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Research Article

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Synergistic inhibition of drug resistant breast cancer cells growth by the combination of luteolin and tamoxifen involves Nrf2 downregulation

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ABSTRACT

Elevated expression of NF-E2-related factor 2 (Nrf2), a nuclear transcription factor, is a frequent genetic abnormality and is an important contributor to chemoresistance in cancer therapy. To further characterize its biological significance, the response of Nrf2 on the synergistic cytotoxic effect of luteolin and tamoxifen was investigated in tamoxifen resistant breast cancer (MCF-7/TAM) cells. Tamoxifen-resistant human breast cancer cells (MCF7/TAM) were treated with tamoxifen and luteolin alone and in combination at different concentration for 24 h. The cell viability was detected by 3-(4,5-dimethiylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The transfection of siRNA was performed using Lipofectamine 2000 Reagent and the expression levels of Nuclear factor erythroid-2 p45-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) were detected by Western blotting. Our results showed that combination treatment significantly sensitizes MCF-7/TAM cells to tamoxifen, which was accompanied by suppression of Nrf2 activation and decreased expression of HO-1. While overexpression of Nrf2 in tamoxifen resistant breast cancer cells conferred protection against the cytotoxicity caused by their combination, knockdown of Nrf2 expression using siRNA techniques enhanced their cytotoxic effect. These results suggested that luteolin in combination with tamoxifen can reversed tamoxifen resistance in MCF-7/TAM cells in synergic manner, at least in part, through suppression of Nrf2 signaling.

Keywords: luteolin, Nrf2 siRNA, tamoxifen resistant breast cancer cell

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INTRODUCTION

The ability to reduce breast tumor growth through the administration of anti-estrogen agents has played a key role in the endocrine therapy of breast cancer. A non-steroidal antiestrogen, tamoxifen (TAM), is the most widely used in estrogen receptor-positive breast cancer patients [1]. Although most patients are initially responsive, resistance to TAM is a critical problem for anti-estrogen therapy [2]. Therefore, it is urgently needed to develop new adjuvants that enhance the efficacy of TAM based chemotherapy and circumvent chemoresistance. Nuclear factor erythroid-2 p45-related factor 2 (Nrf2), a cap'n'collar basic leucine zipper transcription factor, was identified as a critical intracellular regulator in the adaptive response via regulation of a wide array of cytoprotective enzymes [e.g. NADPH quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), aldo-keto reductase family 1, member C1 (AKR1C) and glutathione S-transferase [3]. Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1) whereby Keap1 maintains Nrf2 at low concentrations in the cytoplasm [4]. However, in several types of human cancers, recent studies have demonstrated that high Nrf2 expression results in enhanced resistance to toxic effects of chemotherapeutic drugs and that suppression of endogenous Nrf2, either by transfecting Nrf2-specific

siRNA or overexpressing Keap1, renders these cells more susceptible to therapy[5, 6]. These observations suggest that the Nrf2 signal pathway may function as a cell survival pathway that protects cancer cells against drug-induced cell death. Therefore, if Nrf2 inhibitors as adjuvant to chemotherapeutic drugs can be identified to maximize cancer cells death, this could have significant therapeutic potential.

Luteolin (3, 4, 5, 7-tetrahydroxy flavone), a flavonoid with antioxidant, anti-inflammatory, cardiovascular protection and anti-cancer effects has been identified as a potential Nrf2 inhibitor [7-9]. Luteolin can promote the degradation of Nrf2 mRNA, leading to down-regulation of the antioxidant response element (ARE)-gene battery and enhancing the sensitivity of A549 cells to anti-cancer drugs [10]. Based on these findings, the present study was designed to investigate the combined effects of luteolin and chemotherapeutics agent tamoxifen on drug resistant breast cancer cells and the role of Nrf2 in protecting cells against injury.

EXPERIMENTAL SECTION

2.1 Reagents

Luteolin was obtained from Sigma–Aldrich (St. Louis, MO), and dissolved in DMSO (The DMSO concentration in all drug-treated cell culture medium was 0.1%) and were used in all experiments unless otherwise indicated. Tamoxifen, 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma–Aldrich (St. Louis, MO). Primary antibodies for NQO1, HO-1, β-actin, α-tubulin, Nrf2 siRNA, and control siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents were used in this study were of analytical grade.

2.2. Cell culture

The human breast adenocarcinoma cells (MCF-7) was purchased from National Cell Bank of Iran (NCBI), Pasteur Institute of Iran (Tehran, Iran), and maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown as monolayers at 37°C in humidified atmosphere with 5% CO_2 and 95% air. Tamoxifen-resistant MCF-7 cells (MCF-7/TAM) were established using the methodology reported elsewhere [11]. Briefly, MCF-7 cells were washed with PBS, and the culture medium was changed to phenol-red-free DMEM containing 10% charcoal-stripped, steroid depleted fetal bovine serum (Hyclone, Logan, UT) and tamoxifen (0.1 μM). The cells were continuously exposed to this treatment regimen for 2 weeks and the concentration of tamoxifen was gradually increased to 3 μM over a 9-month period. Initially, the cell growth rates were reduced. However, after exposure to the medium for 9 months, the rate of cell growth gradually increased, showing the establishment of a tamoxifen-resistant cell line [12].

2.3. Cell viability assay

Cell viability was determined using the MTT assay. Briefly, 2.5×10^4 cells in logarithmic phase were seeded in 96-well plates at 37°C with 5% CO_2 for overnight incubation and treated with appropriate concentrations of test samples for the indicated times. The cells were then incubated with a serum-free medium containing MTT at a final concentration of 0.5 mg/ml for 4h. The dark blue formazan crystals formed in intact cells were solubilized in dimethyl sulfoxide, and the absorbance was measured at 570 nm. Results were expressed as the percentages of reduced MTT, assuming the absorbance of control cells as 100%.

2.4. Determination of drug resistance

To determine the drug resistance of MCF-7/TAM cells to tamoxifen, MCF-7, and MCF-7/TAM cells were plated into 96-well plates at approximately 8,000 cells/well in 100 μL medium, then treated with various concentrations of tamoxifen for 24 h. Cell viability was assessed with MTT assay and cell survival ratio was calculated using $A_{treated}/A_{control} \times 100\%$, where $A_{treated}$ and $A_{control}$ were the absorbance from treated and control cells after 24 h incubation, respectively. The IC_{50} was taken as the concentration that caused 50% inhibition of cell proliferation and the degree of resistance was estimated by resistant index (RI), which was calculated by IC_{50} of MCF-7/TAM cells/IC_{50} of MCF-7 cells [13].

2.5. Preparation of nuclear and cytoplasmic extracts

Nuclear extracts were prepared according to the instructions provided in the NE-PER® nuclear and cytoplasmic extraction kit (Pierce). Briefly, cells were resuspended in 10 vol of CER I solution, after which they were incubated in a CER II solution on ice for 1 min and homogenized. Nuclei were recovered by centrifugation at 14,000 rpm for 5

min, and the supernatant was kept as the cytoplasmic extract. The nuclear fraction was extracted for 40 min on ice in NER solution. After centrifugation, the supernatant was used as the nuclear extract.

2.6. Western blot analysis

Cell lysates were prepared using RIPA buffer. Proteins (30 μg per lane) were separated on NuPAGE 4-12% Bis-Tris polyacrylamide gels (Invitrogen) and then electrophoretically transferred to Immuno-Blot PVDF membranes. The membranes were incubated for 2 h at room temperature with a 1:500 dilution of anti-Nrf2 (Santa Cruz Biotechnology), and anti-HO-1 (Santa Cruz Biotechnology). Next, HRP-conjugated secondary antibody was applied at a dilution of 1: 5,000 and the signal was visualized using an ECL detection kit (Santa Cruz Biotechnology). All blots were stripped and probed with polyclonal anti-β-actin antibody to ascertain equal loading of the proteins.

2.7. Nrf2 siRNA transfection

The transfection of siRNA was performed using Lipofectamine 2000 Reagent (Invitrogen, Car-lsbad, CA) according to the manufacturer's protocol. Briefly, a total of 20×10^4 cells were seeded into 6 well plates and transfected the next day with a 100 nM final concentration of siRNA, using 5 μl Lipofectamine 2000. Cells were harvested 48 h after transfection for western blot analysis. To measure the effect of siRNA and luteolin treatment together, the cells were treated with luteolin for another 24 h before determining cell viability and apoptosis.

2.8. Statistical Analysis

All results shown represent means ± SD from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analysis was performed using one-way ANOVA. See details of each statistical analysis used in Figures and Figure legends.

RESULTS AND DISCUSSION

3.1. Establishment of a tamoxifen resistant breast cancer cell line

Breast cancer is the leading cause of cancer death in women worldwide. Despite advances in detection and chemotherapy, many women with breast cancer continue to die of this malignancy [14]. Therefore, an understanding of the molecular mechanisms involved in breast cancer formation and progression should be helpful in developing more effective treatments for breast cancer. Drug resistance during chemotherapy is the major obstacle to the successful treatment of many cancers. Tamoxifen remains a commonly-prescribed drug for the treatment and prevention of ER-positive breast cancer, as the drug increases survival and helps maintain disease-free status [15]. Accumulating data in cancer studies indicate that Nrf2 is directly involved in the resistance against various drugs, such as cisplatin in lung cancer cells [16], cisplatin and paclitaxel in endometrial cancer [5], and 5-fluorouracil (5-FU) in esophageal squamous cancer [17]. Many Nrf2 downstream genes have been shown to contribute to the observed Nrf2-dependent chemoresistance [18, 19]. For instance, treatment of the pancreatic cancer cells with gemcitabine or radiation strongly induced HO-1 expression and enhanced expression was found to be closely associated with the development of cellular resistance to therapy [20].

In this study, the acquisition of tamoxifen resistance in MCF-7/TAM cells was confirmed through MTT assay. The cell viability assay revealed that the percentage of surviving cells decreased significantly in a dose-dependent manner. Tamoxifen (10 μM) treatment in control MCF-7 cells significantly inhibited cell proliferation but not in MCF-7/TAM cells (more than 80 % of live cells) (Fig. 1). The 50 percent inhibition concentration (IC_{50}) for tamoxifen in the MCF-7 and MCF-7/TAM cells was 3.0±0.2 μM VS 10.2±0.9 μM, respectively ($P<0.05$). To verify the drug resistance phenotype of MCF-7/TAM cells, the resistant index (RI) was calculated by IC_{50} of MCF-7/TAM cells/IC_{50} of MCF-7 cells. The RI was 3.4, indicating that MCF-7/TAM cell line is tamoxifen-resistant. Our results manifested that MCF-7/TAM cells could be relatively resistant to tamoxifen treatment and suitable for our further studies.

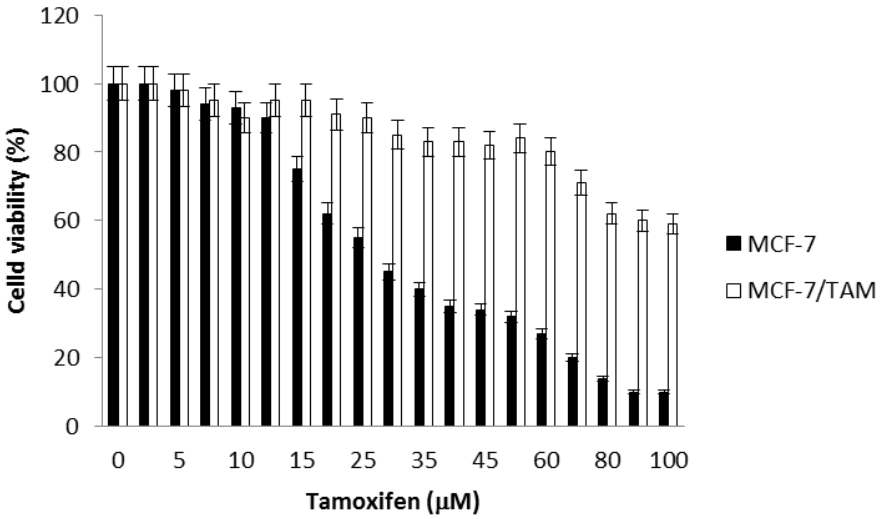
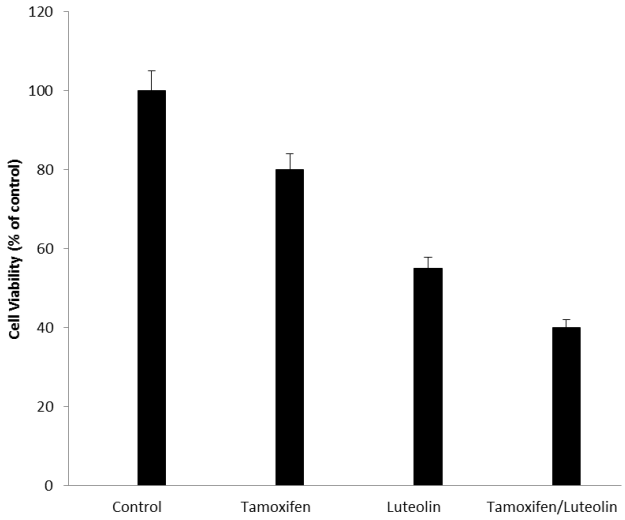


Fig. 1. The inhibitory effect of tamoxifen on MCF-7 and MCF-7/TAM cells proliferation. Data were expressed as means ± SEM of three independent experiments

3.2. Effects of luteolin and tamoxifen, alone and in combination, on cell viability and Nrf2 induction

To evaluate the efficacy of luteolin in combination with the chemotherapeutic agent tamoxifen on the growth of MCF-7/TAM cells, we treated the cells with luteolin and tamoxifen, alone and in combination. After treatment with tamoxifen (80 µM) and luteolin (50 µM) for 24 h, cell viability was decreased to 40% by a combination of tamoxifen and luteolin (Fig. 2A). To investigate whether the synergistic inhibition of cell growth by the combination of tamoxifen and luteolin is associated with the Nrf2 activity, we assessed the effect of luteolin and tamoxifen, alone or in combination, on the nuclear accumulation of Nrf2 protein and its expression. The transcriptional activity of Nrf2 was confirmed by measuring the level of HO-1 protein, a target gene product for Nrf2. Fig. 2B clearly demonstrates that Nrf2 level in whole cell lysate was potently inhibited by the combination of tamoxifen and luteolin, whereas it was decreased slightly in cells treated with tamoxifen or luteolin alone. Such effect was accompanied by a decrease in HO-1 level (Fig. 2B). In combination with tamoxifen, the ability of luteolin to reduce the amount of Nrf2 and HO-1 proteins was also evident in the dose-dependent experiment (Fig. 2C).

A



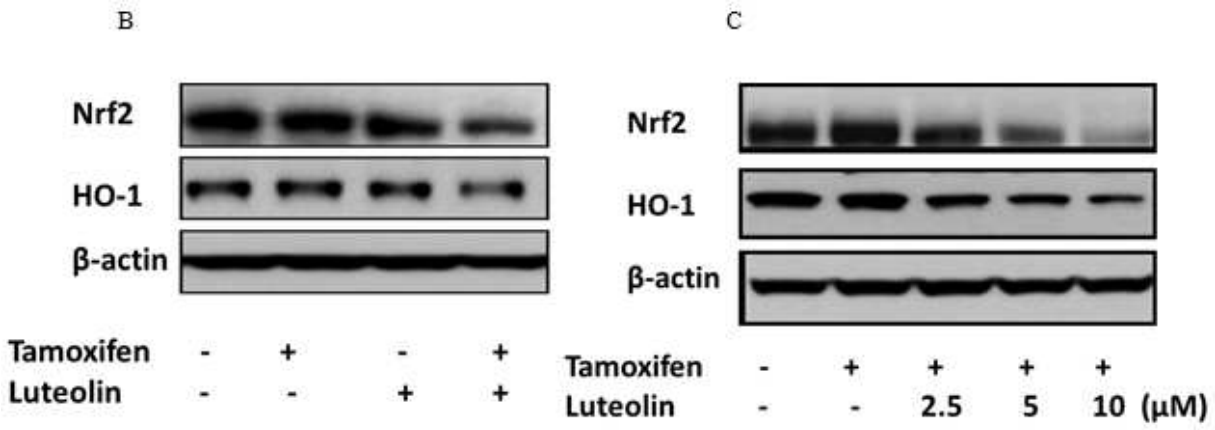


Fig. 2. Effects tamoxifen and luteolin on cell viability (A) and Nrf2 levels (B, C). Error bars represent the mean ± SEM for three independent experiments.

Flavonoids are polyphenolic compounds that occur ubiquitously in food plants and vegetables. Flavonoids are generally safe and are associated with low toxicity, making them ideal candidates for cancer chemopreventive agents. Several flavonoid compounds have been reported to be potent Nrf2 inhibitors, such as epigallocatechin 3-gallate, luteolin and brusatol [21, 10, 22]. In this study, we observed that the combined treatment of tamoxifen and luteolin inhibited the Nrf2 signaling pathway by reducing the nuclear localization of Nrf2 and decreasing the Nrf2 and HO-1 protein levels. It is possible that suppression of Nrf2 activation by the combination treatment is mediated by a mechanism that inhibits its stabilization as well as the regulation via its synthesis, thus decreasing the intracellular level of Nrf2 and subsequent transactivation of its downstream target genes.

3.3. Effect of tamoxifen and luteolin, alone or in combination, on cell viability in Nrf2 knockdown cells

To investigate the biologic relevance between Nrf2 and drug resistance, we examined whether Nrf2 knockdown affected sensitivity to combined treatment. When siRNA-Nrf2-transfected cells were treated with tamoxifen and luteolin in combination, the cell viability was significantly decreased to approximately 25% compared with that of control siRNA, and expression of endogenous Nrf2 and HO-1 proteins was also effectively suppressed (Fig. 3).

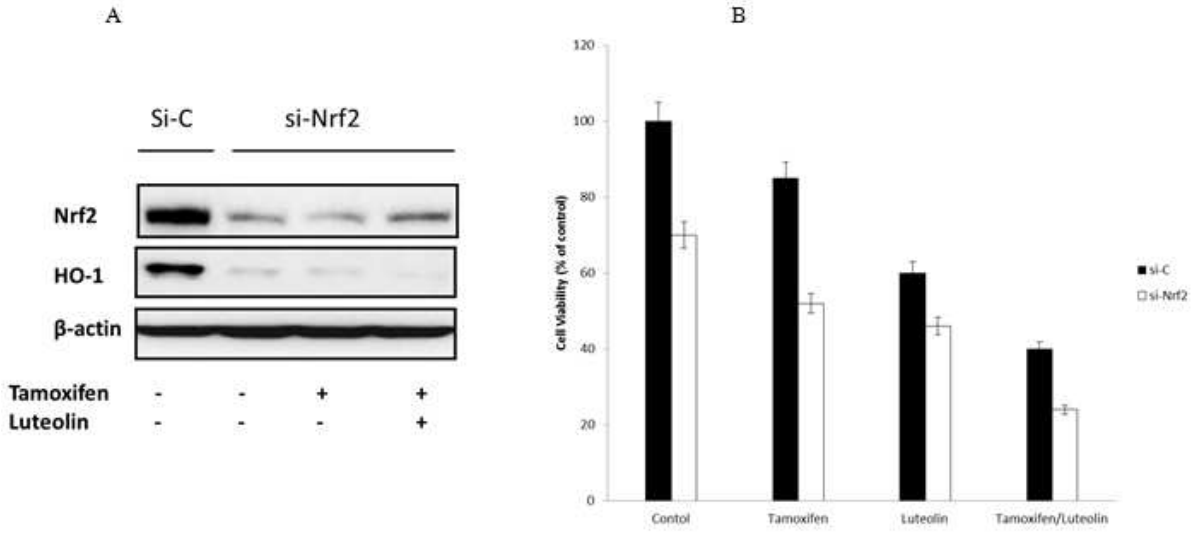


Fig. 3. Effects of Nrf2 knockdown on cell viability (A) and Nrf2 level (B).

CONCLUSION

In conclusion, the present study demonstrates that MCF-7/TAM cells acquiring resistance to chemotherapeutic agents due to Nrf2 overexpression become susceptible by the drug treatment combined with luteolin. Because of the

complexity of cancer, combination therapy is becoming increasingly important to overcome multidrug resistance in cancer and to enhance apoptosis.

Although additional studies in animal models as well as in human clinical trials are necessary to test the efficacy of co-treatment of luteolin and chemotrophic agents as well as other Nrf2 inhibitors during chemotherapy.

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