

Tanshinone IIA inhibits human breast cancer MDA-MB-231 cells by decreasing LC3-II, Erb-B2 and NF- κ Bp65

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Abstract. The ability of tanshinone IIA (Tan-IIA) to inhibit the proliferation of human breast cancer cell lines *in vitro* and *in vivo* is well documented. However, the molecular mechanisms have not been fully elucidated. In the present study, MDA-MB-231 cells were treated with different concentrations of Tan-IIA for 48 h, followed by protein extraction for western blotting. For an *in vivo* study, MDA-MB-231 cells were implanted directly into female SCID mice which were divided randomly into three groups to be treated with vehicle, Tan-IIA (20 mg/kg) and Tan-IIA (60 mg/kg) every other day orally, with treatment starting 4 weeks after inoculation with the MDA-MB-231 cells. The results showed that Tan-IIA inhibited the proliferation of MDA-MB-231 cells and decreased the protein expression of LC3-II and Erb-B2 *in vitro*. Treatment with Tan-IIA (20 or 60 mg/kg) for 90 days resulted in a reduction in tumor size and weight compared to the control group. The protein expression of NF- κ Bp65 was reduced, while caspase-3 was up-regulated compared to the control group. These findings indicate that Tan-IIA inhibits tumor growth in a MDA-MB-231 xenograft animal model. One of the molecular mechanisms may be through a decrease in NF- κ Bp65 and an increase in caspase-3 expression.

Introduction

Based on the GLOBOCAN 2008 estimates, breast cancer is the leading cause of cancer-related death in women worldwide. New cases of breast cancer are estimated to be 1,383,500 with 458,400 deaths (1). Many prescriptions for chemotherapy have

been derived from plants (2). Danshen (*Salviae miltiorrhizae* radix) has been used in traditional Chinese medicine for cardiovascular disease and appeared in the Chinese book, Shennong Bencao Jing (ca. 100 A.D.) (3). Tanshinone IIA (Tan-IIA) was extracted from danshen and was first described in 1968. It is known to have antioxidant (4) and anti-inflammatory properties (5). Our previous studies showed that Tan-IIA induces apoptosis in certain human cancer cells, such as human colon cancer colo 205 cells (6,7), human hepatocellular carcinoma hep-J5 cells (8), small-cell lung cancer H146 cells (9) and non-small-cell lung cancer A549 cells (10). Our previous study also showed that Tan-IIA inhibited MDA-MB-231 cells through inducing apoptosis by increasing Bax to Bcl-xL ratios and the protein levels of p21 and caspase-8 *in vitro* (11). The anticancer effects of Tan-IIA on human breast cancer cell lines *in vitro* and *in vivo* have been well documented (12-15). However, the molecular mechanisms have not been fully elucidated. In the present study, we evaluated the effects and molecular mechanisms of Tan-IIA on MDA-MB-231 cells *in vitro* using a human breast cancer xenograft model in SCID mice.

Materials and methods

Chemicals. Tan-IIA (molecular formula: C₁₉H₁₈O₃, purity >96% by HPLC) was purchased from Herbasin Co. (Shenyang, China). Corn oil, aprotinin, antipain, sodium deoxycholate, leupeptin, sodium orthovanadate, Triton X-100, Tris-HCl, ribonuclease-A and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). L-15 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). The MDA-MB-231 human breast cancer cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan).

Cell culture. The MDA-MB-231 human breast cancer cells were placed into 75-cm³ tissue culture flasks and grown at 37°C in a humidified atmosphere (CO₂ was not present) in L-15 medium (Sigma Chemical Co.) containing 10%

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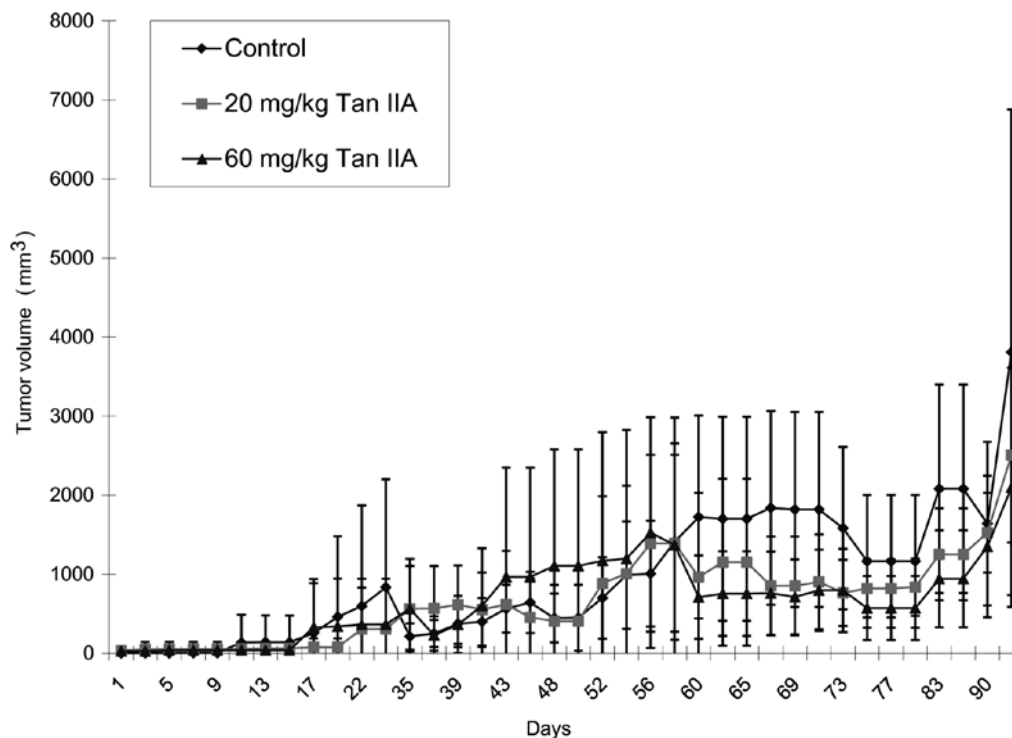


Figure 1. Three-week-old female nude SCID mice were xenografted with MDA-MB-231 cells. On day 28 onwards, the mice were divided randomly into three groups (each group $n=7$) to be treated with Tan-IIA (20 mg/kg) and Tan-IIA (60 mg/kg). As a control, mice were treated with corn oil (0.1 ml/10 g body weight). Each agent was administered orally every other day. At the end of the 3-month dosing schedule, the SCID mice were sacrificed by CO_2 inhalation on the 97th day. After xenograft transplantation, mice exhibiting tumors were monitored and the tumor size was measured once every 2 days using calipers. The results showed that Tan-IIA significantly decreased MDA-MB-231 cell xenograft tumor size.

heat-inactivated FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin). The data presented herein are from a minimum of three independent experiments.

The protein expression of LC3-II and Erb-B2 in MDA-MB-231 cells treated with Tan-IIA was determined by western blotting. The cytotoxicity of Tan-IIA on MDA-MB-231 cells has been well documented (11). The result showed that the proliferation of MDA-MB-231 cells was inhibited by Tan-IIA in a dose- and time-dependent manner. The IC_{50} was $11.85 \pm 0.29 \mu\text{g/ml}$ when MDA-MB-231 cells were treated with Tan-IIA for 48 h.

Protein preparation. Approximately 1×10^6 cells/10-cm dish were incubated with Tan-IIA at 0, 3, 10 and 25 $\mu\text{g/ml}$ concentrations for 48 h before the cells were harvested by centrifugation. Protein was extracted as described (16). Briefly, cell pellets were resuspended in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 $\mu\text{g/ml}$ aprotinin, 5 $\mu\text{g/ml}$ leupeptin and 5 $\mu\text{g/ml}$ antipain) for 30 min at 4°C . Lysates were immediately centrifuged at $13,000 \times g$ for 20 min at 4°C , and the supernatant was collected, aliquoted (50 $\mu\text{g/tube}$) and stored at -80°C before being assayed. The protein concentrations were measured using the Bradford method (17).

Effects of Tan-IIA on the expression of LC3-II, Erb-B2 and β -actin in MDA-MB-231 cells. All samples were separated by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel

electrophoresis (Bio-Rad Life Science Products, Hercules, CA, USA) as previously described (16). The SDS separated proteins were followed by equilibration in transfer buffer (50 mM Tris, pH 9.0, 40 mM glycine, 0.375% SDS and 20% methanol) and electro-transferred to Immobilon-P Transfer membranes (Millipore Corporation, Bedford, MA, USA). The blot was then blocked with a solution containing 5% nonfat dry milk in Tris-buffered saline [10 mM Tris and 150 mM NaCl (Sigma Chemical Co.)], containing 0.05% Tween-20 for 1 h. It was then washed and incubated with antibodies to Erb-B2 (R&D), LC3-II (Sigma Chemical Co.) and β -actin (Upstate, Lake Placid, NY, USA) at 4°C overnight. After incubating with anti-mouse peroxidase-conjugated antibody (Santa Cruz, Santa Cruz, CA, USA), the signal was visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). The detection of β -actin was used as an internal control in all the data for western blotting.

***In vivo* MDA-MB-231 cell tumor xenograft model.** Three-week-old female nude SCID mice (NOD.CB 17-Prkdcscid/Tcu) were xenografted with MDA-MB-231 human breast cancer cells ($3 \times 10^6/0.2$ ml) and maintained in a pathogen-free environment (Laboratory Animal Center of Tzu Chi University, Hualien, Taiwan). On day 28 onwards, the mice were divided randomly into three groups (each group $n=7$) to be treated with Tan-IIA (20 and 60 mg/kg of body weight, dissolved in corn oil). As a control, xenografted tumors were separately treated with corn oil (0.1 ml/10 g body weight). Each agent was administered orally every other day. At the end of the 3-month dosing schedule, the SCID mice were

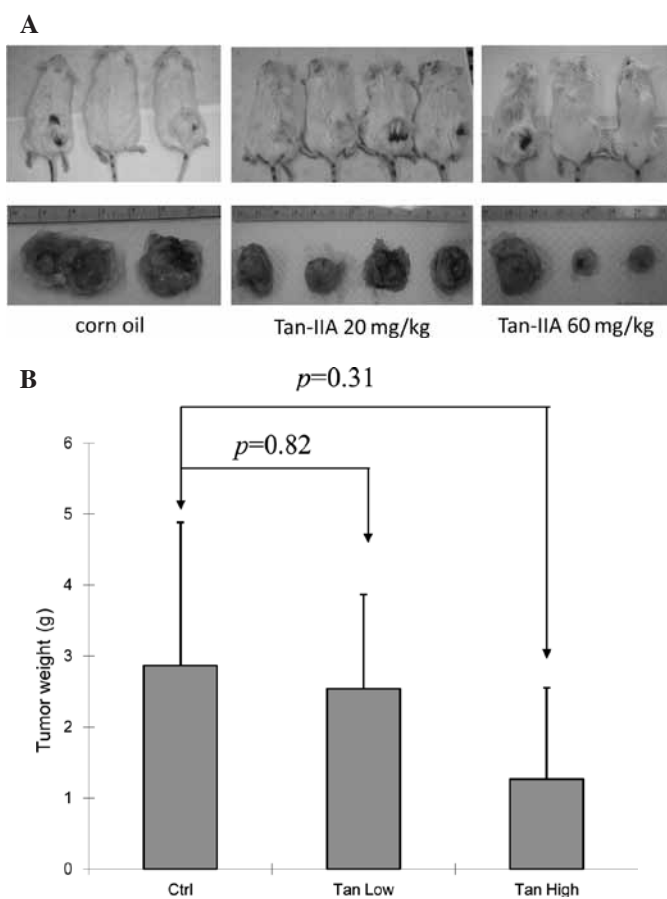


Figure 2. Mice bearing MDA-MB-231 cell xenograft tumors were treated with low dose (20 mg/kg) and high dose (60 mg/kg) Tan-IIA for 90 days. As a control, mice were treated with corn oil. (A) At the end of the 3-month dosing schedule, the SCID mice were sacrificed by CO₂ inhalation on the 97th day, and the xenograft tumors were dissected and weighed. (B) The results also showed that high dose (60 mg/kg) of Tan-IIA significantly decreased the weight of MDA-MB-231 cell xenograft tumors when compared to the control group.

sacrificed by CO₂ inhalation on the 97th day. After xenograft transplantation, mice exhibiting tumors were monitored, and the tumor size was measured once every 2 days using calipers. The tumor volume in each animal was estimated according to the formula: Tumor volume (mm³) = L x W²/2 (where L is the length and W is the width), with the final measurement taken 3 months after tumor cell inoculation. At the same time, the body weight of each animal was measured once every 2 days. The xenograft tumors were dissected and weighed individually, then the proteins were extracted for western blotting. The protein expression of NF-κBp65, caspase-3 and β-actin was measured by western blotting. Immunoreactive bands were scanned and analyzed using a digital scanning densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

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

Results and Discussion

The results showed that Tan-IIA significantly decreased the MDA-MB-231 cell xenograft tumor size (Fig. 1). An illustra-

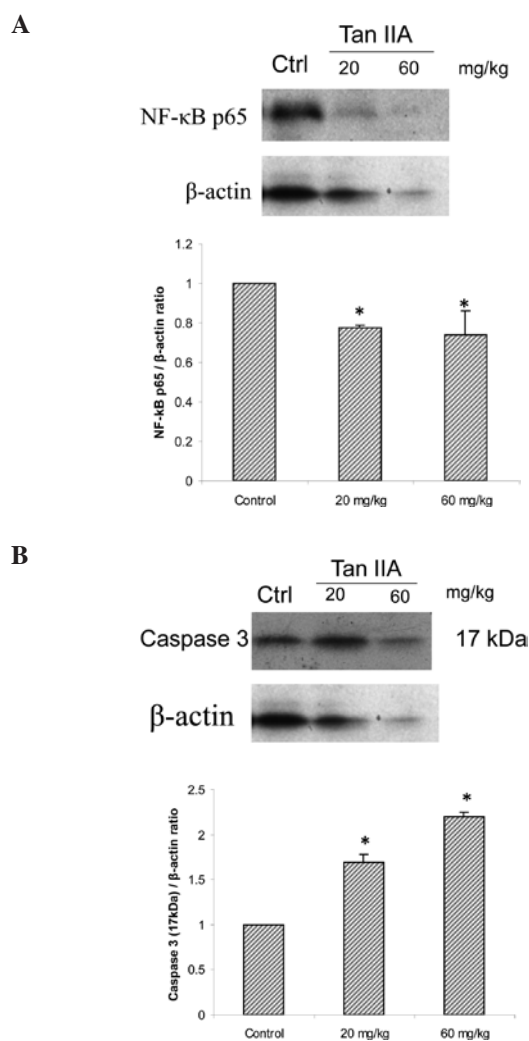


Figure 3. MDA-MB-231 cell xenograft model treated with Tan-IIA decreased NF-κBp65 expression (A), but increased caspase 3 expression (B) when compared to the control group. Values are expressed as the means ± SD. *Significant difference at p<0.05 vs. the control group.

tion of a representative animal treated with Tan-IIA relative to the control is shown in Fig. 2A. The results also showed that Tan-IIA (60 mg/kg) significantly decreased the weight of MDA-MB-231 cell xenograft tumors when compared to the control group (Fig. 2B). All MDA-MB-231 cell xenograft tumors appeared only at the inoculation sites (data not shown). Based on these results, Tan-IIA treatment (both 20 and 60 mg/kg body weight/per day) by oral administration inhibited MDA-MB-231 cell xenograft tumor growth. In the present study, mice with MDA-MB-231 xenograft tumors received Tan-IIA treatment; these tumors continue to grow slowly, indicating that multiple treatments are necessary to achieve a complete response. However, the results showed that a high dose of Tan-IIA had therapeutic potential in MDA-MB-231 cells *in vivo*. This is in agreement with other studies (12,13). Our results showed that MDA-MB-231 cell xenograft tumors treated with Tan-IIA decreased NF-κBp65 (Fig. 3A), but increased caspase 3 expression (Fig. 3B) when compared to the control group. Our results also showed that MDA-MB-231 cells treated with Tan-IIA for 48 h decreased

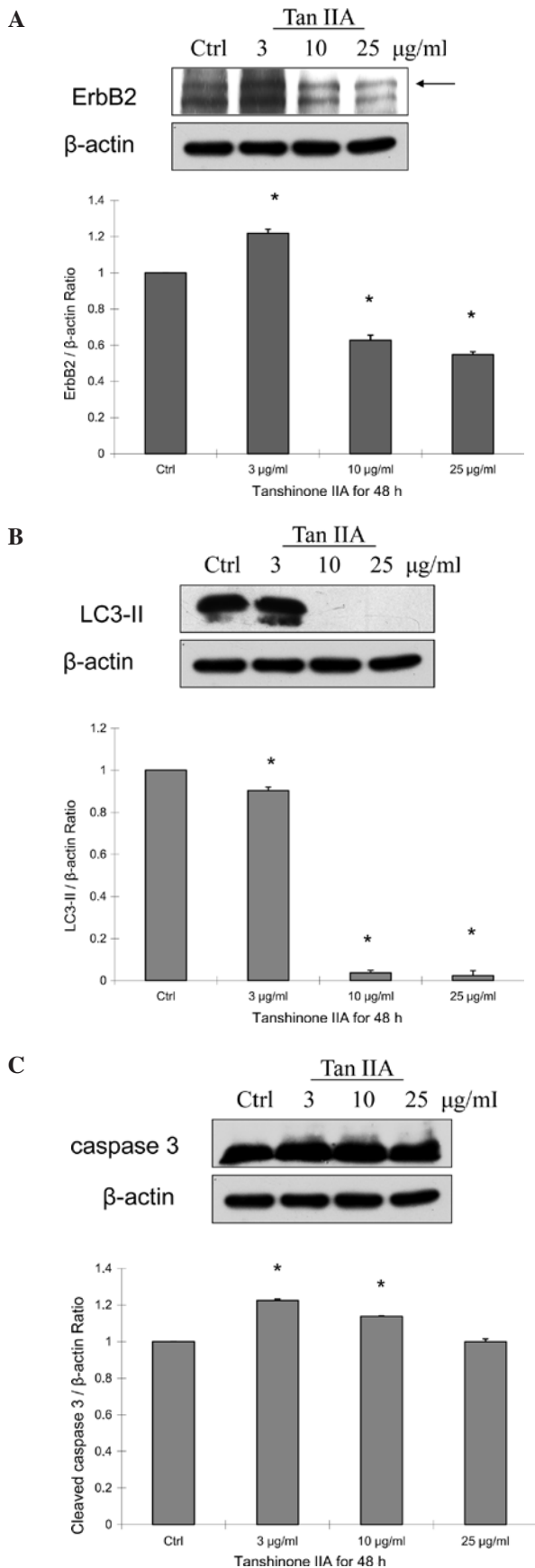


Figure 4. Protein expression of Erb-B2, LC3-II, caspase 3 and β -actin in MDA-MB-231 cells treated with Tan-IIA for 48 h. The results showed that MDA-MB-231 cells treated with Tan-IIA for 48 h down-regulated the protein expression of Erb-B2 (A) and LC3-II (B), but increased caspase 3 expression (C) *in vitro*. Values are expressed as the means \pm SD. *Significant difference at $p < 0.05$ vs. the control group.

the protein expression of Erb-B2 (Fig. 4A) and LC3-II (Fig. 4B), but increased caspase 3 (Fig. 4C) expression. This is not in agreement with other studies, which showed that xenograft tumors treated with Tan-IIA decreased P53 and bcl-2 expression, but not of cerbB-2 (12,13). The present study provides the first report of Tan-IIA anti-human breast cancer activity through a decrease in Erb-B2 and LC3-II expression *in vitro* and a decrease in NF- κ Bp65 expression in an MDA-MB-231 cell xenograft tumor model.

Acknowledgements

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