

# Tanshinone IIA induces TRAIL sensitization of human lung cancer cells through selective ER stress induction

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**Abstract.** Although tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promised anticancer medicine targeting only the tumor, most cancers show resistance to TRAIL-induced apoptosis. For this reason, new therapeutic strategies to overcome the TRAIL resistance are required for more effective tumor treatment. In the present study, potential of Tanshinone IIA as a TRAIL sensitizer was evaluated in human non-small cell lung cancer (NSCLC) cells. NSCLC cells showed resistance to TRAIL-mediated cell death, but combination treatment of Tanshinone IIA and TRAIL synergistically decreased cell viability and increased apoptosis in TRAIL-resistant NSCLC cells. Tanshinone IIA greatly induced death receptor 5 (DR5), but not death receptor 4 (DR4). Furthermore, DR5 knockdown attenuated the combination treatment of Tanshinone IIA with TRAIL-mediated cell death in human NSCLC cells. Tanshinone IIA

also increased CHOP and activated the PERK-ATF4 pathway suggesting that Tanshinone IIA increased DR5 and CHOP by activating the PERK-ATF4 pathway. Tanshinone IIA also downregulated phosphorylation of STAT3 and expression of survivin. Taken together, these results indicate that Tanshinone IIA increases TRAIL-induced cell death via upregulating DR5 and downregulating survivin mediated by, respectively, selective activation of PERK/ATF4 and inhibition of STAT3, suggesting combinatorial intervention of Tanshinone IIA and TRAIL as a new therapeutic strategy for human NSCLC.

## Introduction

Lung cancer is the most common cancer with high incidence and mortality worldwide. Approximately 80% of all lung cancers are non-small cell lung carcinomas (NSCLC) and adenocarcinoma is the most common type (1). Despite increased survival rate via improved diagnosis and treatment of NSCLC, there are still many issues to be resolved including resistance, rapid disease recurrence and progression (2-4). Therefore, new therapeutic strategies to overcome resistance and improve effective treatment of NSCLC patients are urgently required.

The endoplasmic reticulum (ER) participates in various cellular processes, such as protein folding, lipid biosynthesis and calcium storage. Among them precise control of protein folding is a fundamental function of ER to maintain cellular homeostasis (5,6). When misfolded proteins or unfolded proteins are generated during metabolic processes or from various stresses, polypeptide binding protein (BiP or GRP78, sensor) is dissociated from three major ER membrane resident proteins (mediator), inositol-requiring kinase 1 (IRE1), double-strand RNA-activated protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6) leading to activation of the unfolded protein response (UPR) signal (6-9). Dimerization of PERK by UPR activation increases the phosphorylation on Ser 51 of eIF2 leading to attenuation of the global translation initiation (10). On the contrary, eIF2 phosphorylation selectively increases the translation of *ATF4* mRNA encoding transcriptional activator of genes involved in the UPR. One of the ATF4 target genes is CCAAT/enhancer-binding protein homologues protein (*CHOP*), which is another preferential translation target of eIF2 phosphoryla-

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**Abbreviations:** TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; NSCLC, non-small cell lung cancer; TR, TRAIL-resistant; DR5, death receptor 5; DR4, death receptor 4; CHOP, CCAAT/enhancer-binding protein homologues protein; ER, endoplasmic reticulum; PERK, pancreatic ER kinase; ATF4, activating transcription factor 4; ROS, reactive oxygen species; UPR, unfolded protein response; IRE1, inositol-requiring kinase 1; BiP, polypeptide binding protein; eIF2, eukaryotic initiation factor 2

**Key words:** Tanshinone IIA, tumor necrosis factor-related apoptosis-inducing ligand, ATF4, CHOP, death receptor 5, endoplasmic reticulum stress, non-small cell lung cancer

tion. Increased CHOP during ER stress and UPR activation induces expression of apoptosis-related genes including death receptors DR4 and/or DR5 (11,12). For these reasons, various types of anticancer drugs to modulate proteins involved in the ER stress response have been developed (13).

To develop novel cancer therapeutics to overcome drug resistance, multi-targeting strategies has been recently explored (14). Especially, combination therapy targeting multiple anti-apoptotic proteins and/or pro-apoptotic proteins has been considered effective strategy to overcome TRAIL resistance in most types of cancer including NSCLC and increase the anticancer capacity in treatment of cancers (15). Various compounds isolated from herbal plants have been studied for combination therapy with TRAIL or conventional anticancer drugs (16-20). Particularly, Tanshinone IIA isolated from the dried root of *Salvia miltiorrhiza* Bunge (*Lamiaceae*) is one of well-documented natural compounds in terms of combination effects as well as its own anticancer mechanism (21-25). Tanshinone IIA has shown cytotoxic effects in several types of human cancer cells *in vitro* and *in vivo* (26,27).

A recent study revealed that Tanshinone IIA induces apoptosis through inhibition of JAK/STAT3 signaling in myeloid leukemia cells (28). In the present study, we focused on anti-tumor effects of Tanshinone IIA to sensitize TRAIL-mediated apoptosis in human NSCLC cells through modulation of ER stress response and apoptosis signals.

## Materials and methods

**Cell culture and reagents.** NSCLC cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). A549, H596, H1299, Calu-1 and H460 cell lines were grown in RPMI-1640 medium (HyClone Laboratories, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (HyClone Laboratories) in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C, and the viability of cultured cells was monitored by a LUNA-FL automated cell counter (Logos Biosystems, Anyang, Gyeonggi-do, Republic of Korea). Tanshinone IIA (M.W.=328, T4952, ≥97% -HPLC) was purchased from Sigma Aldrich (St. Louis, MO, USA).

**MTT assay.** Cytotoxicity of Tanshinone IIA, TRAIL or combination with Tanshinone IIA and TRAIL was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical) assay in NSCLCs. Cells were seeded in a 96-well-microplate at a density of 1x10<sup>4</sup> cells/well and treated with various concentrations of Tanshinone IIA. After the indicated time, the medium was removed, and fresh medium containing 1 mg/ml MTT was added to each well. The cells were incubated at 37°C for 2 h and then an equal volume of MTT lysis buffer was added to each well. The cells were incubated at 37°C overnight. Optical density (OD) was measured using a microplate reader (Tecan Austria GmbH, Grödig, Austria) at 570 nm.

**Western blotting.** Whole cell lysates were prepared, and western blotting was performed as previously described (28). In brief, total proteins from A549, H596, H1299 and Calu-1 cells were extracted with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% deoxycholic acid-

Na, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF) including protease inhibitor cocktail (Roche). Primary antibodies for CHOP, DR5, ATF4, GRP78, calnexin, PERK, PDI, IRE-1α (Cell Signaling Technology, Beverly, MA, USA), DR4, PARP, cleaved caspase-3, caspase-3, survivin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-β-actin (Sigma-Aldrich) and secondary antibodies coupled to horseradish peroxidase (HRP) from Vector Laboratories Inc. (Burlingame, CA, USA) were used for immunoblotting. Expression was visualized by using enhanced chemiluminescence (ECL) Western blotting detection reagent (Amersham Pharmacia, Piscataway, NJ, USA).

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was isolated from the NSCLC cells with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (2 μg) isolated from cells was reverse transcribed to cDNA using oligo-dT and random primers. Reverse transcription was done using a thermal program of 50°C for 60 min and 85°C for 5 min. The cDNA was amplified by PCR using the following specific primers: DR4 (forward, 5'-GGCTGAGGACAATGCTCACA-3' and reverse, 5'-TTGCTGCTCAGAGACGAAAGTG-3'); DR5 (forward, 5'-GACTCTGAGACAGTGCTTCGATGA-3' and reverse, 5'-CCATGAGGCCCAACTTCTCT-3'); CHOP (forward, 5'-CAACTGCAGAGAATTCAGCTGA-3' and reverse, 5'-ACTGATGCTCTAGATTGTTTCAT-3'); GAPDH (forward, 5'-CCACTCCTCCACCTTTGAC-3' and reverse, 5'-ACCCTGTTGCTGTAGCCA-3'); XBP-1 (forward, 5'-CTGGAACAGCAAGTGGTAGA-3' and reverse, 5'-CTGGGTCCTTCTGGGTAGAC-3'). All primers were synthesized by Bioneer, Co. (Daejeon, Republic of Korea).

**Apoptosis assay.** NSCLCs were seeded in 6-well plates, treated with indicated compounds for 24 h and stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) kit (BioVision Technologies Inc., Golden, CO, USA). The cells were then analyzed using fluorescence-activated cell sorting FACSCalibur flow cytometry.

**siRNA transfection.** Transfection of control or DR5 siRNA (Bioneer) was performed using transfection reagent INTERFERin (Polyplus-transfection, New York, NY, USA) according to the manufacturer's protocol.

**Validation of synergy between tanshinone IIA and TRAIL.** To determine the synergy between Tanshinone IIA and TRAIL, cytotoxicity assay was performed in four NSCLC cell lines by the gradual increase with a same constant ratio between Tanshinone IIA and TRAIL. The values from viability assays were analyzed by CalcuSyn software (Biosoft, Ferguson, MO, USA). The fraction of living cells in each concentration was applied for the analysis of synergism between Tanshinone IIA and TRAIL by CalcuSyn software.

**Statistical analysis.** Data are presented as mean ± standard deviation (SD) from at least three independent experiments in triplicate and analyzed for statistical significance using the unpaired Student's t-test. P<0.05 was considered statistically significant.

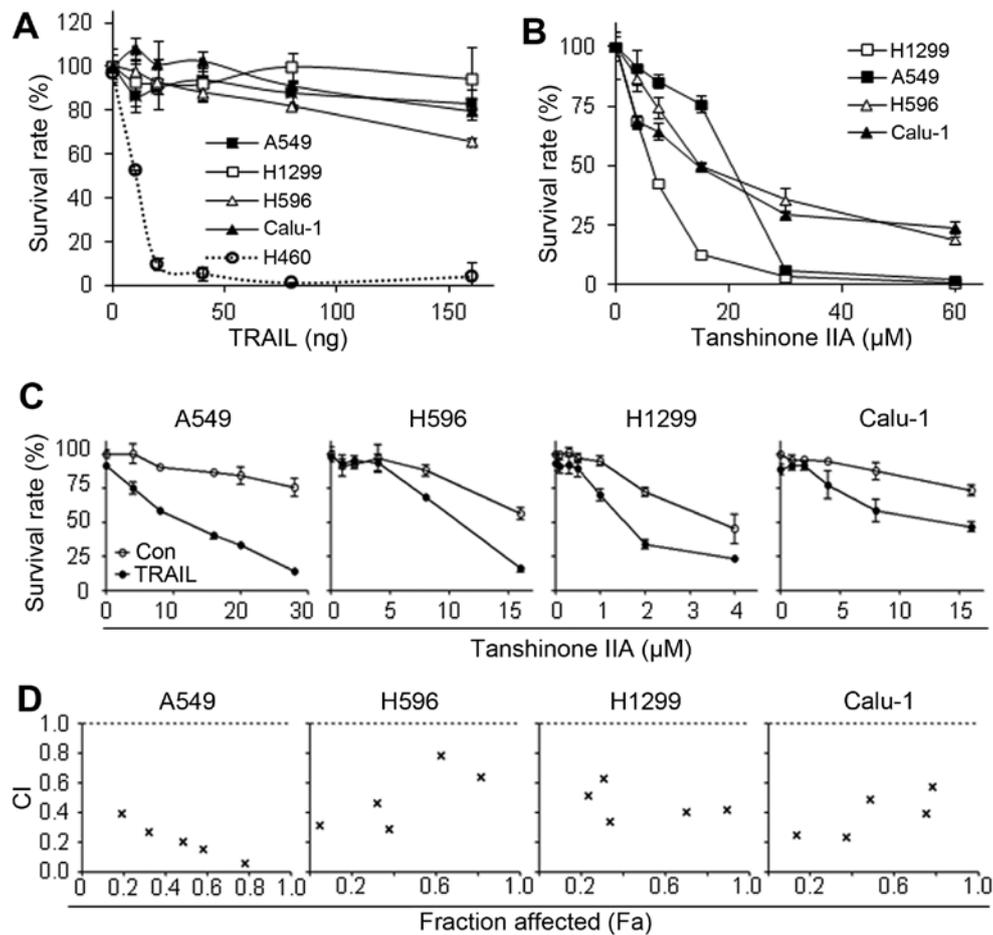


Figure 1. Synergistic effect of tanshinone IIA and TRAIL on cytotoxicity of human TRAIL-resistant NSCLC cells. (A and B) NSCLC cells were treated with TRAIL and/or tanshinone IIA for 24 h as indicated. (C) NSCLC cells were treated with 25 ng/ml of TRAIL and/or tanshinone IIA for 24 h as indicated. Cell viability was then analyzed by the MTT assays. (D) Combination index (CI) values with fraction affected (Fa) between Tanshinone IIA and TRAIL in human NSCLC cells were calculated using Calcsyn software.

## Results

**Combination treatment of Tanshinone IIA and TRAIL synergistically increases the cytotoxicity in TRAIL-resistant NSCLCs.** In order to investigate the possible therapeutic effect of Tanshinone IIA and TRAIL in NSCLC, we first examined whether single treatment of Tanshinone IIA or TRAIL affects viability of human NSCLC cells. Consistent with previous reports (29,30), A549, H596, H1299 and Calu-1 showed high resistance to TRAIL, while H460 was very sensitive to TRAIL with ~12 ng/ml  $IC_{50}$  (Fig. 1A). Tanshinone IIA was cytotoxic in all the tested NSCLC cell lines with  $IC_{50}$  values of ~10-30  $\mu$ M (Fig. 1B). Furthermore, in evaluation of combination effect of Tanshinone IIA and TRAIL, co-treatment synergistically decreased cell viability (Fig. 1C). Surprisingly, the combination index (CI) values below 0.8 at all fractions affected (Fa) points, suggesting that the combination treatment showed strong synergy in all four tested TRAIL resistant NSCLC cell lines (Fig. 1D).

**Combination treatment effectively induces apoptosis in TRAIL-resistant NSCLCs.** Canonical cell death signals through TRAIL receptors trigger extrinsic apoptosis which occurred by extracellular death signals (31). To verify whether

synergistic effect of Tanshinone IIA on cytotoxicity is mediated by apoptotic cell death, we analyzed apoptosis in Tanshinone IIA and TRAIL co-treated NSCLC cells. As shown in Fig. 2A, Tanshinone IIA/TRAIL combination treatment intensively upregulated cleaved-caspase-3, -8 and cleaved-PARP levels, and even combination with half dose of each single treatment showed more increased cleaved forms indicating induction of apoptosis. Consistently, Annexin V and PI double staining revealed that apoptotic population was markedly increased in co-treated NSCLC cells compared to control or single treated A549 and H1299 cells (Fig. 2B). These data indicate that Tanshinone IIA may be a novel TRAIL sensitizer in NSCLC cells.

**Tanshinone IIA-mediated cell death is through DR5 induction, but not DR4 in TRAIL-resistant NSCLCs.** Cancer cells usually acquire TRAIL resistance via downregulation of DR4 and/or DR5 (32,33). To confirm if Tanshinone IIA overcomes the TRAIL resistance by modulation of death receptors, we evaluated the expression of the proteins. Tanshinone IIA treatment significantly increased DR5, but not DR4 in A549, H596 and H1299 cells (Fig. 3A). Since CHOP is one of major transcriptional activators of DR5 (34,35), we next examined the effect of Tanshinone IIA on the expression of CHOP. As shown

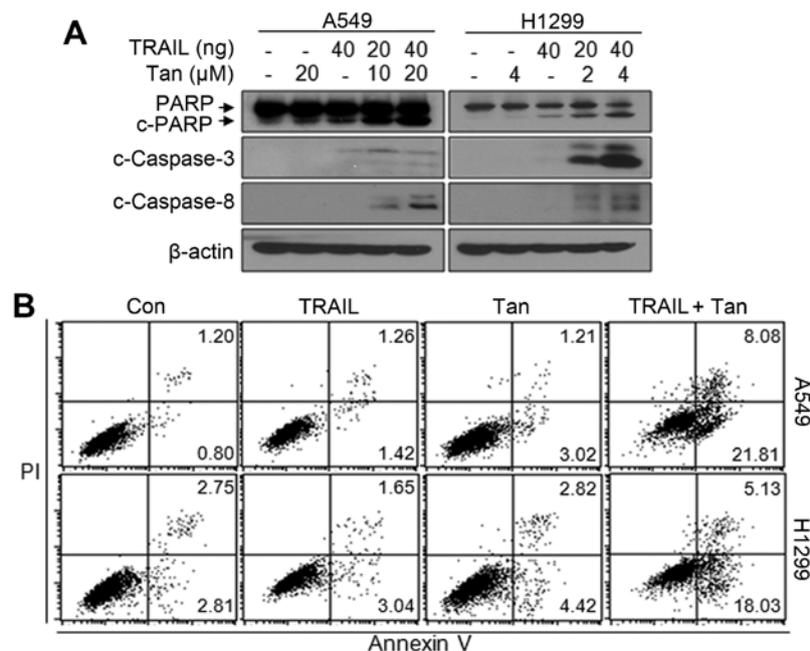


Figure 2. Synergistic effect of Tanshinone IIA and TRAIL on apoptosis induction in human TRAIL-resistant NSCLC cells. (A) Cells were treated with Tanshinone IIA and TRAIL for 24 h as indicated. Cells lysates were prepared and subjected to western blotting with the indicated antibodies.  $\beta$ -actin was used as an internal standard. (B) Cells were treated with Tanshinone IIA and/or TRAIL as following: A549 cells with 10  $\mu$ M of Tanshinone IIA/40 ng/ml of TRAIL and H1299 cells with 4  $\mu$ M of Tanshinone IIA/ 40 ng/ml of TRAIL. After staining with Annexin V-FITC and PI, the apoptotic cells were analyzed by a dot plot using a flow cytometer. The numbers in each plot indicate the percentage of apoptotic cells.

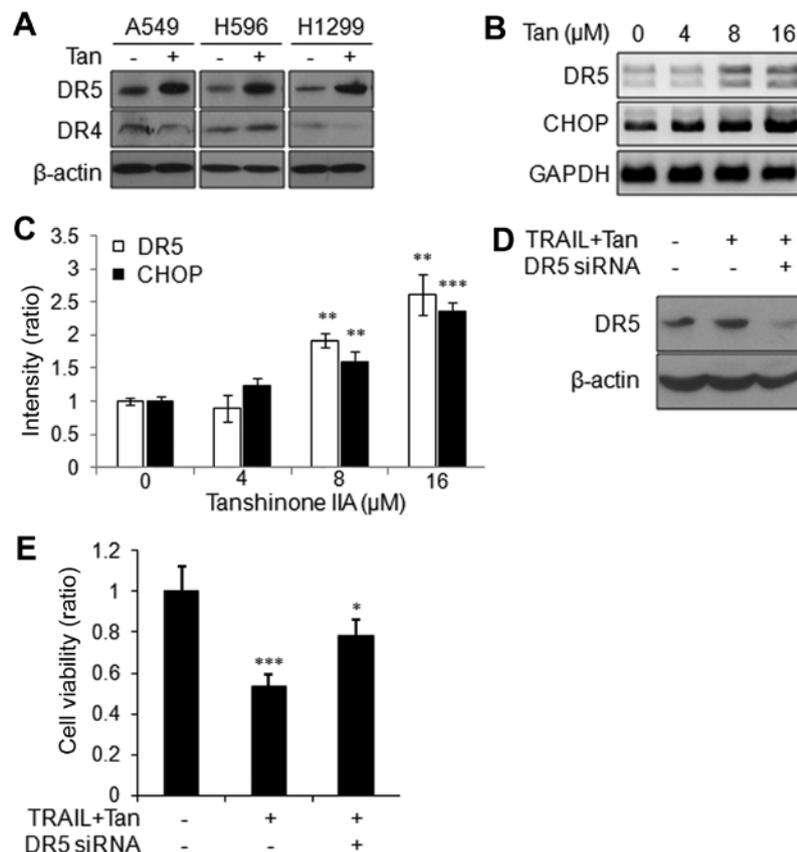


Figure 3. Effect of Tanshinone IIA on the expression of DR5 in human TRAIL-resistant NSCLC cells. (A) A549, H596 and H1299 cells were treated with 20, 20 and 4  $\mu$ M of Tanshinone IIA, respectively. Total cell protein extracts were subjected to immunoblotting with the indicated antibodies.  $\beta$ -actin was used as an internal control. (B) A549 cells were treated with Tanshinone IIA for 24 h as indicated. Total RNA was isolated and RT-PCR analysis was performed as described in Materials and methods. (C) Representation of quantified band intensity in B. (D and E) A549 cells were transfected with DR5 siRNA or control siRNA. Forty-eight hours after transfection, cells were treated with 20  $\mu$ M of Tanshinone IIA and 10 ng/ml of TRAIL for 24 h, and (D) the cell lysates were subjected to immunoblotting with the indicated antibodies and (E) the viability was then analyzed by the MTT assays. \*\*\* $P$ <0.001 and \* $P$ <0.05 compared to control and TR/Tan, respectively. (TR, TRAIL; Tan, Tanshinone IIA).

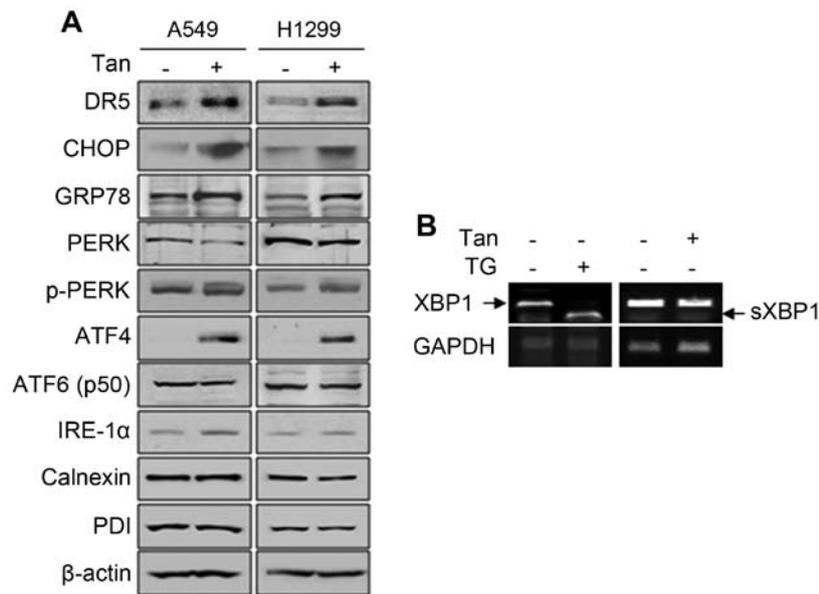


Figure 4. Effect of Tanshinone IIA on the ER stress signaling pathways in human TRAIL-resistant NSCLC cells. (A) A549 and H1299 cells were treated with 20  $\mu$ M of Tanshinone IIA for 24 h. Cells lysates were subjected to western blotting with the indicated antibodies. (B) A549 cells were treated with 20  $\mu$ M of Tanshinone IIA (Tan) for 24 h or thapsigargin (Tg) for 8 h. Total RNA was isolated and RT-PCR was performed to detect the spliced form (sXBP-1) and unspliced form of XBP-1 mRNA. PCR products were separated on a 3% agarose gel.

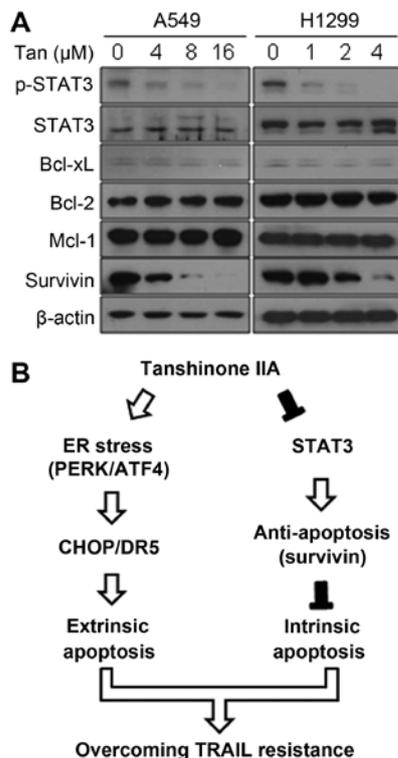


Figure 5. Effect of Tanshinone IIA on STAT3 and anti-apoptotic molecules. (A) A549 and H1299 cells were treated with Tanshinone IIA for 24 h as indicated. Whole cell extracts were prepared and analysed by western blotting using indicated antibodies.  $\beta$ -actin was used as the internal standard. (B) Schematic diagram indicating the effect of Tanshinone IIA on ER stress and STAT3 in NSCLC cells.

in Fig. 3B and C, Tanshinone IIA transcriptionally induced CHOP and also DR5 in a dose-dependent manner. In order to confirm the role of DR5 in the Tanshinone IIA-mediated

TRAIL sensitization of NSCLC cells, we depleted DR5 with specific siRNA (Fig. 3D). As shown in Fig. 3E, inhibition of DR5 restored the TRAIL and Tanshinone IIA-mediated decrease of cell viability. These results indicated that Tanshinone IIA significantly increased expression of DR5, leading to increase of TRAIL sensitivity in human NSCLC cells.

*Tanshinone IIA selectively induces the PERK/ATF4 pathway of ER stress response signals causing induction of DR5.* Since CHOP is transcriptionally induced by ER stress response signals (36), we then examined whether Tanshinone IIA can regulate signaling molecules involved in ER stress response in NSCLCs. As shown in Fig. 4A, Tanshinone IIA significantly induced GRP78 which is an ER stress sensor protein. Tanshinone IIA also activated the PERK/ATF4 signal which is one of three ER stress inducers, but the other two pathways, ATF6 and IRE-1 $\alpha$  signals, were not affected at all (Fig. 4A). In addition, Tanshinone IIA did not induce the splicing of XBP1, while treatment of a well-known ER stress inducer thapsigargin (TG) greatly increased the spliced form of XBP1 mRNA (Fig. 4B). Furthermore, Tanshinone IIA did not affect the expression of other ER resident chaperons contributing protein folding (Fig. 4A). These data indicate that Tanshinone IIA increases DR5 and CHOP by selective activation of the PERK/ATF4 signal.

*Tanshinone IIA suppresses STAT3 and its downstream target survivin.* Because tumor cells with TRAIL resistance usually possess increased levels of anti-apoptotic proteins (37,38), these proteins have been regarded major targets for development of cancer therapeutics. As shown in Fig. 5, Tanshinone IIA decreased phosphorylation of STAT3, which is known to induce anti-apoptotic proteins including survivin, while total STAT3 expression was not changed at all. Furthermore, Tanshinone IIA suppressed survivin in a dose-dependent

manner (Fig. 5). However, other anti-apoptotic proteins Bcl-xL, Bcl-2 and Mcl-1 were not affected by Tanshinone IIA (Fig. 5A). These results suggest that Tanshinone IIA inhibits the STAT3 pathway leading to suppression of survivin in human NSCLC cells.

## Discussion

Even though activation of the TRAIL receptor pathway is a promising therapeutic strategy to selectively remove cancer cells, most cancers including NSCLC have various ways to evade the TRAIL-mediated cell death. Therefore, combination treatment strategies to overcome TRAIL resistance have been intensively studied. Particularly, herbal compounds targeting death receptors are considered effective interventions to increase the TRAIL sensitivity in tumor cells. Recent studies have proven that several natural compounds such as cycloanthranilylproline, cryptotanshinone, kurarinone, curcumin and rocoglamide increased the TRAIL sensitivity in various types of cancer resistant to TRAIL (39-43).

In the present study, we evaluated whether another herbal compound, tanshinone IIA can overcome the TRAIL resistance in human NSCLC cells. Consistent with previous reports (44,45), tanshinone IIA significantly increased DR5 through CHOP induction (Fig. 3). Furthermore, we newly revealed that Tanshinone IIA selectively activated the PERK/ATF4 pathway (Fig. 4). These results suggest that Tanshinone IIA induces DR5 by increasing CHOP through selective activation of the PERK/ATF4 pathway, one of the ER stress response signals. Reactive oxygen species (ROS), chemically reactive molecules, play roles in regulating cell signaling and homeostasis (46). ROS is generated during oxygen usage in all cellular compartments, such as the mitochondrial respiratory chain and ER (47) suggesting ROS as one of ER stress inducers. Based on our previous report, decursin has also similar functions, such as induction of selective ER stress via ROS generation and TRAIL sensitivity in lung cancer cells (18). Recent report indicated that Tanshinone IIA induces considerable amount of ROS formation in A549, but not H596 cells, which lack the NAD(P)H:quinone oxidoreductase (NQO1) (48). Consistently, we also detected ER stress response signals in Tanshinone IIA-treated A549 cells, but not in H596 (data not shown), indicating that ER stress induction by Tanshinone IIA may be mediated by ROS generation. However, more detailed molecular mechanisms how Tanshinone IIA induces synergistic cytotoxic effect with TRAIL in H596 cells should be further elucidated.

Because most tumors are resistant to TRAIL, targeting the main factors associated with the TRAIL resistance is a common strategy to effectively increase the TRAIL sensitivity in treating cancer. For example, inhibition of cell survival factors including survivin and/or induction of anti-apoptotic factors have been used for overcoming the TRAIL resistance (49-55). STAT3 is a well-known transcription factor regulating genes encoding proteins involved in cell survival including survivin and Bcl-2. Major function of STAT3 is to convey signals from the cell surface to the nucleus on activation by cytokines and growth factors (56,57). Phosphorylation of STAT3 by activation of receptor tyrosine kinases (RTKs), such as EGFR, leads to its

dimerization and then translocation to the nucleus. Numerous studies have proved that hyperphosphorylated STAT3 are widely detected in various types of human tumor specimens and is required for malignant transformation of cultured cells (58-62). In this regard, diverse novel STAT3 inhibitors have been developed (60,63). Tanshinone IIA significantly downregulated phosphorylation of STAT3, probably leading to suppression of survivin in human NSCLC cells (Fig. 5).

Taken together, these results clearly demonstrate that Tanshinone IIA selectively induces the ER stress, PERK/ATF4/CHOP pathway leading to significant induction of DR5 and reduces survivin via inactivating STAT3. Furthermore, combination treatment of Tanshinone IIA and TRAIL showed synergistic induction of apoptotic cell death in human NSCLC cells resistant to TRAIL (Fig. 5B), suggesting that Tanshinone IIA is a promising TRAIL sensitizer in NSCLCs and that combination of Tanshinone IIA with TRAIL would be a good therapeutic strategy for treating NSCLCs.

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