

Tanshinone IIA can inhibit MiaPaCa-2 human pancreatic cancer cells by dual blockade of the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways

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Abstract. Tanshinone IIA (Tan-IIA; C₁₉H₁₈O₃) is derived from Danshen (the roots of *Salvia miltiorrhiza*), and has been reported to possess anti-inflammatory and antioxidant activities. Tan-IIA can inhibit BxPC-3 human pancreatic cancer cells *in vitro* through inducing endoplasmic reticulum stress and apoptosis via mitochondrial pathways. However, the efficacy and molecular mechanisms of Tan-IIA in human pancreatic cancer have not yet been elucidated. The transmembrane tyrosine kinases, including insulin-like growth factor 1 receptor (IGF1R), vascular endothelial growth factor receptor (VEGFR) or epidermal growth factor receptor (EGFR) have been implicated in the survival and metastasis of cancer. In addition, the Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathways are the most commonly dysregulated kinase cascades in human cancer. The present study aimed to investigate the efficacy and molecular mechanisms of Tan-IIA in MiaPaCa-2 human pancreatic carcinoma cells. The protein expression levels of EGFR, IGF1R, VEGFR, Ras, PI3K, AKT, mTOR, Raf, MEK, ERK and phosphatase and tensin homolog (PTEN) were detected in Tan-IIA-treated MiaPaCa-2 cells by western blotting. The results demonstrated that the protein expression levels of EGFR, IGF1R, VEGFR, Ras, Raf, MEK, ERK, PI3K, AKT, mTOR and PTEN were decreased in MiaPaCa-2 cells treated with various concentrations of Tan-IIA for different durations. In conclusion, these findings indicated that Tan-IIA may inhibit MiaPaCa-2 human pancreatic cancer cells; the molecular mechanisms underlying this inhibitory effect may

be involved in downregulating EGFR, IGF1R and VEGFR expression, and dual blockade of the Ras/Raf/MERK/ERK and PI3K/AKT/mTOR pathways.

Introduction

Pancreatic cancer was revealed to be the fourth leading cause of cancer-associated mortality in the USA in 2016, and the estimated number of deaths caused by pancreatic cancer for that year is 21,450 in men and 20,330 in women (1). Numerous efforts and chemotherapeutic advancements have been made to improve the efficacy of pancreatic cancer treatment; however, the results remain unsatisfactory (2-4). Therefore, there is an urgent need to develop novel therapeutic agents for pancreatic cancer.

It is well documented that transmembrane tyrosine kinases are strongly associated with the proliferation and metastasis of numerous types of human cancer (5,6). The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) and Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathways are two of the most frequently dysregulated kinase cascades in human cancer. These pathways signify important signal transduction mechanisms, which accelerate the proliferation and survival of cancers via activation of growth factor receptors, such as insulin-like growth factor 1 receptor (IGF1R), vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) (7,8). Either through somatic mutations or epigenetic modifications, the individual downstream constituents of these signaling cascades can induce tumorigenesis and resistance to anticancer treatments, and these constituents have been reported to be frequently transformed in malignant tumors (9).

Tanshinone-IIA (Tan-IIA) is an active component of the plant-derived traditional Chinese medicine Danshen, which has been reported to possess anticancer potential (2,3). Tan-IIA is able to inhibit the protein expression levels of MCL1, B-cell lymphoma 2 (Bcl-2) family apoptosis regulator; tumor protein, translationally-controlled 1 and Bcl-extra large, in order to destroy mitochondrial function and increase Bcl-2-associated

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X protein and caspase-3 expression, thus inducing apoptosis of BxPC-3 human pancreatic cancer cells (10). Furthermore, Tan-IIA increases protein kinase R-like endoplasmic reticulum kinase, activating transcription factor 6, caspase-12 and CCAAT-enhancer-binding protein homologous protein expression, in order to induce apoptosis of BxPC-3 pancreatic cancer cells *in vitro* (11). It has also been reported that Tan-IIA may exert cytotoxic effects on MiaPaCa-2 human pancreatic cancer cells (12). The present study aimed to detect the protein expression levels of IGF1R, VEGFR, EGFR, PI3K, AKT, mTOR, Ras, Raf, MEK, ERK and phosphatase and tensin homolog (PTEN) in MiaPaCa-2 human pancreatic cancer cells following treatment with Tan-IIA.

Materials and methods

Chemicals and reagents. Tan-IIA (molecular formula, $C_{19}H_{18}O_3$; CAS No., 568-72-9) was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The MiaPaCa-2 human pancreatic cancer cell line (BCRC No. 60139) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). MTT, sodium deoxycholate, leupeptin, Triton X-100, Tris/HCl, RNase A, sodium orthovanadate, sodium pyruvate, HEPES and mouse anti- β -actin (cat. no. A5441, MW 43 kDa) were obtained from Sigma-Aldrich; Merck KGaA. Dimethyl sulfoxide (DMSO), potassium phosphate and TE buffer were purchased from Merck KGaA. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, penicillin-streptomycin and glutamine were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Buffer (10X TG-SDS), Tween-20 and glycine were obtained from Amresco, LLC (Solon, OH, USA). BioMax film was obtained from Kodak (Rochester, NY, USA). Other materials and reagents not specified were obtained from Sigma-Aldrich; Merck KGaA or Merck KGaA.

Antibodies. Anti-VEGFR (cat. no. NB100-527, MW 151 kDa) was obtained from Novus Biologicals, LLC (Littleton, CO, USA); anti-EGFR (cat. no. 2239, MW 175 kDa), anti-IGF1R (cat. no. 3018, MW 95 kDa), anti-Ras (cat. no. 3339, MW 21 kDa), anti-Raf (cat. no. 12552, MW 75 kDa), anti-MEK (cat. no. 9126, MW 45 kDa), anti-ERK (cat. no. 4695, MW 42-44 kDa), anti-PI3K (cat. no. 4292, MW 85 kDa), anti-AKT (cat. no. 3063, MW 60 kDa), anti-mTOR (cat. no. 2983, MW 289 kDa) and anti-PTEN (cat. no. 9559, MW 54 kDa) antibodies were all obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. The MiaPaCa-2 cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin (10,000 U/ml penicillin, 10 mg/ml streptomycin) at 37°C in a humidified atmosphere containing 5% CO₂.

Cytotoxicity assay. The cytotoxicity of Tan-IIA in MiaPaCa-2 cells was evaluated by MTT assay in triplicate, as previously described (10). Briefly, the MiaPaCa-2 cells were plated in 96-well plates at a density of 1×10^4 cells/well and were treated with various concentrations (0, 1, 3, 9, 15, 30 and 60 μ g/ml) of Tan-IIA for different durations (24, 48 and 72 h). Subsequently,

the cells were incubated with 100 μ l MTT (1 mg/ml) in fresh complete DMEM for 2 h. The surviving cells converted MTT to formazan, as presented by a blue-purple color when dissolved in DMSO at 37°C for 2 h. Absorbance was measured using an ELISA microplate reader at 590 nm. 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) x 100, where OD test and OD blank are the optical density of the test substance and the blank control, respectively.

Western blotting. The effects of Tan-IIA on the protein expression levels of EGFR, IGF1R, VEGFR, Ras, PI3K, AKT, mTOR, Raf, MEK, ERK and PTEN were detected in MiaPaCa-2 cells. The MiaPaCa-2 cells were treated with various concentrations of Tan-IIA (0, 3, 6 and 12 μ g/ml) for 48 h, or with various concentrations of Tan-IIA (0, 1.5, 3 and 6 μ g/ml) for 72 h, after which the expression levels of EGFR, IGF1R, VEGFR, Ras, PI3K, AKT, mTOR, Raf, MEK, ERK and PTEN were evaluated by western blotting. Alternatively, MiaPaCa-2 cells were treated with Tan-IIA (6 μ g/ml) for various durations (0, 24, 48 and 72 h), after which the proteins expression levels of EGFR, IGF1R, VEGFR, Ras, PI3K, AKT, mTOR, Raf, MEK, ERK and PTEN were evaluated by western blotting.

Western blot analysis was conducted, as previously described (10). Briefly, after treatment, the cells were lysed in ice-cold whole cell extract buffer containing protease inhibitors (cat. no. 20-188; Merck KGaA). The lysate was agitated for 30 min at 4°C and centrifuged at $12,281 \times g$ for 10 min. Protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein (10 μ g) were then subjected to electrophoresis using 10-15% SDS-polyacrylamide gels. To verify equal protein loading (10 μ g) and transfer, proteins were transferred to polyvinylidene difluoride membranes, which were blocked overnight 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 with the specific primary antibodies (1:1,000) for 2 h at 25°C and were washed three times with Tris-buffered saline-0.05% Tween-20 (TBST). Subsequently, the membranes were incubated with anti-rabbit (cat. no. sc-2004) or anti-mouse (cat. no. sc-2005) immunoglobulin G-horseradish peroxidase-conjugated secondary antibodies (1:5,000; Santa Cruz Biotechnology Inc., Dallas, TX, USA). The membranes were then washed a further three times for 10 min with TBST. Finally, the protein bands were visualized on X-ray film and were analyzed using the enhanced chemiluminescence detection system (PerkinElmer, Inc., Waltham, MA, USA) and ImageJ 1.4.4p (National Institutes of Health, Bethesda, MD, USA) software analysis. β -actin was used as an internal control in all western blots. Results are presented as the means \pm standard deviation of three experiments.

Statistical analysis. The statistical significance of the differences between groups was assessed using SPSS software version 20 (IBM Corp., Armonk, NY, USA). Data were analyzed using one-way analysis of variance followed by Dunnett's test. $P < 0.05$ was considered to indicate a statistically significant difference.

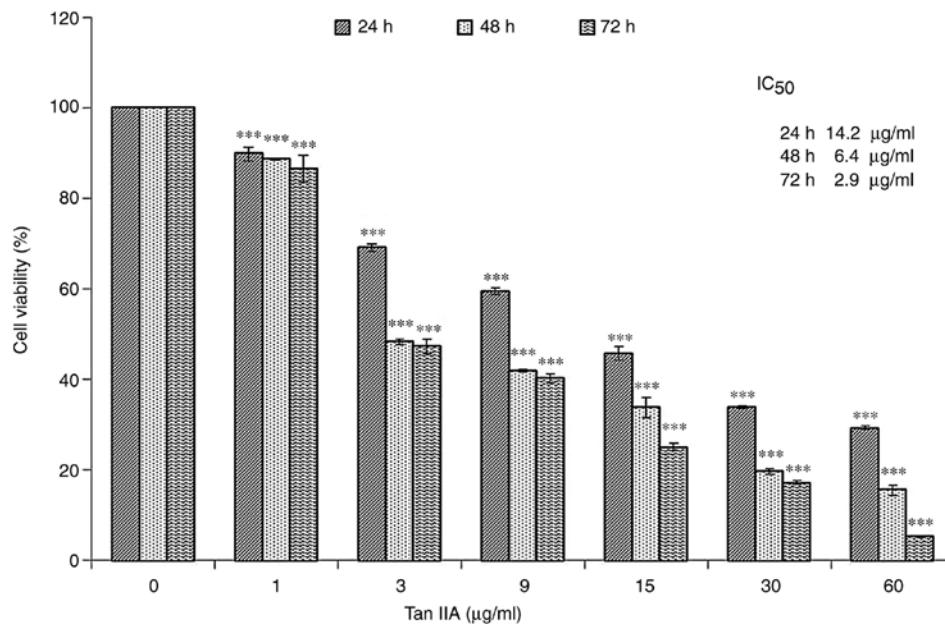


Figure 1. Cytotoxicity of Tan-IIA in MiaPaCa-2 cells. Cytotoxicity of Tan-IIA in MiaPaCa-2 cells was determined using the MTT assay. MiaPaCa-2 cells were cultured with various concentrations (0, 1, 3, 9, 15, 30 and 60 µg/ml) of Tan-IIA for different durations (24, 48 and 72 h). The results revealed that Tan-IIA inhibited the proliferation of MiaPaCa-2 human pancreatic cancer cells in a time- and dose-dependent manner. The IC₅₀ values were 14.2, 6.4 and 2.9 µg/ml, respectively. Results are presented as the means ± standard deviation of three experiments. ***P<0.001 vs. the control group. IC₅₀, half-maximum inhibitory concentration; Tan-IIA, Tanshinone-IIA.

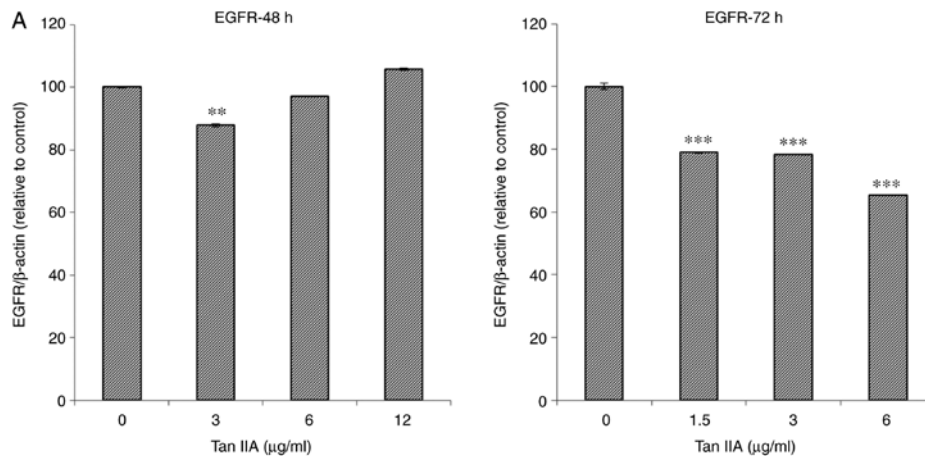


Figure 2. Dose-dependent effects of Tan-IIA on the protein expression levels of EGFR, IGF1R, VEGFR, Ras, Raf, MEK, ERK, PI3K, AKT, mTOR and PTEN in MiaPaCa-2 cells. The MiaPaCa-2 cells were treated with various concentrations (0, 3, 6 and 12 µg/ml) of Tan-IIA for 48 h or with various concentrations (0, 1.5, 3 and 6 µg/ml) of Tan-IIA for 72 h, and the protein expression levels were evaluated by western blot analysis. Protein expression levels of (A) EGFR. **P<0.01, ***P<0.001 compared with the control group. AKT, protein kinase B; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IGF1R, insulin-like growth factor 1 receptor; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; VEGFR, vascular endothelial growth factor receptor.

Results

Cytotoxicity of Tan-IIA in MiaPaCa-2 cells. MiaPaCa-2 cells were cultured with various concentrations (0, 1, 3, 9, 15, 30 and 60 µg/ml) of Tan-IIA for different durations (24, 48 and 72 h). Following Tan-IIA treatment for 24, 48 and 72 h, the half maximum inhibitory concentration values for Tan-IIA were 14.2, 6.4 and 2.9 µg/ml, respectively. These results revealed that Tan-IIA may inhibit the proliferation of MiaPaCa-2 human pancreatic cancer cells in a time- and dose-dependent manner (Fig. 1).

Dose-dependent effects of Tan-IIA on the protein expression levels of EGFR, IGF1R, VEGFR, Ras, Raf, MEK, ERK, PI3K, AKT, mTOR and PTEN in MiaPaCa-2 cells. MiaPaCa-2 cells were treated with various concentrations (0, 3, 6 and 12 µg/ml) of Tan-IIA for 48 h or with various concentrations (0, 1.5, 3 and 6 µg/ml) of Tan-IIA for 72 h, after which the protein expression levels were evaluated by western blot analysis (Fig. 2). The results revealed that Tan-IIA significantly decreased the protein expression levels of Ras (Fig. 2D and M), Raf (Fig. 2E and L), MEK (Fig. 2F and M), ERK (Fig. 2G and M), AKT (Fig. 2I and L) and PTEN (Fig. 2K and L) at 48 and 72 h. However, the

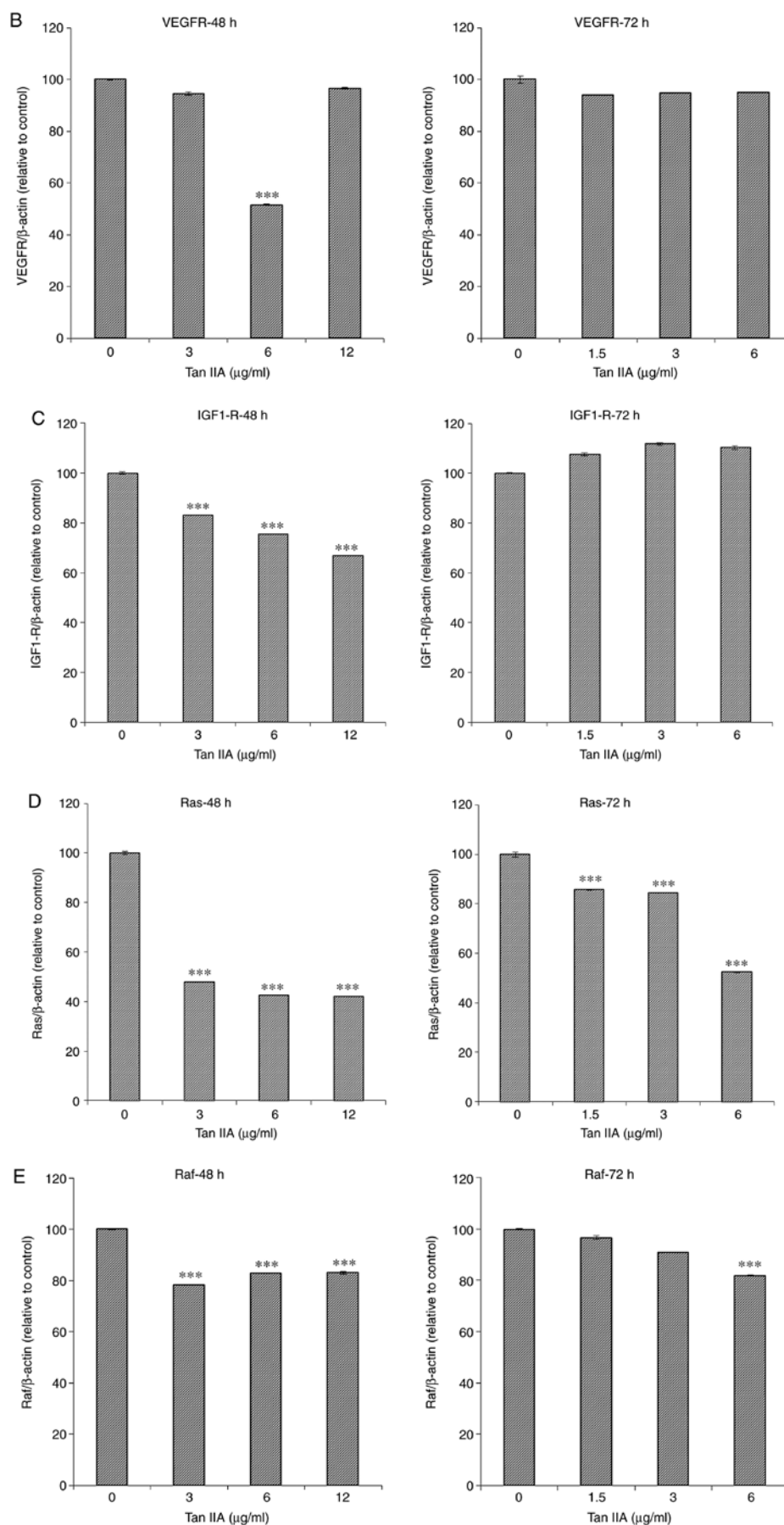


Figure 2. Continued. Protein expression levels of (B) VEGFR, (C) IGF1R, (D) Ras, (E) Raf. *** $P < 0.001$ compared with the control group. AKT, protein kinase B; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IGF1R, insulin-like growth factor 1 receptor; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; VEGFR, vascular endothelial growth factor receptor.

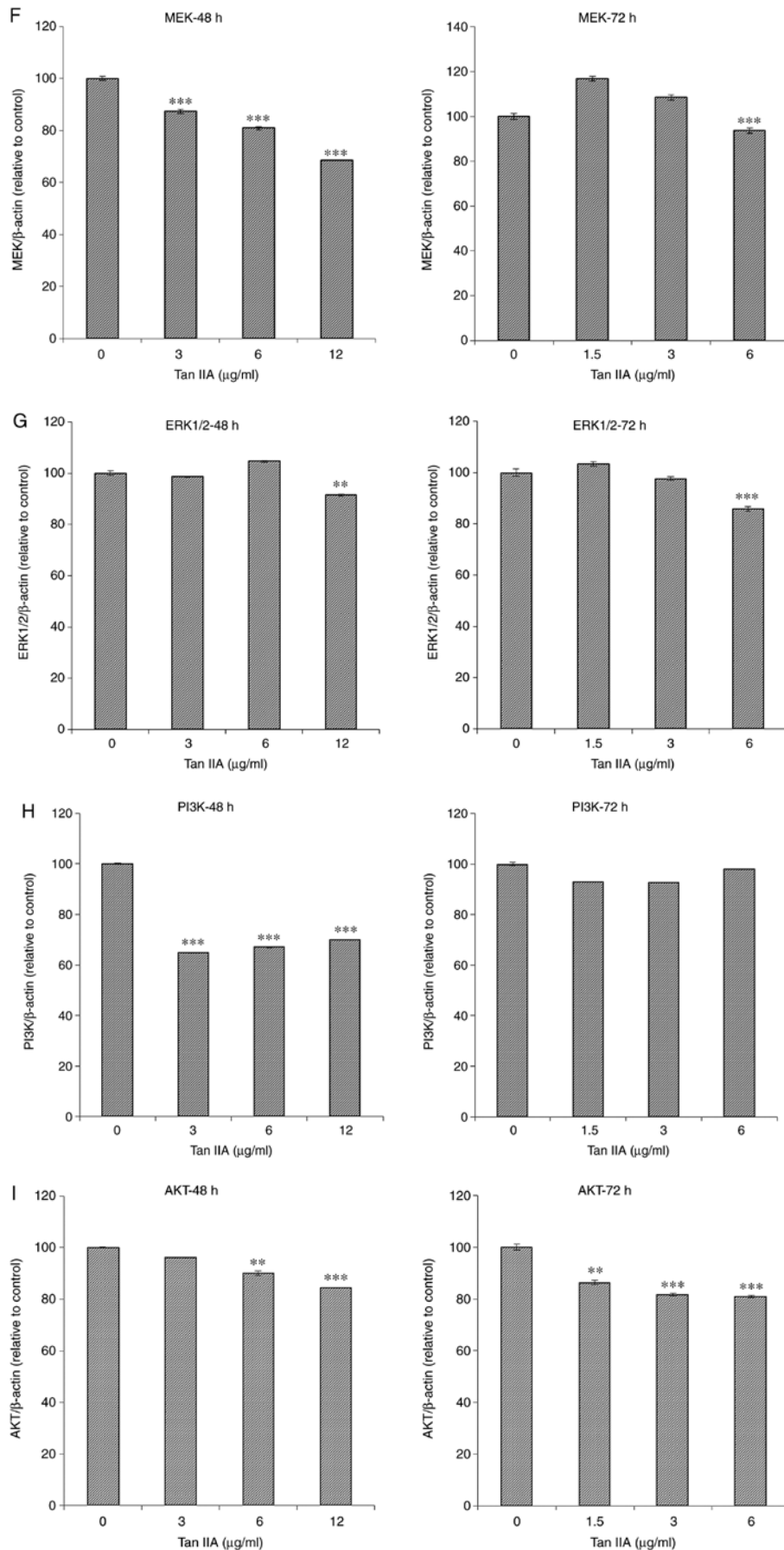


Figure 2. Continued. Protein expression levels of (F) MEK, (G) ERK, (H) PI3K, (I) AKT **P<0.01, ***P<0.001 compared with the control group. AKT, protein kinase B; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IGF1R, insulin-like growth factor 1 receptor; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; VEGFR, vascular endothelial growth factor receptor.

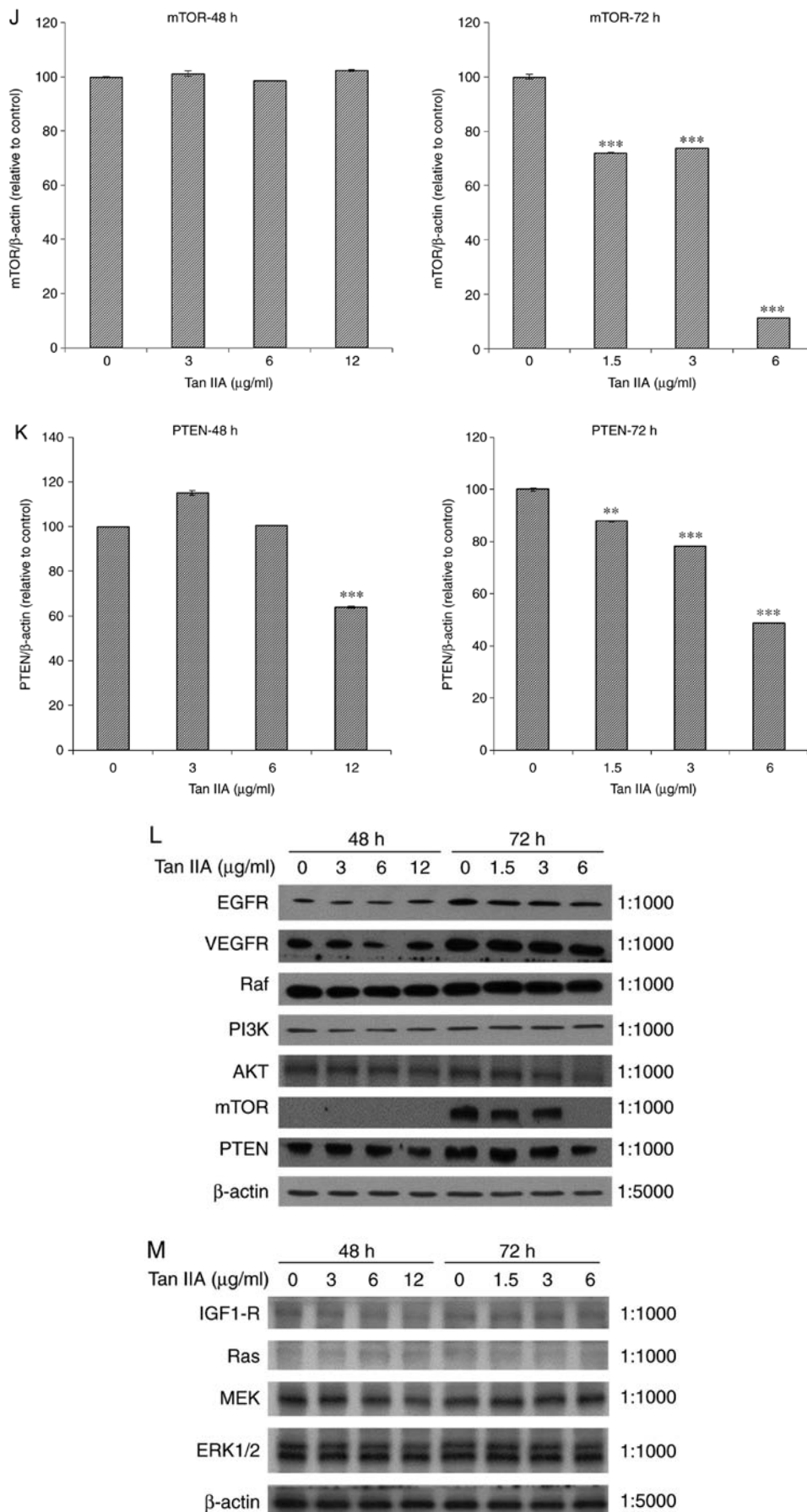


Figure 2. Continued. (J) mTOR and (K) PTEN. (L and M) Representative images of western blots. **P<0.01, ***P<0.001 compared with the control group. AKT, protein kinase B; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IGF1R, insulin-like growth factor 1 receptor; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; VEGFR, vascular endothelial growth factor receptor.

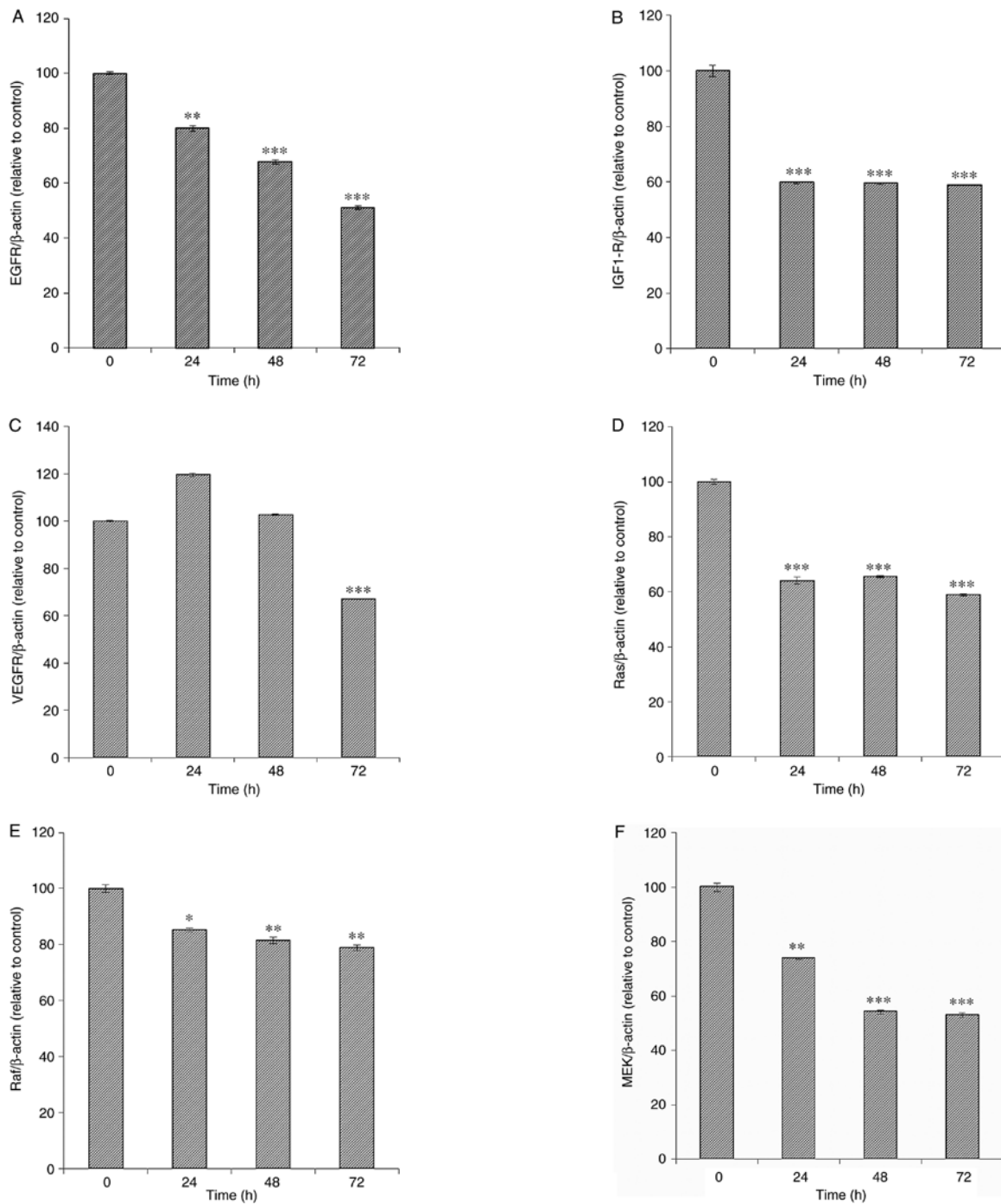


Figure 3. Time-dependent effects of Tan-IIA on the protein expression levels of EGFR, IGF1R, VEGFR, Ras, Raf, MEK, ERK, PI3K, AKT, mTOR and PTEN in MiaPaCa-2 cells. MiaPaCa-2 cells were treated with Tan-IIA (6 $\mu\text{g/ml}$) for various durations (0, 24, 48 and 72 h) and the protein expression levels were evaluated by western blotting. Protein expression levels of (A) EGFR, (B) IGF1R, (C) VEGFR, (D) Ras, (E) Raf, (F) MEK * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control group. AKT, protein kinase B; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IGF1R, insulin-like growth factor 1 receptor; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; VEGFR, vascular endothelial growth factor receptor.

protein expression levels of IGF1R (Fig. 2C and M) and PI3K (Fig. 2H and L) were significantly decreased only in response to 48 h treatment. The protein expression levels of mTOR (Fig. 2J and L) were significantly decreased only in response to 72 h treatment. The protein expression levels of EGFR (Fig. 2A and L) were significantly decreased in response to all concentrations of Tan-IIA after 72 h and in response to 3 $\mu\text{g/ml}$ Tan-IIA after 48 h.

Time-dependent effects of Tan-IIA on the protein expression levels of EGFR, IGF1R, VEGFR, Ras, Raf, MEK, ERK, PI3K, AKT, mTOR and PTEN in MiaPaCa-2 cells. MiaPaCa-2 cells were treated with Tan-IIA (6 $\mu\text{g/ml}$) for various durations (0, 24, 48 and 72 h) and the protein expression levels were evaluated by western blot analysis (Fig. 3). The results revealed that Tan-IIA significantly decreased the protein expression levels of EGFR (Fig. 3A and L), IGF1R (Fig. 3B and L),

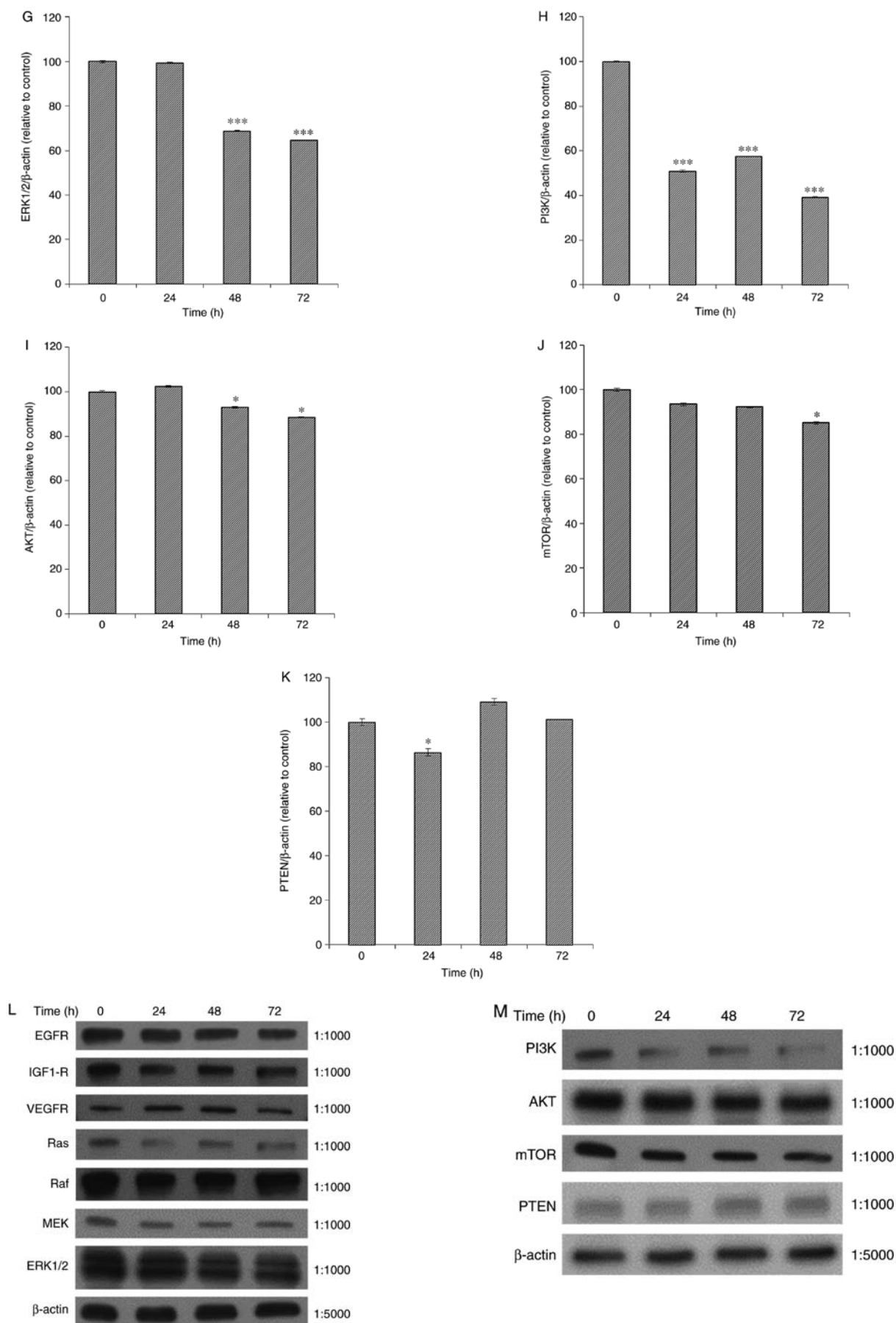


Figure 3. Continued. Protein expression levels of (G) ERK, (H) PI3K, (I) AKT, (J) mTOR and (K) PTEN. (L and M) Representative images of western blots. * $P < 0.05$, *** $P < 0.001$ compared with the control group. AKT, protein kinase B; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IGF1R, insulin-like growth factor 1 receptor; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; VEGFR, vascular endothelial growth factor receptor.

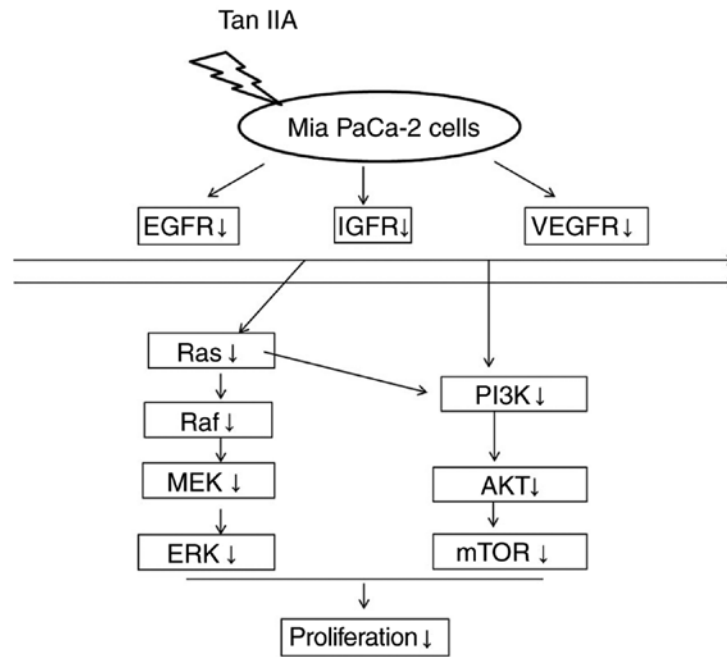


Figure 4. Proposed model for the molecular mechanisms underlying the inhibitory effects of Tan-IIA on Mia PaCa-2 cells. AKT, protein kinase B; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IGF1R, insulin-like growth factor 1 receptor; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; VEGFR vascular endothelial growth factor receptor.

Raf (Fig. 3E and L) and MEK (Fig. 3F and L) in a time-dependent manner. However, the protein expression levels of ERK (Fig. 3G and L) and AKT (Fig. 3I and M) were significantly decreased only at 48 and 72 h; the protein expression levels of VEGFR (Fig. 3C and L) and mTOR (Fig. 3J and M) were significantly decreased only at 72 h; and PTEN (Fig. 3K and M) was only significantly decreased at 24 h. Tan-IIA significantly decreased the protein expression levels of Ras (Fig. 3D and L) and PI3K (Fig. 3H and M) at 24, 48 and 72 h, but expression was slightly higher at 48 h than at 24 and 72 h.

Discussion

Ras is the most frequently mutated oncogene in human cancer (13). ERK belongs to the mitogen-activated protein kinase (MAPK) pathway, and dysregulation of the Ras/Raf/MAPK signal pathway has been well documented in tumorigenesis (14-16). AKT has an important role in apoptosis and cell production. Furthermore, PI3K can stimulate cellular survival and proliferation, and is required for activation of AKT (7,8). It is well known that the PI3K/Akt/mTOR pathway enables the proliferation and survival of cancer driven by growth factor receptors. The PI3K/Akt/mTOR pathway is another of the most frequently dysregulated kinase cascades in human cancer (9). Novel PI3K/AKT/mTOR pathway-targeting drugs can overcome everolimus resistance in pancreatic neuroendocrine tumors and cell lines (17). In pre-clinical and clinical studies, both Ras/Raf/MEK/ERK and PI3K/Akt/mTOR cascade inhibitors have been examined for possible uses (18-20). Natural products and their derivatives can overpower MAPK signaling and the PI3K pathways activated by oncogenic Ras (21).

The present results demonstrated that MiaPaCa-2 cells treated with Tan-IIA exhibited decreased protein expression levels of EGFR, IGF1R, VEGFR, Ras, Raf, MEK, ERK, PI3K, AKT and mTOR. These findings indicated that one of the molecular mechanisms through which Tan-IIA inhibits MiaPaCa-2 cells may be via decreasing EGFR, IGF1R and VEGFR expression, and inhibiting the Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathway cascades. To the best of our knowledge, the present study is the first to reveal that Tan-IIA may inhibit MiaPaCa-2 pancreatic cancer cells through decreasing the protein expression levels of EGFR, IGF1R and VEGFR, and inducing a dual blockade of Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathways (Fig. 4). Although advances have been made in systemic therapies, the use of adjuvant treatment in pancreatic cancer remains uncertain; and the 5-year survival rate for patients with early stage pancreatic cancer remains <25% (22). Further *in vivo* studies regarding the chemotherapeutic potential of Tan-IIA in human pancreatic cancer are required.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author contributions

CCS was involved in designing, performing the experiments and manuscript submission.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The author declares that they have no competing interests.

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