Suppression of AKT Potentiates Synergistic Cytotoxicity of Apigenin with TRAIL in Anaplastic Thyroid Carcinoma Cells

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Abstract. *Background: We studied the effect of apigenin in combination with tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) on cell survival and the influence of AKT inhibition on the combined effect of apigenin with TRAIL in anaplastic thyroid carcinoma (ATC) cells. Materials and Methods: The human 8505C and CAL62 ATC cell lines were used. Results: Apigenin in combination with TRAIL, compared to apigenin alone, reduced cell viability and Bcl2 protein levels, elevated the percentage of dead cells, as well as the protein levels of cleaved PARP and phospho-ERK1/2. The protein levels of Bcl-xL, Bax, Bid, total ERK1/2, and total and phospho-AKT were unchanged. Administration of wortmannin further reduced cell viability, and elevated the percentage of dead cells, cytotoxic activity and cleaved PARP protein levels. Conclusion: Apigenin synergizes with TRAIL through regulation of Bcl2 family proteins in inducing cytotoxicity, and suppression of AKT potentiates synergistic cytotoxicity of apigenin with TRAIL in ATC cells.*

Anaplastic thyroid carcinoma (ATC) is a highly aggressive malignant tumor arising from the thyroid gland presenting extra-thyroidal invasion and distant metastasis with a very poor prognosis (1). Clinically, the treatment of patients with ATC is challenging because of unresponsiveness to conventional therapies, and thus new therapeutic strategies to improve the outcome of patients are under investigation (1).

Apigenin (4',5,7-trihydroxyflavone), a flavonoid derivative, is plentiful in fruits and vegetables, and has anti-oxidant, anti-inflammatory and anti-carcinogenic properties (2). In cancer cells, apigenin exhibits a potent cytotoxic activity, and thereby leads to reduction of tumor volume and abolishment of distant metastasis (2, 3). In this regard, we recently reported that apigenin induced c-Myc-mediated death of FRO ATC cells, and the $BRAF^{V600E}$ inhibitor PLX4032 augmented apigenin-induced death of ATC cells harboring $BRAF^{V600E}$ (4, 5).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of TNF superfamily, is a ligand of death receptor (DR), and involves cell death through activation of caspases in cancer cells (6-8). While TRAIL results in DR-mediated death of sensitive cancer cells, thyroid cancer cells are relatively resistant to DR-mediated cytotoxicity (9, 10). Although apigenin in combination with TRAIL enhances cytotoxicity induced by each agent in various cancer cells, the combined effect of apigenin with TRAIL on survival of ATC cells has not been evaluated (11-15).

PI3K/AKT signaling modulates various cellular processes including survival, growth, proliferation, differentiation and migration (16). PI3K/AKT signaling is de-regulated, and facilitates survival of cancer cells including ATC cells (16, 17). In this regard, we recently reported that AKT participated in the vascular endothelial growth factor receptor (VEGFR) inhibitor SU5416-induced death, and repression of AKT potentiated the Src family kinase (SFK) inhibitor SU6656-induced caspase-independent death of FRO ATC cells (18, 19). In addition, suppression of AKT multiplied the combined effect of apigenin with PLX4032 in induction of death of ATC cells harboring $BRAF^{V600E}$ (5).

In the present study, we evaluated the effect of apigenin in combination with TRAIL on cell survival, and investigated the influence of inhibition of AKT on the combined effect of apigenin with TRAIL in ATC cells.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and streptomycin/penicillin were obtained from Life Technologies (Gaithersburg, MD, USA). Apigenin was purchased from Sigma (St. Louis, MO, USA), and dissolved in

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Key Words: Anaplastic thyroid carcinoma, apigenin, TRAIL, AKT, Bcl2.

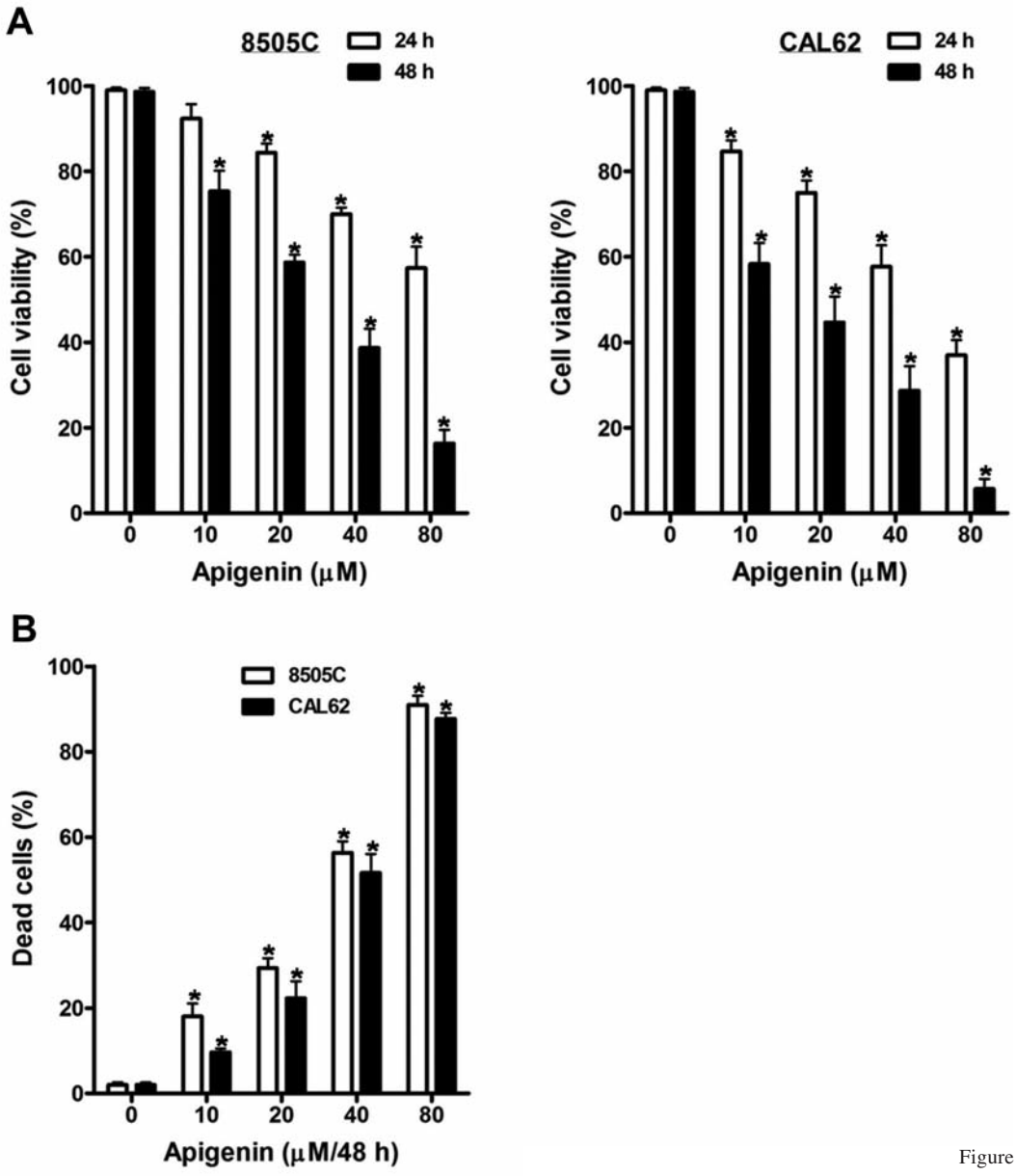

Figure 1. *Continued*

dimethylsulfoxide (DMSO). The final concentration of the vehicle DMSO in the control did not exceed 0.1% in all experiments. TRAIL was obtained from BioVision (Linda, CA, USA). The primary antibodies raised against Bcl-xL, Bcl2, Bax, Bid, cleaved poly (ADP-ribose) polymerase (PARP), and total and phospho-ERK1/2 (Thr402/Tyr404) were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). The primary antibodies raised against total and phospho-AKT (Ser473) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the primary antibody raised against β-actin were from Sigma. All other reagents were purchased from Sigma unless otherwise stated.

Cell culture. For experiments, human ATC cell lines of 8505C and CAL62 cells were used. 8505C and CAL62 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ GmbH, Braunschweig, Germany), and grown in DMEM supplemented with 10% heat-inactivated FBS and 1% streptomycin/penicillin. Cells received fresh medium at regular intervals. Treatments and experiments were performed using cells that were 70% confluent.

Cell viability assay. Cell viability was determined by the CCK-8 Assay Kit (Dojindo laboratories, Kumamoto, Japan). Cells (5×10^3/100 μl) in each well on 96-well plates were incubated overnight, and treated with the drugs for an additional 4 h at 37˚C. Absorbance was measured at 450 nm using a spectrophotometer (Molecular Devices, Palo Alto, CA, USA). All experiments were performed in triplicate.

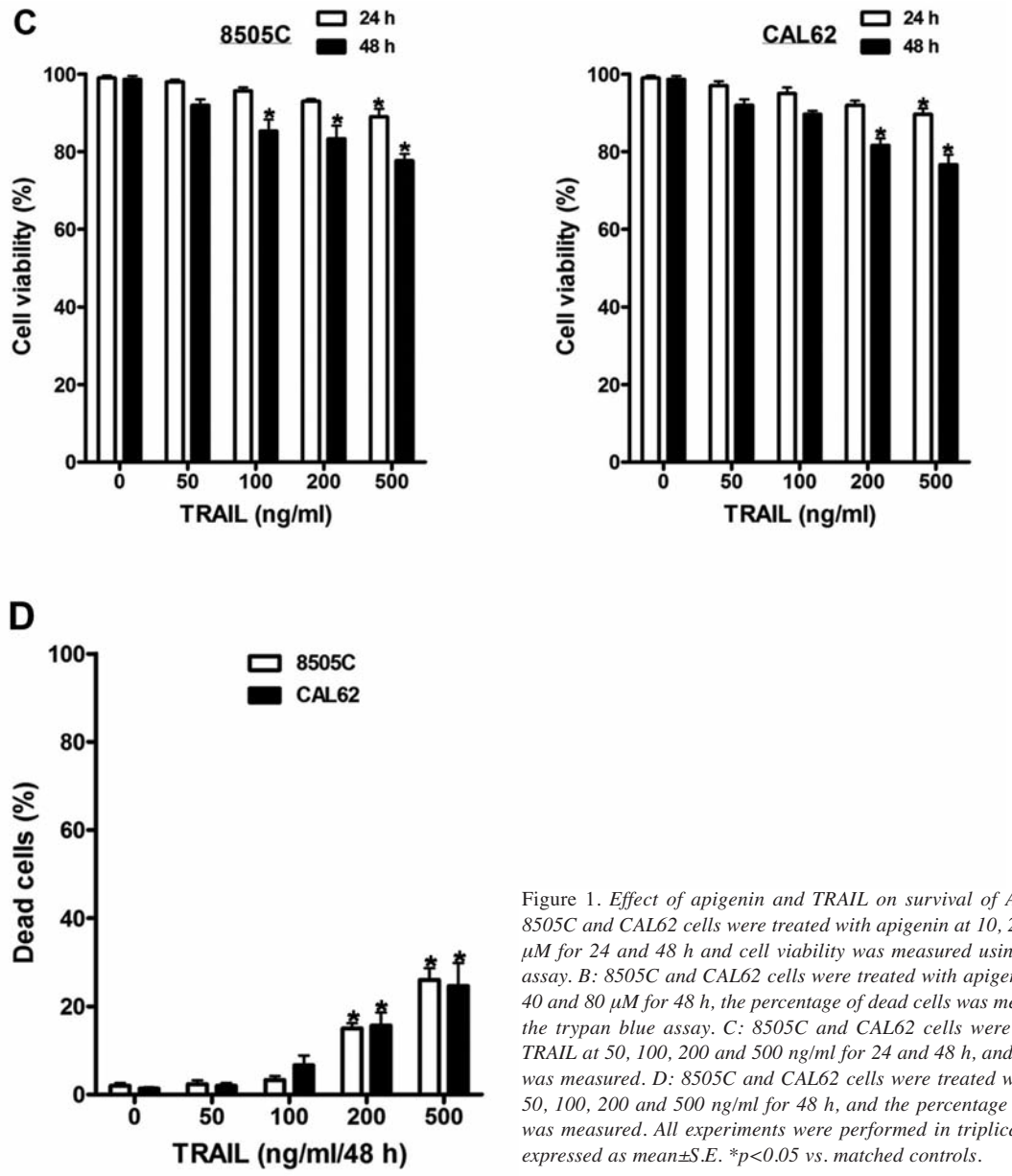

Figure 1. *Effect of apigenin and TRAIL on survival of ATC cells. A: 8505C and CAL62 cells were treated with apigenin at 10, 20, 40 and 80 μM for 24 and 48 h and cell viability was measured using the CCK-8 assay. B: 8505C and CAL62 cells were treated with apigenin at 10, 20, 40 and 80 μM for 48 h, the percentage of dead cells was measured using the trypan blue assay. C: 8505C and CAL62 cells were treated with TRAIL at 50, 100, 200 and 500 ng/ml for 24 and 48 h, and cell viability was measured. D: 8505C and CAL62 cells were treated with TRAIL at 50, 100, 200 and 500 ng/ml for 48 h, and the percentage of dead cells was measured. All experiments were performed in triplicate. Data are expressed as mean±S.E. *p<0.05 vs. matched controls.*

Trypan blue assay. Cells (1×10^4/500 μl) in each well on 12-well plates were incubated, and mixed with trypan blue dye at 37˚C. Stained cells were counted using a hemocytometer. All experiments were performed in triplicate.

Cytotoxicity assay. Cytotoxic activity was measured by the LDH Cytotoxicity Assay Kit (BioVision, Linda, CA, USA). Cells (5×10^3/100 μl) in each well on 96-well plates were incubated, and centrifuged at 250 g for 10 min. Supernatant of 100 μl was transferred in clear 96-well plates. After addition of reaction mixture (2.5 μl Catalyst solution in 112.5 μl Dye solution), cells were incubated for 30 min at room temperature. Absorbance was measured at 495 nm using a spectrophotometer. All experiments were performed in triplicate.

Western blotting. Cells were lysed in RIPA buffer (Sigma) containing 1× cocktail of protease inhibitors and 1 x cocktail of phophatase inhibitors set V (Calbiochem, La Jolla, CA, USA). Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Equivalent amounts of protein (50 μg) were separated by 10% SDS-PAGE, and transferred to Immobilon-P Membrane (Millipore, Bedford, MA, USA). Western blotting was performed using specific primary antibodies and horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies. Bands were detected using ECL Plus Western Blotting Detection System (Thermo Fisher Scientific, Rockford, IL, USA). All reactions were performed in triplicate. The protein levels were quantified by densitometry using ImageJ software (NIH).

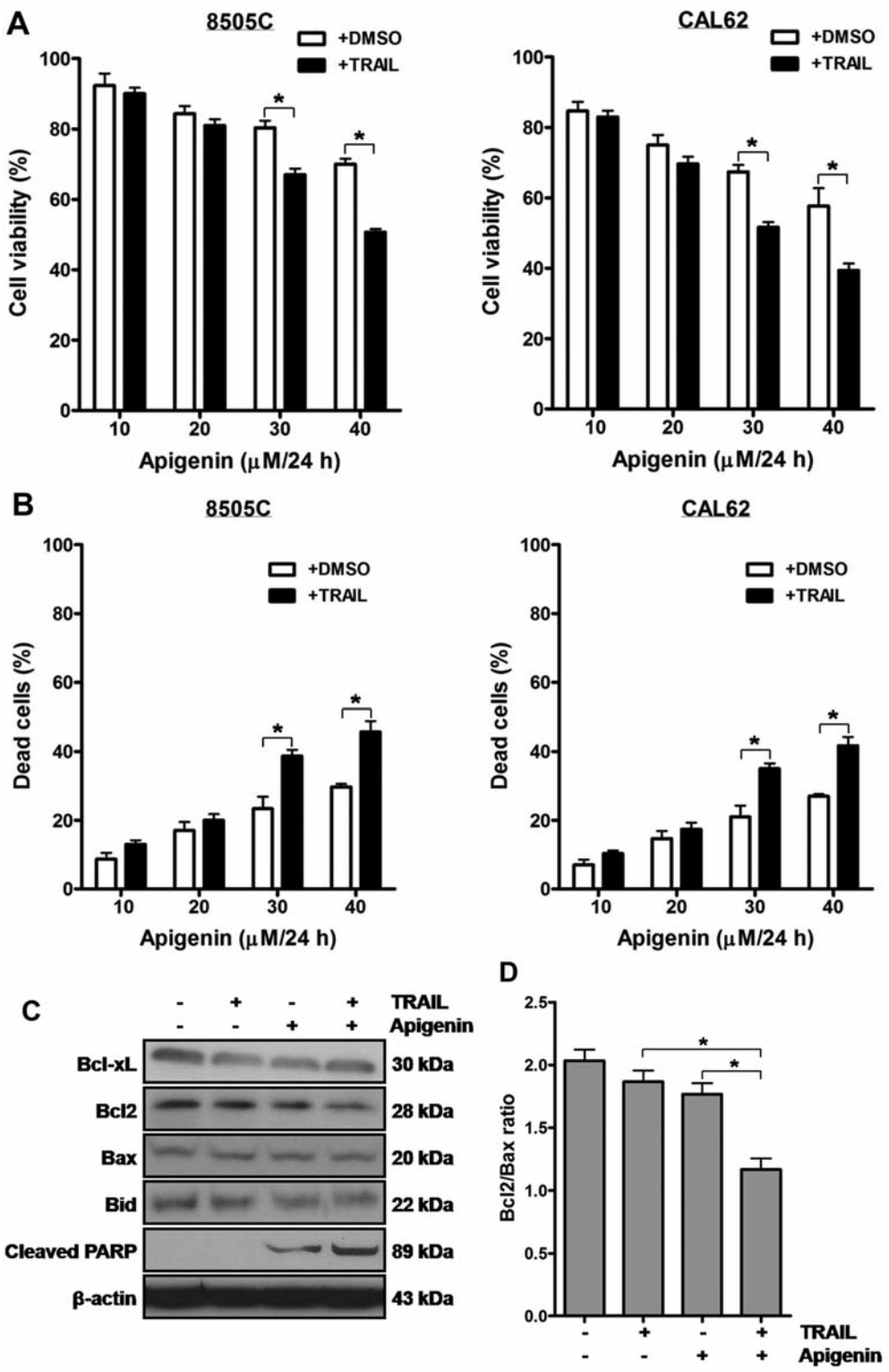

Figure 2. *Influence of apigenin in combination with TRAIL on survival of ATC cells. A and B: 8505C and CAL62 cells were simultaneously treated with apigenin at 10, 20, 30 and 40 μM, and TRAIL at 200 ng/ml for 24 h. Cell viability (A) and the percentage of dead cells (B) were measured. C and D: 8505C cells were treated with apigenin at 40 μM with or without TRAIL at 200 ng/ml for 24 h. The protein levels of Bcl-xL, Bcl2, Bax, Bid and cleaved PARP were measured (C), and the protein levels of Bcl2 and Bax were quantified by densitometry, and Bcl2/Bax ratio was calculated (D). All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean±S.E. *p<0.05.*

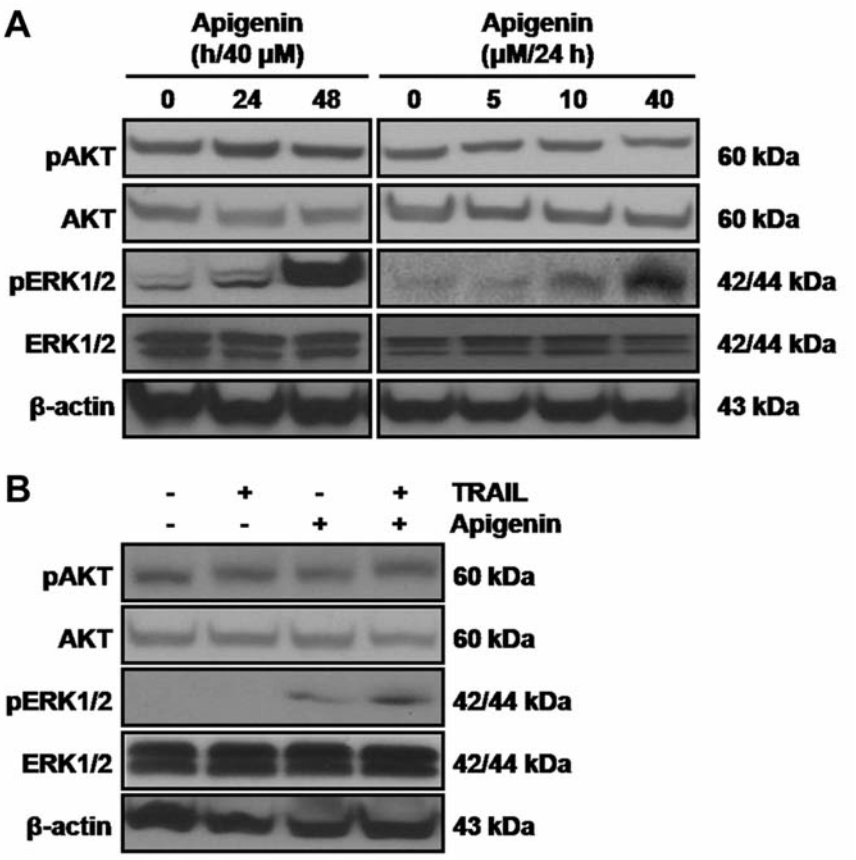

Figure 3. *The involvement of PI3K/AKT and ERK signaling in synergistic cytotoxicity of apigenin with TRAIL in ATC cells. A: 8505C cells were treated with apigenin at 40 μM for 24 and 48 h, and at 5, 10 and 40 μM for 24 h, and the protein levels of total and phospho-AKT, and total and phospho-ERK1/2 were measured. B: 8505C cells were simultaneously treated with apigenin at 40 μM and TRAIL at 200 ng/ml for 24 h, and the protein levels of total and phospho-AKT, and total and phospho-ERK1/2 were measured. All experiments were performed in triplicate. The blots are representative of independent experiments.*

Statistical analysis. All data are expressed as mean±standard error (S.E). Data were analyzed by unpaired Student's *t*-test or ANOVA as appropriate. A *p*-value less than 0.05 was considered to be statistically significant. All analyses were performed using SPSS version 21.0 (SPSS, Chicago, IL, USA).

Results

Apigenin and, to a lesser extent, TRAIL induce death of ATC cells. In order to evaluate the effect of apigenin on cell survival, 8505C and CAL62 cells, were treated with apigenin at 10, 20, 40 and 80 μM for 24 and 48 h and cell viability was measured using the CCK-8 assay (Figure 1A). In addition, cells were treated with apigenin at 10, 20, 40 and 80 μM for 48 h and the percentage of dead cells was measured using the trypan blue assay (Figure 1B). After treatment, cell viability was reduced in a time- and dose-dependent manner, and the percentage of dead cells was elevated in a dose-dependent manner.

To investigate the influence of TRAIL on cell survival, the viability of cells treated with TRAIL at 50, 100, 200 and 500 ng/ml for 24 and 48 h, and the percentage of death of cells treated with TRAIL at 50, 100, 200 and 500 ng/ml for 48 h were measured (Figure 1C and D). The reduction of cell viability and elevation of the percentage of dead cells were concurrently significant only in treatment of TRAIL at 200 and 500 ng/ml for 48 h.

Apigenin synergizes with TRAIL through modulation of Bcl2 family proteins in induction of death of ATC cells. In order to identify the impact of apigenin in combination with TRAIL on cell survival, cells were simultaneously treated with apigenin at 10, 20, 30 and 40 μM (variable doses), and TRAIL at 200 ng/ml (fixed dose) for 24 h (Figure 2A and B). In cells treated with apigenin at 30 and 40 μM, combination of TRAIL diminished cell viability, and enhanced the percentage of dead cells, suggesting that

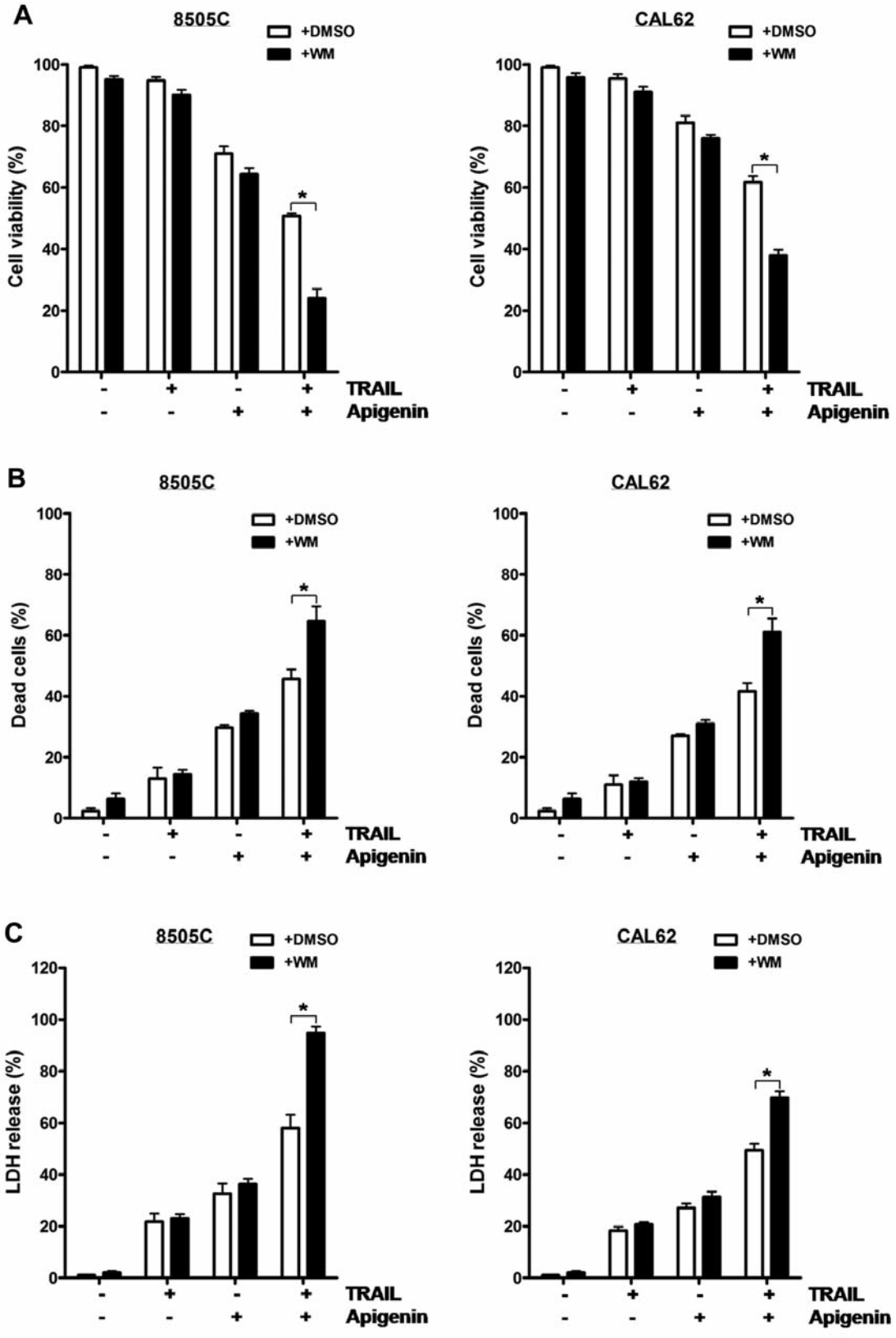

Figure 4. *Continued*

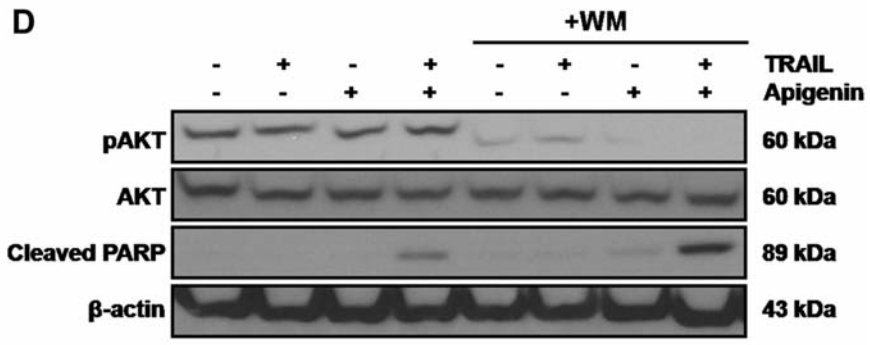

Figure 4. *The impact of suppression of PI3K/AKT signaling on synergistic cytotoxicity of apigenin with TRAIL in ATC cells. A-D: 8505C and CAL62 cells were administered the PI3K inhibitor wortmannin (2 μM, 24 h) before co-treatment of apigenin at 40 μM and TRAIL at 200 ng/ml for 24 h. Cell viability (A), the percentage of dead cells (B), cytotoxic activity using cytotoxicity assay (C), and the protein levels of total and phospho-AKT, and cleaved PARP (D) were measured. All experiments were performed in triplicate. The blots are representative of independent experiments in 8505C cells. Data are expressed as mean±S.E. *p<0.05. WM, wortmannin.*

apigenin has a synergistic activity with TRAIL in inducing cytotoxicity in ATC cells.

When cells were simultaneously treated with apigenin at 40 μM (fixed dose) and TRAIL at 200 ng/ml (fixed dose) for 24 h, cleaved PARP protein levels were enhanced, as depicted in Figure 2C. In cells treated with apigenin and TRAIL, compared to apigenin-alone, Bcl2 protein levels were diminished, while the protein levels of Bcl-xL, Bax and Bid were unchanged. As shown in Figure 2D, Bcl2/Bax ratio was diminished by co-treatment of apigenin and TRAIL, suggesting that the synergism between apigenin and TRAIL is associated with regulation of Bcl2 family proteins in ATC cells.

Synergistic cytotoxicity of apigenin with TRAIL is involved in activation of ERK in ATC cells. To examine the effect of apigenin on PI3K/AKT and ERK signaling, cells were treated with apigenin at 40 μM for 24 and 48 h, and at 5, 10 and 40 μM for 24 h (Figure 3A). As a result of treatment, phospho-ERK1/2 protein levels were multiplied, whereas the protein levels of total and phospho-AKT, and total ERK1/2 were not altered, suggesting that apigenin induced cytotoxicity in ERK in ATC cells.

To explore the influence of apigenin in combination with TRAIL on PI3K/AKT and ERK signaling, when cells were simultaneously treated with apigenin at 40 μM and TRAIL at 200 ng/ml for 24 h, phospho-ERK1/2 protein levels were multiplied, while protein levels of total and phospho-AKT, and total ERK1/2 were unchanged in cells treated with apigenin and TRAIL, compared to apigenin-alone (Figure 3B), suggesting that the synergism between apigenin and TRAIL is relevant to stimulation of ERK in ATC cells.

Inactivation of PI3K/AKT signaling potentiates synergistic cytotoxicity of apigenin with TRAIL in ATC cells. To document the impact of suppression of PI3K/AKT signaling on the synergistic action of apigenin with TRAIL in induction of cell death, cells were administered with the PI3K inhibitor wortmannin (2 μM, 24 h) before co-treatment with apigenin at 40 μM and TRAIL at 200 ng/ml for 24 h. Cell viability (Figure 4A), the percentage of dead cells (Figure 4B), and cytotoxic activity measured using cytotoxicity assay (Figure 4C), and the protein levels of total and phospho-AKT, and cleaved PARP (Figure 4D) were measured. Administration of wortmannin under co-treatment of apigenin and TRAIL further decreased cell viability, and further increased the percentage of dead cells, cytotoxic activity and cleaved PARP protein levels, suggesting that inhibition of PI3K/AKT signaling augments the synergism between apigenin and TRAIL in ATC cells.

Discussion

Apigenin induces cell death involving disintegration of mitochondrial membrane, acceleration of mitochondrial dysfunction and stimulation of proteolytic activity in cancer cells (2). In thyroid cancer cells, apigenin induces c-Myc-mediated death of FRO ATC cells, and inhibits growth of sodium/iodide symporter-transfected FTC133 follicular thyroid carcinoma cells (4, 20). In the present study, apigenin caused death of human 8505C and CAL62 ATC cell lines in a time- and dose-dependent manner. These data are consistent with our previous results, suggesting that apigenin is a candidate as a therapeutic agent in human ATC (4, 5). However, *in vivo* studies to verify the potential for clinical application of apigenin should be undertaken in the future.

Whereas TRAIL leads to DR-mediated death of sensitive cancer cells, thyroid cancer cells are relatively resistant to DR-mediated cytotoxicity (9, 10). In this regard, it was reported

that ATC cells were not sensitive to TRAIL treatment (6). To sensitize ATC cells to TRAIL, the combination of cytotoxic agents with TRAIL was attempted, and some combination regimens were effective in inducing cell death (6, 9, 21). Although the combination of apigenin with TRAIL potentiates cytotoxicity induced by each agent in various cancer cells, whether the combination has synergistic cytotoxicity in ATC cells has not been assessed (11-15). In the present study, TRAIL resulted in cell death only at 200 and 500 ng/ml for 48 h, similar to previous results, showing that TRAIL did not affect the viability of 8505C cells at 1 μg/ml for 24 h (6). Meanwhile, co-treatment of apigenin and TRAIL, compared to treatment of apigenin or TRAIL alone, decreased cell viability and increased the percentage of dead cells. Our data indicate that the combination of apigenin with TRAIL synergistically augments cell death induced by each agent in ATC cells. In addition, these results imply that apigenin has synergistic cytotoxicity with TRAIL in ATC cells.

Bcl2 family proteins are pivotal modulators for cell survival (22). Relative expression of the pro-survival protein Bcl2 and the anti-survival protein Bax, called the Bcl2/Bax switch, has an influence on the fate of cancer cells (23). In this regard, it has been reported that the combination of apigenin with TRAIL caused reduction of Bcl2/Bax ratio in human hepatoma cells and rheumatoid arthritis fibroblast-like synoviocytes (12, 24). In the present study, in order to elucidate a molecular mechanism for the synergism between apigenin and TRAIL, the expression of Bcl2 family proteins was examined. In cells treated with apigenin and TRAIL, compared to apigenin or TRAIL alone, the protein levels of Bcl2 were reduced, whereas those of Bcl-xL, Bax and Bid were not altered. Moreover, Bcl2/Bax ratio was reduced by co-treatment of apigenin and TRAIL, connoting that the synergism between apigenin and TRAIL is involved in regulation of Bcl2 family proteins in ATC cells.

ERK, a member of mitogen-activated protein kinase, modulates various cellular processes including survival, growth, proliferation and differentiation (13). ERK has a critical role in sensitizing cancer cells to TRAIL by overexpression of DR (13). With regard to the role of ERK in the combination of apigenin with TRAIL, it was reported that apigenin sensitized human hepatoma cells to TRAIL through ERK-dependent overexpression of DR5 (13). In the present study, apigenin led to cell death with a concomitant increase of phospho-ERK1/2 protein levels. Furthermore, co-treatment of apigenin and TRAIL, compared to treatment of apigenin alone, increased phospho-ERK1/2 levels. These results denote that the cytotoxicity induced by apigenin and the synergism between apigenin and TRAIL are relevant to the activation of ERK in ATC cells.

In cancer cells, de-regulation of PI3K/AKT signaling promotes cell survival, and results in resistance to chemotherapy-induced cell death (16, 17). In our recent studies, by using FRO ATC cells, AKT engaged in the VEGFR inhibitor SU5416-induced cell death, and suppression of AKT enhanced the SFK inhibitor SU6656-induced caspase-independent cell death (18, 19). In addition, repression of AKT magnified the effect of apigenin in combination with the BRAFV600E inhibitor PLX4032 in induction of death of ATC cells harboring BRAFV600E (5). In the present study, apigenin, TRAIL and apigenin in combination with TRAIL did not change AKT protein levels. Meanwhile, inhibition of AKT, by administration of wortmannin, further reduced cell viability, and further elevated the percentage of dead cells, cytotoxic activity and cleaved PARP protein levels in cells treated with apigenin and TRAIL. These results suggest that apigenin alone or in combination with TRAIL induces cytotoxicity without suppression of AKT, and repression of AKT multiplies synergistic cytotoxicity of apigenin with TRAIL in ATC cells. It would be proposed that inactivation of PI3K/AKT signaling amplifies the synergism between apigenin and TRAIL in ATC cells.

In conclusion, our results suggest that apigenin synergizes with TRAIL through regulation of Bcl2 family proteins in inducing death of ATC cells. Moreover, inhibition of AKT potentiates a synergistic cytotoxicity of apigenin with TRAIL in ATC cells. The present study provides clinical implications regarding the combination of apigenin with TRAIL and AKT inhibitors, as an attractive therapeutic regimen in the treatment of human ATC, that is refractory to conventional therapies.

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