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Auriculasin sensitizes primary prostate cancer cells to TRAIL-mediated apoptosis through up-regulation of the DR5-dependent pathway

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ABSTRACT

Primary prostate cancer cells frequently develop resistance toward chemotherapy as well as most chemotherapeutics have been reported to induce undesirable cytotoxicity in normal cells. In this study, we performed sensitizing activity analysis of auriculasin (AC) to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in RC-58T/h/SA#4 primary prostate cancer cells without significant cytotoxicity in RWPE-1 prostate epithelial cells. Combined treatment with AC and TRAIL at optimal concentrations resulted in tumor-specific apoptotic cell death in RC-58T/h/SA#4 cells, characterized by DNA fragmentation, accumulation of apoptotic cell population, and nuclear condensation. Compared to single treatment with AC or TRAIL, co-treatment with AC and TRAIL significantly increased expression of Bax, cleaved PARP, AIF, endo G, and cytochrome *c* but decreased expression of phosphorylation of AKT and mammalian target of rapamycin (mTOR), phosphoinositide 3-kinase (PI3K), Bcl-2 and caspases-9, -8, -3, and -10. The sensitizing effect of AC to TRAIL was well correlated with inhibition of death receptor 5 (DR5) CHOP, and p53 expression. Moreover, pre-treatment with a chimeric blocking antibody for DR5 effectively reduced AC-TRAIL-induced cell death and apoptosis-related protein expression. These results suggest that non-toxic concentrations of AC sensitize TRAIL-resistant primary prostate cancer cells to TRAIL-mediated apoptosis via up-regulation of DR5 and downstream signaling pathways.

1. Introduction

Numerous cancer species are characterized by extremely aggressive growth in early stages of development to nearby and distant organs (Massagué and Obenauf, 2016). Thus, failure to treat primary cancer at an early stage can increase morbidity and mortality of prostate cancer patients (Berger et al., 2011). In recent years, effective cancer strategies applying chemotherapeutics to cancer stem cells or primary cancer cells have attracted attention (Zhang et al., 2018; Zhang et al., 2018). However, drug resistance and normal cell toxicity are known as major hurdles in early stages of primary cancer therapy. Since primary and metastases cancer cells show molecular differences (Ramaswamy et al., 2003), it is also urgent to articulate novel therapeutic strategies for primary prostate cancer with minimal cytotoxicity in normal cells.

Prostate cancer is the most common cancer species in the world as well as a significant cause of cancer-induced death in men. Although various chemotherapy drugs have shown transient efficacy for prostate cancer, significant side effects are induced by continuous utilization of cancer therapeutics (Karavelioglu et al., 2016; Frederiks et al., 2015). To avoid the side effects of conventional chemotherapies for cancer treatment, development of ideal therapeutics that induce cancer-selective apoptosis has been carried out by numerous researchers. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the TNF ligand superfamily of cytokines broadly expressed by neutrophils, natural killer cells, and lymphocytes (Guimarães et al., 2018). TRAIL can bind to death domain-containing receptors, such as death receptor 4 (DR4) and death receptor 5 (DR5), and non-death domain-containing receptors, such as designated decoy receptor 1 (DcR1), DcR2, and DcR3 (Johnstone et al., 2008). Upon binding of TRAIL to extracellular DR4 and DR5, a cascade of downstream proteins is activated after development of death-inducing signal complex (DISC) and FAS-associated protein death domain (FADD) (Bertsch et al., 2014).

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Consequently, TRAIL triggers caspase-dependent and/or -independent apoptosis directly activating caspase-8 as well as truncates BH3-interacting domain death agonist (BID) (Woo et al., 2017).

However, certain types of cancer cells have demonstrated TRAIL resistance caused by mutational inactivation of pro-apoptotic genes (Bax, and Bak), overexpression of anti-apoptotic genes (Bcl-2, and Bcl-$_{XL}$), and dysfunction of death receptors (DR4, and DR5) (Burris HA 3rd, 2013). This diverse range of resistance mechanisms presents new challenges for long-term tumor control. Furthermore, Kim et al. (2011) reported that primary prostate cancer cells are less sensitive to TRAIL-induced apoptosis than metastatic prostate cancer cells, and Lemke et al. (2014) reported that TRAIL can trigger non-apoptotic signaling pathways, which can induce malignancy in some TRAIL-resistant primary cancer cells. To overcome TRAIL resistance in various cancer cells, numerous strategies that exert promising cancer suppressing activity have been developed and are at the pre-clinical stage. Especially, a number of researchers have shown that cancer cells with TRAIL resistance can be sensitized by bioactive compounds from natural products, including curcumin (Jung et al., 2006), methylseleninic acid (Yamagucci et al., 2005), and ursolic acid (Shin and Park, 2013). Therefore, anti-cancer reports using combined treatments with non-toxic concentrations of natural compounds and TRAIL in TRAIL-resistant primary prostate cancer cells have drawn increasing interest.

Auriculasin (AC) is a prenylated isoflavone in various food ingredients, such as roots of *Flemingia philippinensis* (Wang et al., 2013), stem bark of *Erythrina senegalensis* (Oh et al., 1998), and osage orange fruits (Peter and Krammer, 1998). It is known to activate the caspase-independent signaling pathway and inhibit proliferation of prostate cancer cells, confirming its strong anti-tumor activity (Wang et al., 2013; Cho et al., 2018). In our previous study, AC treatment (5 μM) significantly induced caspase-independent apoptosis in LNCaP metastatic prostate cancer cells without significant RWPE-1 prostate epithelial cell toxicity (Cho et al., 2018). Although AC is an interesting candidate to suppress metastatic prostate cancer due to its lack of normal cell cytotoxicity, the molecular mechanisms underlying the anti-cancer activity of AC in TRAIL-resistant primary prostate cancer cells have not been fully elucidated.

The present study investigated the anti-cancer activity of single or combined treatment with AC and TRAIL against TRAIL-resistant primary prostate cancer cells as well as identified potential mechanisms. Interestingly, non-cytotoxic concentrations of AC efficiently induced DR5-mediated apoptosis by TRAIL in RC-58T/h/SA#4 primary prostate cancer cells. Collectively, our results elucidate the mechanisms behind the synergistic anti-cancer activities of AC and TRAIL at non-toxic concentrations in primary prostate cancer cells and could help facilitate the development of promising cancer strategies without significant side effects.

2. Material and method

2.1. Isolation of AC

AC was isolated as described previously (Wang et al., 2013). Briefly, the air-dried root bark of *F. philippinensis* (0.3 kg) was chopped, and extracted with MeOH (3 L × 3) at room temperature for 7 days. The combined filtrate was concentrated in vacuum to yield a dark red gum (51 g, 17.1%). The MeOH extract (25 g) was subjected to column chromatography (CC) on silica gel using a hexane to ethyl acetate gradient (50:1-1:1) to give 8 fractions (A-H, 500 mL/each). Fraction D (3.2 g) was fractionated via RP-MPLC using a C18 column with elution using a gradient of increasing MeOH (0–100%) in H_2O and 20 mL/min of flow rate to afford 45 subfractions (D1-D45). Subfractions D28-35, enriched with AC were combined (226 mg) and chromatographed over a C18 column using MeOH−H_2O (70:30) to afford (102 mg) AC that was identified on the basis of the following spectroscopic data.

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2.2. Chemicals

DMEM, fetal bovine serum (FBS), trypsin-EDTA, penicillin, and antibiotic-antimycotic were purchased from GIBCO BRL Co. (Gaithersburg, MD, USA). Propidium iodide (PI), and RNase were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, USA). The general caspase inhibitor (z-VAD-fmk), PI3K inhibitor (LY294002), and apoptosis-inducing factor (AIF) inhibitor (N-phenylmaleimide, N-PM) was obtained from R&D systems (Minneapolis, MN, USA), Cell signaling Technology Inc. (MA, USA), and Sigma-Aldrich Co. Ltd. (st. Louis, USA). Anti-Bax (sc-7480), anti-Bcl-2 (sc-7382), anti-caspase-3 (sc-7272), anti-caspase-8 (sc-7890), anti-caspase-9 (sc-7885), anti-caspase-10 (sc-393983), anti-endo G (sc-365359), anti-AIF (sc-5586), anti-poly (ADPribose) polymerase-1 (PARP-1) (sc-7150), anti-AKT (sc-5298), anti-mTOR (sc-8319), anti-PI3K (sc-423), and anti-β-actin (sc-47778) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mitochondria isolation kit and bicinchoninic acid (BCA) protein assay kit were purchased from Pierce (Rockford, IL, USA). ECL kit was purchased from Amersham Life Science (Amersham, UK).

2.3. Cell culture and cell proliferation

The RWPE-1 (human prostate epithelial cells) was purchased from American Type Culture Collection (ATCC, Rockville, ND, USA). RC-58T/h/SA#4 cells were obtained from the Center for Prostate Disease Research (Washington, DC, USA). The cells were cultured in Keratinocyte-SFM (RWPE-1), and DMEM (RC-58T/h/SA#4) medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 μg/mL) (Gibco BRL, Life Technologies, Grand Island, NY) in an incubator containing a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. Sulforhodamine B (SRB) assay

Cell proliferation was determined by sulforhodamin B (SRB, Sigma, St. Louis, USA) assay. The cancer cells were seeded at a concentration of 3×10^4 cells/well in 48-well tissue culture plates and incubated with various concentrations of AC and TRAIL for 24 h. After treatment, medium was aspirated and 10% trichloro-acetic acid was added. After 1 h incubation at 4 °C, the plate was washed five times with D.W and air-dried. The cells were stained with 0.4% (w/v) SRB at room temperature for 1 h and then washed five times using 1% acetic acid. Bound SRB was solubilized with 10 mM Tris, and the absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Inc. US).

The influence of z-vad-fmk (caspases inhibitor), N-PM (AIF inhibitor), and LY294002 (PI3K and AKT inhibitor) on cell viability was also determined by SRB assay. The cells were seeded at a densities of 1×10^5 cells per well in a 24-well plate, and then cultured for 24 h in DMEM. The cells were pre-incubated with 10 μM of z-VAD-fmk or 2 μM of N-PM or 10 μM of LY294002 for 2 h and then treated with the indicated concentrations of AC and TRAIL for 24 h. SRB assay was conducted as described above.

2.5. Annexin V staining by flow cytometry

The apoptotic cell death was determined using Muse® Annexin V and dead cell reagent, according to the manufacturer's protocol. 1×10^5 RC-58T/h/SA#4 cells were seeded into 24 well plates and incubated for 24 h. Treatments were given with AC and TRAIL for 24 h. The cells were trypsinized and washed with PBS. Added 100 μL of the reagent to microcentrifuge tubes and then added 10 μL of cell suspension to each tube and incubated for 20 min at room temperature. The cells were analyzed using a Muse cell analyzer (Merck KGaA, Darmstadt, Germany). The flow cytometry data was obtained from

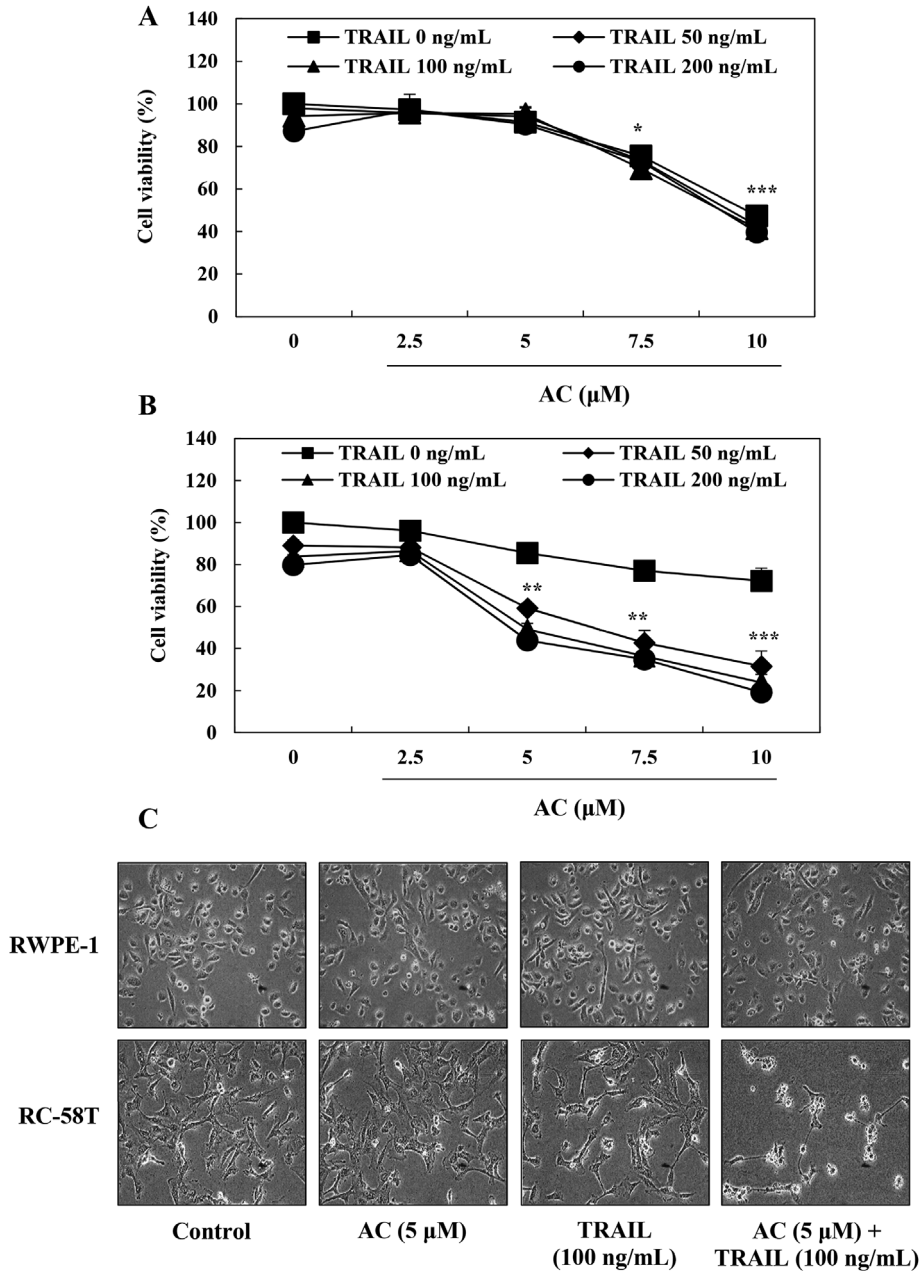

Fig. 1. Cell growth inhibitory effects on prostate cancer and prostate epithelial cells. Cells were treated with various concentrations of AC and/or TRAIL for 24 h. (A) RWPE-1 prostate epithelial cell and (B) RC-58T/h/SA#4 primary prostate cancer viability were measured by SRB assay. (C) Morphological changes were observed under an inverted microscope after 24 h of treatment. Significant differences were compared with the control at $*p < 0.05$ and $**p < 0.01$ using one-way ANOVA.

5000 events (gated cells) per sample. The percentages of cells shown in the figures were calculated from the mean fluorescence intensity in each of the four quadrants. In addition, the coefficient of variation from the mean fluorescence was less than 10%.

2.6. Detection of morphological apoptosis

Characteristic apoptotic morphological changes were assessed by fluorescent microscopy using bis-benzimide (Hoechst 33258) staining. Briefly, the cells were seeded in 6-well plates at a density of 1×10^6 cells per well, followed by treatment with AC and TRAIL for 24 h. After harvesting, the cells were washed twice with PBS and then stained with 200 μL of bis-benzimide (5 μg/mL) for 10 min at room temperature. Then, 10 μL of this suspension was placed on a glass slide and covered with a cover slip. The cells were examined using a fluorescence microscope (Olympus Optical Co. Ltd. Japan) to determine nuclei fragmentation and chromatin condensation.

2.7. Analysis of DNA fragmentation

The cells were seeded at a density of 2×10^6 cells in a 100 mm dish and cultured for 24 h in DMEM. After culturing, the cells were treated with the indicated concentrations of AC and TRAIL for 24 h, followed by centrifugation. The pellets were lysed by lysis buffer (10 mM Tris–HCl, pH 7.5, 10 mM EDTA, pH 8.0, 0.5% Triton X-100, 20% SDS, and 10 mg/mL of proteinase K) and then centrifuged. After extraction with phenol: chloroform: isoamyl alcohol (25:24:1), DNA was precipitated with 2 vol of cold absolute ethanol. The resulting pellets were incubated with TE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, pH 8.0) and RNase (2 mg/mL) for 1 h at 37 °C. Then, separation by

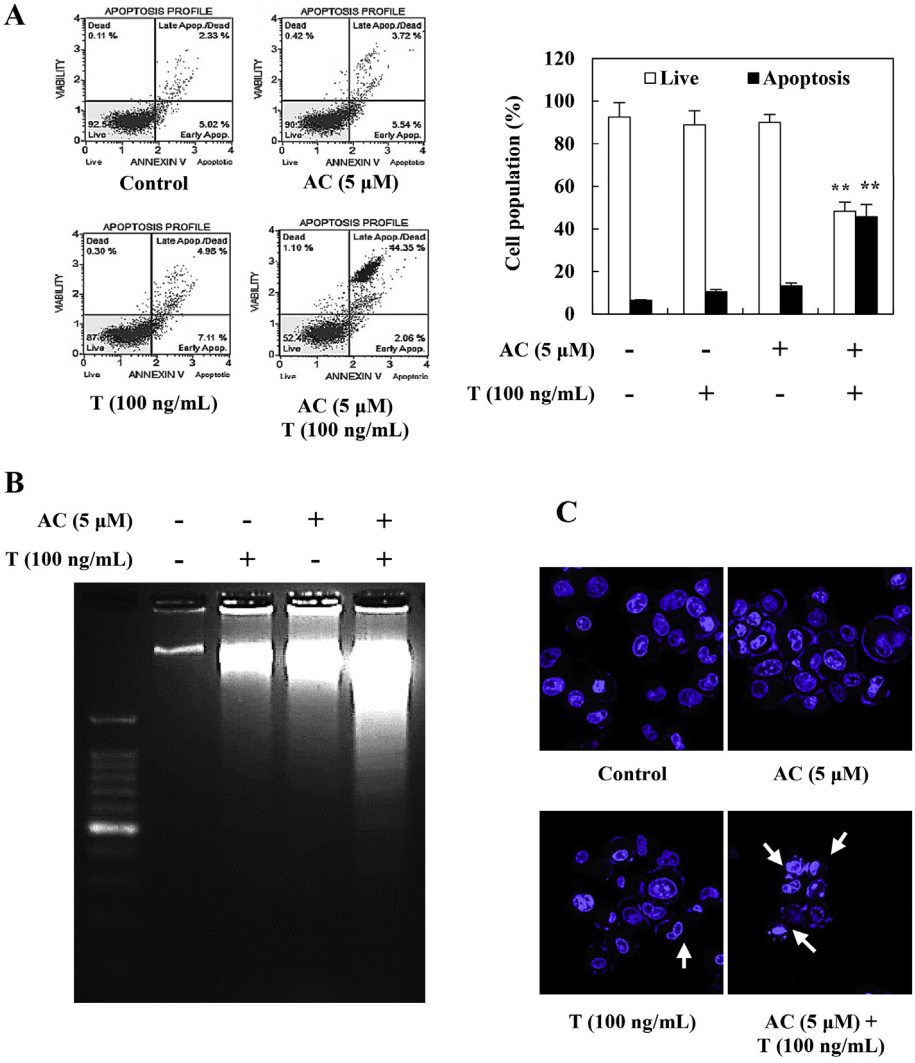

Fig. 2. Combined treatment with AC and TRAIL induces apoptotic cell death in RC-58T/h/SA#4 primary prostate cancer cells. Cells were treated with 5 μM AC and/or 100 ng/mL of TRAIL for 24 h. (A) Apoptotic cell population was quantified by Annexin V staining assay using a Muse cell analyzer. (B) DNA fragmentation was observed by 2% agarose gel electrophoresis. (C) Nuclear condensation was detected by Hoechst staining assay. Significant differences were compared with the control at $*p < 0.05$ and $**p < 0.01$ using one-way ANOVA.

electrophoresis was performed on 2% agarose containing ethidium bromide. The resulting DNA bands were examined using a UV Trans illuminator Imaging System.

2.8. Mitochondria isolation

Mitochondria fraction was isolated from cell lysate using a Mitochondria isolation kit (Pierce, Rockford, USA). Briefly, 800 μL of Reagent A and 10 μL of Reagent B were added to a 2×10^6 cell pellet and incubated on ice for 2 min. The resulting pellets were centrifuged at $700 \times g$ for 10 min at 4 °C. The supernatant was then transferred to a new tube and centrifuged at $12{,}000 \times g$ for 15 min at 4 °C. The supernatant (cytosol fraction) was added to a new tube, and the pellet containing mitochondria received 500 μL of Reagent C followed by centrifugation ($12{,}000 \times g$, 5 min, 4 °C). The mitochondria pellets were lysed by lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM PMSF, and 2 μg/mL of aprotinin) for 30 min on ice. Protein expression in the mitochondria fraction was analyzed by Western blot assay.

2.9. Western blot analysis

The cells were seeded at a density of 5×10^6 cells in a 100 mm dish, and then cultured for 24 h in DMEM. After culturing, the cells were treated with the indicated concentrations of AC and TRAIL for 24 h, followed by centrifugation. The resulting pellets were lysed by lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM PMSF, and 2 μg/mL of aprotinin) for 30 min on ice. The protein content of the supernatant was measured using a BCA protein kit (Pierce, Rockford, IL, USA). The protein samples were then loaded at 10 μg of protein/lane and then separated by 12% SDS-PAGE at 100 V of constant voltage/slab for 1.5 h. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes. After blocking with 2.5% and 5% bovine serum albumin (BSA) for 1 h at 37 °C, the membranes were incubated with primary antibody at 4 °C overnight. Finally, the membranes were treated with horseradish peroxidase-coupled secondary antibodies for 1 h at 4 °C. The membranes were then washed with T-TBS after each antibody binding reaction. Detection of each protein was performed using an ECL kit (Santa Cruz, CA, USA). All protein bands were analyzed 3 times, and the intensity of each band was quantified by Image studio™ Lite software (LI-COR Inc., NE, USA), and the fold of increase was presented comparing with β-

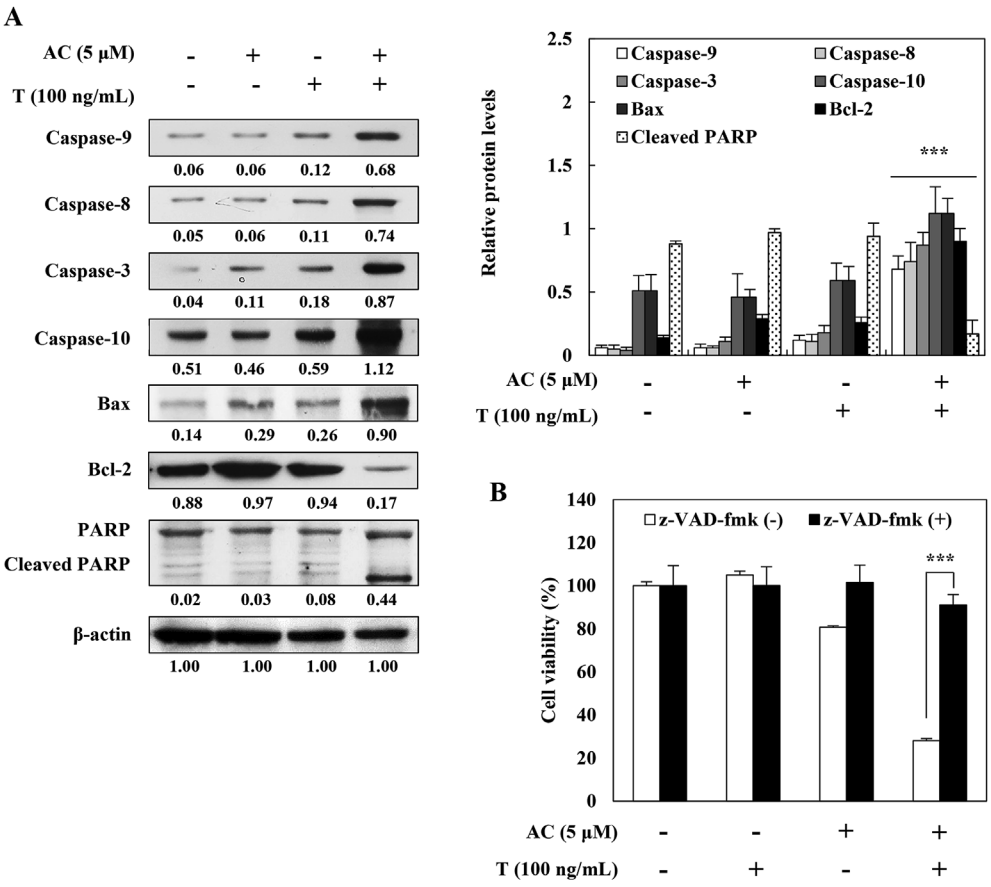

Fig. 3. Combined treatment with AC and TRAIL induces caspase-dependent apoptosis in RC-58T/h/SA#4 cells. Cells were treated with 5 μM AC and/or 100 ng/mL of TRAIL for 24 h. (A) After treatment with AC and/or TRAIL for 24 h, total cell lysates were subjected to detect expression levels of proteins. Expression of caspase-9, -8, -3 and -10, Bax, Bcl-2, and PARP in RC-58T/h/SA#4 cells were analyzed by Western blotting. (B) Cells were pretreated with 10 μM z-vad-fmk, a caspase inhibitor, for 2 h and then incubated with AC and/or TRAIL for 24 h. Cell viability was evaluated by SRB assay. Significant differences were compared with the control at $*p < 0.05$ and $**p < 0.01$ using one-way ANOVA.

actin.

2.10. Statistical analysis

The statistical analyses were evaluated by one-way analysis of variance (ANOVA), with differences analyzed using the Duncan's new multiple-range test. Levels of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ were regarded as statistically significant.

3. Results

3.1. AC sensitizes TRAIL-resistant RC-58T/h/SA#4 cells to TRAIL-induced cell death

In order to determine the inhibitory effect of combined treatment with AC and TRAIL on proliferation of prostate cancer cells, both RC-58T/h/SA#4 and RWPE-1 cells were treated with various doses of AC with or without TRAIL (Fig. 1). AC and TRAIL-induced suppression of cell viability was confirmed by SRB assay. RC-58T/h/SA#4 cells were moderately resistant to 2.5–10 μM AC and 50–200 ng/mL of TRAIL (Fig. 1A). However, co-treatment with AC (5–10 μM) and TRAIL (50–200 ng/mL) significantly reduced viability of RC-58T/h/SA#4 cells, suggesting that TRAIL resistance in RC-58T/h/SA#4 cells could be overcome by addition of AC at low concentration (Fig. 1A, C). Unlike primary prostate cancer cells (i.e. RC-58T/h/SA#4 cells), proliferation of human normal prostate epithelial cells (RWPE-1) was inhibited by a high dose of AC single treatment (7.5–10 μM) (Fig. 1B). However, single or combined treatment with low-dose AC (2.5–5 μM) and TRAIL (50–200 ng/mL) did not have significant cytotoxicity in RWPE-1 cells (Fig. 1B and C). Therefore, these results suggest that combined treatment with non-cytotoxic concentrations of AC (5 μM) and TRAIL (100 ng/mL) exerted a synergistic inhibitory effect against primary cancer cell proliferation.

3.2. AC sensitizes TRAIL-mediated apoptosis in RC-58T/h/SA#4 cells

After binding of TRAIL to extracellular DR4 and DR5, an apoptotic cascade of downstream proteins is activated during assembly of death-inducing signaling complex (DISC) at their intracellular death domains (Bertsch et al., 2014). To determine whether or not AC could sensitize RC-58T/h/SA#4 cells to TRAIL-mediated apoptosis, cells were treated with AC alone or in combination with TRAIL for 24 h. As shown in Fig. 2A, the percentage of apoptotic cells was significantly higher upon co-treatment with AC and TRAIL compared to the control. Furthermore, biological markers of apoptosis such as DNA fragmentation, condensation of chromatin, and expression of apoptotic bodies were significantly enhanced upon combined treatment with AC and TRAIL treatment (Fig. 2B and C). These results show that co-treatment with non-cytotoxic concentrations of AC and TRAIL significantly elevated apoptosis in TRAIL-resistant RC-58T/h/SA#4 cells.

3.3. Sensitization to TRAIL-mediated apoptosis by AC is dependent on caspase proteins

Once TRAIL binds to either DR4 or DR5, the death signal propagates through the TRAIL-mediated signaling pathway, which leads to caspase-8-mediated activation of caspase-3, cleavage of PARP, and caspase-3 activation through caspase-9 (Khaider et al., 2012). To assess the molecular mechanism behind AC in TRAIL-mediated apoptosis, we analyzed several key proteins involved in TRAIL-induced apoptosis in primary prostate cancer cells. Treatment with AC or TRAIL alone slightly affected expression levels of caspases, Bax, Bcl-2, and PARP in RC-58T/h/SA#4 cells (Fig. 3A). However, combined treatment with AC and TRAIL led to significant down-regulation of caspases-8, -9, -3 and

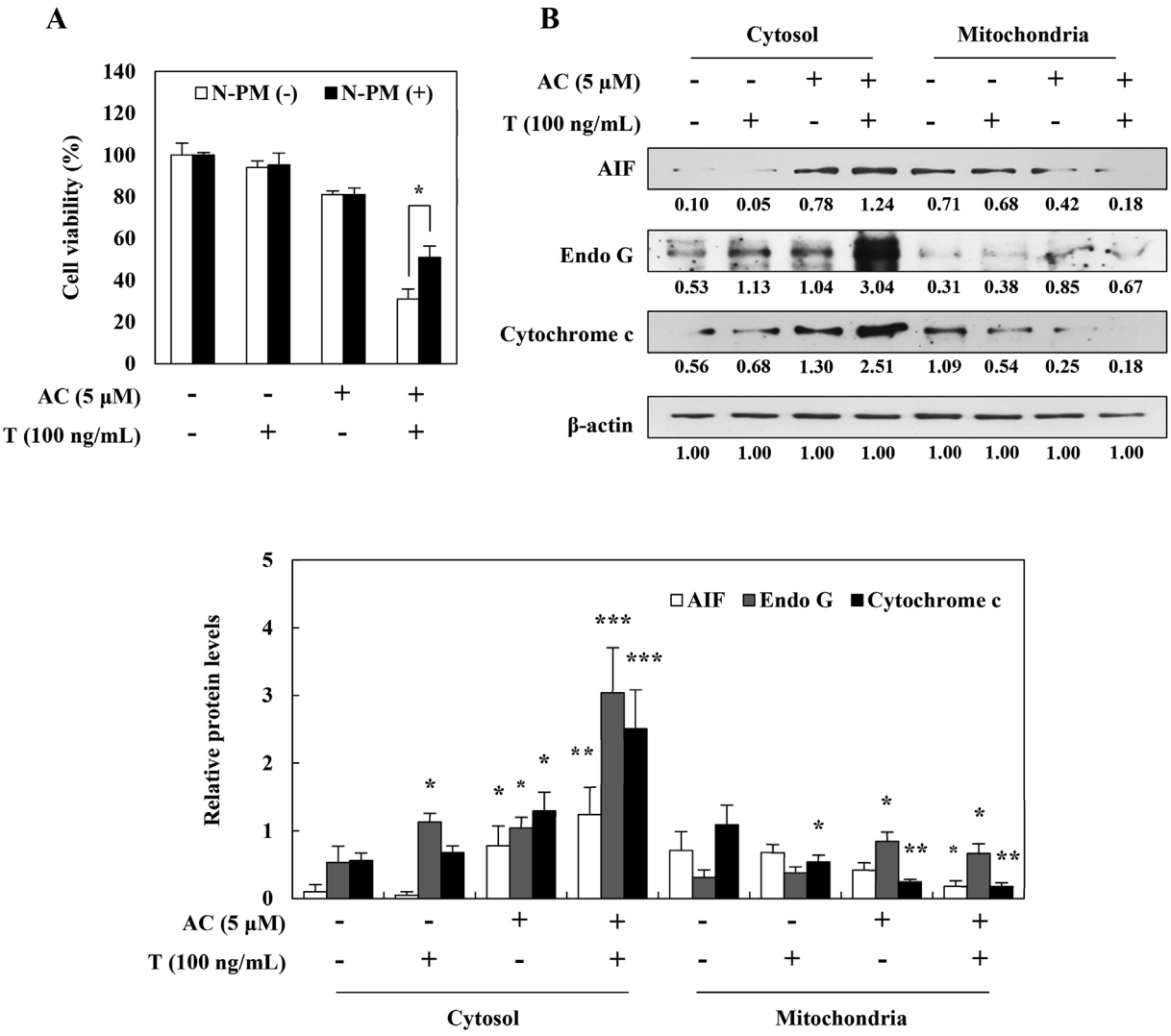

Fig. 4. Combined treatment with AC and TRAIL triggers caspase-independent apoptosis in RC-58T/h/SA#4 cells. (A) Cells were pretreated with 2 μM N-phenylmaleimide (N-PM), an AIF inhibitor, for 2 h and then incubated with AC and/or TRAIL for 24 h. Cell viability was determined by SRB assay. (B) After treatment with AC and/or TRAIL for 24 h, total cell lysates were subjected to detect expression levels of proteins. Expression levels of AIF, Endo G, and cytochrome *c* in RC-58T/h/SA#4 cells were analyzed by Western blotting. Significant differences were compared with the control at $*p < 0.05$ and $**p < 0.01$ using one-way ANOVA.

-10, and Bcl-2 as well as up-regulation of Bax and cleaved PARP in RC-58T/h/SA#4 cells. In addition, pre-treatment with 10 μM pan-caspase inhibitor (z-VAD-fmk) overcame apoptotic death by 62.41% upon co-treatment with AC and TRAIL (Fig. 3B). These results indicate that co-treatment with AC and TRAIL induced synergistic apoptosis via activation of the caspase-dependent pathway.

3.4. Co-treatment with AC and TRAIL triggers caspase-independent cell death

To determine the role of caspase-independent apoptotic regulatory proteins in AC-induced TRAIL sensitization, RC-58T/h/SA#4 cells were cultured in the presence or absence of the AIF inhibitor N-phenylmaleimide (N-PM) (Fig. 4A). Pre-treatment of cells with N-PM significantly blocked apoptotic cell death induced by co-treatment with AC and TRAIL (Fig. 4A). Further, we investigated the effect of co-treatment with AC and TRAIL on release of AIF, endonuclease G (Endo G), and cytochrome *c* proteins into the cytosol from mitochondria (Fig. 4B). Compared to single treatment with AC or TRAIL, expression levels of released AIF, Endo G, and cytochrome *c* into the cytosol were significantly elevated upon combined treatment with AC and TRAIL (Fig. 4B). These findings suggest that AC and TRAIL-induced synergistic cell death utilized a caspase-independent signaling mechanism involving AIF and Endo G.

3.5. AC sensitizes TRAIL-mediated apoptosis through up-regulation of DR5

To further explore the mechanism behind TRAIL-mediated apoptosis sensitized by AC, expression levels of C/EBP homologous protein (CHOP), DR5, DR4, and p53 were evaluated by Western blot analysis. AC treatment elevated expression levels of CHOP, DR5, and p53 proteins in a dose-dependent manner, whereas expression of DR4 was not affected (Fig. 5A). To clarify whether or not this increase in DR5 expression could be responsible for AC and TRAIL-induced cell death, RC-58T/h/SA#4 cells were treated with 50–150 ng/mL of DR5 Fc chimera prior to combined treatment with AC and TRAIL. DR5 Fc chimeric proteins showing a high affinity for DR5 will bind to DR5 instead of TRAIL and can be utilized in studies as DR5 inhibitors to identify receptor bioactivity. Treatment with DR5 Fc chimera markedly reduced AC and TRAIL-induced cell death (Fig. 5B) as well as the apoptotic cell population (Fig. 5C). In addition, DR5 inhibitor pretreatment remarkably blocked expression of AC and TRAIL-induced pro-apoptotic proteins, such as CHOP, DR5, p53, cleaved PARP, cytosolic AIF, Bax, and caspases-9, -8, and -3, whereas anti-apoptotic Bcl-2 protein

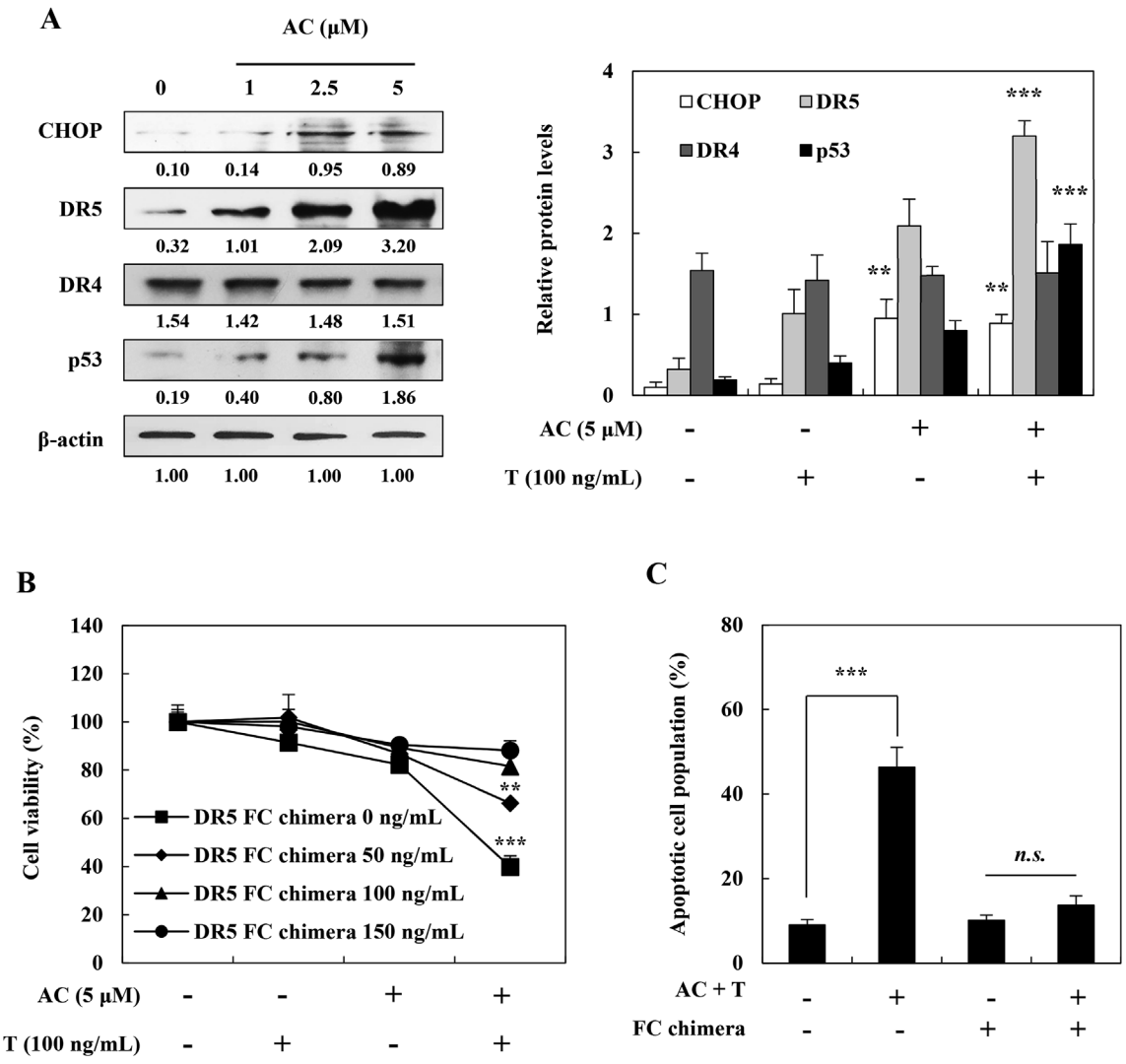

Fig. 5. AC sensitizes TRAIL-mediated apoptotic cell death through DR5 up-regulation in RC-58T/h/SA#4 cells. (A) After treatment with AC for 24 h, total cell lysates were subjected to detect expression levels of proteins. Expression levels of CHOP, DR5, DR4 and p53 in RC-58T/h/SA#4 cells were analyzed by Western blotting. (B) Cells were pretreated with 0–150 ng/mL of DR5 FC chimera protein for 2 h and then incubated with AC and/or TRAIL for 24 h. Cell viability was determined by SRB assay. (C) Apoptotic cell population was quantified by Annexin V staining assay using Muse cell analyzer. Significant differences were compared with the control at $*p < 0.05$ and $**p < 0.01$ using one-way ANOVA.

expression was elevated by AC and TRAIL co-treatment (Fig. 6). Together, these results indicate that AC-induced DR5 up-regulation is the initiating process of AC and TRAIL-induced synergistic apoptosis.

3.6. Co-treatment with AC and TRAIL suppresses phosphorylation of PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR signaling pathway is a well-known regulator of cell survival, proliferation, and metastasis and is often targeted for chemopreventative agent-induced apoptosis (Duan et al., 2016). Inhibition of AKT and mTOR phosphorylation prevents uncontrolled cell proliferation, allowing these proteins to regulate various factors involved in apoptosis (Chang et al., 2003). To assess whether or not combined treatment with AC and TRAIL inhibits the PI3K/AKT/mTOR signaling pathway, protein expression was measured by Western blot analysis. Co-treatment with AC and TRAIL resulted in down-regulation of PI3K, phosphorylated AKT, and phosphorylated mTOR in RC-58T/h/SA#4 cells (Fig. 7A). In addition, pretreatment with PI3K/AKT inhibitor (LY294002) followed by combined treatment of AC and TRAIL significantly facilitated cell death compared to non-LY294002 treated cells at 12 h and 24 h (Fig. 7B). These results demonstrate the partial involvement of the PI3K/AKT/mTOR signaling pathway in RC-58T/h/SA#4 cell growth inhibition induced by co-treatment with AC and TRAIL.

4. Discussion

In the present study, AC isolated from *F. philippinensis* was screened by testing its synergistic apoptosis activity in combination with TRAIL in primary prostate cancer cells. AC was found to improve TRAIL-mediated apoptosis in TRAIL-resistant RC-58T/h/SA#4 primary prostate cancer cells. In addition, involvement of the DR5 apoptosis pathway in co-treatment with AC and TRAIL was investigated for the first time. Hence, the results of the current study, in which AC sensitized primary prostate cancer cells to TRAIL-induced apoptosis, can provide an effective strategy for primary prostate cancer therapy using a food-derived compound.

Numerous studies have attempted to identify ideal therapeutics that selectively induce cancer cell death without significant cytotoxicity in normal cells. In our screening results, a high concentration of AC (7.5–10 μM) treatment significantly reduced RPWE-1 cell proliferation down to 75.49–43.18% compared to the control group, whereas an

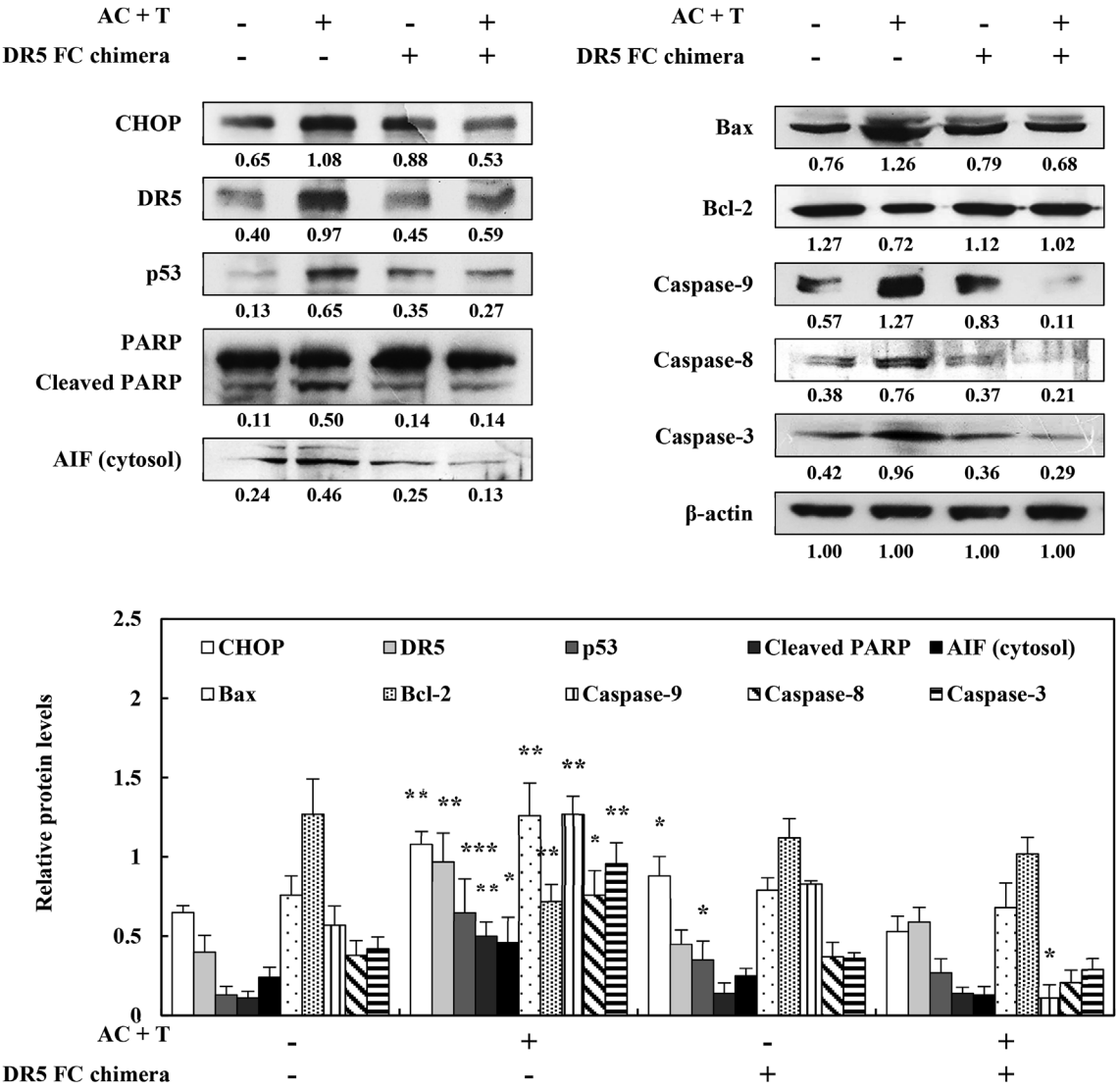

Fig. 6. DR5 down-regulation suppresses apoptotic cell death induced by co-treatment with AC and TRAIL in RC-58T/h/SA#4 cells. Cells were pretreated with 100 ng/mL of DR5 FC chimera protein for 2 h and then incubated with AC and TRAIL for 24 h. After co-treatment with AC and TRAIL for 24 h, total cell lysates were subjected to detect expression levels of proteins. Expression levels of CHOP, DR5, p53, PARP, Bax, Bcl-2, and caspases-9, -8, and -3 in RC-58T/h/SA#4 cells were analyzed by Western blotting. Significant differences were compared with the control at $*p < 0.05$ and $**p < 0.01$ using one-way ANOVA.

equivalent concentration of AC did not affect proliferation of RC-58T/h/SA#4 primary prostate cancer cells. These data suggest that the primary cancer suppressive effect of single AC treatment is accompanied by undesirable cytotoxicity in prostate epithelial cells. Furthermore, some types of prostate cancer cells, including LNCaP, and RC-58T/h/SA#4 cells, are known to be resistant to apoptotic cell death induced by chemotherapeutics (Nesterov et al., 2001; Lee et al., 2014). In addressing tumor species showing resistance to chemotherapeutics, a combination of cancer therapeutics has shown promise for treating cancer (Tang et al., 2016; Jang et al., 2016; Shin and Park, 2013). In the case of advanced cancer, combination chemotherapy resulted in an 18% reduction of death risk, even though toxicity was significantly increased by treatment with poly-chemotherapeutics (Wagner et al., 2010). Furthermore, recent studies reported that a model for synergistic induction of apoptosis by TRAIL and other anti-cancer drugs can activate additional cytotoxic mechanisms against normal human cells, such as hepatocytes, lymphocytes and osteoblasts (Newsom-Davis et al., 2009; Meurette et al., 2006). Interestingly, combined treatment with non-toxic doses of AC (5 μM) and TRAIL (100 ng/mL) significantly induced apoptotic cell death in TRAIL-resistant primary prostate cancer cells without normal cell cytotoxicity. Therefore, drug-sensitizing natural compounds with low cytotoxicity in normal cells are believed to be promising candidates for effectively overcoming various cancer species.

TRAIL is known to promote apoptosis in cancer cells by binding to two death receptors, DR4 and DR5, which are mainly expressed on the surface of cancer cells but not normal cells. However, there is also evidence that some cancer cells escape TRAIL-induced apoptosis via down-regulation of DR4 and/or DR5 on the cell surface (Zhang and Zhang, 2008). Thus, DR5 overexpression is expected to up-regulate apoptosis in cancer cells through effective binding with TRAIL. In this study, AC moderately up-regulated DR5, CHOP, and p53 in RC-58T/h/SA#4 cells, whereas expression of DR4 protein was not affected by AC treatment. Furthermore, treatment with DR5-specific FC chimera protein significantly inhibited AC and TRAIL-mediated apoptotic cell death. Similar to our results, ursolic acid was shown to up-regulate expression of p53, DR5, and CHOP, resulting in TRAIL-induced apoptosis in metastatic prostate cancer cells (Shin and Park, 2013). In our previous study, we also reported that isoegomaketone sensitized TRAIL-resistant primary prostate cancer cells to apoptosis via up-regulation of p53, CHOP, and DR5 (Lee et al., 2014). Since CHOP and p53 have been reported to be involved in transcription of DR5 (Sessler et al., 2013; Lin

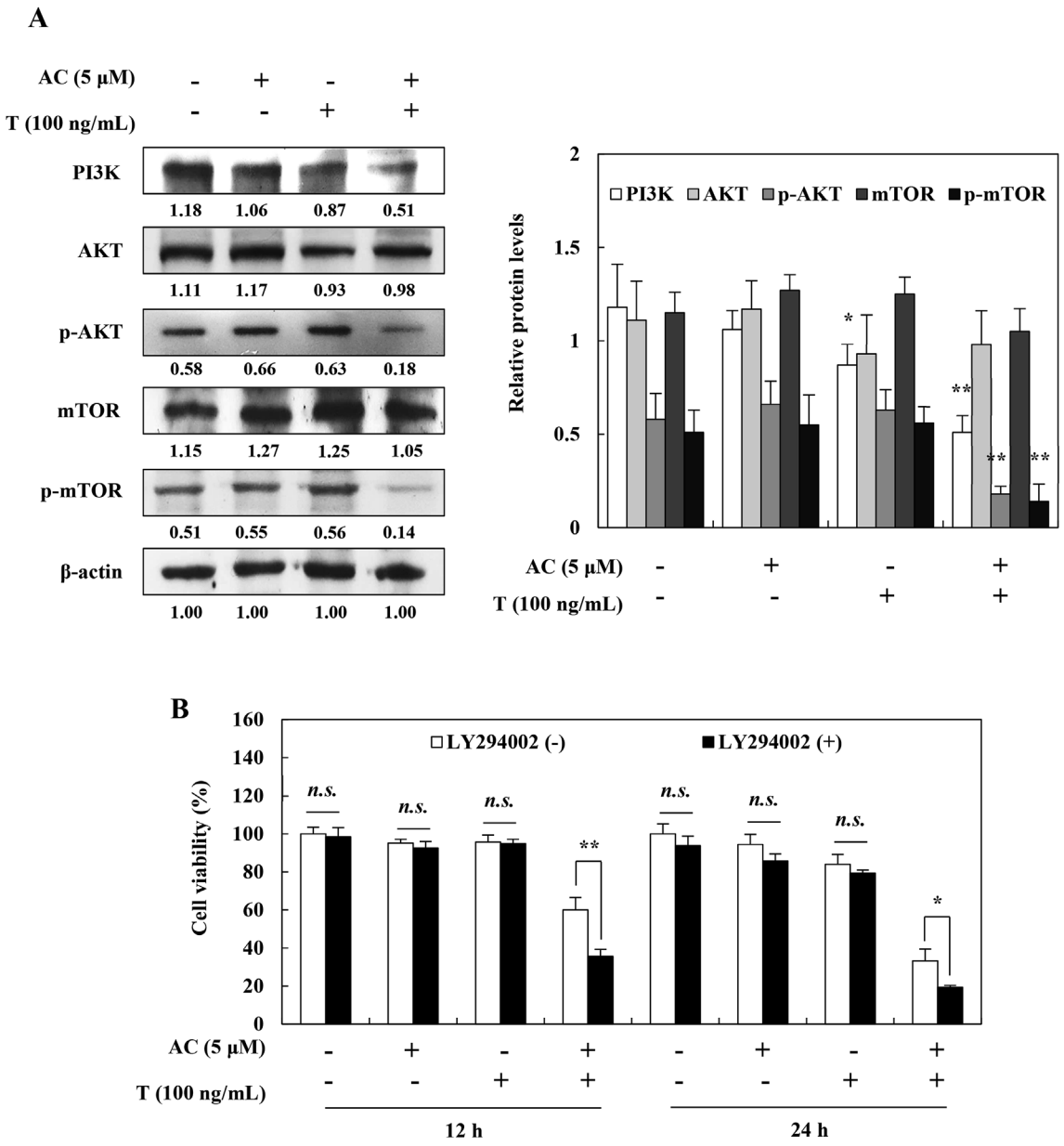

Fig. 7. Combined treatment with AC and TRAIL inhibits the PI3K/AKT/mTOR pathway in RC-58T/h/SA#4 cells. (A) After treatment with AC and/or TRAIL for 12 h, total cell lysates were subjected to detect expression levels of proteins. Expression levels of PI3K, AKT, p-AKT, mTOR, and p-mTOR in RC-58T/h/SA#4 cells were analyzed by Western blotting. (B) Cells were pretreated with 10 μM LY294002, PI3K and AKT inhibitor, for 2 h and then incubated with AC and/or TRAIL for 24 h. Cell viability was evaluated by SRB assay. Significant differences were compared with the control at $*p < 0.05$ and $**p < 0.01$ using one-way ANOVA.

et al., 2011; Yamaguchi and Wang, 2004), our results show that CHOP- and p53-mediated DR5 up-regulation contributed to the TRAIL-sensitizing effect of AC in TRAIL-resistant primary prostate cancer cells.

Apoptosis, a type of programmed cell death, is initiated by two canonical pathways: (1) extrinsic and (2) intrinsic pathways (Elmore et al., 2007). Binding of cancer necrosis factor (TNF)-α, FAS ligand and TRAIL to their receptors triggers the extrinsic apoptosis pathway activating adaptor molecules and caspases. On the other hand, the intrinsic pathway is usually initiated by response to DNA damage, hypoxia, and oncogenes and involves p53, PUMA, Bax, and p21 (Hofseth et al., 2004; Hengartner, 2000). In this study, we provide convincing evidence that AC significantly enhanced TRAIL-induced apoptosis through activation of extrinsic pathway proteins, including DR5, and caspases-8, -10 and −3. Our results also reveal that the apoptotic molecular mechanism induced by co-treatment with AC and TRAIL regulated intrinsic apoptotic pathway proteins such as AIF, Endo G, cytochrome *c*, Bax, Bcl-2, cleaved-bid, and cleaved-PARP. Release of AIF, Endo G, and cytochrome *c* from mitochondria into the cytosol is involved in both the extrinsic and intrinsic apoptotic pathways (Elmore et al., 2007). In addition, natural compounds and TRAIL have been reported to synergistically suppress TRAIL-resistant tumors through the extrinsic and intrinsic apoptosis signaling pathways (Zhang et al., 2016; Jang et al., 2016). Therefore, AC can increase sensitivity to TRAIL-induced apoptosis via a synergistic combination of the extrinsic and intrinsic apoptosis signaling pathways.

The PI3K/AKT pathway is a prototypic survival signaling pathway that can phosphorylate many downstream substrates such as mammalian target of rapamycin (mTOR), NF-κB, Raf, and ASK1 related with proliferation, growth, survival, and metabolism (Hay, 2005). Once PI3K/AKT/mTOR are activated, they directly and/or indirectly regulate many other proteins, including Bax, MDM2, Bad, and FOXO, which are closely associated with drug resistance (Burris HA 3rd, 2013).

Accumulative evidence has shown that several cancer cells are resistant to TRAIL due to high levels of constitutively active AKT and deficiency of death receptor expression (Nesterov et al., 2001; Xu et al., 2010). In our previous study, AC was reported to suppress the PI3K/AKT/mTOR signaling pathways in LNCaP prostate cancer cells (Cho et al., 2018a) and human umbilical vein endothelial cells (HUVECs) (Cho et al., 2018b) Consistent with these studies, co-treatment with AC and TRAIL also suppressed PI3K, phosphorylation of AKT, and phosphorylation of mTOR in RC-58T/h/SA#4, and pretreatment of LY294002 (PI3K and AKT inhibitor) significantly facilitate AC and TRAIL-induced cell death. These results suggest that combined treatment with AC and TRAIL may inhibit the PI3K/AKT/mTOR pathway to facilitate TRAIL-induced proliferation suppressive activity in primary prostate cancer cells.

In conclusion, this study is the first to demonstrate that AC sensitizes primary prostate cancer cells to TRAIL by triggering up-regulation of DR5, leading to caspase-dependent and -independent TRAIL-mediated apoptosis. The present findings suggest that AC may activate the death ligand pathway to treat TRAIL resistance in primary prostate cancer cells without impairing the cancer selectivity of TRAIL.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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