Original Research Article

Inhibitory effects of tamoxifen and tanshinone, alone or in combination, on the proliferation of breast cancer cells via activation of p38 MAPK signalling pathway

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Abstract

Purpose: To investigate the effects of tamoxifen and tanshinone administered individually or in combination, on the proliferation of breast cancer (BC) cells, and the underlying mechanism(s) of action.
Methods: Human breast cancer cell lines (SNU-306, SNU-334 and SNU-1528), and normal primary mammary epithelial cell line (HMEC) were cultured at 37 ℃ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % fetal bovine serum (FBS), l glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified incubator containing 5 % CO_2. Cell proliferation was determined using MTT assay, while real-time quantitative polymerase chain reaction (qRT-PCR) was used to determine the expressions of apoptosis-related genes. The expressions of p38 mitogen-activated protein kinases (p38 MAPK) were determined by Western blotting.
Results: There were only few viable cells in tamoxifen- and tanshinone-treated wells, and cell viability was concentration-dependently reduced. Treatment of SNU-306 cells with tamoxifen (30 µM) or tanshinone (20 µM) alone significantly reduced the expression of Wip1 after 72 h of incubation, and the level of expression was significantly reduced in SNU-306 cells treated with combination of tamoxifen and tanshinones, relative to those treated with tamoxifen or tanshinone alone (p < 0.05). The extent of apoptosis was significantly higher in SNU-306 cells treated with tamoxifen or tanshinone alone or in combination than in control cells (p < 0.05). Expressions of Bax, caspase 3 and p53 were significantly higher in SNU-306 cells than in control cells, and were significantly higher in SNU-306 cells treated with combination of tamoxifen and tanshinone than in those treated with tamoxifen or tanshinone alone (p < 0.05). The level of expression of MAPK was significantly higher in SNU-306 cells treated with tamoxifen or tanshinone alone, and in combination treatment, than in control cells (p < 0.05).
Conclusion: Tamoxifen and tanshinone administered alone or in combination promote apoptosis in BC cells via mechanisms involving the up-regulation and phosphorylation of MAPK.

Keywords: Breast cancer, Tamoxifen, Tanshinone, Expression, Apoptosis

INTRODUCTION

Breast cancer (BC) is a malignant tumor and one of the leading causes of death in women globally [1]. The incidence of BC has greatly increased over the last two decades, and endocrinotherapy is the most commonly used method for its treatment [2-4]. The rapid

invasion and metastasis of BC cells affect its treatment [5,6]. Therefore, the development of new treatment strategies for BC is needed to reduce mortalities associated with BC.

Apoptosis, a programmed cell death regulated by the expressions of various molecules such as Bax, p53 and caspases, plays an important role in the regulation of cell proliferation [7,8]. Apoptosis is inhibited in prostate carcinoma cells on exposure to γ-rays via a mechanism involving the up-regulation of wild-type p53-induced phosphatase 1 (Wip1) [9]. The expression of Wip1 is involved in the activation of various pathways [10-13].

This molecule is involved in the development and metastasis of ovarian carcinoma, and its overexpression prevents the cells from undergoing apoptosis [11-14]. Studies have shown that Wip1 is elevated in kidney and nasopharyngeal carcinoma tissues [15,16]. It has been reported that targeting of the expression of Wip1 is of therapeutic importance.

The present study investigated the effects of tamoxifen and tanshinone administered alone or in combination, on the proliferation of BC cells, and the underlying mechanism(s).

EXPERIMENTAL

Materials

The cell lines SNU-306, SNU-334, SNU-1528 and HMEC were obtained from the American Type Culture Collection (USA); DMEM was a product of Gibco BRL (USA), while Synergy II microplate reader was purchased from Bio-Tek Instruments Inc. (USA). Flow cytometer was purchased from BD Biosciences (USA), and bicinchoninic acid (BCA) assay kit was a product of Sangon Biotech Co., Ltd. Chemiluminescence liquid and autoradiography film were purchased from Bio-Rad Laboratories Inc. (USA). Trizol reagent was a product of Thermo Fisher Scientific Inc. (USA), and SYBR Premix Ex TaqTM II was purchased from Takara Bio Inc. (Japan).

Cell culture

The SNU-306, SNU-334, SNU-1528 and HMEC were cultured at 37 °C in DMEM supplemented with 5 % FBS, l-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified incubator containing 5 % CO_2. Cells in exponential growth phase were used for this study.

MTT assay

This was used to determine the extent of cell proliferation in SNU-306 cells. The cells (2 x $I0^4$ cells/well) at exponential growth phase were seeded into 96-well plates containing DMEM. After 24 h of incubation, the cells were treated with varied concentrations of tamoxifen or tanshinone (10 - 50 µM) and cultured for 72 h. This was followed by the addition of 20 ml of 0.5 % MTT solution within 4 h, after which the culture medium was changed. Dimethylsulfoxide (DMSO, 100 µl) was added in drops to each well, and the wells were placed on an oscillator for 10 min to completely dissolve the formazan crystals. The control wells contained culture medium, MTT solution and DMSO only, and were treated same way as the sample wells. Each well was incubated in the dark for 2 h and absorbance was measured at 495 nm using synergy II microplate reader. The procedure was performed in triplicate and cell proliferation (C) was calculated as in Eq 1.

$$C\ (\%) = (1 - Abs)/Abc \times 100\ \%$$

where *Abs* = absorbance of sample well, and *Abc* = absorbance of control well.

Apoptosis assay

Apoptosis in SNU-306 cells treated with tamoxifen or tanshinone (10 - 50 µM) was determined using a flow cytometer. The cells were incubated for 72 h, harvested and subsequently treated with HEPES binding buffer containing Annexin V-FITC and propidium iodide (PI). The treatment was carried out at room temperature for 20 min, after which the cells were placed on a flow cytometer and read. The measurements were performed in triplicate.

Western blotting

The expressions of Wip I, apoptosis-related genes and p38 MAPK were determined using Western blotting. The cells were lysed using ice-cold radioimmunoprecipitation assay (RIPA) buffer for 2.5 h. The cell lysate was washed twice with phosphate-buffered saline (PBS) and then treated with Nonidet P-40 (1 %), Triton X-100 (0.1 %), Na_2PO_4 (30 mM) mixed with sodium orthovanadate (1 mM), Tris-HCl (2.5 mM), sodium chloride (100 mM), leupeptin (10 µg/ml) and aprotinin for 45 min at 4 °C. The cell suspension was then centrifuged at 13,000 g for 25 min at 4 °C.

The protein concentration of the supernatant was determined using BCA assay kit. A portion of the total cell protein (35 μg) from each sample was separated on a 10 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min. Subsequently, non-fat milk powder (0.05 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C to block non-specific binding of the blot. Thereafter, the blot was incubated with primary antibodies of Wip1, caspase-3, p53, Bax, Bcl-2, p38 MAPK and p-p38 MAPK at a dilution of 1 to 1000 at 4 °C overnight. Thereafter, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The blot was developed using an x-ray film. Grayscale analysis of the bands was performed using ImageJ analysis software (4.6.2). Respective protein expression levels were normalized to that of β-actin, used as a standard reference.

qRT-PCR

Total RNAs were isolated from the treated and control cells using Trizol reagent. The RNAs were reverse-transcribed to cDNAs using random primers at 45 °C for 2 h. The samples were heated at 95 °C for 10 min, and PCR amplification of the reverse transcribed reaction mixture was carried out using 20 μl reaction mixture and equal volume of SYBR Premix Ex TaqTM II. The reaction mixture also contained cDNA (2 μl), mixture of forward and reverse primers (0.8 μl) and double-distilled H_2O (6 μl). The PCR conditions were: pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 3 s, annealing at 60 °C for 34 s, and 40 cycles. The procedure was performed in triplicate. Relative expression was quantified using $2^{-\Delta\Delta Cq}$ method, and β-actin gene was used as internal reference.

Statistical analysis

Data are expressed as mean ± SD, and the statistical analysis was performed using SPSS (17.0). Groups were compared using Student *t*-test, and values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of tamoxifen and tanshinone on SNU-306 cell viability

There were only few viable cells in tamoxifen- and tanshinone-treated wells, and cell viability

was concentration-dependently reduced. Maximum decrease in cell viability was obtained with tamoxifen and tanshinone at 30 and 20 μM, respectively, and cell proliferations at these concentrations were 62 and 58 %, respectively. However, combination of tamoxifen and tanshinone reduced the proliferation of SNU-306 cells to 29 % ($p < 0.05$; Figure 1).
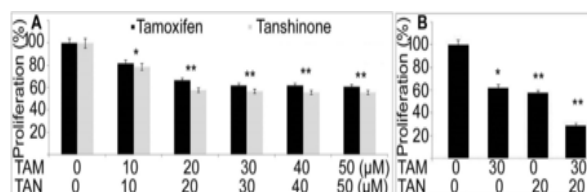

Figure 1: Effect of tamoxifen and tanshinone on the proliferation of SNU-306 cell. **A:** Proliferation of SNU-306 cells following treatment with varied concentration of tamoxifen or tanshinone (10 – 50 μM); **B:** Proliferation of SNU-306 cells following treatment with combination of tamoxifen and tanshinone. $^*p < 0.05$ and $^{**}p < 0.01$, when compared to control cells

Expressions of Wip1 in untreated BC and HMEC cells

Results of qRT-PCR and Western blotting showed that the level of expression of Wip1 was significantly higher in BC cells than in HMEC cells ($p < 0.05$; Figure 2).
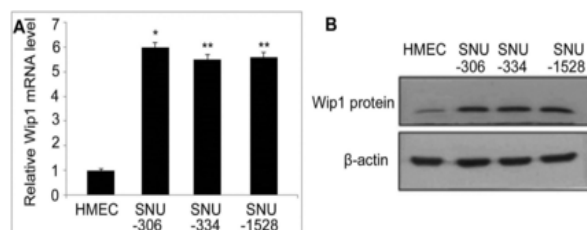

Figure 2: Expressions of Wip1 in BC and HMEC cells. **A:** Expression of Wip1 in BC and HMEC cells as determined using qRT-PCR; **B:** Expression of Wip1 in BC and HMEC cells as determined using Western blotting. $^*p < 0.05$ and $^{**}p < 0.01$, when compared to HMEC cells

Effects of tamoxifen and tanshinone on the expression of Wip1 in SNU-306 cells

Treatment of SNU-306 cells with tamoxifen (30 μM) or tanshinone (20 μM) alone significantly reduced the expression of Wip1; expression level of Wip1 was significantly reduced in SNU-306 cells treated with combination of tamoxifen and tanshinones, when compared with those treated with tamoxifen or tanshinone alone ($p < 0.05$). These results are shown in Figure 3.
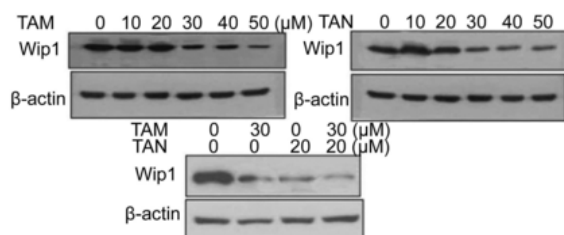
Figure 3: Expression of Wip1 in tamoxifen- and tanshinone-treated SNU-306 cells

Effects of tamoxifen and tanshinone treatment on cell apoptosis

As shown in Figure 4, the extent of apoptosis was significantly higher in SNU-306 cells treated with tamoxifen or tanshinone alone or in combination than in control cells ($p < 0.05$). Apoptosis in SNU-306 cells treated with tamoxifen or tanshinone alone, or in combination were 36.65, 40.98 and 67.32 %, respectively, while that of control was 2.43 %.
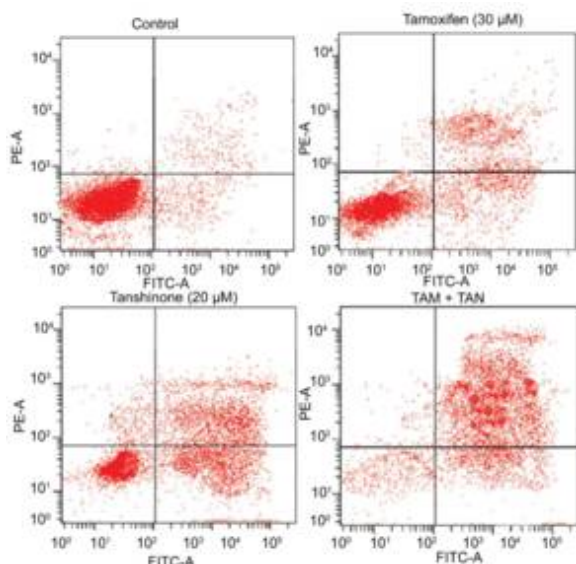

Figure 4: Effects of tamoxifen and tanshinone on apoptosis of SNU-306 cells. $^{*}P < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, when compared to control cells

Effects of tamoxifen and tanshinone on expressions of apoptosis-related genes

The expressions of Bax, caspase 3 and p53 were significantly higher in SNU-306 cells than in control cells, and significantly higher in SNU-306 cells treated with combination of tamoxifen and tanshinone than in those treated with tamoxifen or tanshinone alone ($p < 0.05$). However, the expression of Bcl-2 was significantly lower in the different treatment groups than in control cells ($p < 0.05$; Figure 5).
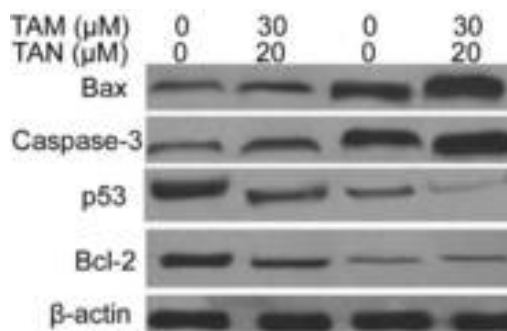

Figure 5: Up-regulation of apoptosis-related genes in SNU-306 cells treated with tamoxifen and tanshinone

Effects of tamoxifen and tanshinone treatment on p38 MAPK

The level of expression of MAPK was significantly higher in SNU-306 cells treated with tamoxifen or tanshinone alone or in combination than in control cells ($p < 0.05$). However, there were no significant differences in the expression levels of ERK and JNK between SNU-306 and control cells. These results are shown in Figure 6.
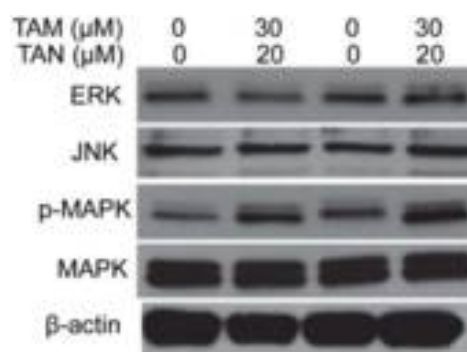

Figure 6: Expression levels of MAPK, ERK and JUN in SNU-306 cells

DISCUSSION

Inhibition of Wip1 expression using UV radiation has been reported to play a key role in the induction of cell apoptosis [17]. Cultures of BC cells exhibit marked increases in apoptosis on exposure to CCT007093, an inhibitor of Wip1 [17].

The present study investigated the effects of tamoxifen and tanshinone administered alone or in combination on the proliferation of BC cells, and the underlying mechanism(s). Tamoxifen or tanshinone administered alone or in combination significantly inhibited proliferation of BC cells, and the inhibition of proliferation was significantly higher in BC cells treated with combination of tamoxifen and

tanshinone. In human chromosomes, Wip1 exists as a serine/threonine phosphatase whose expression is upregulated in various types of cancers [18-21]. Overexpression of Wip1 causes down-regulation of cell apoptosis [22,23]. Studies involving neuronal tissues have shown that the suppression of Wip1 expression by exposure of nerve cells to manganese significantly enhances apoptosis [1]. It has also been reported that the expression of Wip1 in breast tumor is significantly higher than in normal tissues [24]. In this study, the expression of Wip1 was significantly higher in BC cells than in normal cells, an indication that Wip1 may promote the proliferation of BC cells.

In cancer cells, there are usually significant increases in the expressions of factors which inhibit apoptosis, and significant reductions in the expressions of pro apoptotic molecules [22,23]. In prostate carcinoma cells, exposure to γ rays causes up-regulation of Wip1 which in turn catalyses the translocation of Bax to mitochondrion, thereby inhibiting cell apoptosis [9]. In rats, inhibition of Wip1 overexpression has been reported to increase the levels of p53 and Bax, thereby enhancing cell apoptosis [14].

Regulation of the expressions of pro and anti-apoptotic factors has been reported to have immense importance in the treatment of cancers. In this study, the population of apoptotic cells increased significantly on exposure of BC cells to tamoxifen or tanshinone alone or in combination, and the combination increased apoptosis more than when the drugs were administered alone. Expressions of Bax, caspase 3 and p53 were significantly higher in SNU-306 cells than in control cells, and were significantly higher in SNU-306 cells treated with combination of tamoxifen and tanshinone than in those treated with tamoxifen or tanshinone alone. However, the expression of Bcl 2 was significantly lower in the different treatment groups than in control cells.

The phosphorylation of p38 MAPK by various chemotherapeutic agents has been reported to inhibit tumor growth and development via a mechanism involving cell cycle arrest [25 - 28]. In this study, the level of expression of MAPK was significantly higher in SNU-306 cells treated with tamoxifen or tanshinone alone or in combination than in control cells. However, there were no significant differences in the expressions levels of ERK and JNK between SNU-306 and control cells. These results suggest that tamoxifen and tanshinone may exert anti-apoptotic effect via the up-regulation and phosphorylation of MAPK. It is possible that

the down-regulation of Wip1 inhibits growth and development of BC cells by activating MAPK [24].

CONCLUSION

Tamoxifen and tanshinone administered alone or in combination promotes apoptosis in BC cells via mechanisms involving the up-regulation and phosphorylation of MAPK.

DECLARATIONS

Acknowledgement

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Haiyan Chen and Yuntao Wang performed all the experimental work and carried out the literature survey, Xiaoqing Ding designed the study and wrote the paper. The paper was approved by all the authors for publication.

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