RESEARCH ARTICLE

Synergistic Effects of Exemestane and Aspirin on MCF-7 Human Breast Cancer Cells

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Abstract

Objective: The purpose of this study is to investigate the combined effects of exemestane and aspirin on MCF-7 human breast cancer cells. Methods: Antiproliferative effects of exemestane and aspirin, alone and in combination, on growth of MCF-7 human breast cancer cells were assessed using the MTT assay. Synergistic interaction between the two drugs was evaluated in vitro using the combination index (CI) method. The cell cycle distribution was analyzed by flow cytometry and Western blotting was used to investigate the expression of cyclooxygenase-1, cyclooxygenase-2 and Bcl-2. Results: MTT assays indicated that combination treatment obviously decreased the viability of MCF-7 human breast cancer cells compared to individual drug treatment (CI<1). In addition, the combination of exemestane and aspirin exhibited a synergistic inhibition of cell proliferation, significantly arrested the cell cycle in the G_0/G_1 phase and produced a stronger inhibitory effect on COX-1 and Bcl-2 expression than control or individual drug treatment. Conclusion: These results indicate that the combination of exemestane and aspirin might become a useful method to the treatment of hormone-dependent breast cancer. The combination of the two inhibitors significantly increased the response as compared to single agent treatment, suggesting that combination treatment could become a highly effective approach for breast cancer.

Keywords: Exemestane - aspirin - MCF-7 - combination treatment - synergistic effect

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Introduction

Breast cancer is the leading cause of cancer death in females worldwide, accounting for 23% of the total new cancer cases and 14% of the total cancer deaths(Jemal et al., 2011). Approximately 75% of all patients with breast cancer have a tumor expressing the estrogen receptor (ER) and/or the progesterone receptor. Over the last few years ,aromatase inhibitors (AIs) have proved to be effective treatment for ER positive breast cancer. The third generation AIs (letrozole, anastrozole and exemestane) are now widely recommended for adjuvant therapy in postmenopausal breast cancer patients with hormone-sensitive disease (Goldhirsch et al., 2005; Winer et al., 2005; Janicke 2007). Currently, AIs have been used as the standard first-line treatment for patients with advanced estrogen dependent breast cancer (Smith and Dowsett 2003; Geisler and Lonning 2005; Riemsma et al., 2010). Cyclooxygenases (COX)-1 and COX-2 have been found to be over-expressed in breast cancer tissue when compared to normal tissue (Hwang et al., 1998). COX-1 and COX-2 have been causally linked to breast cancer cell proliferation, motility and invasiveness (Timoshenko et al., 2003; Basu et al., 2006; Hiraga et al., 2006; Tsatsanis et al., 2006). Prostaglandin (PG) E2, which has tumor and cell growth promoting activity (Taketo 1998), is produced from arachidonic acid by either COX-1 or COX-2. Breast tumors produce more PGE2 than normal breast tissue (Masferrer et al., 2000). PGE2 have many effects, such as induction of Bcl-2 (which also has a role in inhibition of apoptosis) (Sheng et al., 1998), influencing estrogen biosynthesis by induction of the aromatase gene (Harris et al., 1999), and involvement in angiogenesis (Gately, 2000).

A growing body of experimental and epidemiological evidence suggests that the use of aspirin or other NSAIDs (Non-steroidal anti-inflammatory drugs) may reduce the risk of several cancers, tumor burden, and tumor volume (Chan et al., 2007; Slattery et al., 2007; Van Dyke et al., 2008).

NSAIDs are thought to interfere with breast carcinogenesis mainly via inhibiting the cyclooxygenase 1 and 2 enzymes (COX-1 and COX-2) (Davies et al., 2002). Inhibiting these enzymes may impact breast carcinogenesis via multiple pathways including interference with DNA adduct formation (Abbadessa et al., 2006), angiogenesis (Masferrer et al., 2000) and aromatase production (Diaz-Cruz et al., 2005) and via increasing apoptosis (Leahy et al., 2002). Some previous studies have demonstrated that aspirin, irreversible COX-1 and COX-2 inhibitor,can induce cell apoptosis through COX-dependent and COX-independent pathways. The

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COX-independent effects include alteration of gene transcription (Xu et al., 1999; Lu et al., 2008) , cell cycle arrest, modulation of several protein kinases and other molecular signaling pathways (Dong et al., 1997; Pillinger et al., 1998) .

Strategies using combination drugs to enhance the efficacy of cancer treatment have been proved in recent years. They may react together to give synergistic action to inhibit tumors through regulation of different signaling pathways or compensation for the opposite properties in cancer cell proliferation or apoptosis. Therefore, in this study, we investigated the effects of combined exemestane and aspirin on breast cancer cells, including cell survival, cell cycle, and alterations in signaling pathway.

Materials and Methods

Cell Culture

The human breast cancer cell line MCF-7 was kindly provided by our school's Laboratory of Molecular Biology.The cells were cultured in DMEM containing 10% heat inactivated FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine and maintained in a humidified atmosphere with 5% CO_2 at 37˚C.

Compounds

Exemestane was a gift from Pfizer Inc (New York, USA). Aspirin was purchased as commercial products from our hospital pharmacy. Exemestane and aspirin were dissolved in dimethylsulfoxide (DMSO) and stored at -20˚C in the treatment. 10 M stock of aspirin and 20 mM stock of exemestane were employed in this study. Final concentrations of exemestane ranged from 3.125 to 100 μM and that of aspirin ranged from 125 μM to 5 mM. The final concentration of dimethylsulfoxide in the DMEM medium was kept at less than 0.1% and equal amounts of the solvent were added to control cells.

Anti-Bcl-2 and anti-β-actin antibodies were purchased from Beijing Zhongshan Biotech (Zhongshan, Beijing, China). Antibodies for COX-1 and COX-2 were from Bioworld Technology Inc. The PGE2 EIA-Monoclonal assay kit was also from Beijing Zhongshan Biotech (Zhongshan, Beijing, China).

Cell Proliferation Assay

MCF-7 cell proliferation was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) assay. Cells were plated at $1x10^4$ cells/well in 96-well plates, cultured overnight, and then treated with various concentrations of exemestane or aspirin for 72 h. After cells were exposed to each drug for 72 h in 96-well plates, 20μL of MTT solution (5 mg/ml) was added to each well and the plates were incubated for 4 h at 37˚C, then the coloured formazan product was dissolved using 150 μl of DMSO. The 96-well plates were placed on a shaker for 10 min at room temperature to thoroughly dissolve the MTT color product.Then the optical density (OD) of each well was measured at 490 nm on an ELISA plate reader. The IC_{50} of therapeutic drug was determined as each drug concentration showing 50% cell growth inhibition as compared with the control cell growth. Six replicate wells were used for each drug concentration and the testing was carried out independently three times.

Analysis of Interactions

The level of interaction between exemestane and aspirin was assessed by measuring combination-index(CI), a quantitative representation of pharmacological interaction between two drugs. The combination effect of exemestane and aspirin was assessed using the median effect analysis method, as previously described by Chou and Talalay (Chou and Talalay, 1984). The two drugs were combined in a fixed ratio of doses that typically corresponded to 0.125, 0.25, 0.5, 1, 2, and 4 times that of the individual IC_{50}s. The CI values of interactions between exemestane and aspirin were analyzed according to the Chou and Talaly method using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA):CI<1, CI = 1, and CI>1 indicate synergistic, additive, and antagonistic effects, respectively (Chou and Talalay, 1984).

Cell cycle analysis by flow cytometry

Equal numbers of MCF-7 cells (1×10^6/well) were plated in 6-well dishes and treated with exemestane,aspirin (IC_{50} concentration respectively), or a combination of these two drugs for 72 h. And then the adhered cells were harvested by trypsinization, washed twice with PBS, and fixed overnight in 70% ethanol at 4°C. After the ethanol was removed, the cells were washed twice in PBS and then resuspended in 1 ml of propidium iodide/Triton X-100 staining solution [PBS containing 0.1% Triton X-100 (Sigma), 200μg /ml RNAse A (Sigma), and 50 μg/ml propidium iodide (Sigma)] in the dark for 30 min. The cell cycle was measured by flow cytometry. The cell cycle profiles, including G_0/G_1, S, and G_2/M phases, were calculated using ModFit LTTM software.

Western Blotting

MCF-7 cells growing with or without the drugs were washed with ice-cold phosphate-buffered saline (PBS) solution and scraped in lysis buffer. The lysates were centrifuged at 15,000 rpm for 30 min at 4˚C and the supernatant was collected. Briefly, protein concentration in each sample was determined using the bicinchoninic acid protein assay kit (Beyotime Institute of Biotech., Shanghai, China). Equal amounts of protein from each sample were loaded on 10% SDS-polyacrylamide minigels and electrophoresed. Proteins were transblotted to polyvinylidene difluoride (PVDF) membranes and then blocked with a solution of PBS containing 5% milk and 0.1% Tween 20 for 2 h. The PVDF membranes were probed with specific primary antibodies against COX-1, COX-2 and Bcl-2 diluted 1:500 in TBST at 4˚C overnight . After rinsing with TBST (0.1% Tween-20, TBS) 3 times, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) at room temperature for 1 h. Positive bands were detected using ECL reagents (millipore). β-actin was used as loading control. Human recombinant COX-2 (Cayman) were used as positive controls.

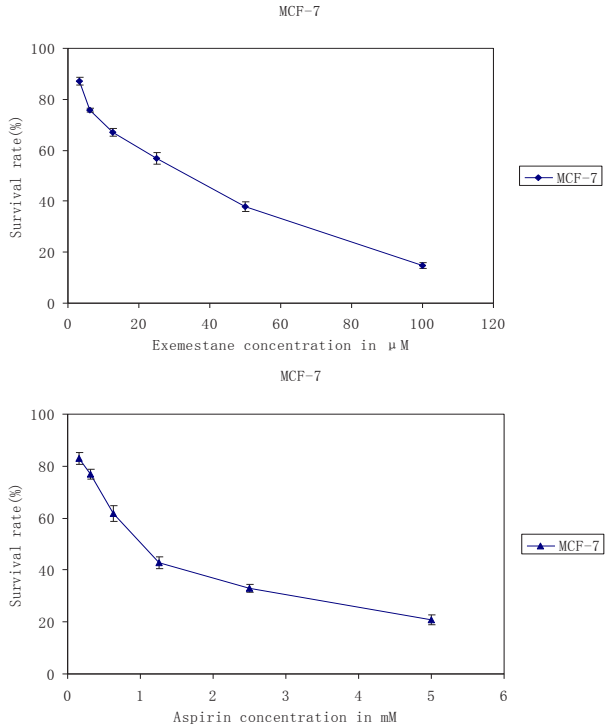

Figure 1. MCF-7 Cell Proliferation Inhibitory Effect of Different Concentrations of Exemestane and Aspirin. Cell proliferation was determined by MTT assay. The cells were exposed to the varying concentrations of exemestane in MCF-7 cells (3.125-100μM) and aspirin in MCF-7 cells (156.25μM-5mM) for 72 h.Three independent experiments are present

Measurement of prostaglandin E2 (PGE2) levels

Cells (5×10^4/well) were initially plated in 6-well dishes and allowed to adhere overnight. After 24 h the growth media were replaced with serum-free medium containing vehicle or exemestane or aspirin or their combination at the concentration of IC_{50} levels. In these particular experiments, media was not replaced at any time after the start of treatment exposure. After 72 h, media was collected and PGE2 concentrations were measured at 450 nm on an ELISA plate reader.

Statistical analysis

All data were assayed in three independent experiments. The results were displayed as the mean ± standard deviation (SD). Student's t test and one-way ANOVA test were used to determine the differences between control and treatment groups. The P value less than 0.05 was considered statistically significant.

Results

Growth inhibition of MCF-7 cells by exemestane and aspirin

The effects of exemestane and aspirin on the proliferation of MCF-7 cells were determined using MTT assay. MCF-7 cells were treated with different doses of exemestane (3.125-100 μM) or aspirin (156.25 μM-5 mM) for 72 h. A dose-dependent decrease in the cell viability was observed with both exemestane and aspirin treatments with IC_{50} value of (24.97±1.773) μM and (0.98±0.098) mM for exemestane and aspirin respectively at 72 h exposure (Figure 1). Hence we used 25μM exemestane and 1 mM aspirin for all the experiments.

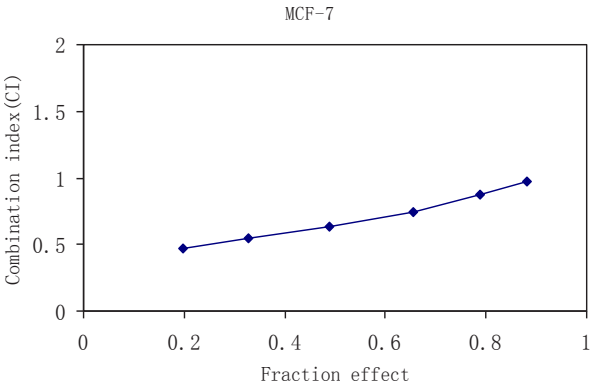

Figure 2. The Combination Index (CI) Value was Calculated Using the Chou-Talalay Method after the Combination of Exemestane with Aspirin Treatment as Described Previously. CI<1, CI = 1, and CI>1 indicate synergistic, additive, and antagonistic effects, respectively

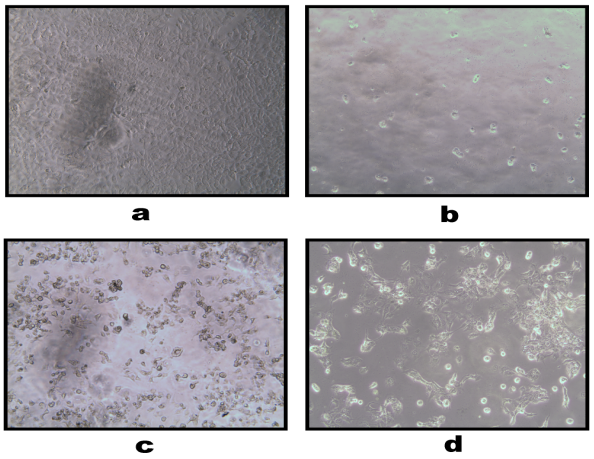

Figure 3. Photomicrographs of MCF-7 Cells in Different Groups after a 3-day Treatment Period. (a) Untreated MCF-7 cells. (b) MCF-7 cells were treated with combined exemestane and aspirin . (c) MCF-7 cell were treated with aspirin (d) MCF-7 cells were treated using exemestane. The concentrations of exemestane and aspirin were used at IC_{50} levels. Magnification in each micrograph is 200×

Synergistic antiproliferative effects of combined exemestane and aspirin treatment

To investigate the effects of combined exemestane and aspirin on MCF-7 cells, they were exposed to various concentrations of exemestane and aspirin concomitantly for 72 hours. Combination treatment induced more cell death than treatment with either exemestane or aspirin. The combination of exemestane with aspirin for 72 h resulted in a synergistic effect (CI < 1) on the inhibition of the growth of MCF-7 cells when CI is between 0.47 and 0.97 for the cell line (Figure 2). In support of this result, photomicrographs show that treatment with exemestane or aspirin alone had only slight effects on MCF-7 cell number and morphology, whereas combined treatment caused a large suppression on cell proliferation after 72 hours treatment (Figure 3).

Cell cycle effects of exemestane and aspirin

To clarify the mechanisms by which exemestane and aspirin inhibit the proliferation of MCF-7 cells,

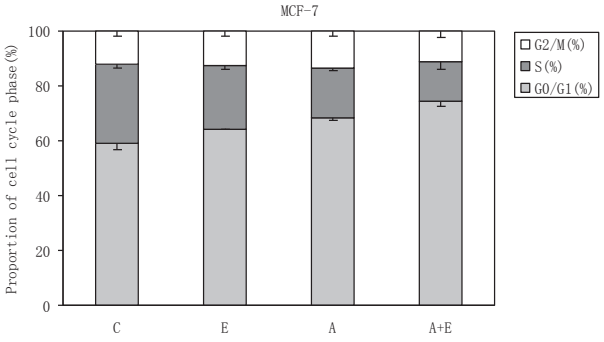

Figure 4. Flow Cytometric Analysis was Applied to Determine the Alterations in Cell Cycle Distributions in MCF-7 Cells after Administrations of Exemestane and Aspirin for 72 h. The concentrations of exemestane and aspirin were used at IC_{50} levels. Columns in the diagram depict cell cycle phase distribution in MCF-7 cells following the administration of the exposure schedules as stated above. C, E, A, and A+E refer to control, exemestane aspirin and concurrent administration respectively

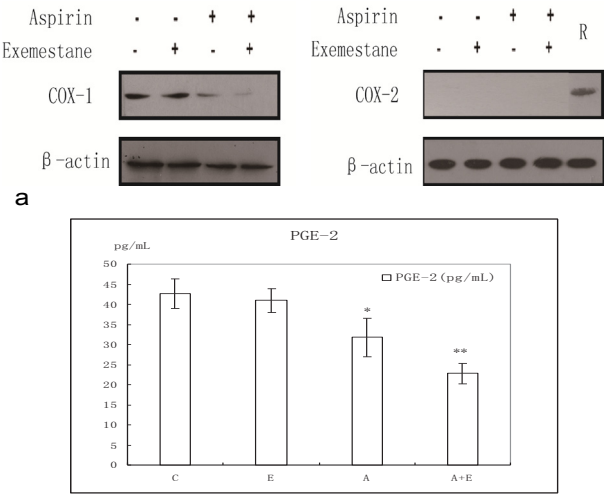

Figure 5. Effects of Exemestane and/or Aspirin on Expression of COX-1, COX-2 and on PGE2 Synthesis in MCF-7 Cells. (a) MCF-7 cells were treated with exemestane, aspirin and their combinations for 72 hours at IC_{50} levels. After treatment cells were harvested and lysed, and equal aliquots of extracted protein were analyzed for COX-1 and COX-2 expression by Western blotting. β-actin was used as loading control. Lane R is an human recombinant COX-2 as positive control. (b) Media was collected from the different treatment groups and prepared for use in the EIA assay for PGE2. Vertical bars indicate the mean cell count±SEM in each treatment group. C,E,A,and A+E refer to control,exemestane aspirin and concurrent administration respectively. *P < 0.05 as compared with control treatment. **P < 0.05 as compared with single agent treatment or control

the cell cycle was analyzed by flow cytom-etry. Cell cycle analysis of MCF-7 cells (Figure 4) demonstrated that treatment with either exemestane or aspirin also increased the population of cells in G_0/G_1 phase with a concomitant decrease of cells in S phase (P<0.05). In addition, combined exemestane and aspirin treatment further increased the fraction of MCF-7 cells in the G_0/G_1 phase than treatment with either exemestane or aspirin (P<0.05), which indicated that these two drugs exerted synergistic growth-inhibitory effects to cause cell cycle arrest at G_0/G_1 phase.

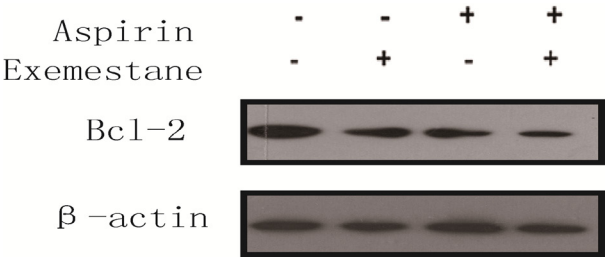

Figure 6. Combination Effects of Exemestane and Aspirin on Bcl-2 Protein Expression in MCF-7 Cells. MCF-7 cells were treated with exemestane, aspirin and their combinations for 72 hours. After treatment cells were harvested and lysed, and equal aliquots of extracted protein were analyzed for Bcl-2 expression by Western blotting. The levels of β-actin were also measured as a normalization control

Effect of exemestane or aspirin or their combination on COX-1, COX-2 expression and PGE2 production in MCF-7 cells

Cyclooxygenases (COX), the enzymes that convert arachidonic acid into prostaglandins, have been causally linked to breast cancer cell proliferation, motility and invasiveness.Therefore,in this study, we analyzed the expression of COX-1 and COX -2 in MCF-7 cell line and assessed PGE2 production (Figure 5). COX-1 and PGE2 expression are found to be suppressed after aspirin and combined aspirin and exemestane treatment. The basal level of COX-2 in MCF-7 was barely undetectable. Whereas there were no significant changes in expression of COX-2 protein in response to exemestane, aspirin alone, or the combination.

Effects of aspirin and exemestane treatment on Bcl-2

To further evaluate the potential synergistic mechanisms of exemestane and aspirin, the expression of Bcl-2 were detected by western blot analysis in MCF-7 cells.As shown in Figure 6, combined treatment with exemestane and aspirin caused a large relative decrease Bcl-2 levels as compared to MCF-7 cells in the control or single-agent treatment.

Discussion

In this study, our results clearly indicated that both exemestane and aspirin inhibited the growth and induced cell cycle arrest of MCF-7 cells. Such effects were concentration-dependent. Combined exemestane with aspirin for 72 h exhibited notably greater cell growth inhibitory than exemestane or aspirin alone. This study also revealed an enhanced effect on cell cycle progression. It is well known that aspirin is associated with reduced risk of cancer development (Ghosh et al., 2010). In the present study,we investigated that aspirin exhibited remarkably anti-proliferative effects and dose dependent growth inhibition on MCF-7 human breast cancer cells. Some preclinical studies have shown that aspirin synergistically enhanced the anti-tumor effect of chemotherapy in ovarian cancer and colon cancer (Kanthamneni et al., 2010; Son et al., 2010).

Overexpression of COX-2 has been shown to be involved in many processes of carcinogenesis, such as tumour aggressiveness and growth (Ristimaki et al.,

2002; Juuti et al., 2006). Although COX-1 is present at a constant level in most cells and tissues, previous studies have shown that COX-1 is highly expressed in several types of tumors,including breast cancer (Chulada et al., 2000; Kirschenbaum et al., 2000; Connolly et al., 2002; Sales et al., 2002; Gupta et al., 2003; Daikoku et al., 2005; Daikoku et al., 2006; Daikoku et al., 2007; Erovic et al., 2008). These data indicated that not only cyclooxygenase-2 (COX-2) but also COX-1 has a role in carcinogenesis. Previously, Howe L.R et al (Howe and Lippman, 2008) reported that the sum of COX-1 and COX-2 activity may be a key determinant in breast carcinogenesis. COX-1 and COX-2 may be present in breast tumors to catalyze the conversion of arachidonic acid to prostaglandins.Prostaglandin E2 have strong tumor and cell growth promoting activity (Taketo, 1998). It has previously been demonstrated that cyclooxygenase activation and prostaglandin production has also been associated with increase in metastasis (Kundu and Fulton, 2002; Surowiak et al., 2005; Tsatsanis et al., 2006). Therefore,we analyzed the expression of COX-1 and COX-2 in MCF-7 cell line and assessed PGE2 production. Liu et al. (1996) proved that the primary COX isoform expressed in MCF-7 cells is COX-1, which is similar to our result. Our data demonstrated that the combination treatment caused a large relative decrease in the expression of COX-1 and PGE2 as compared to in the control or single-agent treatment.Wheras the expression levels of COX-2 was not altered in response to exemestane, aspirin alone, or the combination. Some preclinical studies have also shown that COX-1 selective inhibition exhibited significant inhibitory effects in bladder cancer cells in vitro (Mohseni et al., 2004), ovarian cancer cell lines (Daikoku et al., 2005; Daikoku et al., 2006; Son et al., 2010) and human breast cancer (Connolly et al., 2002; Kundu and Fulton 2002; Ristimaki et al., 2002; McFadden et al., 2006; Howe 2007). These data suggest that COX-1 may play an important role of the anticancer activity in our study.

The results from our study also showed that both exemestane and aspirin decreased Bcl-2. To confirm this,we observed expression level of anti-apoptotic proteins.The Bcl-2 (anti-apoptotic factor) expression was significantly decreased in combination with aspirin treated approach. Bcl-2 is an anti-apoptotic member of Bcl-2 family which promotes cell survival and regulates MMP (Ruvolo et al., 2001). Bcl-2 is a key apoptosis regulator that is down regulated in many cell types in response to treatment leading to activation of apoptosis (Diel et al., 1999). These results indicates that downregulation of the anti-apoptotic proteins Bcl-2 might be involved in combination-induced cell death.

In summary, we demonstrated that exemestane in combination with aspirin synergistically inhibited cell proliferation and induced cell cycle arrest of human breast cancer MCF-7 cells in vitro. The present work suggests that the combination might be a promising approach to treat breast cancer, although more studies are needed.

Acknowledgements

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