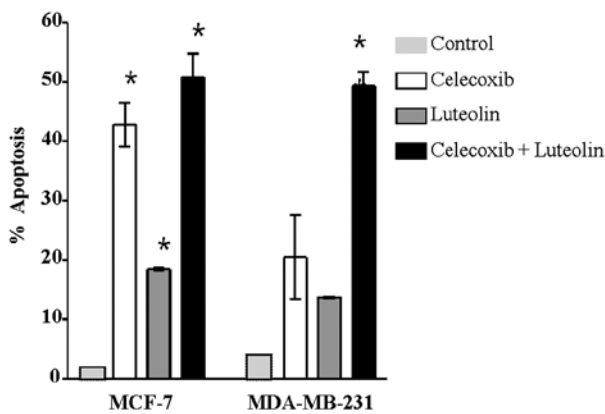


Figure 3. Apoptosis by celecoxib or luteolin or both in MCF-7 and MDA-MB-231 breast cell lines shown by TUNEL assays. The cell lines were treated with celecoxib, luteolin, or the combination. Apoptotic cells were identified by brown staining. Quantification of the apoptotic cells was performed by manual counting (*P<0.05, **P<0.01).

25 µM, luteolin decreased cell viability to 20.0% compared to controls, and at 50 µM, it reduced cell viability to 20.8% compared to controls.

The XTT assays detected no differences in MCF-7 cell viability following treatment with either celecoxib (75 µM) or luteolin (50 µM) after 24 h, but did show decreased viability after 48 h and 72 h (Fig. 2, P=0.04 and 0.02, respectively) MDA-MB-231 cell viability showed no differences following treatment for 24 h, but celecoxib treatment resulted in a decrease in viability after 72 h of treatment, compared to 48 h (P=0.04). Luteolin treatment did not inhibit the viability of MDA-MB-231 cells time-dependently (P=0.13).

As shown in Figs. 1 and 2, the celecoxib and luteolin combination treatment significantly decreased cell viability and demonstrated greater efficiency in inducing tumor cell death after 72 h of treatment, compared to treatment with either alone or the controls in both cell lines (P=0.01). This was observed in a concentration- and time-dependent manner.

Analysis of apoptosis. The induction of apoptosis was evaluated using TUNEL assays and Annexin V- FITC/PI staining. The TUNEL assays demonstrated that the combination treatment resulted in significant increase in apoptosis induction compared with treatment with a single drug or controls (Fig. 3). In the MCF-7 cells, apoptosis was significantly different from

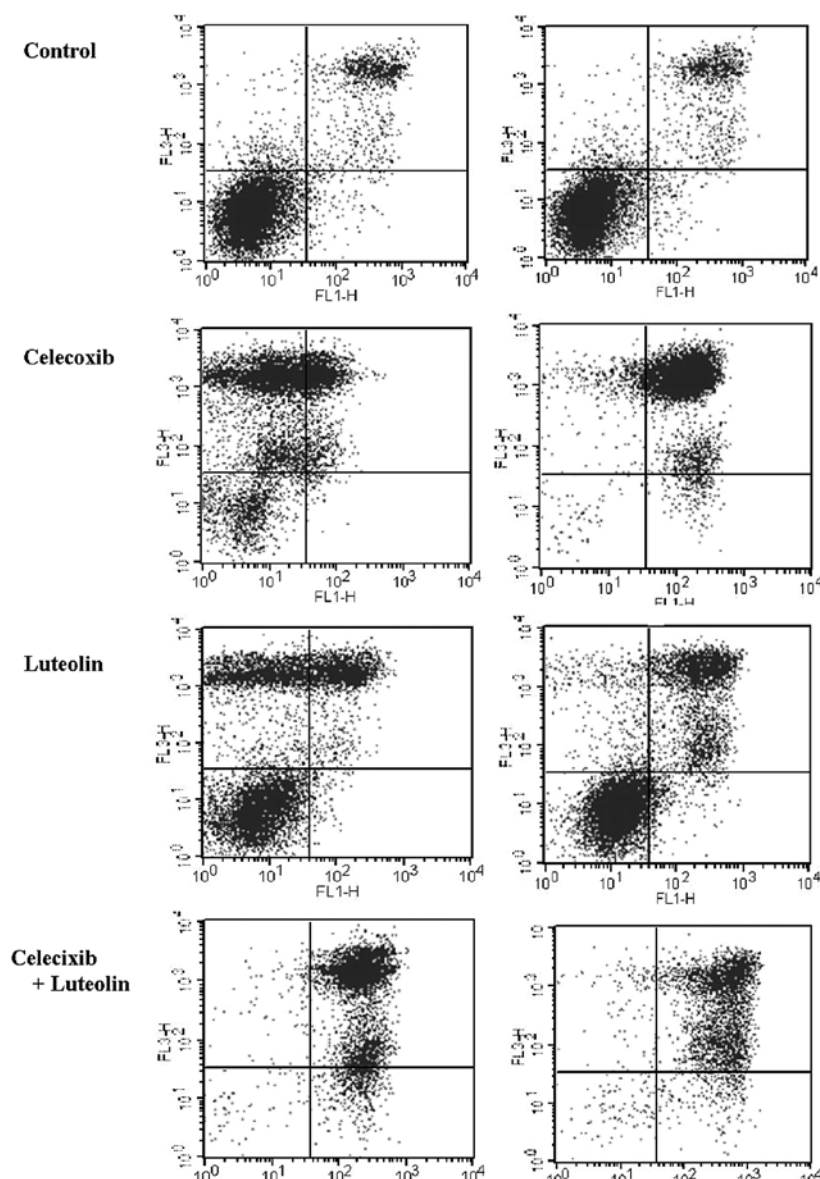


Figure 4. Apoptosis of MCF-7 (left) and MDA-MB-231 (right) breast cancer cell lines. Annexin V-FITC staining is represented on the horizontal axis and PI staining is represented on the vertical axis. The increase in the proportion of cells undergoing early and late apoptosis/necrosis was significantly higher after combination treatment in both cell lines than in the controls ($P=0.01$).

controls after combination treatment ($P=0.03$). Similarly, in the MDA-MB-231 cells, the induction of apoptosis was significantly higher after combination treatment compared to treatment with a single drug or controls ($P<0.01$). As shown in the Annexin V-FITC/PI staining (Fig. 4), the increase in the proportion of cells undergoing early and late apoptosis/necrosis was significantly higher following celecoxib and luteolin combination treatment in both cell lines ($P=0.01$). The quantification of the Annexin V-FITC/PI staining is presented in Table I.

Celecoxib and luteolin combination decreases expression of pAkt. To determine the effect of celecoxib and/or luteolin treatment on the expression of representative pro-survival markers, western blots were performed to analyze the steady-state levels of β -catenin, STAT3, Akt and pAkt (Fig. 5). Decreased levels of Akt phosphorylation (pAkt) were noted after combination treatment compared with β -catenin, STAT3, and Akt ($P<0.05$).

pAkt was significantly decreased in the MCF-7 and MDA-MB-231 cells following combination treatment ($P=0.02$).

Discussion

The effect of celecoxib and luteolin combination treatment for breast cancer has not been reported. However, in the present study, celecoxib and luteolin combination treatment of breast cancer cells resulted in a synergistic effect on the cell death in a concentration- and time-dependent manner when compared to treatment with either drug alone. Celecoxib was developed as a selective COX-2 inhibitor for the treatment of chronic pain in arthritis. However, considering the regulatory role of various antitumor signaling pathways, inhibition of COX-2 by celecoxib can induce proapoptotic effects. Suh *et al* reported that celecoxib significantly decreased MCF-7/HER2-18 and MDA-MB-436 breast tumor growth *in vitro* (15). Basu *et al* reported that celecoxib treatment altered the expression of

Table I. Quantification of the analysis of Annexin V-FITC apoptosis.

	Lt. bottom Quadrant viable cells	Rt. bottom quadrant Early apoptotic cells	Rt. top quadrant Late apoptotic cells
MCF-7			
Control	64.27	1.09	20.51
Celecoxib	13.75	0.69	22.53
Luteolin	50.23	1.15	21.52
Celecoxib + Luteolin	1.14	9.50	87.56
MDA-MB-231			
Control	83.56	1.33	11.52
Celecoxib	4.67	1.96	88.04
Luteolin	59.91	3.67	31.11
Celecoxib + Luteolin	2.45	6.23	88.49

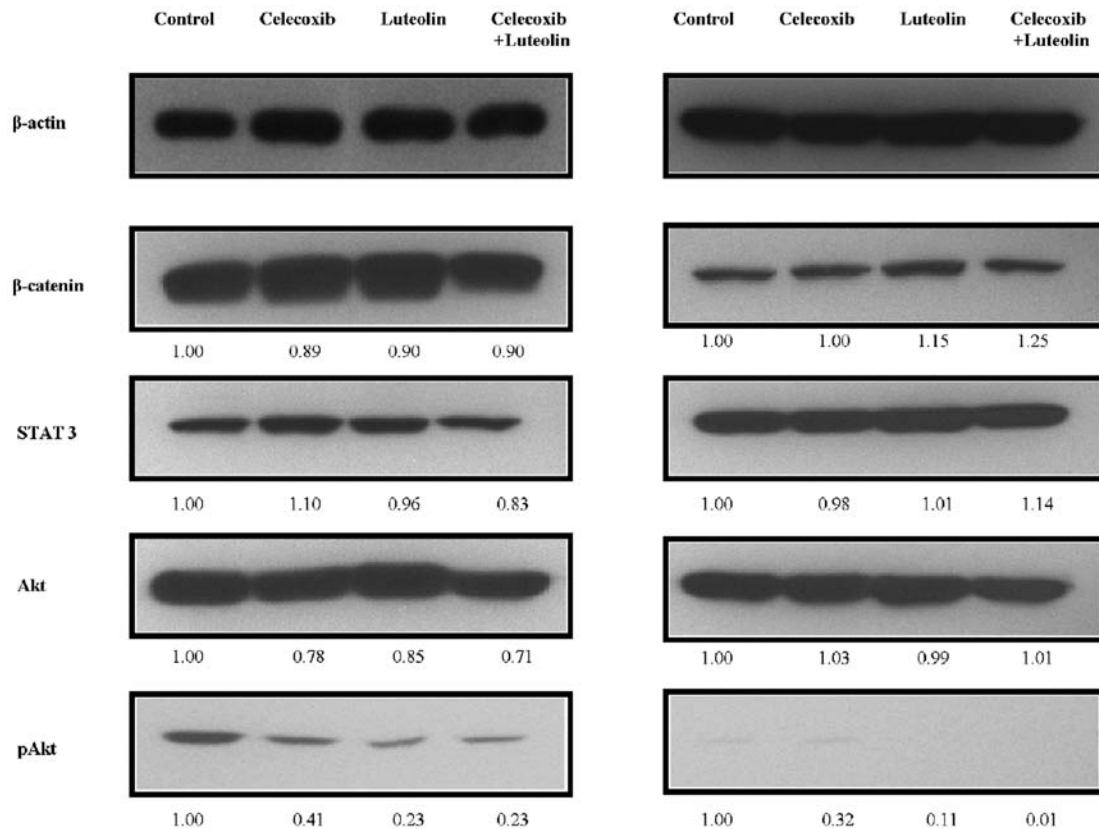


Figure 5. Western blot analysis of breast cancer cells treated with celecoxib, luteolin, or combination treatment. Both cell lines showed decreased expression of pAkt following the combination treatment when compared to controls. β -actin levels are shown to ensure equal protein loading.

genes associated with angiogenesis, proliferation, apoptosis, and the cell cycle (16). *In vitro* results were corroborated *in vivo* in tumor-bearing mice treated with celecoxib. In the present study, we have shown that celecoxib treatment decreases cell viability and increases cell death after 72 h of treatment in both ER-positive and -negative human breast cancer cells ($P < 0.05$). Celecoxib inhibited tumor growth by increasing apoptosis. In the MCF-7 and MDA-MB-231 cell lines, the percentage of cells undergoing apoptosis increased by 45% and 26% from the control, respectively.

Luteolin induces several beneficial effects, including acting as an anti-inflammatory and anticancer agent. Chiang *et al* reported that luteolin significantly suppresses the growth of MCF-7 and MDA-MB-435 breast tumor *in vitro* (17). Shinh *et al* reported that luteolin significantly decreased MCF-7 and MDA-MB-231 breast tumor growth *in vitro* (18). We have shown that the viability of MCF-7 cells was inhibited by luteolin after 72 h of treatment in a time-dependent ($P = 0.02$) and concentration-dependent manner ($P = 0.04$). In the MCF-7 cell line, the percentage of cells undergoing apoptosis increased

by 20% from the control to the treated cells. However, in the MDA-MB-231 cell line, luteolin treatment did not inhibit cell viability concentration-dependently ($P=0.78$) or time-dependently ($P=0.13$).

In the MCF-7 and MDA-MB-231 cells, the celecoxib and luteolin combination treatment significantly decreased cell viability and was more efficient in killing tumor cells after 72 h of treatment, compared to treatment with only one drug or the control ($P=0.01$). The percentage of cells undergoing apoptosis increased by 52 and 50% from the control in the MCF-7 and MDA-MB-231 cells, respectively. The antitumor effect of the celecoxib and luteolin combination treatment was more significant in the MDA-MB-231 cells compared to treatment with either drug alone (celecoxib: 20% and luteolin: no effect, and this effect was more significant than in the MCF-7 cells ($P<0.05$ vs. $P<0.01$)).

The pro-oncogenic mechanism of the COX-2 is not fully understood. Ghosh *et al* showed that prostaglandin E_2 (PGE_2) was the major downstream effector of COX-2 (19). Activation of the epidermal growth factor receptor (EGFR) via PGE_2 could result in activation of Ras and the mitogen-activated protein kinase (MAPK) pathway (20,21). Protein kinase B (Akt/PKB) activity is implicated in K-Ras-induced expression of COX-2, and the stabilization of COX-2 mRNA partly depends upon the activation of Akt/PKB (22). For example, PGE_2 inhibits apoptosis by stimulating the PI3kinase/Akt pathway. Furthermore, celecoxib was able to bind to and inhibit 3-phosphoinositide-dependent protein kinase-1 (PDK1). PDK1 is an essential component of cell growth and survival signaling pathways involving PI3K, upstream of PDK1, and Akt/PKB, downstream of PDK1 (11,23). Since Akt/PKB has a regulatory role of in pro-oncogenic pathways, the discovery of its inhibition by celecoxib provides a suitable explanation for the drug's COX-2 anticancer effects.

As an anticancer agent, luteolin inhibits proliferation of SCC-9 human oral squamous carcinoma cells, blocks DMBA-induced DNA adduct formation and cytotoxicity in MCF-7 cells, and inhibits CYP1A1 and CYP1B1 EROD activity (24-26). Furthermore, luteolin inhibits the proliferation of several cancer cell line via the inhibition of the protein tyrosine kinase activity and autophosphorylation of EGFR, transphosphorylation of the EGFR downstream effector protein enolase and activation of MAPK/ERK (27). Luteolin is able to inhibit IGF-1-induced activation of IGF-1R and Akt, and phosphorylation of Akt (28).

To determine whether celecoxib and luteolin alter prosurvival signaling in breast cancer cells, we used western blotting to examine Akt phosphorylation. Decreased levels of pAkt were noted after treatment with either drug ($P<0.05$). Akt phosphorylation was further reduced by combination treatment in the MCF-7 and MDA-MB-231 cells ($P=0.02$). We demonstrated that enhanced antitumor activity by celecoxib and luteolin combination treatment could result in decreased levels of Akt phosphorylation.

Therapeutic selectivity is important in anticancer treatments. The use of celecoxib has drawn much attention since the review of cardiac safety compared to another COX-2 inhibitor (29). Horinaka *et al* and Chen *et al* showed that luteolin induces marginal cytotoxicity in normal cells (30,31). These results imply that luteolin is relatively safe when used as an anticancer agent. The anticancer properties of luteolin have been tested in

conjunction with other anticancer drugs. Luteolin was observed to increase drug-induced cytotoxicity in a variety of cancer cells (14). These results support the idea that a celecoxib and luteolin combination treatment will provide a better therapeutic strategy to treat breast cancer than treatment with celecoxib alone.

Our study has several limitations. This study examines breast cancer cell lines *in vitro*. Additional studies are required to demonstrate the efficacy of celecoxib and luteolin combination treatment *in vivo*. Even though, the effect of celecoxib and luteolin combination treatment was demonstrated in both ER-positive and -negative human breast cancer cells, breast cancer is a heterogeneous disease, therefore HER2 receptor expression is important for determining definite breast cancer treatment. This can be answered quite soon with our collateral studies. In spite of these limitations, our study demonstrated that a celecoxib and luteolin combination treatment is effective in killing breast cancer cells. To our knowledge, this is first study of a celecoxib and luteolin combination treatment in breast cancer.

In conclusion, we demonstrated that a combination of celecoxib and luteolin could provide superior inhibition of breast cancer cell growth than either celecoxib or luteolin treatment alone. Additional studies, in human breast cancer cells that overexpress HER2 and *in vivo* xenograft models, are needed to demonstrate the effect of celecoxib and luteolin combination in human breast cancer. These results suggest that celecoxib and luteolin combination treatment might be a new treatment modality that could improve prevention of breast cancer and decrease recurrence after early breast cancer treatment. Celecoxib and luteolin in combination with other anticancer drugs may improve the therapeutic value of the combined agents by allowing the use of lower, sub-toxic doses to achieve a more effective cancer treatment.

Acknowledgements

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