

# Tanshinone IIA inhibits Hep-J5 cells by increasing calreticulin, caspase 12 and GADD153 protein expression

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**Abstract.** Tanshinone IIA (Tan-IIA) is extracted from Danshen, *Salviae miltiorrhizae* Radix, which has been widely adopted in traditional herbal medicine to treat cardiovascular and hepatic diseases. Tan-IIA induces apoptosis and inhibits proliferation in human hepatocellular carcinoma (HCC) cells. However, the molecular mechanisms of Tan-IIA on human HCC cells are not understood clearly. In the present study, the cytotoxicity of Tan-IIA as well as its molecular mechanisms in human HCC J5 cells was investigated. The cytotoxicity was assayed by MTT. The protein expression of p53, p21, Bax, Bcl-2, Cdc25c, Cdc2, calreticulin, caspase 12, GADD153, caspase 3 and  $\beta$ -actin in J5 cells were determined by Western blotting. The cell cycles were analyzed by FACS. The protein expression of caspase 12, GADD153 and caspase 3 were detected by immunocytochemical staining. The results showed that Tan-IIA inhibited J5 cells in a dose- and time-dependent manner. The protein expression of p53, p21, Bax, calreticulin, caspase 12, caspase 3 and GADD153 were increased, but Bcl-2, Cdc25c and Cdc2 were decreased in J5 cells. In addition, the results also showed that Tan-IIA arrested J5 cells in the G2/M phase. Immunocytochemistry staining showed that J5 cells treated with Tan-IIA up-regulated the protein expression of caspase 12, 3 and GADD153. Taken together, the findings suggest that Tan-IIA inhibits and induces apoptosis in J5 cells through novel molecular targets, calreticulin, caspase 12 and GADD153.

## Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer in men in the world (1). According to a 2007 report from the 'People's Health Bureau of Taiwan' HCC is the leading cause of cancer related deaths in men (41.4/100,000) in Taiwan

(2). HCC is chemo-resistant to many of the available chemotherapeutic agents (such as 5-fluorouracil, doxorubicin and cisplatin) (3). Because of serious limitations of the surgical and medical treatment available, an ongoing need for more effective chemotherapeutic agents exists for HCC. The endoplasmic reticulum (ER) stress-induced apoptotic pathway has been suggested to be least affected by the resistance mechanisms, becoming a potential target of chemotherapeutic strategy (4). Tanshinone IIA (Tan-IIA; C<sub>19</sub>H<sub>18</sub>O<sub>3</sub>) was extracted from Danshen, *Salviae Miltiorrhizae* Radix (5,6). It has been well documented that Tan-IIA possesses antioxidant properties (7,8), anti-inflammatory activities (9,10) and anti-tumor activity in many human cancer cells (11-13). Our previous studies showed that Tan-IIA induces apoptosis and inhibits proliferation in human lung cancer A549 cells through the induction of ROS and a higher ratio of Bax/Bcl-2 (14). Growth inhibition and apoptosis induction of Tan-IIA on human hepatocellular carcinoma cells are well documented (15-17). However, the molecular mechanisms that cause Tan-IIA to induce apoptosis in human hepatocellular carcinomas via interaction of ER stress have not been clarified. In the present study, we investigated the effects of Tan-IIA in human hepatocellular carcinoma J5 cells.

## Materials and methods

**Chemicals and reagents.** Fetal bovine serum (FBS), sodium pyruvate, HEPES, dimethyl sulfoxide (DMSO), RPMI-1640, MTT, trypsin-EDTA, mouse anti- $\beta$ -actin, Cdc25c and penicillin-streptomycin were obtained from Sigma-Aldrich (St. Louis, MO, USA), 10x TG-SDS buffers, Tris, Tween-20, SDS and glycine were obtained from Amresco (St. Louis, MO, USA). BioMax Film was obtained from Kodak. Mouse anti-caspase 12 and Cdc2 were obtained from Abcam. Mouse anti-caspase 3 was obtained from R&D (Minneapolis, MN, USA), and rabbit anti-Bcl-2 and calreticulin from Cell Signaling Technology.

**Cell culture.** The human hepatocellular carcinoma J5 cell line was kindly provided by Dr H.J. Harn (Graduate Institute of Cancer Biology and Center for Molecular Medicine, China Medical University and Hospital, Taichung, Taiwan).

J5 cells were maintained with RPMI-1640 medium containing 10% FBS, 1% penicillin-streptomycin (10,000 U/ml

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penicillin; 10 mg/ml streptomycin) at 37°C humidified atmosphere containing 5% CO<sub>2</sub>.

**Cell proliferation assay.** Viability was evaluated by MTT assay in triplicate (18). Briefly, J5 cells were plated in 96-well plates at a density of  $2 \times 10^4$  cells/well and allowed to adhere and grow for 24 h. J5 cells were treated with various concentrations (0, 1, 2.5, 5, 10, and 20  $\mu$ g/ml) of Tan-IIA for different durations (24, 48 and 72 h), then 100  $\mu$ l of 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added and incubated for 2 h at 37°C. Subsequently, the medium was removed and 100  $\mu$ l DMSO was added to the wells. Absorbance was measured using an ELISA plate reader at 590 nm. Data were calculated as the percentage of proliferation, using the following formula: Proliferation rate (%) =  $(OD_{\text{test}} - OD_{\text{blank}}) \times 100$ , where  $OD_{\text{test}}$  and  $OD_{\text{blank}}$  are the optical density of the test substances and the blank control, respectively.

**Protein preparation.** Approximately  $1 \times 10^6$  cells/10-cm dish were incubated with various concentrations of Tan-IIA (0, 1, 2.5 and 5  $\mu$ g/ml) for 24 h before cells were harvested by centrifugation. Protein was extracted as described previously (19). Briefly, cells cultured in 10-cm dishes were washed twice with ice-cold PBS and lysed in 100  $\mu$ l of lyses buffer (Pro-Prep buffer, Intron). After incubation on ice for 40 min, cell lysates were centrifuged and then supernatants were collected. Protein concentrations were measured using the Bradford method (20).

**Western blotting.** Western blot analysis was conducted using antibody against caspase 3, Cdc25c, Cdc2, caspase 12, CHOP, Bcl-2, Calreticulin and  $\beta$ -actin (internal controls) to determine loading efficiency. Protein samples containing 40  $\mu$ g of protein were separated on 10-15% SDS-polyacrylamide gels and transferred to immobilon polyvinylidene difluoride membranes (Millipore). 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 nonspecific binding. After incubated with primary Abs, the membranes were washed thrice with TBST buffer followed by incubation with appropriate streptavidin-HRP-conjugated secondary Abs. The immunoreactive bands were visualized with an enhanced chemiluminescence (ECL, Millipore Corporation) detection kit. Immunoreactive bands were scanned (GS-800; Bio-Rad Life Sciences, Hercules, CA, USA) and analyzed using a digital scanning densitometer (Quantity One, v4.4.0; Bio-Rad Life Sciences).

**Cell cycle analysis.** Cell cycle analysis was performed as stated previously (21). Briefly, J5 cells were treated with various concentrations (0, 1, 2.5 and 5  $\mu$ g/ml) of Tan-IIA for 24 h. Cells were harvested and washed with PBS, and then resuspended in 70% ethanol at -20°C overnight. Cells were washed thrice with PBS and stained with 20  $\mu$ g/ml propidium iodide (PI). DNA content was analyzed on a cytofluorimeter by fluorescence-activated cell sorting analysis (FACScan, Becton Dickinson Co., San Jose, CA) and ModFit software (Verity Software House, Turrumurra, NSW, Australia). J5 cells were treated with Tan-IIA (2.5  $\mu$ g/ml) for different

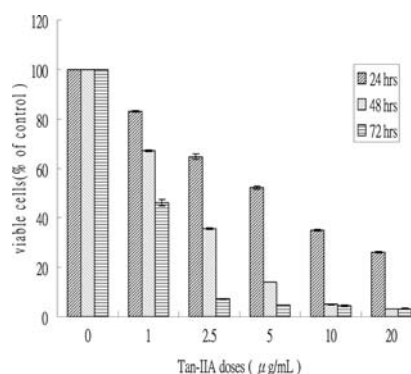


Figure 1. Cytotoxicity of Tanshinone IIA (Tan-IIA) in J5 cells. The cytotoxicity of Tan-IIA in J5 cells was determined using the MTT assay as described in Materials and methods. Tan-IIA significantly inhibited J5 cell growth in a time- and dose-dependent manner. Each point is the mean  $\pm$  SD of three experiments. \* $P < 0.05$  with respect to control.

durations (24, 48 and 72 h), then cells were harvested and washed in PBS, and resuspended in 70% ethanol at -20°C overnight. Cells were washed thrice with PBS and stained with 20  $\mu$ g/ml PI. DNA content was analyzed on a cytofluorimeter by fluorescence-activated cell sorting analysis (FACScan, Becton Dickinson Co.) and ModFit software (Verity Software House).

**Immunocytochemistry.** Immunocytochemistry analysis procedures were described previously (22). Briefly, J5 cells were treated with or without Tan-IIA (2.5  $\mu$ g/ml) for 24 h, and then fixed by 4% paraformaldehyde to allow the detection of anti-caspase 3, caspase 12 and GADD153 antibody (1:200, Cell Signaling Technology), and then FITC-conjugated secondary antibody (1:1,000, Chemicon). Cells were observed with fluorescent microscopy (Zeiss Axioskop 2 plus).

J5 cells were treated with Tan-IIA (2.5  $\mu$ g/ml) for 24 h, then washed with PBS twice. J5 cells were fixed in 4% paraformaldehyde solution for 1 h, then washed with PBS again and then stained with PI (5  $\mu$ g/ml) and/or with DAPI (0.4  $\mu$ g/ml) for 10 min in the dark. Cells were covered with PBS and observed with fluorescent microscopy.

**Inhibition of the effect of caspase 12 by Z-VAD-FMK.** Hep-J5 cells were pretreated with or without caspase 12 inhibitor Z-VAD-FMK (50  $\mu$ M) for 1 h, then treated with or without Tan-IIA (2.5  $\mu$ g/ml) for 24 h. Cell viability was determined with MTT assay as previously described (18).

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

## Results

**Cytotoxicity of Tan-IIA in J5 cells.** J5 cells were exposed to various concentrations of Tan-IIA of 0, 1, 2.5, 5, 10 and 20  $\mu$ g/ml for 24 h. Viable cell percentages relative to control were  $82.94 \pm 0.31$ ,  $64.71 \pm 1.15$ ,  $52.14 \pm 0.69$ ,  $34.99 \pm 0.35$  and

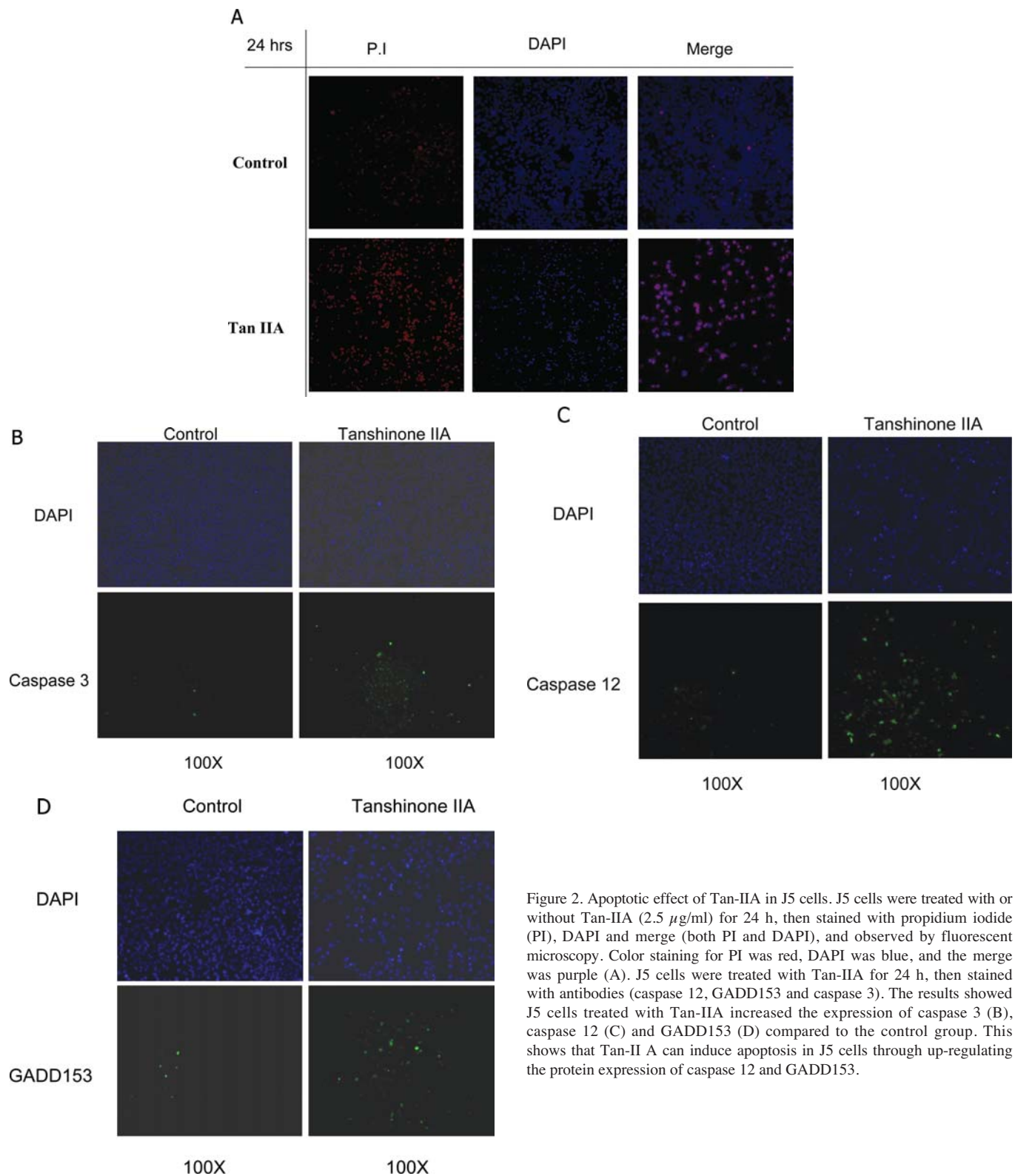


Figure 2. Apoptotic effect of Tan-IIA in J5 cells. J5 cells were treated with or without Tan-IIA (2.5  $\mu\text{g/ml}$ ) for 24 h, then stained with propidium iodide (PI), DAPI and merge (both PI and DAPI), and observed by fluorescent microscopy. Color staining for PI was red, DAPI was blue, and the merge was purple (A). J5 cells were treated with Tan-IIA for 24 h, then stained with antibodies (caspase 12, GADD153 and caspase 3). The results showed J5 cells treated with Tan-IIA increased the expression of caspase 3 (B), caspase 12 (C) and GADD153 (D) compared to the control group. This shows that Tan-II A can induce apoptosis in J5 cells through up-regulating the protein expression of caspase 12 and GADD153.

26.04 $\pm$ 0.3%, respectively. J5 cells were exposed to Tan-IIA at 0, 1, 2.5, 5, 10 and 20  $\mu\text{g/ml}$  for 48 h. Viable cell percentages relative to control were 67.07 $\pm$ 0.37, 35.69 $\pm$ 0.32, 13.99 $\pm$ 0.09, 4.96 $\pm$ 0.11 and 3.15 $\pm$ 0.07%, respectively. J5 cells were exposed to Tan-IIA at 0, 1, 2.5, 5, 10 and 20  $\mu\text{g/ml}$  for 72 h. Viable cell percentages relative to control were 46.04 $\pm$ 1.24, 7.21 $\pm$ 0.08, 4.67 $\pm$ 0.04, 4.57 $\pm$ 0.28 and 3.34 $\pm$ 0.07%, respectively. The results show that the cytotoxicity of Tan-IIA in J5 cells decreases in a dose- and time-dependent manner

(Fig. 1). The half maximal inhibitory concentration ( $\text{IC}_{50}$ ) for 24, 48 and 72 h was 5.62, 1.81 and 0.93  $\mu\text{g/ml}$ , respectively.

**Tan-IIA induces apoptosis in J5 cells.** The apoptotic effect of Tan-IIA in J5 cells was determined by immunocytochemistry. Cells were treated with or without Tan-IIA (2.5  $\mu\text{g/ml}$ ) for 24 h, then stained with PI, DAPI and merge (both PI and DAPI). Then J5 cells were observed with fluorescent microscopy. J5 cells stained with PI were red, DAPI were blue, and



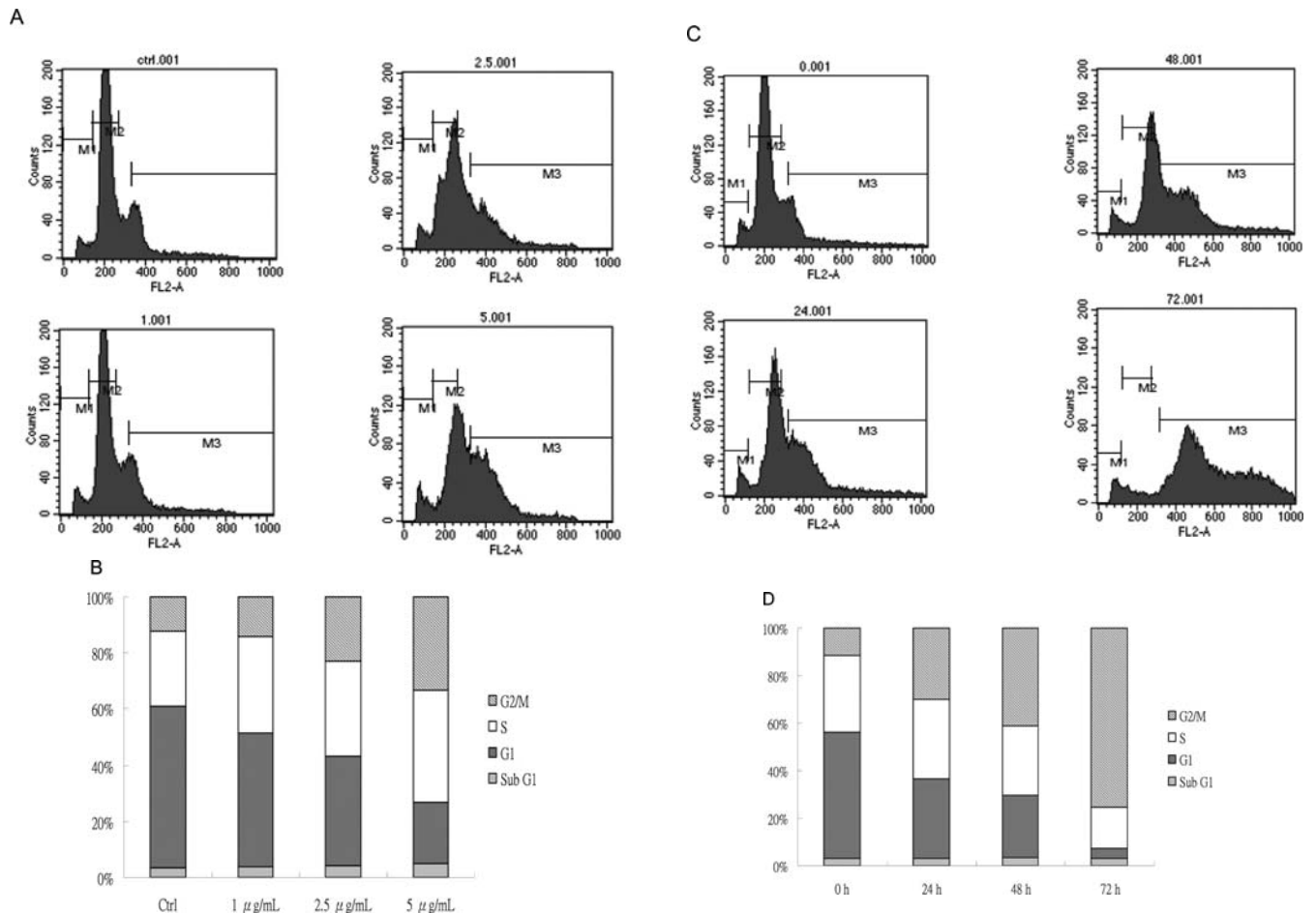


Figure 3. Cell cycle effect of Tan-II A in J5 cells. J5 cells were treated with various concentrations of Tan-II A (0, 1, 2.5 and 5 µg/ml) for 24 h and then stained with 10 µg/ml propidium iodide. The cell cycle was determined by FACS assay as described in Materials and methods. The results showed J5 cells treated with Tan-II A arrest in G2/M phase (A and B). J5 cells were treated with Tan-II A (2.5 µg/ml) for different durations (24, 48 and 72 h), and then stained with 10 µg/ml propidium iodide. The cell cycle was determined by FACS assay as described in Materials and methods. The results showed J5 cells treated with Tan-II A arrest in G2/M phase (C and D).

merge were purple (Fig. 2A). The results show that Tan-II A induced apoptosis.

J5 cells were treated with or without Tan-II A (2.5 µg/ml) for 24 h, then J5 cells were stained with DAPI and caspase 3. J5 cells were observed with fluorescent microscopy. We found that J5 cells treated with Tan-II A significantly increased caspase 3 expression as observed with DAPI staining (Fig. 2B). These results also suggest that Tan-II A induces apoptosis in J5 cells.

J5 cells were treated with or without Tan-II A (2.5 µg/ml) for 24 h, then stained with caspase 12 and GADD153. Then J5 cells were observed with fluorescent microscopy. Tan-II A increased the presentation of caspase 12 (Fig. 2C) and GADD153 (Fig. 2D) compared with the control group. These results suggest that Tan-II A induces ER stress in J5 cells.

**Tan-II A induced J5 cell arrest at G2/M phase.** J5 cells were treated with various concentrations of Tan-II A (0, 1, 2.5 and 5 µg/ml) for 24 h. Then the cell cycle was examined by flow cytometry. The results showed that Tan-II A induced J5 cell arrest at the G2/M phase (Fig. 3A and B). Hep-J5 cells were treated with Tan-II A (2.5 µg/ml) for different durations (24, 48 and 72 h), and then the cell cycle was examined with flow cytometry. Tan-II A also induced J5 cell arrest at the G2/M

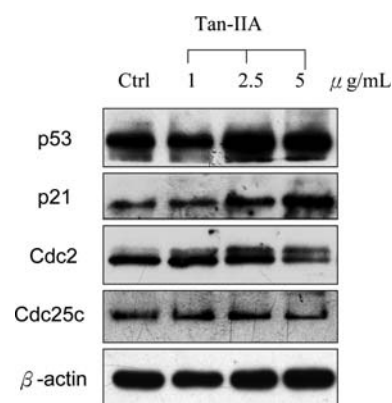


Figure 4. Protein expression of P53, P21, Cdc25c and Cdc2 in Hep-J5. The G2/M phase check-point associated protein expression in J5 cells treated with Tan-II A for 24 h were measured with Western blotting as described in Materials and methods. J5 cells were treated with Tan-II A for 24 h decreased Cdc25c and Cdc2 expression but increased P53 and P21 expression.

phase (Fig. 3C and D). Tan-II A induced J5 cell arrest at the G2/M phase in a time- and dose-dependent manner. In addition, J5 cells were treated with Tan-II A. The cell cycles associated with protein expression such as P53, P21, Cdc2 and

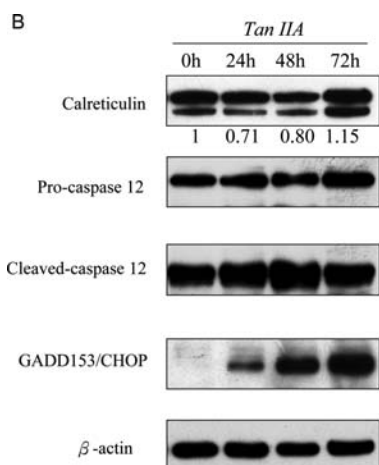
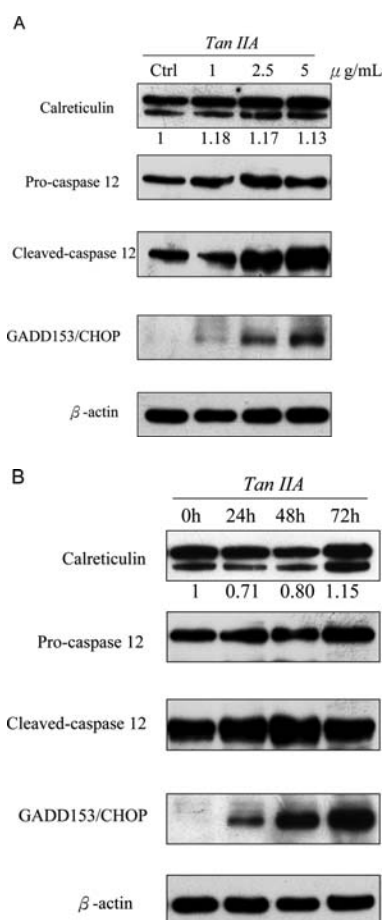


Figure 5. Effects of ER stress associated proteins in Hep-J5 cells. (A) J5 cells were treated with various concentrations of Tan-IIA (0, 1, 2.5 and 5  $\mu$ g/ml) for 24 h. The protein expression of calreticulin, caspase-12, GADD153, Bcl-2 and caspase 3 were measured by Western blotting as described in Materials and methods. (B) J5 cells were treated with Tan-IIA (2.5  $\mu$ g/ml) for different durations (24, 48 and 72 h), and then the protein expression of calreticulin, caspase-12 and GADD153 were measured by Western blotting as described in Materials and methods. Tan-IIA increased the protein expression of calreticulin, caspase-12 and GADD153 in dose- (A) and time- (B) dependent manner.

Cdc25c in J5 cells were measured by Western blotting. The results show that the protein expression of p53 and P21 were up-regulated but the protein expression of Cdc2 and Cdc25c were down-regulated in J5 cells (Fig. 4).

**Tan-IIA induces ER stress through Calreticulin and caspase 12 in J5 cells.** J5 cells were treated with various concentrations of Tan-IIA (0, 1, 2.5 and 5  $\mu$ g/ml) for 24 h, then examined by Western blotting. The results show that Tan-IIA up-regulated calreticulin and cleavage pro-caspase 12 expression, and then stimulated downstream GADD153 increasing in J5 cells (Fig. 5A). Hep-J5 cells were treated with Tan-IIA (2.5  $\mu$ g/ml) for different durations (24, 48 and 72 h), then examined by Western blotting. The results show that Tan-IIA up-regulated calreticulin and the cleavage of pro-caspase 12 expression, and then stimulated downstream GADD153 in J5 cells (Fig. 5B). The results also show that J5 cells treated with Tan-IIA induced GADD153 overexpression suggesting that Tan-IIA induces ER stress in J5 cells.

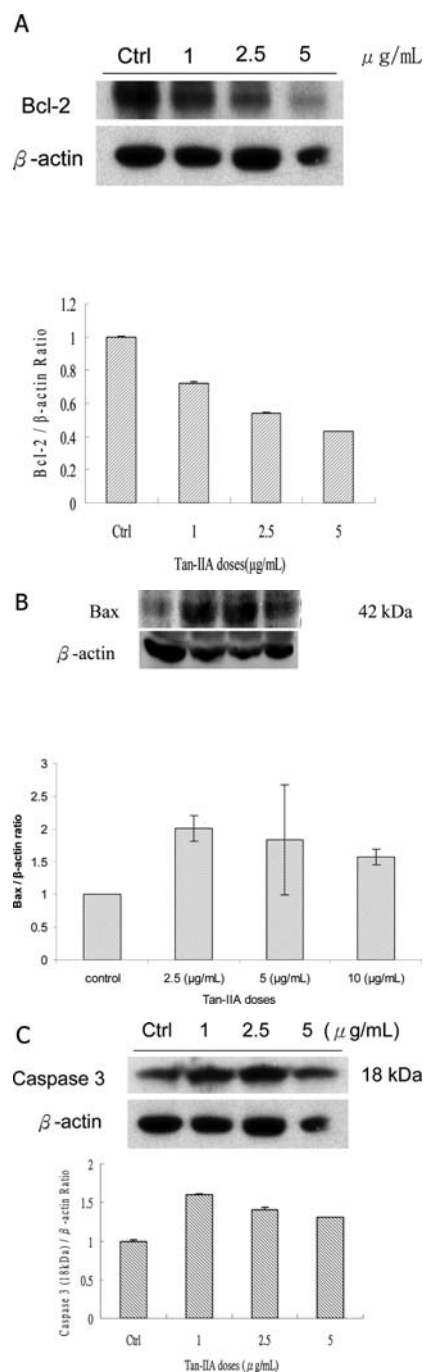


Figure 6. The protein expression of Bax, Bcl-2 and caspase 3 in Hep-J5 cells. (A) J5 cells were treated with various concentrations of Tan-IIA (0, 1, 2.5 and 5  $\mu$ g/ml) for 24 h. The protein expression of Bcl-2 and caspase 3 were measured by Western blotting as described in Materials and methods. (B) J5 cells were treated with various concentrations of Tan-IIA (0, 2.5, 5 and 10  $\mu$ g/ml) for 24 h. The protein expression of Bax was measured by Western blotting as described in Materials and methods. Tan-IIA increased caspase 3 (A) and Bax (B), but decrease Bcl-2 (C) expression.

**Tan-IIA induces apoptosis through mitochondrial dysfunction.** The protein expression of Bax, Bcl-2 and caspase 3 in J5 cells treated with Tan-IIA were measured by Western blotting. Tan-IIA decreased the protein expression of Bcl-2 (Fig. 6A) but increased Bax (Fig. 6B) and caspase 3 expression (Fig. 6C) in J5 cells. These results suggest that Tan-IIA destroys the mitochondrion function of J5 cells to induce apoptosis.

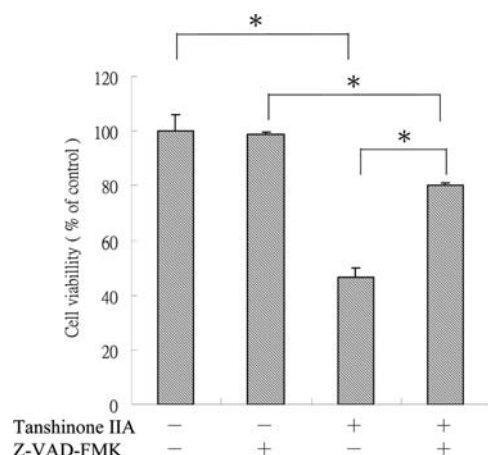


Figure 7. The effects of caspase inhibitor Z-VAD-FMK. J5 cells were pretreated with the caspase inhibitor Z-VAD-FMK (50  $\mu$ M) for 1 h, then treated with Tan-IIA (2.5  $\mu$ g/ml) for 24 h. Cell viability was determined by MTT assay as described in Materials and methods. J5 cells were treated with both Tan-IIA and Z-VAD-FMK. Viable cells increased significantly compared to Tan-IIA alone. Each point is the mean  $\pm$  SD of three experiments. \* $P < 0.05$  with respect to control.

**Inhibition of the effect of caspase 12 by Z-VAD-FMK.** Hep-J5 cells were pretreated with caspase 12 inhibitor Z-VAD-FMK (50  $\mu$ M) for 1 h, then J5 cells were treated with or without Tan-IIA (2.5  $\mu$ g/ml) for 24 h. The viability of J5 cells was determined by MTT assay. The viable cell percentages of J5 cells treated with Tan-IIA and Z-VAD-FMK increased significantly when compared to J5 cells treated with Tan-IIA alone (Fig. 7). J5 cells pretreated with Z-VAD-FMK (50  $\mu$ M) decreased the cytotoxicity of Tan-IIA in J5 cells. The results show that one of the molecular mechanisms for Tan-IIA to inhibit J5 cells may be through increasing caspase 12 expression.

## Discussion

In the present study the results show that Tan-IIA induced apoptosis and inhibited Hep-J5 cells in a time- and dose-dependent manner. Tan-IIA increased the protein expression of P53, P21 and Bax, but decreased Bcl-2 expression. These results are in agreement with previous studies (15). Our results show that Tan-IIA down-regulated the protein expression of Cdc2 and Cdc25c in J5 cells. In addition, Tan-IIA arrested J5 cells at the G2/M phase. It is not consistent with other studies that showed SMMC-7721 treated with Tan-IIA was arrested at the G0/G1 phase (15). ER stress-associated apoptotic molecules such as Calreticulin, caspase 12 and GADD153 are important key factors (23-25). Our results show that Hep-J5 cells treated with Tan-IIA increased the protein expression of calreticulin, caspase 12 and GADD153. Furthermore, the J5 cells pretreated with Z-VAD-FMK (50  $\mu$ M) decreased the cytotoxicity of Tan-IIA. One of the molecular mechanisms for Tan-IIA to inhibit Hep-J5 cells may be through up-regulating the protein expression of caspase 12. Tan-IIA inhibited proliferation and induced apoptosis in hepatocellular carcinoma cell lines by a different pathway, such as decreasing Bcl-2 and increasing Bax expression (15), down-regulating EGF and EGFR protein expression in SMMC-

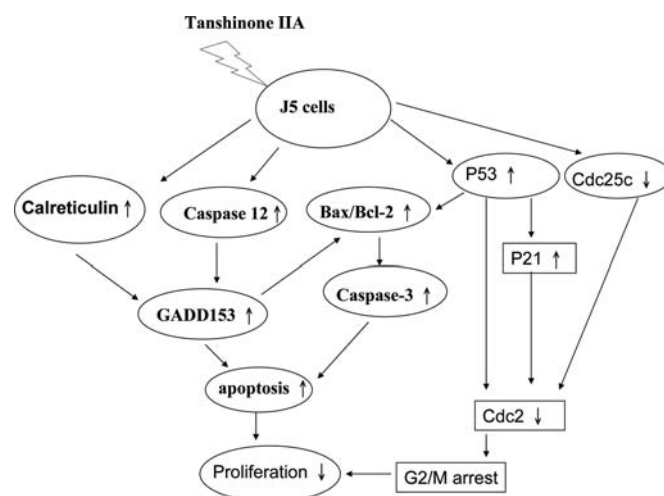


Figure 8. Proposed model for the molecular mechanism of Tanshinone IIA inhibition of the proliferation of J5 cells.

7721 (16) and by ROS-mediated p38 MAPK activation in HepG2 (17). Our results show that Tan-IIA inhibited proliferation and induced apoptosis in hepatocellular carcinoma J5 cells through up-regulating the protein expression of calreticulin, caspase 12 and GADD153. In Fig. 8 the proposed model of Tan-IIA inhibition of HCC J5 cells is reported for the first time.

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