

# *Tussilago farfara* L. augments TRAIL-induced apoptosis through MKK7/JNK activation by inhibition of MKK7-TIPRL in human hepatocellular carcinoma cells

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Received April 24, 2014; Accepted May 23, 2014

DOI: 10.3892/or.2014.3279

**Abstract.** Induction of apoptosis through activation of the TRAIL pathway is considered to be a promising anticancer strategy due to its ability to selectively induce apoptosis in cancer cells. However, the ability of cancer cells to acquire TRAIL resistance has limited the clinical translation of this approach. We previously reported that the TOR signaling pathway regulator-like (TIPRL) protein contributes to the resistance to TRAIL-induced apoptosis by inhibiting the MKK7-c-Jun N-terminal kinase (JNK) pathway via MKK7-TIPRL interaction. In the present study, we identified *Tussilago farfara* L. (TF) as a novel TRAIL sensitizer among 500 natural products using an ELISA system that specifically detects the MKK7-TIPRL interaction, and we validated candidates by GST-pull down assay. Co-treatment of Huh7

cells with TF and TRAIL induced apoptosis via inhibition of the MKK7-TIPRL interaction and an increase in MKK7/JNK phosphorylation. This is the first report to describe TF as a novel TRAIL sensitizer, unveiling a potentially novel therapeutic strategy in cancer therapy.

## Introduction

Acquisition of apoptosis resistance is a hallmark of cancer cells and limits the treatment options in cancer patients (1,2). Therefore, co-treatment with several chemotherapy agents has been considered in order to reduce the incidence of acquired apoptosis resistance in cancer cells (3). TRAIL is considered to be a promising anticancer agent due to its ability to selectively induce apoptosis in cancer cells; Walczak *et al* reported that injection of TRAIL into mouse xenografts transplanted with human tumor cells resulted in growth inhibition of the transplanted tumors. In contrast, no TRAIL-induced toxicity was observed in normal tissue cells such as hepatocytes (4).

Binding of TRAIL to DR4 or DR5 is known to initiate apoptosis through the formation of death-inducing signaling complexes (DISCs) via the recruitment of FAS-associated protein and caspase-8. Through the formation of the DISC complex, proteolytic cleavage and activation of caspase-3 occur, eventually resulting in the biochemical and morphological hallmarks of apoptosis. However, depending on the cell type and external stimuli, caspase-8 also activates caspase-9 through the cleavage of pro-apoptotic proteins, such as BH3-interacting domain death agonist. Subsequently, pro-apoptotic molecules such as cytochrome *c* are released from the mitochondria to amplify the apoptotic response by mediating the simultaneous activation of the extrinsic and intrinsic apoptotic pathways (5). However, other decoy receptors can inhibit TRAIL-induced apoptosis by competing with DR4 or DR5, while dysfunction of DISC components such as

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**Abbreviations:** ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; HAECs, human aortic endothelial cells; HCC, hepatocellular carcinoma; JNK, c-Jun N-terminal kinase; MKK7, mitogen protein kinase kinase 7; MMP, mitochondrial membrane potential; TIPRL, TOR signaling pathway regulator-like protein; TMRE, tetramethylrhodamine; TF, *Tussilago farfara* L.; TRAIL, TNF-related apoptosis-inducing ligand; PARP, poly (adenosine diphosphate-ribose) polymerase

**Key words:** TRAIL resistance, HCC, TIPRL, *Tussilago farfara* L., MKK7, apoptosis

fas-associated protein with death domain (FADD), caspase-8 and cellular FLICE-like inhibitory protein (cFLIP), and mutational inactivation of pro-apoptotic genes Bax or Bak can also lead to TRAIL resistance (6). One strategy that has been considered for overcoming TRAIL resistance is the combination of different chemotherapeutic agents with TRAIL. The combinatorial approach of treatment with various genotoxic drugs, such as 5-fluorouracil and cisplatin, in the presence of TRAIL has been successful in animal models *in vivo*, and clinical trials in humans are currently underway (7).

We recently demonstrated that TOR signaling pathway regulator-like (TIPRL) is highly upregulated in hepatocellular carcinoma (HCC) cells and contributes to TRAIL resistance by forming the complex MKK7/PP2Ac/TIPRL, which blocks the phosphorylation of JNK, thus inhibiting the apoptosis cascade induced by TRAIL. In support of this, the combination of TIPRL knockdown in the presence of TRAIL sensitizes cancer cells to TRAIL-induced apoptosis via sustained MKK7 phosphorylation and JNK activation (8). In the present study, we aimed to identify compounds that inhibit the interaction between MKK7/TIPRL and could thus be used as TRAIL sensitizers. Using an ELISA system which detects MKK7 and TIPRL interaction *in vitro*, we determined that *Tussilago farfara* L. (TF; commonly known as coltsfoot) specifically inhibited the TIPRL-MKK7 interaction. This inhibition led to an apoptotic/cytotoxic effect on Huh7 cells after co-treatment with TF and TRAIL.

*Tussilago farfara* L. belongs to the family Asteraceae, which is known to have antioxidant, anti-inflammatory effects (9), antimicrobial activity and an  $\alpha$ -glucosidase inhibitory effect (10). *In vitro* studies indicate that TF has significant antiproliferative activity in cancer cells (11). In a study by Park *et al.*, sesquiterpenoids isolated from TF inhibited the growth of HCC HepG2 cells via inactivation of acyl CoA: diacylglycerol acyltransferase (DGAT) (11). However, the function of TF as a TRAIL sensitizer has never been demonstrated. Therefore, in this study, we sought to investigate the role of TF in TRAIL-induced apoptosis and to evaluate it as a novel strategy to overcome the resistance of cancer cells to apoptosis.

## Materials and methods

**Preparation of a methanol fraction of *Tussilago farfara* L. herb.** TF from leaves and stems (International Biological Material Research Center, Daejeon, Korea) with voucher no. FBM026-99 (9) was extracted with methanol for three days at room temperature. Concentrated and lyophilized TF was dissolved in dimethyl sulfoxide (DMSO) (stock 200 mg/ml); the final concentration of DMSO in the culture medium was controlled at 0.1% (v/v).

**High-performance liquid chromatography (HPLC) analysis.** HPLC profiles of TF were analyzed using a photodiode array system (PDA) as a detector under the following conditions: column, BEH 1.7  $\mu$ M (2.1x100 mm); mobile phase, solvent A H<sub>2</sub>O + 0.1% formic acid and solvent B acetonitrile (ACN) + 0.1% formic acid; the flow rate, 0.4 ml/min, oven temperature 35°C; injection volume, 2  $\mu$ l. The solution was filtered and analyzed by HPLC in a similar way.

**Reagents.** Antibodies against phospho(p)-MKK7 (Ser271/Thr261), MKK7, p-JNK (Thr183/Tyr185), JNK, caspase-8, caspase-9, caspase-3 and PARP were purchased from Cell Signaling Technology (Beverly, MA, USA); anti-cytochrome *c* antibody was purchased from BD Pharmingen (San Diego, CA, USA). Antibodies against glutathione S-transferase, hemagglutinin, GAPDH and HRP-conjugated secondary antibodies were obtained from AbFrontier Co. (Seoul, Korea). Z-VAD-fmk and SP600125 were purchased from Calbiochem (Darmstadt, Germany). The human TRAIL was a kind gift from Dr C. Choi (KAIST, Korea) and was also purchased from Apotech (Chemin Des Croisettes, Switzerland).

**Cell culture.** Huh7 and 293T cells and human aortic endothelial cells (HAECs) (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM and EBM-2, respectively, supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere in a 5% CO<sub>2</sub> incubator at 37°C.

**Cell viability assay.** Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) assay. The cells were seeded at a density of 1x10<sup>4</sup> cells/well and were treated with TF and/or TRAIL. After 24 h, MTT (2 mg/ml) was added to each well, and the absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. The cell viability was calculated as a percentage of viable cells in drug-treated group vs. the untreated control by the following equation: Cell viability (%) = [OD (drug) - OD (blank)]/[OD (control) - OD (blank)] x 100.

**Crystal violet cell growth assays.** The cells were fixed and stained with crystal violet solution [40% ethanol, 60% phosphate-buffered saline (PBS) and 0.5% crystal violet]. After 20 min, 1 ml of 10% acetic acid was added to each well, and the absorbance was read at 590 nm.

**Cell cycle analysis.** The cells were fixed in 70% ethanol at -20°C and treated with 10 mg/ml RNase A for 1 h at 37°C. Then the pellets were suspended in 1 ml of propidium iodide (PI) solution [50  $\mu$ g/ml PI, 1 mg/ml RNase A and 0.1% (w/v) Triton X-100] in 3.8 mM sodium citrate. Samples were analyzed using CellQuest Software (BD Biosciences, San Jose, CA, USA).

**Caspase-3 activity assay.** Caspase-3 activity was measured using the Caspase-3 Luminescent assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Caspase-3 activity was measured using a Luminescence Plate Reader (Molecular Devices Co., Sunnyvale, CA, USA).

**Measurement of mitochondrial membrane potential (MMP).** The cells were stained with tetramethylrhodamine (TMRE) (Molecular Probes, Eugene, OR, USA), for 30 min at 37°C. MMP was determined by flow cytometry using CellQuest software.

**ELISA assay.** The full-length cDNAs of human TIPRL and MKK7 were provided by the Korea Human Gene Bank (Korea Research Institute of Biosciences and Biotechnology,

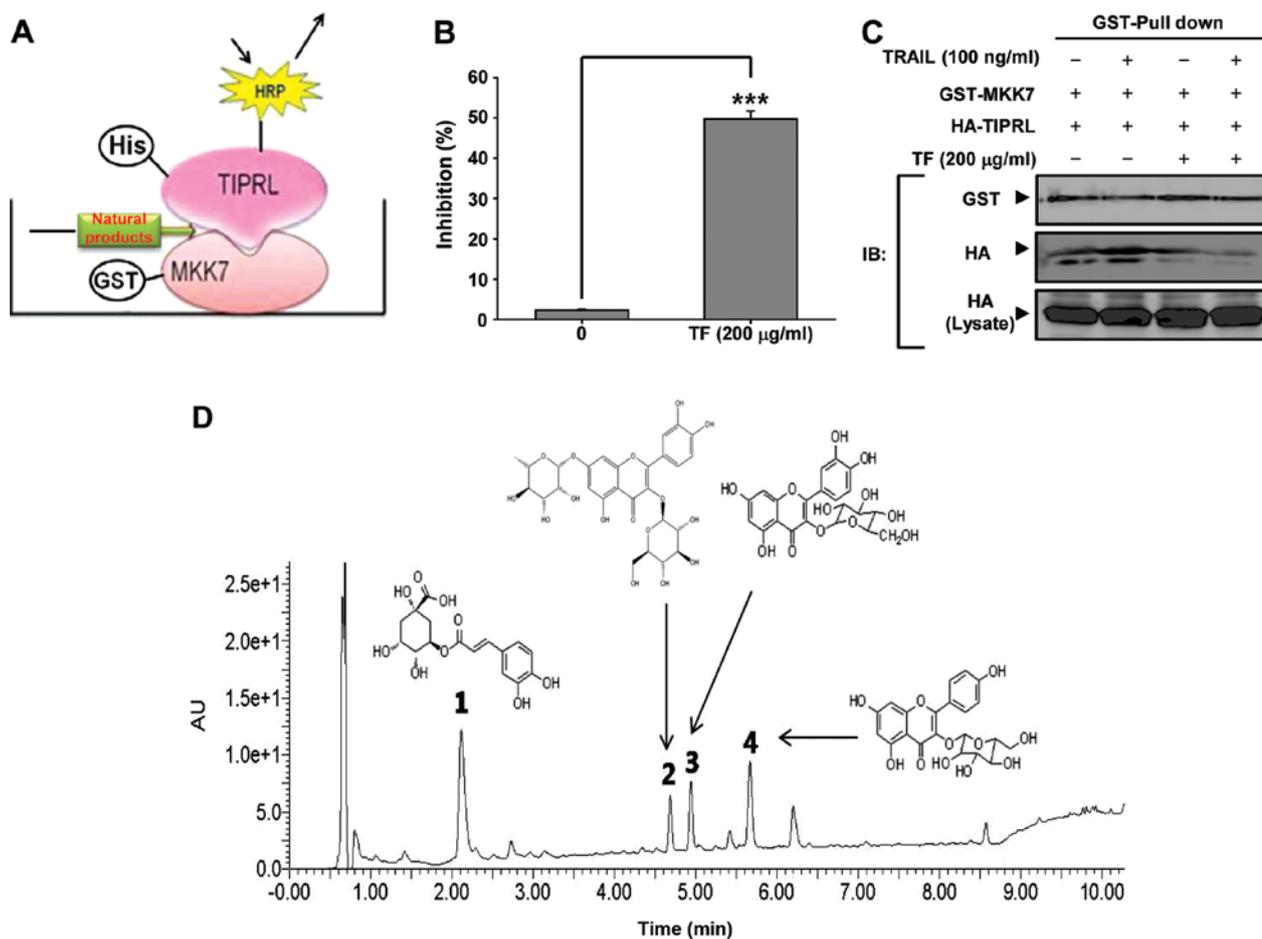


Figure 1. Inhibition of MKK7-TIPRL interaction by TF. (A) Schematic diagram of an ELISA assay designed to detect the MKK7-TIPRL interaction. (B) The percentage of inhibition of MKK7-TIPRL interaction by TF was assayed by the ELISA system that was performed using MKK7 for capturing and TIPRL as a detector. The results are expressed as the mean  $\pm$  SD of triplicate measurements (\*\*\*)  $p < 0.001$ . (C) GST-pull down assay. 293T cells were cotransfected with GST-MKK7 and HA-TIPRL vectors and then treated with TF and/or TRAIL for 24 h. (D) HPLC profiles of TF. Several commercially available compounds were used under the same HPLC conditions as standard materials. The peaks shown in the HPLC profiles: **1**, chlorogenic acid; **2**, quercetin 3-O-glucoside 7-O-rhamnoside; **3**, quercetin-3-O-glucopyranoside; and **4**, astragaline. MKK7, mitogen protein kinase kinase 7; TIPRL, TOR signaling pathway regulator-like protein; TF, *Tussilago farfara* L.; GST, glutathione S-transferase; HPLC, high-performance liquid chromatography.

Korea). TIPRL and MKK7 were subcloned into the PCGN vector (W. Herr, Cold Spring Harbor Laboratory), and pEBG containing GST tag (kindly provided by Y. Liu, NIA, National Institutes of Health) vector in order to construct the HA-tagged and the GST-tagged plasmid, respectively. Purified TIPRL was conjugated with HRP. To screen natural products in a high-throughput system (HTS), an ELISA system that detects the TIPRL and MKK7 interaction was constructed. Briefly, 96-well ELISA plates were coated with GST-MKK7 dissolved in PBS-T buffer (Tween-20 0.05% and BSA 0.5%), followed by the addition of 100  $\mu$ l of TIPRL-HRP dissolved in PBS-T buffer. After 1 h, 100  $\mu$ l of TMB solution was added to each well, and the absorbance was read at 450 nm.

**GST-pull down assay.** 293T cells were transfected with expression vector constructs using TurboFect transfection reagent (Thermo Scientific, Lafayette, CO, USA), lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF and 1X protease inhibitor cocktail) containing complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Whole-cell extract was incubated with Glutathione

Sepharose 4B (GE Healthcare, Uppsala, Sweden), and the eluted GST-tagged proteins were subjected to western blotting using the indicated antibodies.

**Western blotting.** The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF and 1X protease inhibitor cocktail) and spun down at 14,000  $\times$  g for 20 min at 4°C. The protein samples were subjected to western blotting with the indicated antibodies.

**Subcellular fractionation.** The cells were harvested in ice-cold lysis buffer (20 mM HEPES, 2 mM EDTA, 0.1 mM PMSF, 10  $\mu$ g/ml of pepstatin A and leupeptin), homogenized and centrifuged for 10 min at 3,500  $\times$  g. The supernatant was collected for the cytosol fraction.

**Statistical analysis.** All data are presented as the mean  $\pm$  standard deviation (SD) of three independent experiments. Statistical significance was verified by a Student's t-test using SigmaPlot software (Systat Software Inc., San Jose, CA, USA).

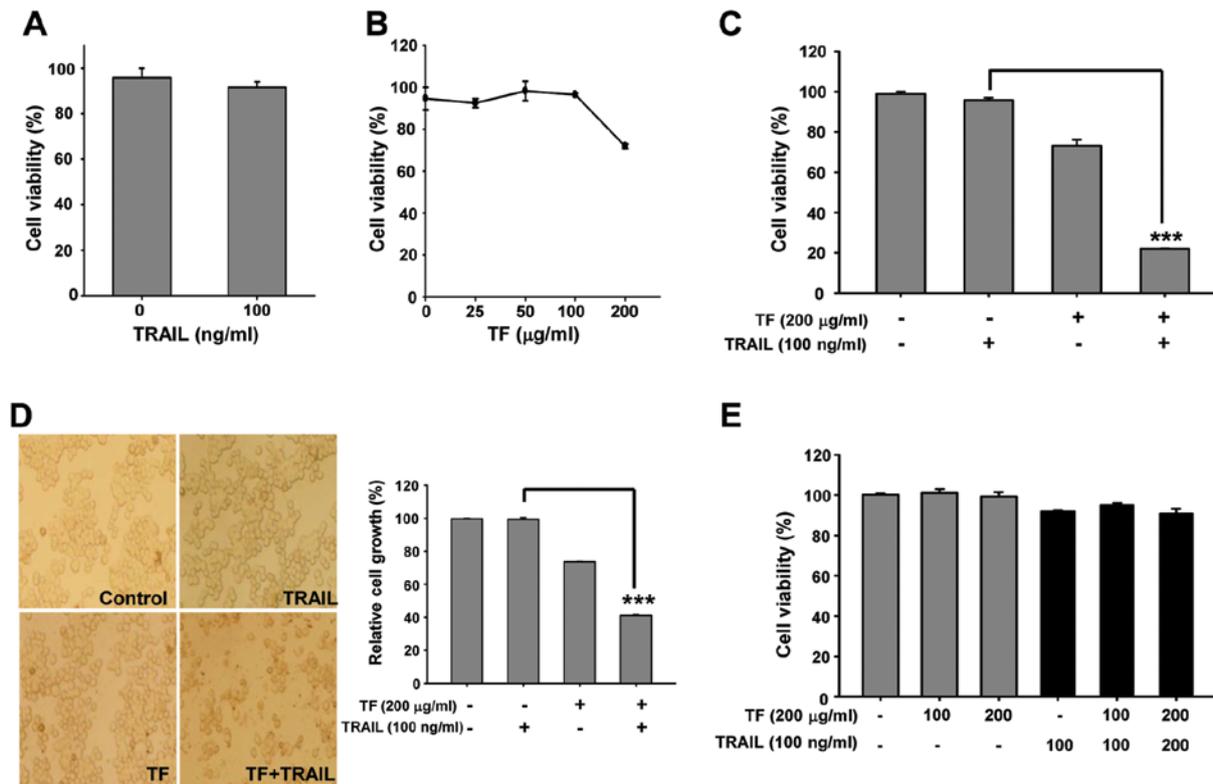


Figure 2. Synergistic effects of TF and TRAIL on cell viability. Huh7 cells were treated with (A) TRAIL, (B) dose-dependent TF or (C) a combination of TF (200 µg/ml) and TRAIL (100 ng/ml) (C) for 24 h (\*\*\*)  $p < 0.001$ , TRAIL vs. TF/TRAIL). (D) The morphology of cells was observed under a microscope. Images were captured at an original magnification of x200 (left panel). The cell growth was assessed using crystal violet staining (right panel) (\*\*\*)  $p < 0.001$ , TRAIL vs. TF/TRAIL). (E) HAECs were treated with TRAIL and/or TRAIL for 24 h. TF, *Tussilago farfara* L.; HAECs, human aortic endothelial cells.

## Results

*TF specifically inhibits the MKK7-TIPRL interaction.* To screen out bioactive natural products that could potentially abolish TRAIL resistance, we constructed an ELISA system that specially detects the MKK7-TIPRL interaction (Fig. 1A). GST-MKK7 was coated on a polyvinyl chloride microtiter plate and was then exposed to react with His-TIPRL-conjugated with HRP. For the quantification of inhibition of the MKK7-TIPRL interaction, natural products were introduced before the addition of TIPRL. Among 500 natural products in our library (data not shown), TF showed 50% inhibition of the interaction between MKK7 and TIPRL (Fig. 1B). To further validate the inhibition of the MKK7/TIPRL interaction by TF, GST pull down assay was conducted (Fig. 1C). The MKK7-TIPRL interaction was significantly decreased by TF in the presence of TRAIL compared with either TRAIL or TF alone, supporting the possibility that TF specifically inhibits the MKK7-TIPRL interaction.

The HPLC profile of the TF extract was examined using photodiode array detector (PDA) (12). Given that the retention times and UV spectra in TF were identical to those of commercially purchased standards, our HPLC data showed that major constituents of the TF extract were phenolic compounds such as chlorogenic acid, quercetin 3-*O*-glucoside 7-*O*-rhamnoside, quercetin-3-*O*-glucopyranoside and astragalins (Fig. 1D). We postulated that the phenolic compounds in TF inhibit the interaction of TIPRL-MKK7 upon TRAIL treatment.

*Augmentation of TRAIL-induced cytotoxicity by TF in Huh7 cells.* Next, we examined whether TF enhances the sensitivity of Huh7 cells to TRAIL. Fig. 2A shows that 100 ng/ml of TRAIL did not affect the viability of Huh7 cells. In addition, when Huh7 cells were treated with 200 µg/ml TF alone for 24 h, 73% cell viability was observed (Fig. 2B), implying that Huh7 cells are resistant to TRAIL and TF has low cytotoxicity. However, the combination of TF and TRAIL treatment decreased cell viability to 25% (Fig. 2C), and colony formation assays using crystal violet confirmed that TF exhibited cytotoxicity in the presence of TRAIL (Fig. 2D). However, HAECs, normal endothelial cells, were not harmed by TF or TRAIL alone, or by the combination of TF and TRAIL (Fig. 2E). These findings indicate that TF can be a novel sensitizer of apoptosis induction in TRAIL-resistant Huh7 cells.

*Activation of caspase-dependent apoptosis in Huh7 cells treated with TF and TRAIL.* To investigate the mechanism involved in TF-TRAIL-induced apoptosis in Huh7 cells, immunoblot analysis was performed using anti-caspase-8, anti-caspase-9, anti-caspase-3 and anti-PARP antibodies. Co-treatment of TF with TRAIL induced the activation of caspase-8, caspase-9, caspase-3 and PARP (Fig. 3A). When Huh7 cells were treated with TF (200 µg/ml) and/or TRAIL (100 ng/ml), caspase-3 activity was significantly enhanced in the cells co-treated with TF and TRAIL compared to cells treated with TF or TRAIL only (Fig. 3B). In addition, cell cycle analysis showed that sub-G1 DNA contents increased

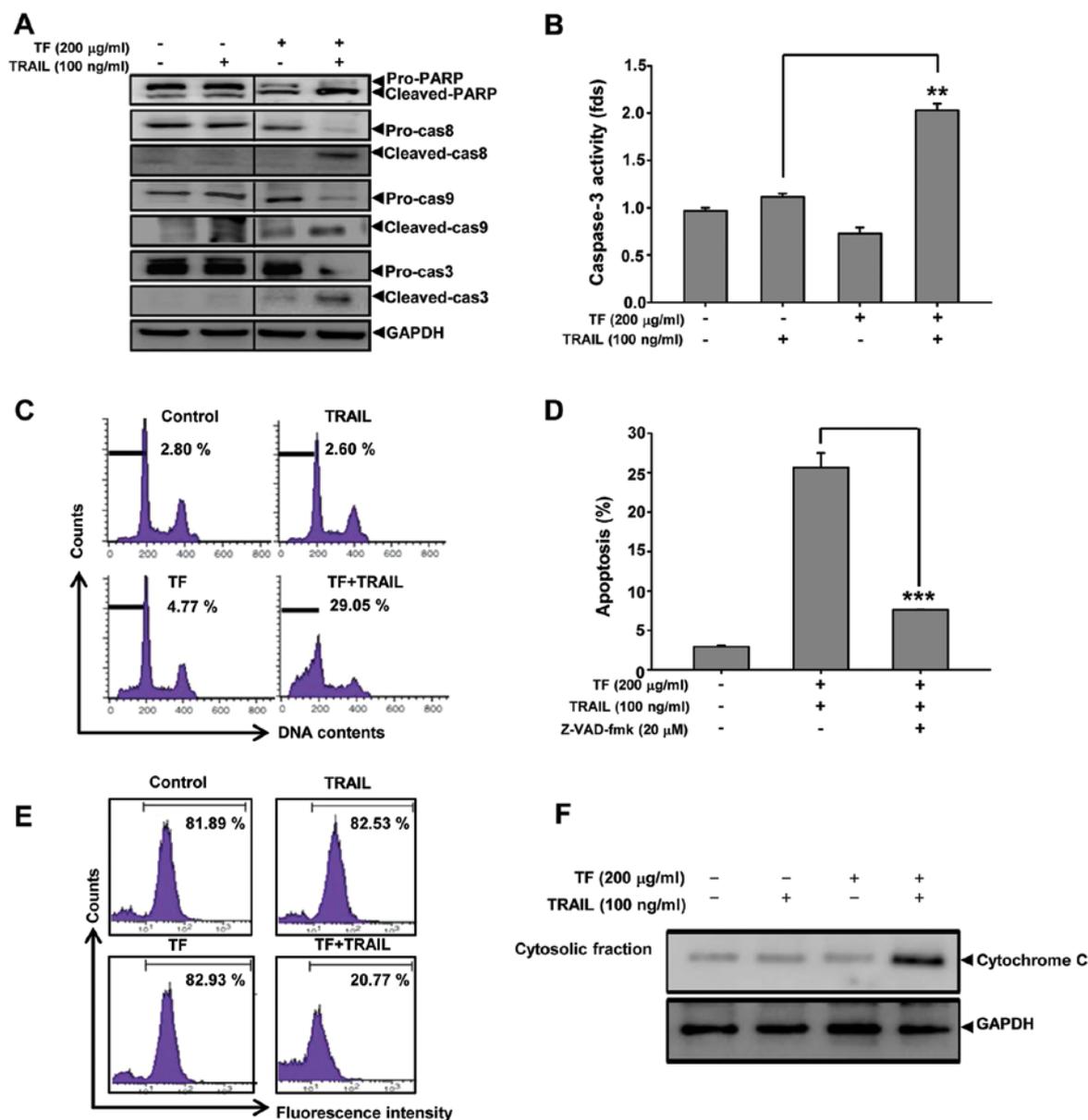


Figure 3. Augmentation of TRAIL-induced apoptosis by TF. Cells were treated with TF (200  $\mu$ g/ml) and/or TRAIL (100 ng/ml) for 24 h. (A) The cell lysates were subjected to western blotting using the indicated antibodies. GAPDH was used as the loading control. (B) Caspase-3 activity was measured using fluorimetric caspase-3 luminometric assay kit (\*\* $p$ <0.01, TRAIL vs. TF/TRAIL). (C and D) The cell cycle distribution was analyzed by flow cytometry. Bar graphs represent the percentages of sub-G1 DNA contents in cells undergoing apoptosis (\*\* $p$ <0.001, TF/TRAIL vs. TF/TRAIL/z-VAD-fmk; significant difference between the apoptotic ratio of cells). (E) The cells were harvested, stained with TMRE and analyzed for MMP by flow cytometry. (F) The cytosolic fraction of cell pellets was subjected to western blot analysis using the anti-cytochrome *c* antibody. GAPDH was used for loading control. TF, *Tussilago farfara* L.; TMRE, tetramethylrhodamine; MMP, mitochondrial membrane potential.

from 2.60 (TRAIL only) to 29.05% (TF/TRAIL treatment). Notably, TF increased the sub-G1 percentage to 27.05% in the presence of TRAIL, which was suppressed to nearly 7.64% with pretreatment with the pan-caspase inhibitor z-VAD-fmk for 1 h (Fig. 3C and D). These results imply that TF sensitizes Huh7 cells to TRAIL-induced cell death through the activation of the apoptotic machinery.

Next, we evaluated the MMP in Huh7 cells treated with TF and TRAIL using TMRE solution as a fluorescent potential-dependent indicator, and observed that the MMP of cells that were co-treated with TF and TRAIL was 20.77%. In contrast, the MMP of cells treated with 200  $\mu$ g/ml TF and 100 ng/ml TRAIL was 82.93 and 82.53%, respectively, implying

that TF/TRAIL-induced apoptosis is mediated through the mitochondrial/extrinsic apoptotic pathway (Fig. 3E). To further confirm the TF/TRAIL-induced apoptosis via mitochondria, we assessed the level of cytochrome *c* in the cytoplasmic fraction of Huh7 cells co-treated with TF and TRAIL, and found upregulation of cytochrome *c* in the Huh7 cells co-treated with TF and TRAIL compared to cells treated with TF or TRAIL alone (Fig. 3F). Collectively, the combined treatment of TF and TRAIL induced apoptosis through the intrinsic/extrinsic pathway.

*Involvement of MKK7/JNK activation in TF/TRAIL-induced apoptosis.* Previously, we reported that activation of MKK7/

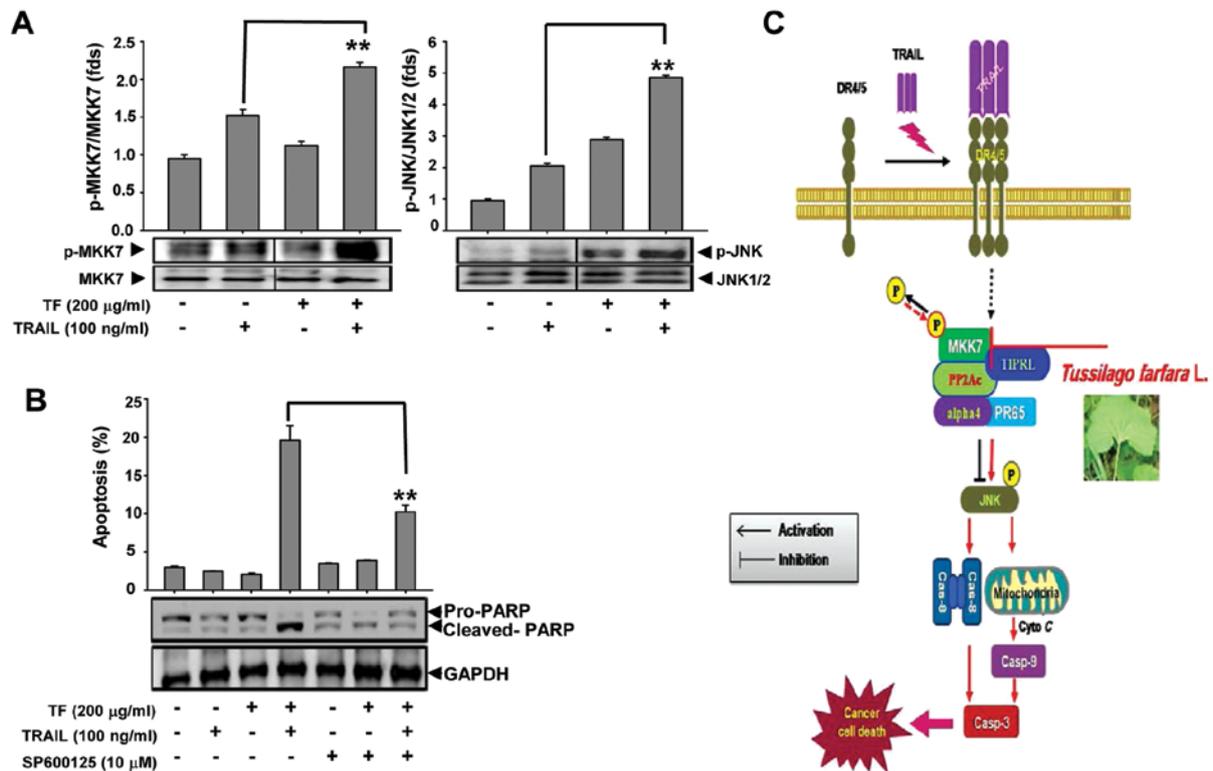


Figure 4. Involvement of MKK7/JNK in TF/TRAIL-induced apoptosis. Cells were treated with TF (200 µg/ml) and/or TRAIL (100 ng/ml) for 24 h. (A) The cell lysates were subjected to western blot analysis using the indicated antibodies. Bar graphs represent the relative level of phospho(p)-MKK7/MKK7 (left panel) and p-JNK/JNK (right panel) (\*\* $p < 0.01$ , significant difference between cells treated with TF/TRAIL and TRAIL). (B) The cells were pre-treated with SP600125 for 1 h and were then treated with TF and/or TRAIL for an additional 24 h. The bar graphs represent the percentages of the sub-G1 apoptotic DNA fraction (\*\* $p < 0.01$ , significant difference between the apoptotic portion of cells treated with TF/TRAIL/SP600125 and with TF/TRAIL only). (C) In TRAIL-resistant cancer, the dephosphorylation of MKK7 by PP2Ac via TIPRL leads to the inhibition of MKK7/JNK, the caspase cascade and apoptosis, eventually contributing to TRAIL resistance in cancer cells. However, TF interferes with the MKK7-TIPRL interaction, subsequently inducing phosphorylation/activation of MKK7/JNK, and finally activating the caspase cascade including extrinsic and intrinsic pathways and cell death. MKK7, mitogen protein kinase kinase 7; JNK, c-Jun N-terminal kinase; TF, *Tussilago farfara* L.; TIPRL, TOR signaling pathway regulator-like protein.

JNK is involved in TRAIL-induced apoptosis in HCCs after TIPRL knockdown (8). Based on our results, we examined the involvement of MKK7/JNK activation in TF/TRAIL-induced apoptosis, and found an increase in the phosphorylation status of MKK7/JNK in cells co-treated with TF and TRAIL (Fig. 4A). Furthermore, as shown in Fig. 4B, pretreatment of Huh7 cells with SP600125, a JNK-specific inhibitor, led to reduction in the apoptotic portion and cleaved PARP following TF/TRAIL treatment. Our results strongly imply that combined TF and TRAIL treatment induced the apoptosis of Huh7 cells through the activation of MKK7/JNK.

## Discussion

Molecular targeted therapies against cancer cells are currently being studied with the hope of reducing side-effects and increasing target specificity for cancer patients (13,14). We previously reported that the TIPRL protein contributes to TRAIL resistance of HCC cells by interacting with MKK7, and that knockdown of TIPRL by siRNA and TRAIL treatment leads to apoptosis via activation of MKK7 and its downstream JNK (8). In the present study, we aimed to identify natural compounds which overcome TRAIL resistance by inhibition of the MKK7 and TIPRL interaction. This is the first report suggesting *Tussilago farfara* L. (TF) as a novel TRAIL sensi-

tizer that induces apoptosis by inhibiting the MKK7-TIPRL interaction.

To identify bioactive natural products that inhibit the interaction between MKK7 and TIPRL, we constructed an ELISA system with GST-MKK7 and His-TIPRL recombinant proteins, and selected TF as the most potent inhibitor of the MKK7/TIPRL interaction among 500 natural products. We further confirmed the inhibition of MKK7-TIPRL interaction by TF via a GST-pull down assay, which revealed that TF specifically inhibited the MKK7-TIPRL interaction in the presence of TRAIL (Fig. 1). TF, which is also known as coltsfoot, is a herbal remedy currently used as an antioxidant and anti-inflammatory agent (15). Many chemicals have been reported as constituents in TF, including phenolic acids, such as ferulic, p-hydroxybenzoic, caffeic, and caffeotartaric acids, and flavonoids, such as quercetin, kaempferol, quercetin-3-arabinoside, kaempferol-3-flucosides, kaempferol-3-arabonsides and quercetin glucoside (16). Our PDA study also determined that TF contains flavonoid-related compounds such as chlorogenic acid, quercetin 3-*O*-glucoside 7-*O*-rhamnoside, quercetin-3-*O*-glucopyranoside and astragaline. Given reports that flavonoids exert antitumor effects on several types of cancers such as ovarian, breast, cervical, pancreatic and prostate cancer (17), we hypothesized that these flavonoid-related compounds contained in TF may have a role in overcoming

TRAIL resistance by inhibition of the MKK7-TIPRL interaction.

As it is well known that HCC shows resistance to TRAIL treatment (18,19), we examined the viability of Huh7 cells following treatment with a high concentration of TRAIL (100 ng/ml), and observed resistance of Huh7 cells to TRAIL. However, TF caused synergistic cytotoxicity in Huh7 cells co-treated with TRAIL. Song *et al* reported that TIPRL knockdown induces TRAIL-induced cell death via a caspase/mitochondrial-dependent pathway (8). Similarly in the present study, the combined treatment of TF with TRAIL induced the activation of caspase-9, caspase-8, caspase-3 and PARP and enhanced the sub-G1 apoptotic population. These results were confirmed by the finding that TF/TRAIL-induced apoptosis was reduced by pretreatment with z-VAD-fmk. MMP and cytochrome *c* levels were also increased, supporting induction of apoptosis through the intrinsic pathway. In addition, co-treatment of TF and TRAIL also induced the phosphorylation of MKK7/JNK, which was suppressed by pretreatment with SP600125. These results strongly indicate that TF augments TRAIL-induced apoptosis via activation of MKK7/JNK in Huh7 cells, and that it could be used as a TRAIL sensitizer.

Resistance of apoptosis is one of the hallmarks of cancer and is a major obstacle that needs to be overcome for the successful application of cancer therapies. Resistance to TRAIL-induced apoptosis is one of the main mechanisms of apoptosis resistance. We identified TF as a potential TRAIL sensitizer among 500 natural products, and we elucidated the molecular mechanism of TF/TRAIL-induced apoptosis in Huh7 cells (Fig. 4C). However, further studies are warranted to identify the specific chemical component in TF that is responsible for the inhibition of MKK7-TIPRL interaction and to validate these results as well as the safety of TF in *in vivo* models.

### Acknowledgements

This study was supported by the Mid-Career Researcher Program through the National Research Foundation of Korea, funded by the Ministry of Education, Science and Technology, and by the KRIBB Research Initiative Program.

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