

Tanshinone IIA inhibits gastric carcinoma AGS cells by decreasing the protein expression of VEGFR and blocking Ras/Raf/MEK/ERK pathway

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Abstract. The RAS/RAF/MEK/ERK pathway is one of the most frequently dysregulated kinase cascades in human cancer, that facilitate the proliferation and survival of cancers driven by growth factor receptors. Tanshinone IIA (Tan-IIA) was extracted from Danshen (*Salviae Miltiorrhizae* Radix). Tan-IIA inhibition of the proliferation of gastric cancer are well documented, but the molecular mechanisms of Tan-IIA inhibition of gastric cancer have not been well elucidated. We evaluated the protein expression of vascular epidermal growth factor receptor (VEGFR), human epidermal growth factor receptor 2 (HER2), Ras, Raf, MEK, ERK, PARP, caspase-3 and β -actin in AGS cells by western blotting. The results showed that AGS cells treated with Tan-IIA upregulated the protein expression of PARP and caspase-3 but decreased VEGFR, HER2, Ras, Raf, MEK and ERK time- and dose-dependently. These findings demonstrated that Tan-IIA inhibited human gastric cancer AGS cells; one of the molecular mechanisms may be through decreasing the protein expression of VEGFR and HER2, then blocking the Ras/Raf/MEK/ERK pathway to induce the activation of PARP and caspase-3 to induce apoptosis.

Introduction

Tanshinone IIA (Tan-IIA; C₁₉H₁₈O₃), is one of the active components in Radix *Salviae miltiorrhizae* (1,2), with antioxidant properties (3,4) and anti-inflammatory activities (5,6). Tan-IIA can inhibit many human cancer cell lines through different molecular mechanisms, such as colon cancer colo205 cells (7), breast cancer MDA-MB-231 cells (8),

non-small cell lung cancer A549 cells (9), small cell lung cancer H146 cells (10), hepatocellular carcinoma Hep-J5 cells (11), breast cancer BT-20 cells (12) and pancreatic cancer BxPC-3 cells *in vitro* (13,14). The transmembrane tyrosine kinase has been strongly implicated in the growth, survival, and metastasis of a wide variety of human tumors (15,16). The PI3K/AKT/mTOR and RAS/RAF/MEK/extracellular signal-regulated kinase (ERK) pathways are two of the most frequently dysregulated kinase cascades in human cancer are well documented (17,18). Both pathways represent important signal transduction mechanisms that facilitate the proliferation and survival of cancers driven by growth factor receptors, such as human epidermal growth factor receptor 2 (HER2) or epidermal growth factor receptor (EGFR). The individual downstream components of these signaling cascades either through somatic mutation or epigenetic modification, are also known to be frequently altered in cancer, thus contributing to tumorigenesis and resistance to anticancer therapies (19). It is well documented that Tan-IIA can inhibit the proliferation of AGS cells through inducing ER stress and intrinsic pathway to induce apoptosis (20). Tan-IIA also can inhibit human gastric cancer AGS cells through inducing G2/M phase arrest, and extrinsic pathway to induce apoptosis (21). These indicate that Tan-IIA may be one of the complementary medicines for gastric cancer. But the molecular mechanisms of Tan-IIA in gastric cancer cells remain unclear. In the present study, we investigated the protein expression levels of VEGFR, HER2, Ras, Raf, MEK, ERK, PARP and caspase-3 in human gastric cancer AGS cells treated with Tan-IIA.

Materials and methods

Tan-IIA was obtained from Sigma-Aldrich (St. Louis, MO, USA) (CAS no. 568-72-9); The HER2 (#2165, MW 185 kDa), Ras (#3339, MW 21 kDa), Raf (#12552, MW 75 kDa), MEK (#9126, MW 45 kDa), and caspase-3 (#9661, MW 17 kDa) antibodies were all obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). VEGFR (N100-527, MW 150 kDa) antibodies were both obtained from Novus Biologicals (Littleton, CO, USA). ERK (sc-94, MW 44 kDa) and PARP (sc-7150, MW 116, 89 kDa) antibodies were all obtained from Santa Cruz

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Key words: Tanshinone IIA, gastric carcinoma AGS cells, vascular epidermal growth factor receptor, Ras/Raf/MEK/ERK pathway

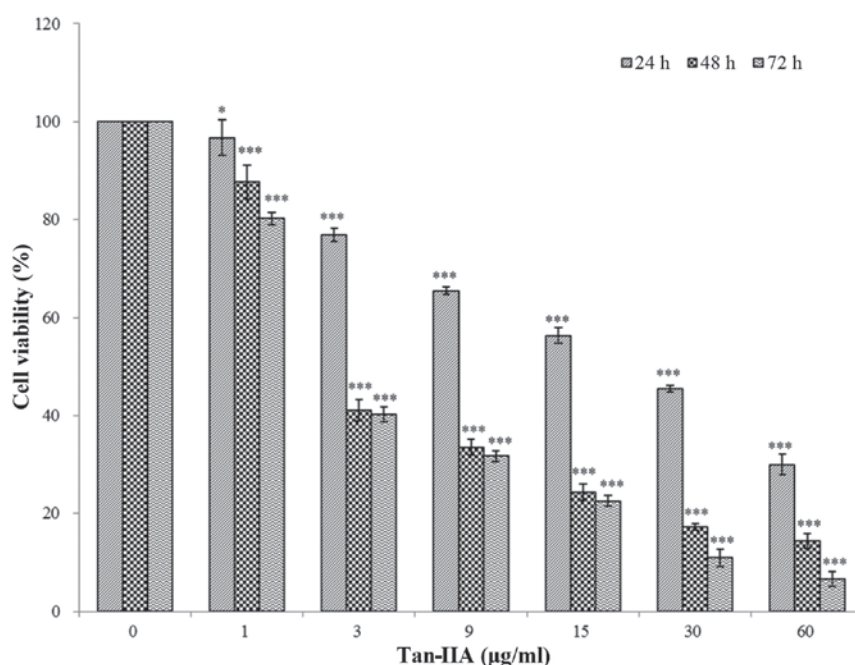


Figure 1. Cytotoxic effects of tanshinone IIA (Tan-IIA) on AGS cells. The cytotoxic effects of Tan-IIA on AGS cells were determined by MTT assay as described in Materials and methods. Each point is the mean \pm SD of 3 experiments. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Biotechnology, Inc. (Dallas, TX, USA). F-12K medium, fetal bovine serum (FBS), penicillin-streptomycin and glutamine were obtained from Gibco-BRL (Grand Island, NY, USA). Potassium phosphate and 0.2 mm PVDF membranes were purchased from Merck Co. (Darmstadt, Germany); BioMax film was from Kodak (Rochester, NY, USA). Sodium deoxycholate, leupeptin, Triton X-100, Tris-HCl, ribonuclease-A, sodium pyruvate, HEPES, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Tween-20, mouse anti- β -actin were from Sigma-Aldrich. The AGS human gastric adenocarcinoma cell line (BCRC no. 60102) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan).

Cell culture. The human gastric adenocarcinoma AGS cells were obtained from the Food Industry Research and Development Institute. The cell culture procedure was as previously described (20,21). Briefly, the AGS cells were placed into 75-cm² tissue culture flasks and maintained in F-12K contained with 10% heat-inactivated FBS (Gibco-BRL, Grand Island, NY, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO₂. All data presented are from at least three independent experiments.

Cytotoxicity assay. The cytotoxicity of Tan-IIA for AGS cells was evaluated by MTT assay in triplicate as previously described (20,21). Briefly, The AGS cells were plated in 96-well plates at a density of 2×10^4 cells/well for 16–20 h. Thereafter, the cells were treated with various concentrations (0, 1, 3, 9, 15, 30 and 60 μ g/ml) of Tan-IIA for 24, 48 and 72 h. Subsequently, the cells were incubated with 1 mg/ml of MTT in fresh complete F-12K medium for 1 h. The surviving cells converted MTT to formazan by forming a blue-purple color when dissolved in dimethyl sulfoxide. The intensity of

formazan was measured at 590 nm using a microplate reader. The relative percentage of cell viability was calculated by dividing the absorbance of treated cells by that of the control in each experiment, using the following formula: proliferation rate (%) = (OD test - OD blank) \times 100, where OD test and OD blank are the optical density of the test substances and the blank control, respectively.

Western blot analysis. The western blot procedures followed previous reports (20,21). Briefly, AGS cells were treated with various concentrations of Tan-IIA for different durations, and then the cells were lysed in ice-cold whole cell extract buffer containing the protease inhibitors. The lysate was vibrated for 30 min at 4°C and centrifuged at $12,281 \times g$ for 10 min. Protein concentration was measured by BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of proteins were subjected to electrophoresis using 12% sodium dodecyl sulfate-polyacrylamide gels. To verify equal protein loading and transfer, proteins were then transferred to polyvinylidene difluoride membranes and the membranes were blocked for 1 h at 4°C using blocking buffer (5% non-fat dried milk in solution containing 50 mM Tris/HCl (pH 8.0), 2 mM CaCl₂, 80 mM sodium chloride, 0.05% Tween-20 and 0.02% sodium azide). The membranes were then incubated for 2 h at room temperature with specific primary antibody followed by anti-rabbit or anti-mouse immunoglobulin G-horseradish peroxidase conjugated secondary antibodies. The membranes were washed three times for 10 min with washing solution. Finally, the protein bands were visualized on the X-ray film using the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, MA, USA).

Statistical analysis. Values are presented as the means \pm SD. The Student's t-test was used to analyze statistical significance.

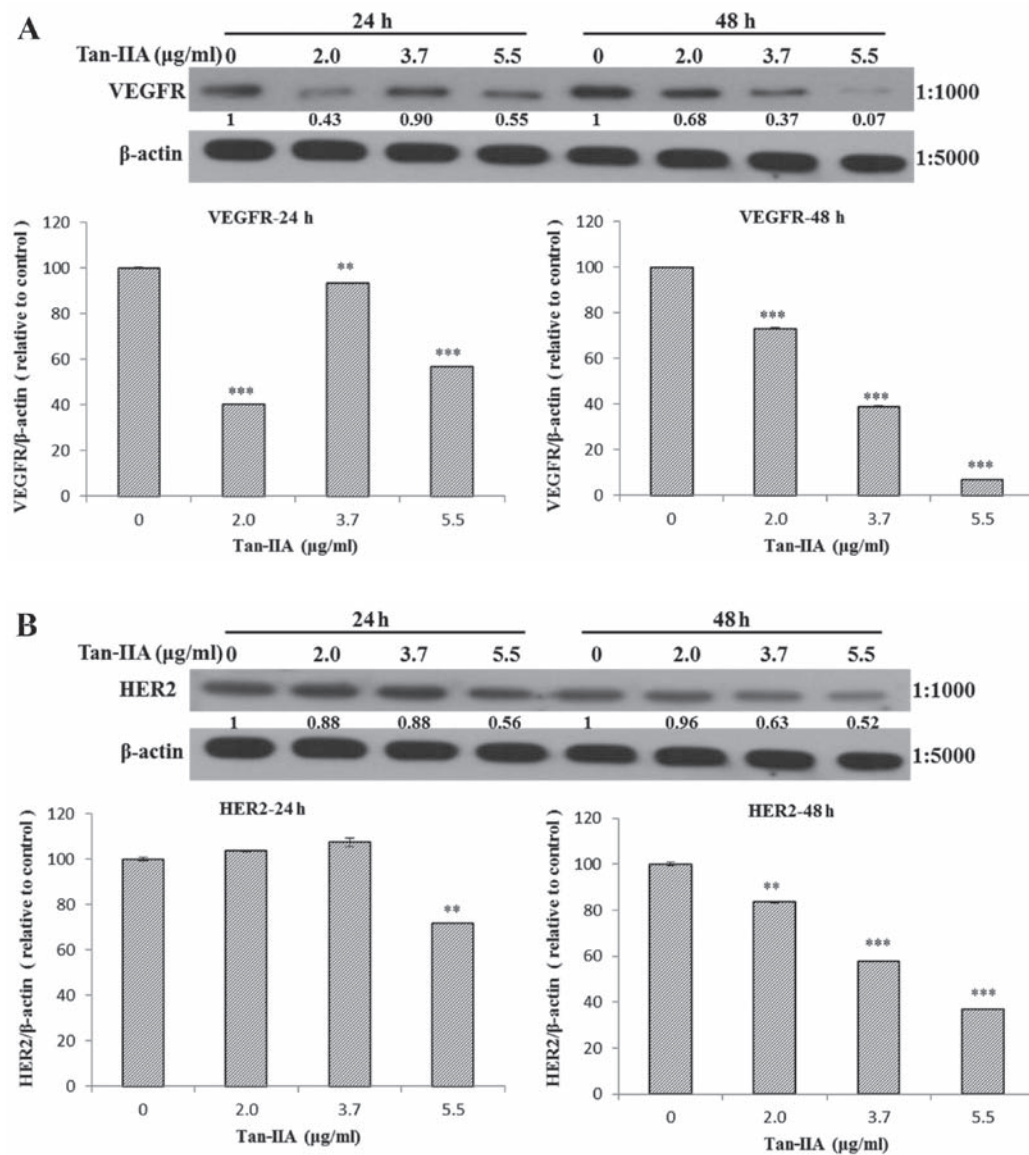


Figure 2. Protein expression of vascular epidermal growth factor receptor (VEGFR), human epidermal growth factor receptor 2 (HER2) and β -actin in AGS cells. The AGS cells were treated with various concentrations of tanshinone IIA (Tan-IIA) (0, 2.0, 3.7 and 5.5 μ g/ml) for 24 or 48 h and then the protein expression levels were evaluated by western blot analysis as described in Materials and methods. The results showed that Tan-IIA decreased the protein expression level of (A) VEGFR and (B) HER2 significantly. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

P -value < 0.05 , was considered to indicate a statistically significant difference for all the tests, at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

Results

Effects of Tan-IIA in the viability of AGS cells. The results revealed that Tan-IIA can inhibit AGS cells in a time- and dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) was 5.5, 3.7 and 3.5 μ g/ml at 24, 48 and 72 h, respectively (Fig. 1), this is in agreement with our previous studies (20,21).

Effects of Tan-IIA on the protein expression of VEGFR, HER2, Ras, Raf, MEK, ERK, PARP, caspase-3 and β -actin in AGS cells. The AGS cells were treated with various concentrations of Tan-IIA (0, 2.0, 3.7 and 5.5 μ g/ml) for 24 or 48 h and then the protein expression levels of VEGFR, HER2, Ras,

Raf, MEK, ERK, PARP, caspase-3 and β -actin were evaluated by western blot analysis. The results showed that Tan-IIA can decrease the protein expression levels of VEGFR (Fig. 2A), HER2 (Fig. 2B), Ras (Fig. 3A), Raf (Fig. 3B), MEK (Fig. 3C) and ERK (Fig. 3D), but increase PARP (Fig. 4A) and caspase-3 (Fig. 4B) levels significantly.

Effects of Tan-IIA on the protein expression of VEGFR, HER2, Ras, Raf, MEK, ERK, PARP, caspase-3 and β -actin in AGS cells. The AGS cells were treated with Tan-IIA (3.7 μ g/ml) for different durations (0, 24 and 48 h) and then the protein expression levels of VEGFR, HER2, Ras, Raf, MEK, ERK, PARP, caspase-3 and β -actin were evaluated by western blot analysis. The results showed that Tan-IIA can decrease the protein expression levels of VEGFR (Fig. 5A), HER2 (Fig. 5B), Ras (Fig. 5C), Raf (Fig. 5D), MEK (Fig. 5E) and ERK (Fig. 5F), but increased PARP (Fig. 5G) and caspase-3 (Fig. 5H) levels significantly.

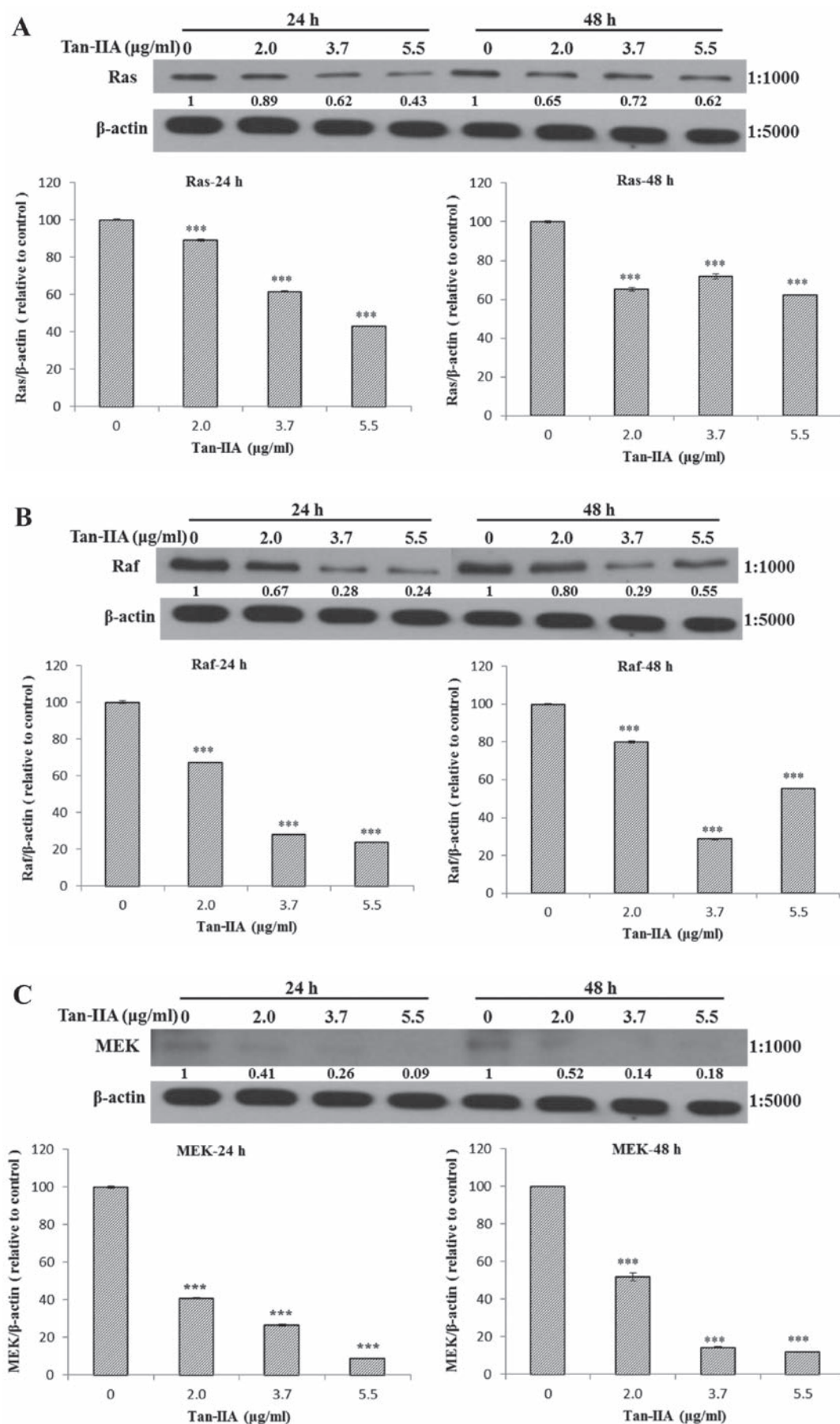


Figure 3. (A-C) Protein expression of Ras, Raf, MEK, extracellular signal-regulated kinase (ERK) and β-actin in AGS cells. The AGS cells were treated with various concentrations of tanshinone IIA (Tan-IIA) (0, 2.0, 3.7 and 5.5 μg/ml) for 24 or 48 h and then the protein expression levels were evaluated by western blot analysis as described in Materials and methods. The results show that Tan-IIA decreased the protein expression levels of (A) Ras, (B) Raf, (C) MEK levels significantly. *P<0.05; **P<0.01; ***P<0.001.

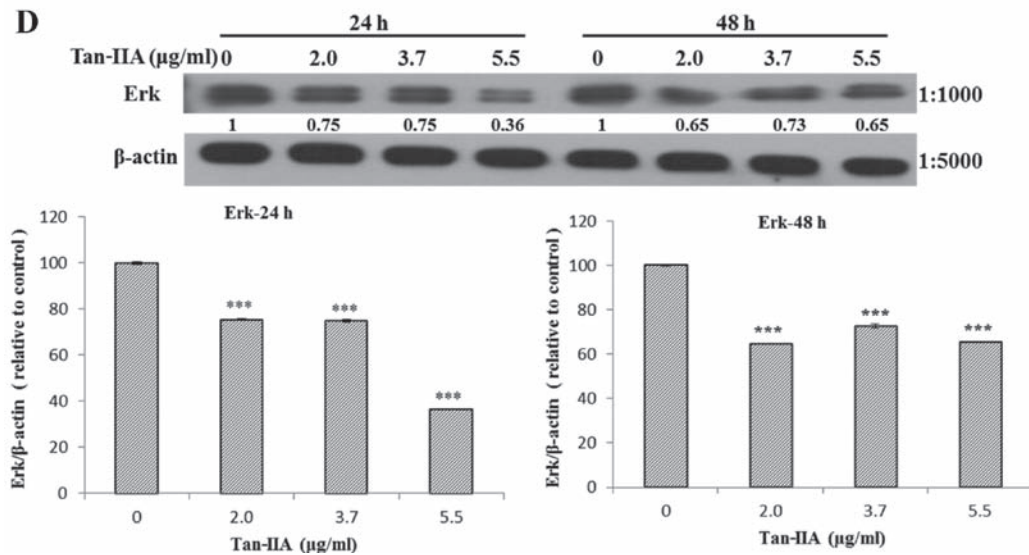


Figure 3 Continued. (D) The results showed that Tan-IIA can decrease the protein expression levels of ERK levels significantly.

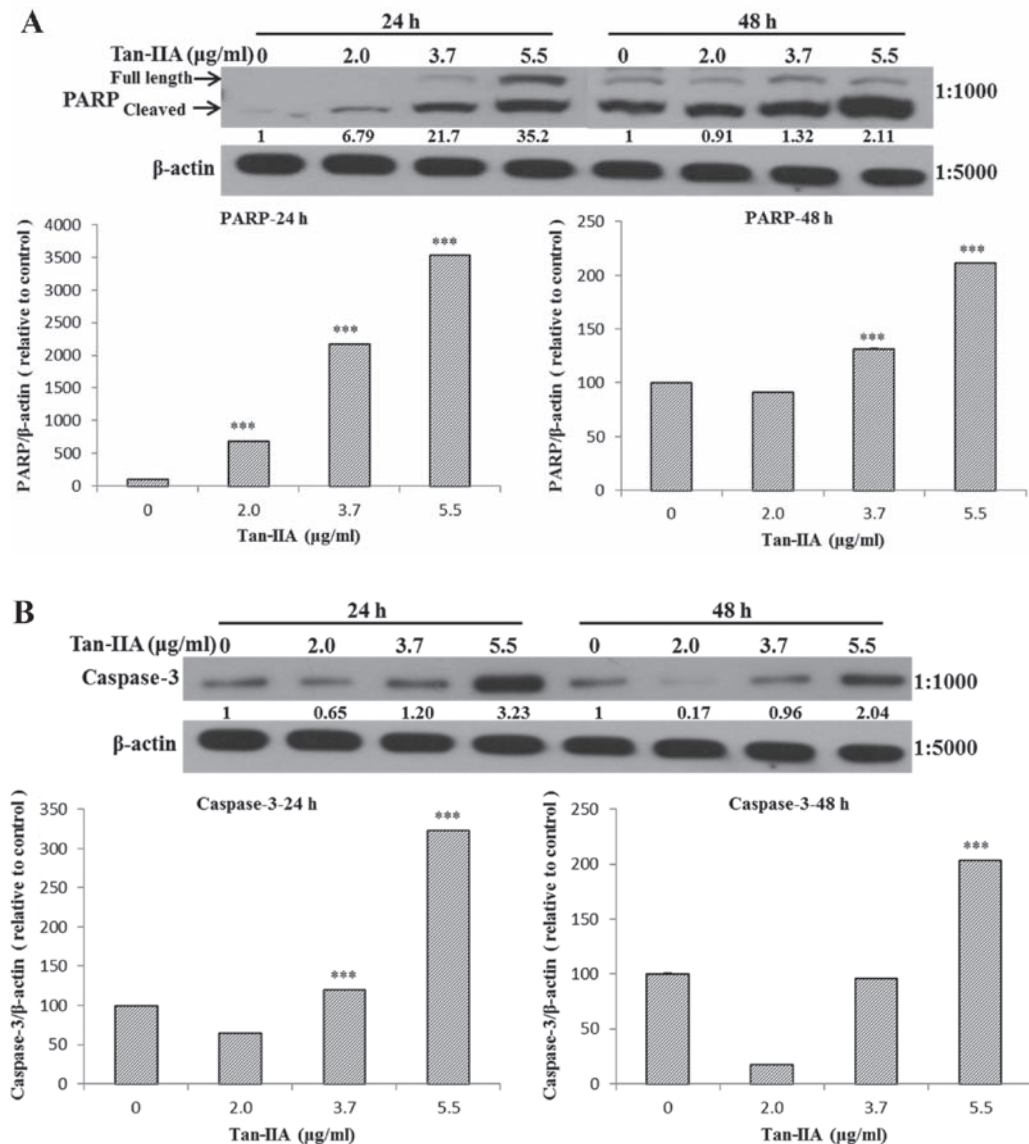


Figure 4. Protein expression of PARP, caspase-3 and β -actin in AGS cells. The AGS cells were treated with various concentrations of tanshinone IIA (Tan-IIA) (0, 2.0, 3.7 and 5.5 $\mu\text{g/ml}$) for 24 or 48 h and then the protein expression levels were evaluated by western blot analysis as described in Materials and methods. The results show that Tan-IIA increased (A) PARP and (B) caspase-3 levels significantly. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

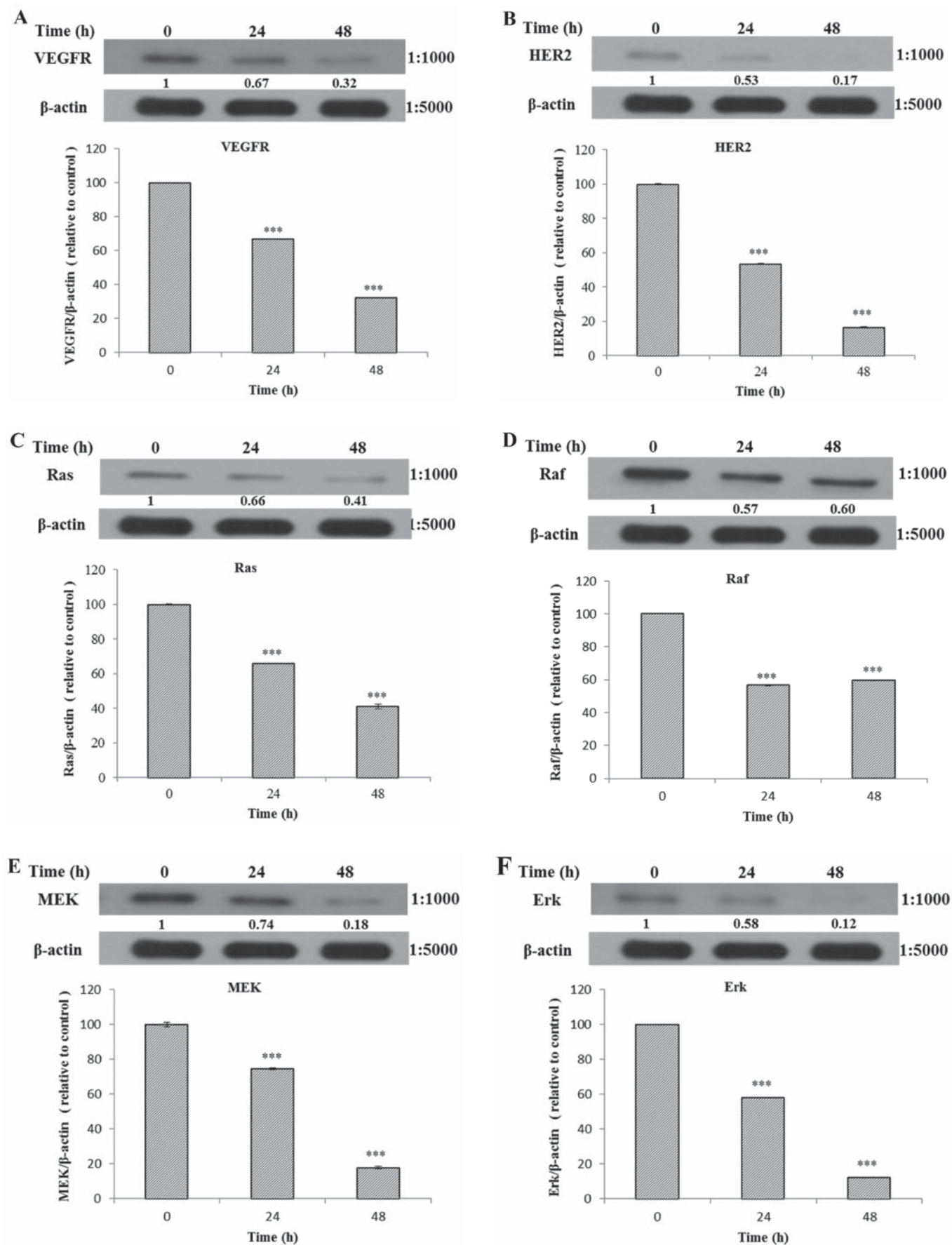


Figure 5. (A-F) Protein expression of vascular epidermal growth factor receptor (VEGFR), human epidermal growth factor receptor 2 (HER2), Ras, Raf, MEK, extracellular signal-regulated kinase (ERK), PARP, caspase-3 and β -actin in AGS cells. The AGS cells were treated with tanshinone IIA (Tan-IIA) ($3.7 \mu\text{g/ml}$) for different durations (0, 24 and 48 h) and then the protein expression levels of VEGFR, HER2, Ras, Raf, MEK, ERK, PARP, caspase-3 and β -actin were evaluated by western blot analysis. The results showed that Tan-IIA can decrease the protein expression levels of (A) VEGFR, (B) HER2, (C) Ras, (D) Raf, (E) MEK and (F) ERK levels significantly. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

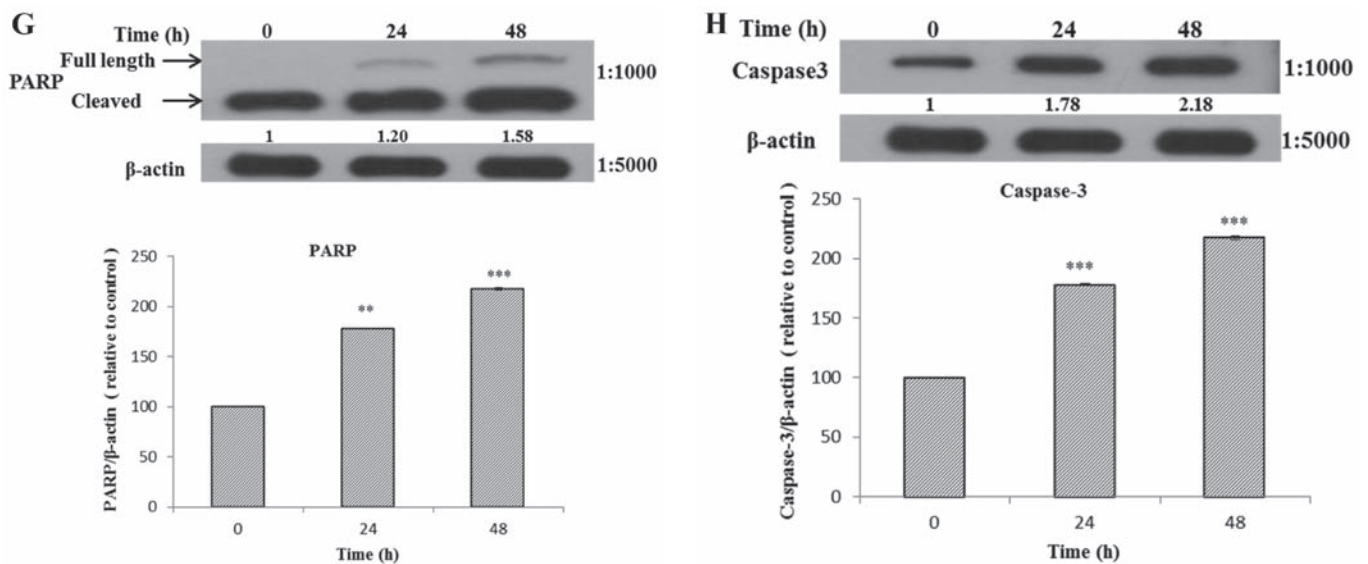


Figure 5 Continued. The results showed that Tan-IIA can increase (G) PARP and (H) caspase-3 levels significantly.

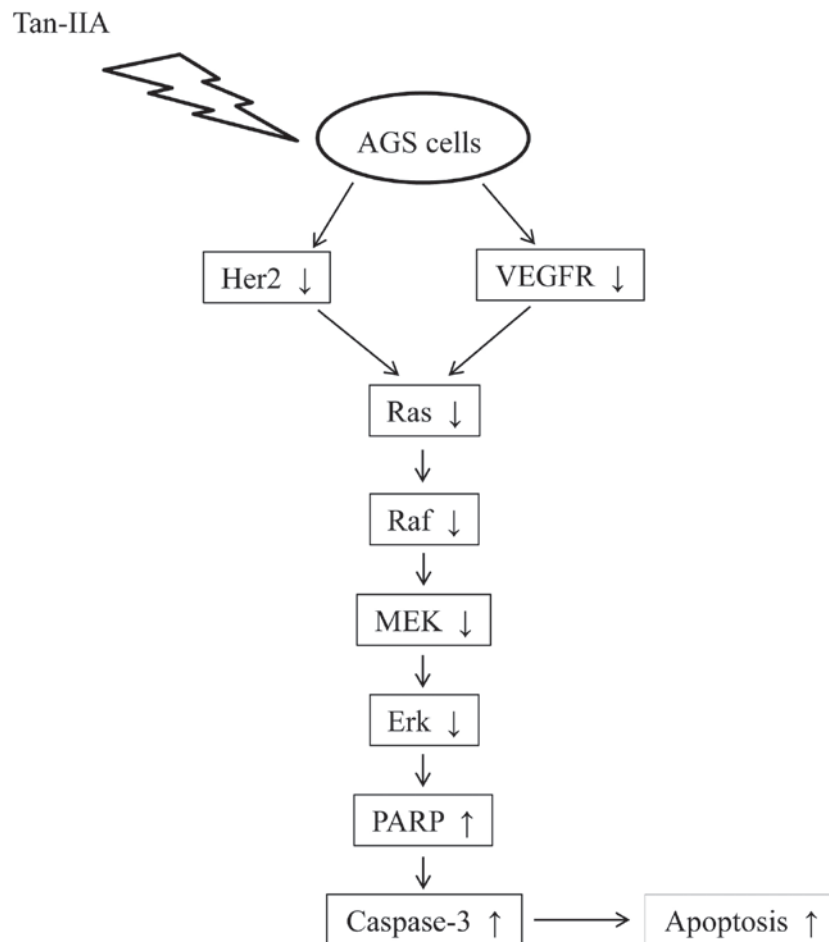


Figure 6. The proposed model for tanshinone IIA (Tan-IIA) to inhibit the proliferation of AGS cells. Tan-IIA inhibited human gastric cancer AGS cells through decreasing the protein expression of vascular epidermal growth factor receptor (VEGFR), human epidermal growth factor receptor 2 (HER2) and blocking Ras/Raf/MEK/extracellular signal-regulated kinase (ERK) pathway.

Discussion

It is well documented that Tan-IIA inhibits the proliferation of gastric cancer SGC7901 cells time- and dose-dependently

through inducing apoptosis and G0/G1 phase arrest (22,23). Tan-IIA also triggered the intrinsic apoptotic signaling pathway and arrested in G2/M phase to inhibit the proliferation of gastric cancer MKN-45 cells (24). Our results also showed

that Tan-IIA inhibited human gastric cancer AGS cells in a time- and dose-dependent manner *in vitro*.

It is well documented that Ras/Raf/MAPK pathway regulates a variety of cellular functions, and is also involved in the formation of new blood vessels, wound healing and tissue repair, cell cycle regulation, and cell migration. Ras is the most frequently mutated oncogene in human cancer. Dysregulation of Ras/Raf/MAPK pathway is a common event in cancer, signaling through this pathway is important for tumorigenesis (25-28). MAPK, which in mammals is also called MEK, is a serine/threonine kinase activated in response to growth factors and cytokines to promote cell apoptosis and survival (29). ERK belongs to mitogen-activated protein kinase (MAPK) pathway, and is activated via phosphorylation in response to cytokines, growth factors and stress. Phospho-ERK (p-ERK) can inhibit apoptosis (30,31). Our results demonstrated that AGS cells treated with Tan-IIA can decrease the protein expression level of VEGFR, HER2, Ras, Raf, MEK, ERK, time- and dose-dependently. Our results also demonstrated that the treatment of AGS cells with Tan-IIA can increase the protein expression of PARP and caspase-3. These findings indicate Tan-IIA can induce apoptosis to inhibit the proliferation of AGS cells. The proposed model for tan-IIA to inhibit the proliferation of AGS cells is shown in Fig. 6. This is the first report that Tan-IIA could inhibit gastric carcinoma AGS cells by decreasing the protein expression of VEGFR, Her2 and blocking the Ras/Raf/MEK/ERK pathway to induce apoptosis. The chemotherapeutic potential of Tan-IIA for human gastric cancer warrants further study.

Acknowledgements

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Competing interests

The author declares there is no competing interest.

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