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RESEARCH ARTICLE

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Angelicin potentiates TRAIL-induced apoptosis in renal carcinoma Caki cells through activation of caspase 3 and down-regulation of c-FLIP expression

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Abstract

Angelicin is a furocoumarin derived from *Psoralea corylifolia* L. fruit that has anti-inflammatory and anti-tumor activity. In the present study, the effect of angelicin in enhancing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptotic cell death was studied in Caki (renal carcinoma) cells. Angelicin alone and TRAIL alone had no effect on apoptosis, but in combination these compounds markedly induced apoptosis in the cancer cell lines while not inducing apoptosis in normal cells. The combination treatment induced accumulation of the sub-G1 population, DNA fragmentation, and activated caspase 3 activity in Caki cells, induced down-regulation of c-FLIP expression post-translationally, and over-expression of c-FLIP markedly blocked apoptosis induced by combined treatment with angelicin plus TRAIL. This study provides evidence that angelicin might be a TRAIL sensitizer.

KEYWORDS

angelicin, apoptosis, caspase, c-FLIP, TRAIL

1 | INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can induce apoptosis in a wide variety of cancer cells but not in most normal cells (Pan et al., 1997; Sheridan et al., 1997). However, treatment with TRAIL may be insufficient for cancer therapy because some cancer cells are resistant to TRAIL-induced apoptosis (Ganten et al., 2005; Hernandez, Smith, Wang, Wang, & Evers, 2000). TRAIL-resistant cancer cells can be sensitized by chemopreventive agents, suggesting that combined treatment with TRAIL sensitizers may represent an approach to overcome TRAIL resistance.

Angelicin, a furocoumarin found in *Psoralea corylifolia* L. fruit, is a traditional Chinese medicine that has anti-inflammatory and anti-viral

Abbreviations: ATF4, Activating transcription factor 4; CHOP, CCAAT-enhancer-binding protein homologous protein; PARP, poly (ADP-ribose) polymerase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

effects (Cho et al., 2013; Liu et al., 2013; Wei et al., 2016). Angelicin can induce apoptosis in neuroblastoma cells via the intrinsic caspase-mediated pathway and modulation of apoptosis-related genes (Rahman, Kim, Yang, & Huh, 2012). Angelicin suppresses proliferation of human lung carcinoma cells by promoting G2/M arrest and induction of apoptosis (Li et al., 2016). However, whether angelicin has sensitizing effects against TRAIL-mediated apoptotic cell death is unknown.

In the present study, angelicin was evaluated for its ability to enhance TRAIL-induced apoptotic cell death in cancer cells.

2 | MATERIALS AND METHODS

2.1 | Cell cultures and materials

Human renal carcinoma cells (Caki) were obtained from the American Type Culture Collection (Manassas, VA). Normal human skin fibroblasts (HSFs) cells were purchased from Korea Cell Line Bank (Seoul, Korea). The culture medium used in these experiments was Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 20 mM HEPES buffer, and 100 μg/mL gentamicin. Recombinant human TRAIL and z-VAD-fmk were purchased from R&D Systems (Minneapolis, MN). *N*-Acetyl-L-cysteine (NAC) and trolox were obtained from Calbiochem (San Diego, CA). Anti-Mcl-1, anti-Bcl-2, anti-Bcl-xL, anti-survivin, anti-Bim, anti-cIAP2, anti-CHOP, and anti-ATF4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-3 and anti-Grp78 antibodies were purchased from ENZO (Ann Arbor, MI). Anti-XIAP and anti-c-FLIP antibodies were purchased from BD Biosciences (San Jose, CA) and ALEXIS Corporation (San Diego, CA), respectively. Anti-PARP, anti-cleaved caspase-3, and anti-DR5 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-actin antibody was obtained from Sigma–Aldrich (St. Louis, MO). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2 | Flow cytometry analysis

For flow cytometry, the cells were resuspended in 100 μL of phosphate-buffered saline (PBS), and 200 μL of 95% ethanol was added while the cells were vortexed. The cells were then incubated at 4°C for 1 hr, washed with PBS, resuspended in 250 μL of 1.12% sodium citrate buffer (pH 8.4) with 12.5 μg of RNase and incubated for an additional 30 min at 37°C. Cellular DNA was stained by adding 250 μL of a propidium iodide solution (50 μg/mL) to the cells for 30 min at room temperature. The stained cells were analyzed by fluorescent-activated cell sorting on a FACS flow cytometer to determine the relative DNA content, which was based on the red fluorescence intensity.

2.3 | Western blot analysis

Cells were washed with cold PBS and lysed on ice in 50 μL of lysis buffer (50 mM Tris-HCl, 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pH 7.5) (Jo and Shin 2017; Park, Kwon, & Chun, 2017). Lysates were centrifuged at 10,000*g* (15 min at 4°C), and the supernatant fractions collected. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilon-P membrane. Specific proteins were detected using an enhanced chemiluminescence (ECL) Western blot kit according to the manufacturer's instructions.

2.4 | 4′,6′-Diamidino-2-phenylindole staining for nuclei condensation and fragmentation

To examine cellular nuclei, cells were fixed with 1% paraformaldehyde on glass slides for 30 min at room temperature. After fixation, cells were washed with PBS and 4′,6′-diamidino-2-phenylindole (DAPI, 300 nM; Roche, Mannheim, Germany) was added to the fixed cells for 5 min. After the nuclei were stained, cells were examined by fluorescence microscopy.

2.5 | Cell death assessment by DNA fragmentation assay

The cell death detection ELISA plus kit (Boehringer Mannheim, Indianapolis, IN) was used for assessing apoptotic activity by detecting fragmented DNA in cells treated with TRAIL, angelicin, and their combination. Briefly, each culture plate was centrifuged for 10 min at 200 g, the supernatant removed, and the pellet lysed for 30 min. After recentrifuging the plate at 200 g for 10 min, the supernatant that contained the cytoplasmic histone-associated DNA fragments was collected and incubated with an immobilized anti-histone antibody. The reaction products were incubated with a peroxidase substrate for 5 min and measured by spectrophotometry at 405 and 490 nm (reference wavelength) with a microplate reader. Signals in the wells containing the substrate alone were subtracted as the background.

2.6 | DEVDas (Asp-Glu-Val-Asp-ase) activity assay

To evaluate DEVDase activity, cell lysates were prepared after their respective treatments with TRAIL in the presence or absence of angelicin. Assays were performed in 96-well microtiter plates by incubating 20 μg of cell lysates in 100 μL of reaction buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10% glycerol) containing a caspase substrate [Asp-Glu-Val-Asp-chromophore-*p*-nitroanilide (DEVD-pNA)] at 5 μM. Lysates were incubated at 37°C for 2 h and the absorbance at 405 nm measured spectrophotometrically.

2.7 | c-FLIP constructs and stable cells

The human c-FLIP expression vector was constructed, as described previously (Kim et al., 2008) and Caki cells were transfected in a stable manner with the pcDNA 3.1-c-FLIP plasmid using Lipofectamine as described by the manufacturer (Invitrogen, Carlsbad, CA). After 48 hr of incubation, transfected cells were selected in cell culture medium containing 700 μg/mL G418 (Invitrogen, Carlsbad, CA). After 2 or 3 weeks, single independent clones were randomly isolated, and each individual clone plated separately. After clonal expansion, cells from

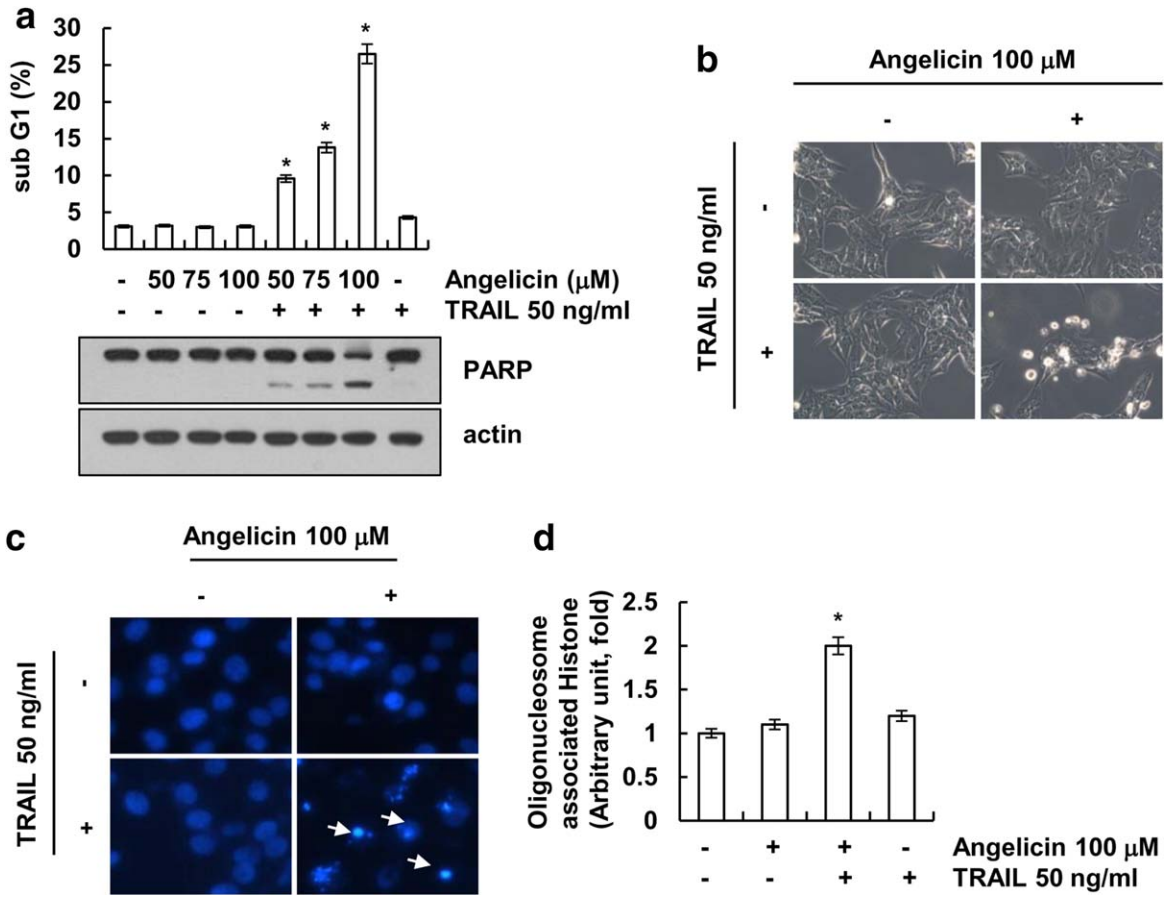

FIGURE 1 Angelicin sensitizes TRAIL-induced apoptosis in Caki cells. (a) Caki cells were treated with 50 ng/mL TRAIL in the presence or absence of the indicated concentrations with angelicin for 24 hr. Apoptosis was analyzed as a sub-G1 population by flow cytometer. The protein levels of PARP and actin were determined by Western blotting. The level of actin was used as a loading control. (b-d) Caki cells were treated with 50 ng/mL TRAIL in the presence or absence of 100 μM angelicin for 24 hr. The cell morphology was examined using interference light microscopy (b). The condensation and fragmentation of the nuclei were detected by DAPI (c). The DNA fragmentation detection kit determined the fragmented DNA (d). The values in (a,d) represent the mean ± SD from three independent samples. $^*p < .01$ compared to the control. [Color figure can be viewed at wileyonlinelibrary.com]

each independent clone were tested for c-FLIP expression by immunoblotting and used in this study.

2.8 | Reverse transcription polymerase chain reaction

Total RNA was isolated using TriZol reagent (Life Technologies; Gaithersburg, MD). cDNA was prepared using M-MLV reverse transcriptase (Gibco-BRL; Gaithersburg, MD) according to the manufacturer's instructions. The following primers were used for the amplification of human c-FLIP and actin: c-FLIP (sense) 5′-CGG ACT ATA GAG TGC TGA TGG-3′ and (antisense) 5′-GAT TAT CAG GCA GAT TCC TAG-3′, and actin (sense) 5′-GGC ATC GTC ACC AAC TGG GAC-3′ and (anti-sense) 5′-CGA TTT CCC GCT CGG CCG TGG-3′. The PCR amplification was performed using the following cycling conditions: 94°C for 3 min followed by 17 (actin) or 23 cycles (c-FLIP) of 94°C for 45 s, 58°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. The amplified products were separated by electrophoresis on a 1.5% agarose gel and detected under UV light.

2.9 | Statistical analysis

Data were analyzed using a one-way ANOVA and post-hoc comparisons (Student–Newman–Keuls) using the Statistical Package for Social Sciences 22.0 software (SPSS Inc., Chicago, IL).

3 | RESULTS

3.1 | Effect of angelicin on TRAIL-induced apoptotic cell death in Caki cells

We investigated whether angelicin could enhance TRAIL-induced apoptotic cell death in human renal carcinoma Caki cells. Cells were treated with angelicin alone (50, 75, 100 μM), TRAIL alone (50 ng/mL), and co-treated with angelicin and TRAIL. Apoptotic cell death was assayed by flow cytometry analysis and Western blotting. Angelicin or TRAIL alone did not induce apoptotic cell death. However, the combination markedly induced accumulation of a sub-G1 cell population and cleavage of poly (ADP-ribose) polymerase (PARP) (Figure 1a) producing apoptotic morphology, e.g., apoptotic

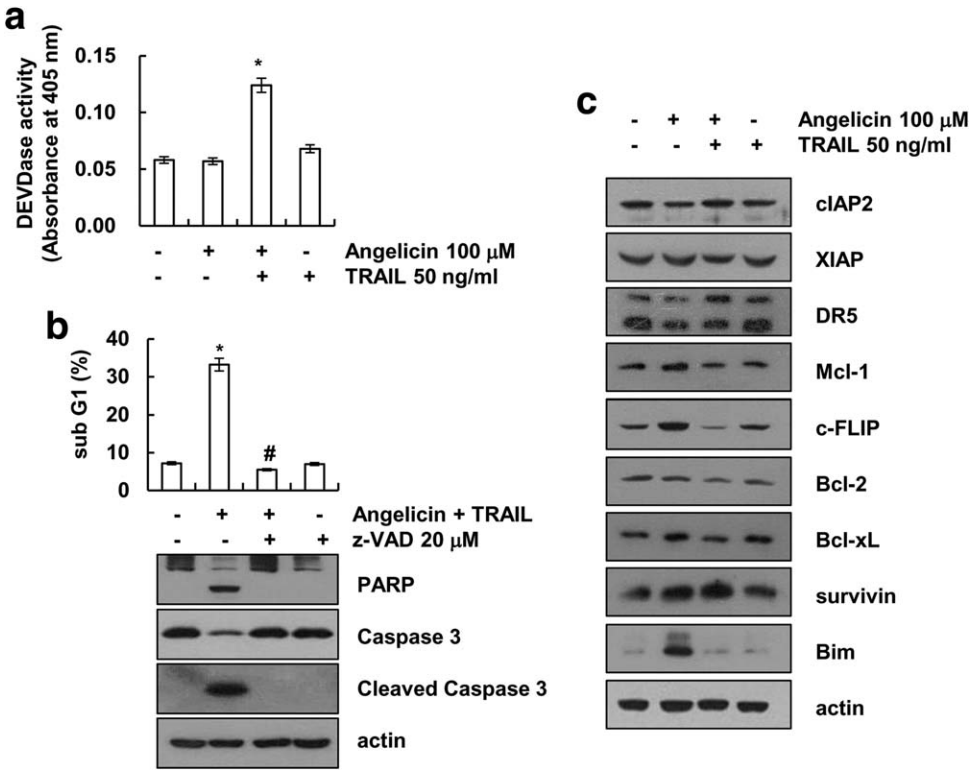

FIGURE 2 Combined treatment with angelicin plus TRAIL induces caspase-mediated apoptosis in Caki cells. (a) Caki cells were treated with 50 ng/mL TRAIL in the presence or absence of the indicated concentrations with angelicin for 24 hr. Caspase activities were determined with colorimetric assays using caspase-3 DEVDase assay kits. (b) Caki cells were treated with 100 μM angelicin plus 50 ng/mL TRAIL for 24 hr in the presence or absence of 20 μM z-VAD-fmk (z-VAD). The sub-G1 fraction was measured by flow cytometry. The protein expression levels of PARP, caspase-3, cleaved caspase-3, and actin were determined by Western blotting. The level of actin was used as a loading control. (c) Caki cells were treated with 50 ng/mL TRAIL in the presence or absence of 100 μM angelicin for 24 hr. The protein expression levels of cIAP2, XIAP, DR5, Mcl-1, c-FLIP, Bcl-2, Bcl-xL, survivin, Bim, and actin were determined by Western blotting. The level of actin was used as a loading control. $^*p < .01$ compared to the control. $\#p < .01$ compared to the angelicin plus TRAIL

body formation, cell shrinkage, and cell detachment on the plate (Figure 1b). In addition, we assessed nuclear condensation and DNA fragmentation, which is the hallmark of apoptosis. The combined treatment with angelicin plus TRAIL induced nuclear condensation and DNA fragmentation while each compound alone was without effect (Figure 1c,d).

3.2 | Angelicin-induced apoptosis is caspase-dependent in Caki cells

We then examined the expression and activity of caspase-3 in Caki cells to determine whether the angelicin (100 μM) plus TRAIL (50 ng/mL)-induced apoptosis was associated with the activation of caspase-3. Treatment with the angelicin/TRAIL combination increased caspase-3 activity (Figure 2a). To confirm the roles of caspase activation in the angelicin plus TRAIL-induced apoptosis, we assessed whether a pan-caspase inhibitor, z-VAD-fmk, could attenuate apoptosis. As shown in Figure 2b, pre-treatment with z-VAD-fmk attenuated inhibition of sub-G1 population and cleavage of PARP and caspase-3. Next, we examined the effect of the combination treatment on the expression levels of apoptosis-related proteins in Caki cells. The combination treatment induced down-regulation of c-FLIP expression, whereas expression of apoptosis-related proteins (cIAP1, XIAP, DR5, Mcl-1, Bcl-2, Bcl-xL, and survivin) was unchanged (Figure 2c). Interestingly, angelicin alone induced Bim expression. However, the combined treatment did not, suggesting that TRAIL attenuated the effect of angelicin and that expression of c-FLIP expression plays a critical role in the combination treatment.

3.3 | Combined treatment of angelicin and TRAIL induces down-regulation of c-FLIP expression

To investigate the potential mechanisms underlying the down-regulation of c-FLIP protein in angelicin plus TRAIL-induced apoptosis, we examined c-FLIP mRNA levels, expression of which was unchanged (Figure 3a). We then examined whether combined treatment with angelicin plus TRAIL regulated protein stability using an inhibitor of de novo protein synthesis, cycloheximide (CHX; 20 μg/mL). The expression levels of c-FLIP were gradually decreased within 6 hr in cells treated with CHX alone. However, combined treatment with angelicin plus TRAIL more rapidly reduced c-FLIP protein expression (Figure 3b). These results suggested that the angelicin/TRAIL combination induced down-regulation of c-FLIP expression at the post-translational level. To further investigate the role of the down-regulation of c-FLIP protein in the combined treatment-induced apoptosis, c-FLIP-overexpressing cells

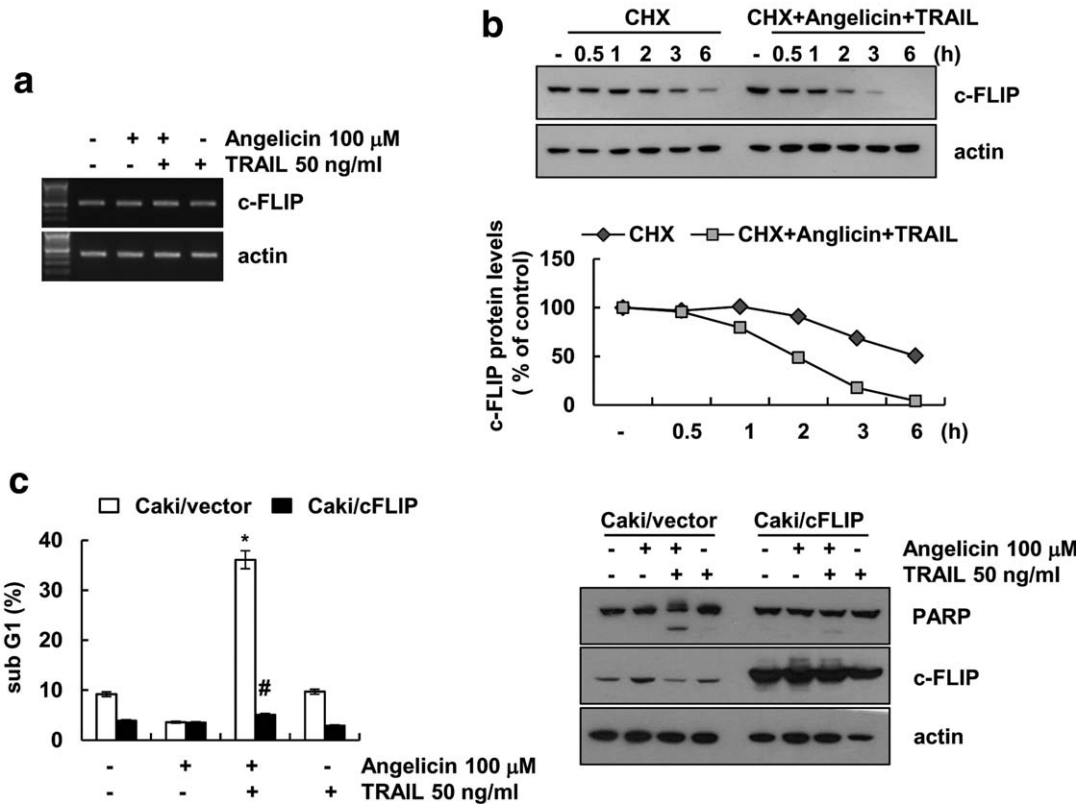

FIGURE 3 Down-regulation of c-FLIP is associated with the induction of combined treatment-induced apoptosis. (a) Caki cells were treated with 50 ng/mL TRAIL in the presence or absence of 100 μM angelicin for 24 hr. c-FLIP mRNA expression levels were determined by **reverse transcription polymerase chain reaction**. The level of actin was used as a loading control. (b) Caki cells were treated with or without angelicin plus TRAIL in the presence of CHX (20 μg/mL) for the indicated time periods. The c-FLIP and actin protein levels were determined by Western blotting. The band intensity of the c-FLIP protein was measured using ImageJ (public domain JAVA image-processing program; http://rsb.info.nih.gov/ij). (c) Vector cells (Caki/vector) and c-FLIP overexpressed cells (Caki/c-FLIP) were treated with 50 ng/mL TRAIL in the presence or absence of 100 μM angelicin for 24 hr. The sub-G1 fraction was measured by flow cytometry. The protein expression levels of PARP, c-FLIP, and actin were determined by Western blotting. The level of actin was used as a loading control. The values in (c) represent the mean ± SD from three independent samples. $^{*}p < 0.01$ compared to the control. $\#p < 0.01$ compared to the angelicin plus TRAIL-treated Caki/vector

were used. Ectopic expression of c-FLIP inhibited combined treatment-induced apoptosis and PARP cleavage (Figure 3c) suggesting that the down-regulation of c-FLIP expression plays a critical role in the apoptosis induced by the combined treatment.

3.4 | Angelicin-mediated TRAIL sensitization is not associated with induction of endoplasmic reticulum stress and ROS signaling pathway

Induction of endoplasmic reticulum (ER) stress is linked to TRAIL-mediated apoptosis (Jiang et al. 2007; Martin-Perez, Niwa, & Lopez-Rivas, 2012). Therefore, we investigated whether angelicin can induce ER stress. Angelicin induced Grp78 protein in a dose-dependent manner (Figure 4a). However, the key transcriptional factors of ER stress (ATF4 and CHOP) were unaltered in response to angelicin (Figure 4a). In addition, we investigated whether reactive oxygen species (ROS) were involved in angelicin-mediated TRAIL sensitization. As shown in Figure 4b, the ROS scavengers (NAC, GEE (glutathione ethyl ester), and Trolox) had no effect on angelicin plus TRAIL-induced apoptosis. Angelicin-mediated TRAIL sensitization thus appears independent of ER stress and ROS signaling.

3.5 | Combined treatment with angelicin and TRAIL induces apoptosis in other cancer cells, but not normal cells

Next, we examined whether combined treatment could enhance apoptosis in other types of cancer cells and normal cells. The combination of angelicin plus TRAIL enhanced the sub-G1 population and PARP cleavage in Sk-hep1 (hepatocellular carcinoma cells; Figure 5a) and MDA-MB-361 cells (breast cancer cells; Figure 5b). In contrast, angelicin plus TRAIL had no effect on morphological changes and apoptotic cell death in HSF cells and mouse renal tubular epithelial (TCMK-1) cells (Figure 5c). These data indicate that angelicin enhances TRAIL-mediated apoptotic cell death in cancer cells, but not in normal cells.

4 | DISCUSSION

In the present article, we demonstrated the mechanisms underlying combined treatment with angelicin plus TRAIL-induced apoptotic cell death in human renal carcinoma Caki cells. Combined treatment induced down-regulation of c-FLIP through modulation of protein

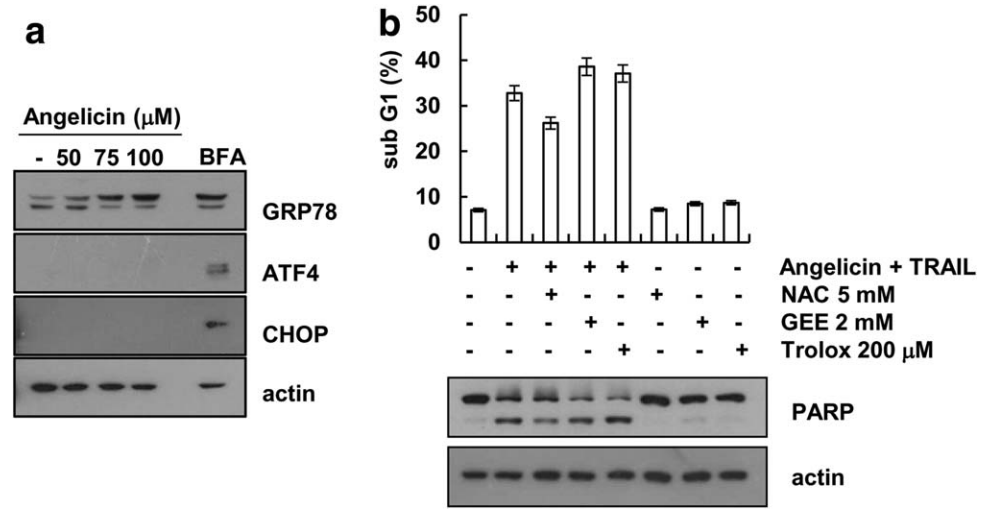

FIGURE 4 TRAIL sensitization by angelicin is independent of ER stress and ROS signaling pathway. (a) Caki cells were treated with the indicated concentrations of angelicin or 2 μM brefeldin A (positive control) for 6 hr. The protein expression levels of Grp78, ATF4, CHOP, and actin were determined by Western blotting. The level of actin was used as a loading control. (b) Caki cells were pretreated with 5 mM NAC, 2 mM GEE, and 200 μM Trolox for 30 min, and then added with 50 ng/mL TRAIL plus 100 μM angelicin for 24 hr. The sub-G1 fraction was measured by flow cytometry. The protein expression levels of PARP and actin were determined by Western blotting. The level of actin was used as a loading control. The values in (b) represent the mean ± SD from three independent samples

stability and markedly induced apoptotic cell death in cancer cells without inducing apoptosis in normal cells. These data suggest that angelicin may be an effective TRAIL sensitizer and that combined treatment could be an effective anti-cancer strategy.

Recently, Rahman et al. (2002) reported that angelicin induced apoptosis in human SH-SY5Y neuroblastoma cells via down-regulation of anti-apoptotic proteins (Bcl-1, Bcl-xL, and Mcl-1) and a caspase-dependent pathway. They showed cellular cytotoxicity with an IC_{50}

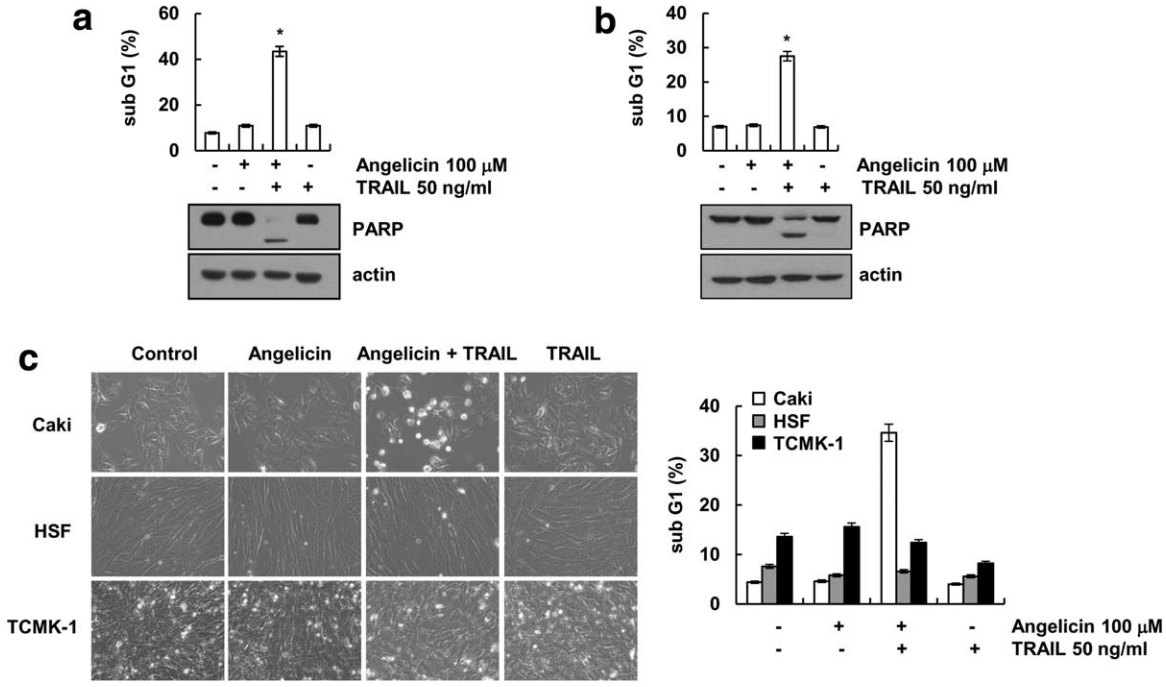

FIGURE 5 The effects of combined treatment with angelicin and TRAIL on apoptosis in other carcinoma and normal cells. (a) Sk-hep1 (hepatocellular carcinoma) and (b) MDA-MB-361 (breast carcinoma) cells were treated with 50 ng/mL TRAIL in the presence or absence of 100 μM angelicin for 24 hr. The level of apoptosis was measured by the sub-G1 fraction using flow cytometry. The protein expression levels of PARP and actin were determined by Western blotting. Actin was used as a loading control. (c) Caki, HSF, and normal mouse kidney (TCMK-1) cells were treated with 50 ng/mL TRAIL in the presence or absence of 100 μM angelicin for 24 hr. Cell morphology was examined using interference light microscopy. The level of apoptosis was measured by the sub-G1 fraction using flow cytometry. The values in (a, b, and c) represent the mean ± SD from three independent samples. *$p < .01$ compared to the control

value of 49.56 μM at 48 hr of incubation. In our study, a high concentration of angelicin (100 μM) did not induce apoptotic cell death at 24 hr in Caki cells. However, combined treatment angelicin (50–100 μM) and TRAIL (50 ng/mL) caused apoptotic cell death in Caki, Sk-hep1, and MDA-MB-361 cells, but not normal cells. Li et al. (2016) also reported that angelicin inhibited growth and migration via regulation of JNK and ERK pathways. In our study, one mechanism of angelicin-mediated TRAIL sensitization is down-regulation of c-FLIP expression. Our group also reported that combined treatment with thioridazine and curcumin induced down-regulation of c-FLIP through up-regulation of ROS-mediated PSMA5 expression (Seo, Kim, et al., 2017). In addition, inhibition of cathepsin S induced down-regulation of c-FLIP expression via up-regulation of Cbl expression in a ROS dependent manner (Seo, Min, et al., 2017). ROS plays a key role in post-translational regulation of c-FLIP protein through multiple mechanisms (Chanvorachote et al., 2005; Wilkie-Grantham, Matsuzawa, & Reed, 2013). However, the ROS scavengers (NAC, GEE, and Trolox) failed to inhibit the effects of the combined treatment of angelicin plus TRAIL-induced apoptosis (Figure 4b). Further experiments are required to identify the mechanism of c-FLIP down-regulation in the combination treatment with angelicin plus TRAIL. Ectopic expression of c-FLIP markedly blocked combined treatment with angelicin plus TRAIL-induced apoptosis. Overexpression of c-FLIP has been observed in several types of human cancer and inhibits cell death receptor-mediated apoptosis (Li et al., 2008; Oyarzo et al., 2006; Ryu, Lee, Chi, Kim, & Park, 2001; Zhou et al., 2004). Collectively, our data showed that angelicin enhanced TRAIL-induced apoptotic cell death in cancer cells, but not in normal cells. Furthermore, we demonstrated that the combination of angelicin plus TRAIL down-regulated c-FLIP expression post-translationally. These data suggest that angelicin might be an efficient sensitizer for TRAIL resistance cancer cells.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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