Tanshinone IIA inhibits BT-20 human breast cancer cell proliferation through increasing caspase 12, GADD153 and phospho-p38 protein expression

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Received November 28, 2011; Accepted January 16, 2012

DOI: 10.3892/ijmm.2012.908

Abstract. Breast cancer is the leading cause of cancer-related deaths in women worldwide. Tanshinone IIA (Tan-IIA) is one of the pure compounds from Salviae miltiorrhizae radix (Danshen). Tan-IIA can inhibit human breast cancer cells but the molecular mechanisms are not well understood. Our previous study showed that Tan-IIA can inhibit hep-J5 human hepatocellular carcinoma cells through the endoplasmic reticulum (ER) stress-induced apoptotic pathway. In the present study, we evaluated the effects of Tan-IIA on BT-20 human breast cancer cells and assessed the involvement of the ER-stress-apoptotic pathway. The cytotoxicity of Tan-IIA in BT-20 cells was measured by the MTT assay. The cell cycles were analyzed by flow cytometry. The expression of ER stress-related proteins in BT-20 cells treated with Tan-IIA were evaluated by western blotting and immunocytochemical staining. These results showed that Tan-IIA can inhibit BT-20 cells and increase the sub-G1 phase in a time- and dose-dependent manner. Tan-IIA could increase the protein expression of caspase 12, GADD153, caspase 3, phospho-JNK, phospho-p38 and Bax, but decreased Bcl-xl and phospho-ERK expression in BT-20 cells. These findings indicate that Tan-IIA possesses therapeutic potential for human breast cancer BT-20 cells; one of the molecular mechanisms may be through inducing ER stress and the MAPK pathway to induce apoptosis and inhibit proliferation.

Introduction

Tanshinone IIA (Tan-IIA) is one of the pure compounds from Salviae miltiorrhizae radix (Danshen). It is well documented that Tan-IIA has antioxidant (1-3), and anti-inflammatory properties (4-6). In addition, Tan-IIA could inhibit multiple types of human cancer cells, such as human hepatocellular carcinoma cells (7), human breast cancer (8) and acute promyelocytic leukemia cells (9). Our previous studies have demonstrated that Tan-IIA inhibits the proliferations of human breast cancer MDA-MB-231 cells (10) human colon cancer Colo-205 cells (11,12), small cell lung cancer H146 cells (13), non-small cell lung cancer A549 (14) and human hepatocellular carcinoma hep-J5 cells (15). Breast cancer is the leading cause of cancer-related death in women worldwide (16). Tan-IIA inhibition of human breast cancer cells is well-documented (8,17-19). However, the molecular mechanisms are not understood clearly. Our previous study showed that Tan-IIA can inhibit human hepatocellular cancer hep-J5 cells through the endoplasmic reticulum (ER) stress-induced apoptotic pathway (14). In the present study, we assessed the effects of Tan-IIA in human breast cancer BT-20 cells and evaluated the involvement of the ER stress pathway and of other pathways in the Tan-IIA effects.

Materials and methods

Chemicals and reagents. Fetal bovine serum (FBS), sodium pyruvate, HEPES, dimethyl sulfoxide (DMSO), RPMI-1640, MTT, trypsin-EDTA, mouse anti-β-actin, and penicillin-streptomycin, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Buffer (10X TG-SDS), Tris, Tween-20, SDS and glycine, were obtained from Amresco (St. Louis, MO, USA). BioMax film was obtained from Kodak. The p38 inhibitor, SB203580 and the ERK inhibitor, were obtained from Cell Signaling Technology (Beverly, MA, USA). The caspase inhibitor, ZVAD-FMK was obtained from R&D Bioscences. Other materials and reagents not specified were obtained from Sigma or Merck.

Cell culture. The human breast cancer BT-20 cells are an estrogen receptor negative human breast cancer cells, obtained...
from the American Type Culture Collection (ATCC, HTB-19). BT-20 was established from a 74 year-old Caucasian female by E.Y. Lasfargues and L. Ozzello in 1958. The BT-20 cells were maintained in RPMI-1640 medium containing 10% FBS, 1% penicillin/streptomycin (10,000 U/ml penicillin, 10 mg/ml streptomycin) at 37°C in a humidified atmosphere containing 5% CO₂ (20).

Cell proliferation assay. The BT-20 cells were plated in 96-well plates at a density of 1x10⁵ cells/well and allowed to adhere and grow for 24 h. The medium was then replaced with 100 µl/well of fresh medium containing various concentrations of Tan-IIA (0, 0.25, 0.5, 1, 2, 4 and 8 µg/ml) and the cells were cultured for different durations (24, 48 and 72 h). Then, 100 µl of 1 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) were added and then the cells were incubated for 2 h at 37°C. Subsequently, the medium was removed and 100 µl DMSO was added to the wells. The absorbance was measured using an ELISA plate reader at 560 nm. Data were calculated as the percentage of proliferation by using the following formula: proliferation (%) = (ODtest−ODblank) × 100, where ODtest and ODblank are the optical densities of the test substances and the blank controls, respectively (15).

Cell cycle analysis. The BT-20 cells were plated in 12-well plates at a density of 6x10⁴ cells/well and allowed to adhere and grow for 24 h, then were treated with Tan-IIA (3 µg/ml) for different durations (24, 48 and 72 h) or treated with various concentrations of Tan-IIA (0, 1.5, 3 and 4.5 µg/ml) for 24 h. The cells were harvested and washed with PBS and then resuspended in 70% ethanol at -20°C overnight. The cells were washed thrice with PBS and then stained with 20 µg/ml propidium iodide (PI) buffer (0.1% Triton X-100, 0.2 µg/ml RNase A, 40 µg/ml PI). The DNA content was analyzed by fluorescence-activated cell sorting analysis (Beckman Coulter, FC500 flow cytometer) using CXP software (CXP cytometer and CXP analysis) (21).

Protein preparation. Approximately 1.5x10⁶ cells/6-cm dish were incubated with Tan-IIA (3 µg/ml) for different durations (0, 1, 3, 6 and 24 h; 24, 48 and 72 h) before the cells were harvested by centrifugation. The BT-20 cells were washed twice with ice-cold PBS and lysed in 100 µl of lysis buffer (Pro-Prep buffer; Intron). After incubation on ice for 30 min, the cell lysates were centrifuged (13,000 rpm, 4°C for 10 min) and the supernatants were collected. The protein concentration of the samples was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA) (10).

Western blotting. Western blot analysis was conducted using antibodies against calreticulin, caspase 12, cleaved caspase 3, GADD153, Bax, Bcl-xl, p-JNK, JNK, p-p38, p38, p-ERK and ERK. β-actin was used as the internal control to determine loading efficiency. Protein samples (containing 30 µg of protein) were separated on 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to immobilon polyvinylidene difluoride membrane (PVDF) membranes (Millipore, Bedford, MA). The membranes were incubated in TBST buffer [0.1 M Tris-HCl (pH 7.4), 0.9% NaCl, 0.1% Tween-20] supplemented with 5% dry non-fat milk for 1 h to block non-specific binding. After incubation with the primary antibodies, the membranes were washed thrice with TBST buffer followed by incubation with the appropriate streptavidin-HRP-conjugated secondary antibodies. The immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (Millipore). Immunoreactive bands were scanned (2000R; Kodak, Image Station) and analyzed by using ImageJ software (http://rsb.info.nih.gov/ij/) (22). Antibodies for calreticulin (no. 2891; 1:1,000), caspase 12 (no. 2202; 1:1,000), GADD153 (no. 2895; 1:2,000), full length caspase 3 (no. 9662; 1:1,000), phospho-ERK (no. 4730; 1:1,000), total-ERK (no. 9212; 1:1,000), total-JNK (no. 9252; 1:1,000), phospho-p38 (no. 9212; 1:2,000), total-p38 (no. 3491; 1:1,000), Bax (no. 2774; 1:2,000) and Bcl-xl (no. 2764; 1:1,000) were from Cell Signaling Technology. Antibodies for cleaved caspase 3 (no. GTX61024, 1:500) and phospho-JNK (no. GTX24821, 1:1,000) were from GeneTex, Inc.(San Antonio, TX, USA). The antibody for β-actin was from Sigma (no. A5441; 1:10,000). HRP-conjugated horse anti-mouse and goat anti-rabbit IgG antibodies were obtained from Cell Signaling Technology. Other materials and reagents not specified were obtained from Sigma or Merck.

Immunocytochemical staining. The immunocytochemical analysis procedures have been previously described (6). Briefly, the BT-20 cells were treated with Tan-IIA (3, 6 and 9 µg/ml) for 24 h, and were then fixed with 4% paraformaldehyde to allow the detection of caspase 12 (Cell Signaling Technology, no. 2202, 1:200), caspase 3 antibodies (GeneTex, Inc., 1:20). No treatment was used as the control, for the detection of the FITC-conjugated secondary antibody (1:500; Chemicon). The cells were then observed under a fluorescent microscope (Olympus, Olympus Model 1X81 microscope system).

Evaluate the effects of p38, ERK and caspase by SB203580, PD98059 and Z-VAD-FMK. BT-20 cells were pretreated with p38 inhibitor (50 µM) (SB203580, Cell Signaling Technology, no. 5633), ERK inhibitor (50 µM) (PD98059, Cell Signaling Technology, no. 9900), caspase inhibitor (50 µM) (ZVAD-FMK, RD, no. FMK001) for 1 h, respectively, then were treated with Tan-IIA (3 µg/ml) for 24 h. Cell viability was determined by the MTT assay as previously described (23).

Statistical analysis. Values are presented as the means ± SD. The Student's t-test was used to analyze statistical significance. A P-value <0.05 was considered statistically significant for all the tests.

Results and Discussion

The effects of Tan-IIA on the viability of BT-20 cells. The BT-20 cells were plated in 96-well plates at a density of 1x10⁵ cells/well and treated with various concentrations of Tan-IIA (0, 0.25, 0.5, 1, 2, 4 and 8 µg/ml) for different durations (24, 48 and 72 h) as previously described. The viable cell percentages relative to the control were 98.97±6.32, 99.32±2.55, 81.08±5.48, 53.76±0.56, 36.4±3.89 and 30.88±1.2% respectively, when cultured with 0, 1.5, 3 and 4.5 µg/ml Tan-IIA for 24 h, respectively. The viable cell percentages relative to the control were...
98.83±6.21, 92.14±8.06, 49.5±8.35, 26.32±3.63, 20.52±1.49 and 17.8±0.58%, respectively, when cultured with 0.25, 0.5, 1, 2, 4 and 8 µg/ml Tan-IIA for 48 h. The viable cell percentages relative to control were 79.67±3.35, 61.43±3.79, 34.7±1.26, 15.11±1.67, 10.78±0.89 and 7.08±0.32%, respectively, when cultured with 0.25, 0.5, 1, 2, 4 and 8 µg/ml for 72 h (Fig. 1). The IC\textsubscript{50} of Tan-IIA for BT-20 cells treated for different durations (24, 48 and 72 h) were 3.3, 1.87 and 0.67 µg/ml, respectively. These results showed that Tan-IIA could inhibit BT-20 cells in a dose- and time-dependent manner (Fig. 1).

Tan-IIA induced apoptosis in BT-20 cells. The BT-20 cells were plated in 12-well plates at a density of 6x10\textsuperscript{5} cells/well and then were treated with Tan-IIA (3 µg/ml) for different durations (24, 48 and 72 h), and then stained with propidium iodide. The cell cycle was determined by FACS assay as described in Materials and methods. The percentages of sub-G1 cells were 0.8, 2.9, 4.7 and 8.5% at 0, 24, 48 and 72 h, respectively. The BT-20 cells were plated in 12-well plates at a density of 6x10\textsuperscript{5} cells/well and then were treated with various concentrations of Tan-IIA (0, 1.5, 3 and 4.5 µg/ml) for 24 h. The cell cycle distributions were analyzed by FACS. The results showed that the percentages of sub-G1 cells were 3.3, 5.2, 8.8 and 13.8% after treatment with 0, 1.5, 3 and 4.5 µg/ml Tan-IIA, respectively.
Figure 4. Protein expression of caspase 12, caspase 3, GADD153 and calreticulin in BT-20 cells. The expression levels of ER stress-associated proteins in BT-20 cells treated with Tan-IIA (3 µg) for different durations (24, 48 and 72 h) were measured by western blotting as described in Materials and methods. The results show that Tan-IIA increases the protein expression of (A) caspase 12, (B) GADD153 and (C) cleaved caspase 3 but (D) decreases calreticulin expression.

Figure 5. Protein expression of Bax, Bcl-xl and β-actin in BT-20 cells. BT-20 cells were treated with Tan-IIA (3 µg/ml) for different durations (1, 3, 6 and 24 h). The protein expression levels were measured by western blotting as described in Materials and methods. The results show that Tan-IIA could increase the ratio of Bax/Bcl-xl at the 6 and 24 h time points.
Figure 6. Protein expression of p-p38, p38 and β-actin in BT-20 cells. The expression levels of the MAPK pathway associated proteins in BT-20 cells treated with Tan-IIA (3 µg) for different durations (1, 3, 6 and 24 h) were measured by western blotting as described in Materials and methods. The results show that Tan-IIA decreases the protein expression of (A) total-p38 but (B) increases the protein expression of p-p38, especially at the 6 h time point.

Figure 7. Protein expression of p-ERK, ERK and β-actin in BT-20 cells. The MAPK pathway associated protein expression in BT-20 cells treated with Tan-IIA (3 µg) for different durations (1, 3, 6 and 24 h) were measured by western blotting as described in Materials and methods. The results show that Tan-IIA can decrease the protein expression of (A) total-ERK (44 kDa) and (B) p-ERK, especially at the 24 h time point.
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1.5, 3 and 4.5 µg/ml Tan-IIA for 24 h (Fig. 3). These results showed that Tan-IIA can induce apoptosis in a time- and dose-dependent manner.

The effects of Tan-IIA on the protein expression of calreticulin, caspase 12, cleaved caspase 3 and GADD153 in BT-20 cells. BT-20 cells were treated with Tan-IIA (3 µg/ml) for different durations (24, 48 and 72 h) and the proteins expression levels were evaluated by western blotting. The results showed that Tan-IIA increased the protein expression levels of caspase 12 (Fig. 4A), GADD153 (Fig. 4B) and cleaved caspase 3 (Fig. 4C), but decreased calreticulin expression (Fig. 4D). These results suggest that Tan-IIA can induce ER stress in BT-20 cells.

The effects of Tan-IIA on the protein expression of Bax, Bcl-xl, p-JNK, JNK, p-p38, p38, p-ERK and ERK in BT-20 cells. BT-20 cells were treated with Tan-IIA (3 µg/ml) for different durations (1, 3, 6 and 24 h) and the protein expression levels were evaluated by western blotting. The results showed that Tan-IIA could increase the ratio of Bax/Bcl-xl at the 6 and 24 h time points (Fig. 5). The results also showed that Tan-IIA decreased the protein expression levels of total-p38 (Fig. 6A) but increased the protein expression levels of p-p38 (Fig. 6B). Tan-IIA decreased the protein expression levels of total-ERK (44 kDa) (Fig. 7A) and p-ERK (Fig. 7B). Tan-IIA also increased the protein expression levels of total-JNK (54 and 46 kDa) (Fig. 8A) and p-JNK (54 and 46 kDa) (Fig. 8B).

Immunocytochemical analysis. BT-20 cells were treated with Tan-IIA (0, 3, 6 and 9 µg/ml) for 24 h, and then were fixed with 4% paraformaldehyde to allow for the detection of caspase 12 and caspase 3 by staining with the respective antibodies. BT-20
cells treated with Tan-IIA showed increased expression of caspase 12 (Fig. 9A) and caspase 3 (Fig. 9B), and the effect was dose-dependent. These results suggest that Tan-IIA induces ER stress and apoptosis in BT-20 cells.

**Effects of SB203580, PD98059 and ZVAD-FMK in BT-20 cells.** BT-20 cells were pretreated with the p38 inhibitor (50 µM) SB203580, the ERK inhibitor (50 µM) PD98059, Cell Signaling Technology) and the caspase inhibitor (50 µM) ZVAD-FMK, for 1 h, followed by treatment with Tan-IIA (3 µg/ml) for 24 h. Cell viability was determined by the MTT assay. The results showed that the viable cell percentages of BT-20 cells treated with SB203580 and with Tan-IIA significantly increased when compared to BT-20 cells treated with Tan-IIA alone. The results showed that the viable cell percentages of BT-20 cells treated with the p38 inhibitor SB203580 and with Tan-IIA significantly increased when compared to BT-20 cells treated with Tan-IIA alone (Fig. 10). Pretreatment of BT-20 cells with SB203580 or PD98059 decreased the cytotoxicity of Tan-IIA in BT-20 cells. These results showed that one of the molecular mechanisms for Tan-IIA to inhibit BT-20 cells may be through the MAPK pathway.

In conclusion, Tan-IIA could inhibit human breast cancer BT-20 cells in a time- and dose-dependent manner. This is in agreement with other reports (8,10,17,18). It is well-documented that activation of ER stress activates the upstream element caspase 12, followed by the upregulation of the target protein C/EBP-homologous protein (CHOP; also known as GADD153) (24). In the present study, the results showed that Tan-IIA could increase the protein expression levels of caspase 12, GADD153 and caspase 3. These results indicate that Tan-IIA could induce ER stress and apoptosis in BT-20 cells. It is well-documented that when GADD153 is upregulated Bcl-2 expression is suppressed (25,26). Our results also

Figure 9. Immunocytochemical analysis. BT-20 cells were treated with Tan-IIA (0, 3, 6 and 9 µg/ml) for 24 h, and then were fixed with 4% paraformaldehyde and stained with antibodies (caspases 12 and 3). Color staining for PI was red, and for caspases 12 and 3 were green. The results showed that BT-20 cells treated with Tan-IIA had increased expression of (A) caspase 12, and (B) caspase 3 when compared to the control group.
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showed that Tan-IIA increased the ratio of Bax/Bcl-xl. It was suggested that Tan-IIA induces ER stress to inhibit proliferation through increasing the ratio of Bax/Bcl-xl in BT-20 cells. The MAPK pathway (JNK, p38 and ERK) is one of the molecular mechanisms to regulate cancer cell activity. Phospho-p38 can induce apoptosis and phospho-ERK can inhibit apoptosis (27-29). Our results showed that Tan-IIA increased the protein expression levels of p-JNK (especially after 1 h) and p-p38 (especially after 6 h). Our results also showed that Tan-IIA decreased the p-ERK protein expression levels, especially at 24 h. The proposed model of the interactions between ER stress and the MAPK pathway in BT-20 cells treated with Tan-IIA is shown in Fig. 11.

This is the first report to demonstrate that Tan-IIA inhibits human breast cancer BT-20 cells through increasing caspase 12, GADD153, p-p38, p-JNK and caspase 3 protein expression levels and by decreasing p-ERK expression. These findings indicate that Tan-IIA has therapeutic potential in human breast cancer BT-20 cells. One of the molecular mechanisms for the Tan-IIA effects may be through inducing ER stress and the MAPK pathway to induce apoptosis and inhibit proliferation. It is necessary to perform an in vivo study to further confirm the effects of Tan-IIA observed in the in vitro breast cancer cell line.

Acknowledgements

This study was supported by grant 100-CCH-IRP-96 from the Research Section of the Changhua Christian Hospital, Changhua, Taiwan, R.O.C.

References