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 G₀/G₁ 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

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% CO₂ 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 1x10⁴ 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 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 IC₅₀. The CI values of interactions between exemestane and aspirin were analyzed according to the Chou and Talalay method using Compusyn software (Compusyn, 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 (1x10⁴/well) were plated in 6-well dishes and treated with exemestane, aspirin (IC₅₀ concentration respectively), or a combination of these two drugs for 72 h. 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), 200 μg/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 G₀/G₁, S, and G₂/M phases, were calculated using ModFit LT™ 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.
Synergistic Effects of Exemestane and Aspirin on MCF-7 Human Breast Cancer Cells

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 (5x10^4/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.

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,
the cell cycle was analyzed by flow cytometry. Cell cycle analysis of MCF-7 cells (Figure 4) demonstrated that treatment with either exemestane or aspirin also increased the population of cells in G0/G1 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 G0/G1 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 G0/G1 phase.

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


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