RESEARCH ARTICLE

Naringenin up-regulates the expression of death receptor 5 and enhances TRAIL-induced apoptosis in human lung cancer A549 cells

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Scope: While TRAIL is relatively non-toxic to normal cells, it can selectively induce apoptosis in many types of transformed cells. Nevertheless, some non-small cell lung cancer (NSCLC) cells are particularly resistant to the effects of TRAIL. Here, we report that in combination with naringenin exposure to TRAIL induced apoptosis in TRAIL-resistant NSCLC A549 cells with no detectable inhibitory effects on cell proliferation of normal lung fibroblast cells.
Methods and results: Cytotoxicity was evaluated by MTT assay. Apoptosis was detected using DAPI staining, and flow cytometry. The protein levels were determined by Western blot analysis. Caspase activity was measured using a colorimetric assay. For knockdown of Bid and DR5 expression, Bid and DR5 siRNAs were transfected into cells via lipofection. We could show that following exposure to naringenin, DR5 proteins were up-regulated and knockdown of DR5 expression by siRNA attenuated naringenin plus TRAIL-induced apoptosis. Naringenin and TRAIL effectively induced Bid cleavage and siRNA-mediated silencing of Bid reduced the sensitizing effect of naringenin. Furthermore, co-treatment with naringenin and TRAIL resulted in reduction of the clonogenic capacity of A549 cells, and surviving clones could be re-sensitized for repeated TRAIL treatment.
Conclusion: Our results indicate that treatment with a combination of TRAIL and naringenin may be a safe strategy for treatment of resistant NSCLC.

Keywords:
A549 / Apoptosis / Death receptor 5 / Naringenin / Tumor necrosis factor-related apoptosis-inducing ligand

1 Introduction

For both men and women, lung cancer is the most frequent cause of cancer-related death in the United States, whereas non-small cell lung cancer (NSCLC) constitutes 75–80% of lung cancers [1]. Clinical trials have demonstrated that the benefit of combination chemotherapy among the fittest patients with advanced NSCLC is marginal [2]. Thus, to

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Abbreviations: DAPI, 4,6-diamidino-2-phenylindile; **DR5,** death receptor 5; **IAP,** inhibitor of apoptosis protein; **JC-1,** 5,5′,6,6′-tetrachloro1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; **JNK,** c-Jun NH2-terminal kinase; **MMP** mitochondrial membrane potential; **MTT,** 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; **NSCLC,** non-small cell lung cancer; **PARP,** poly(ADP-ribose) polymerase**PI,** propidium iodide; **TRAIL,** tumor necrosis factor-related apoptosis-inducing ligand; **XIAP,** X chromosome encoded IAP

improve clinical management of this serious disease, novel treatment strategies are urgently needed.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor 5 (DR5, also named TRAIL-R2) locates at the cell surface, becomes acivated or oligomerized upon binding to its ligand TRAIL or overexpression, and then signals apoptosis through caspase-8-mediated rapid activation of caspase cascades [3, 4]. Through a mitochondrial-regulated apoptotic pathway, the Bcl-2 family member Bid can be recruited by active caspase-8 to kill cells. [5]. Active caspase-8 can also directly activate executioner caspases for the induction of cell death through a mitochondria-independent pathway [6]. In the mitochondria-dependent apoptotic pathway, caspase-8-mediated cleavage of Bid generates a truncated form of Bid (tBid) that translocates to the mitochondria and promotes release of apoptogenic factors in concert with proapoptotic Bax and Bak proteins [7–9]. Inhibitor of apoptosis proteins (IAPs), including cIAP1, cIAP2 and X chromosome-encoded IAP (XIAP), negatively regulates TRAIL-induced apoptosis through the inhibition of caspases [10]. Recently, DR5 has attracted even more attention because its ligand TRAIL preferentially induces apoptosis in transformed or malignant cells, demonstrating its potential as a tumor-selective apoptosis-inducing cytokine for cancer treatment [11]. Certain cancer therapeutic agents induce expression of DR5 in cancer cells and are thereby able to augment TRAIL-induced apoptosis or to initiate apoptosis [12, 13].

Naringenin is a flavonoid that is believed to have a bioactive effect on human health [14, 15]. Several previous studies have demonstrated that naringenin inhibits CYP3A4 activity, and it exhibits aorta dilatory, antioxidant, antiestrogenic, antiproliferative and antimetastatic effects [16–25]. Stimulation with naringenin has been shown to reduce hepatitis C virus secretion in infected cells by 80%. Moreover, naringenin is effective at concentrations that are an order of magnitude below the toxic threshold in primary human hepatocytes and in mice [18], suggesting its potential activity as a chemopreventive agent. Clinically, total cancer incidence was significantly lower with higher naringenin intake, mainly because of a lower risk of lung cancer in men [19].

In the present study, we investigated whether naringenin could promote TRAIL-mediated apoptotic death in TRAIL-resistant NSCLC A549. We found that naringenin potently sensitizes TRAIL-induced apoptosis in A549 cells through DR5 induction and abolishes resistance to Bid-mediated cleavage of TRAIL, which leads to the significant induction of TRAIL-mediated signaling and cell death in A549 cells.

2 Materials and methods

2.1 Reagents and antibodies

Propidium iodide (PI), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindile (DAPI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The pan-caspase inhibitor, z-VAD-fmk and the specific mitochondrial dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), were obtained from Calbiochem (San Diego, CA, USA). Naringenin (95% purity) was purchased from Sigma-Aldrich and dissolved in DMSO. TRAIL was purchased from Koma Biotech (Seoul, Korea) and was dissolved in PBS. All other chemicals not specifically cited here were purchased from Sigma-Aldrich. Anti-actin antibody was obtained from Sigma-Aldrich. Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were purchased from GE Healthcare (Buckinghamshire, UK). All other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Cell culture and viability assay

A549, H460 and human normal lung fibroblast WI-38 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). All cells were cultured in RPMI1640 medium purchased from Invitrogen (Carlsbad, CA, USA), or in a minimum essential medium containing 10 μM of non-essential amino acids. For the cell viability assay, the cells were treated with naringenin and TRAIL for the indicated conditions. Cell viability was quantified by MTT assay at the indicated times [26].

2.3 Nuclear staining with DAPI

For DAPI staining, the cells were washed with PBS and fixed with 3.7% paraformaldehyde (Sigma-Aldrich) in PBS for 10 min at room temperature. The fixed cells were washed with PBS and stained with 2.5 μg/mL DAPI solution for 10 min at room temperature. The cells were then washed twice with PBS and analyzed by fluorescence microscopy (Carl Zeiss, Germany).

2.4 Detection of mitochondrial membrane potential (MMP) and apoptosis by flow cytometry analyses

For analysis of apoptosis, the cells were collected, washed with cold PBS and then resuspended in annexin-V-binding buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$ according to the manufacturer's protocol. Aliquots containing cells were incubated with annexin-V FITC, mixed and incubated for 15 min at room temperature in the dark. PI at a final concentration of 5 μg/mL was added to identify necrotic cells. Apoptotic cells were measured using a fluorescence-activated cell sorter analysis in a flow cytometer (Becton Dickinson, San Jose, CA, USA). To measure the MMP, the dual-emission fluorescent dye

JC-1 was used. JC-1 is internalized and concentrated by respiring mitochondria and can reflect changes in MMP in living cells. There are two excitation wavelengths 527 nm (green) for the monomer form and 590 nm (red) for the JC-1 aggregate form. Briefly, the cells were collected and incubated with 10 μM JC-1 for 30 min at 37°C. The cells were subsequently washed once with cold PBS and analyzed using a DNA flow cytometer.

2.5 Western blot analysis and caspase activity

For Western blot analysis, an equal amount of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) by electroblotting. Caspase activity was determined by colorimetric assays using caspase-3 and caspase-8 activation kits (R&D systems, Minneapolis, MN, USA), according to the manufacturer's protocols.

2.6 Treatment with siRNA

The cells were transfected with Bid and DR5 siRNA (Dharmacon, Chicago, IL, USA), or an equal amount of non-specific control RNA for use as a control (Dharmacon) using LipofectAMINE 2000 (Invitrogen), according to the manufacturer's protocol. Following siRNA transfection, the cells were incubated for 24 or 40 h, followed by incubation under the indicated conditions.

2.7 Statistical analysis

All data are presented as mean ± SD. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $^{*}p < 0.05$ was accepted as an indication of statistical significance. All the figures shown in this article were obtained from at least three independent experiments.

3 Results

3.1 Naringenin sensitizes TRAIL-induced apoptosis in TRAIL-resistant A549 cells

As shown in Fig. 1A, treatment with 10–10 000 ng/mL TRAIL induced limited cell death over a period of 24 h in A549 cells, compared with those in H460 cells treated with the same agent (Fig. 1A), suggesting that A549 cells were resistant to TRAIL. Next, we examined the antiproliferative effect of naringenin alone or in combination with TRAIL in A549 cells. Naringenin concentrations up to 500 μM still inhibited cell proliferation, although cellular activity in reduction of MTT was slightly decreased at the highest concentration. However, cell viability was markedly reduced by the indicated condition (Fig. 1B). Furthermore, co-treatment of A549 cells with 100 μM naringenin and 100 ng/mL TRAIL for 12 h significantly increased apoptotic bodies, whereas treatment with naringenin or TRAIL alone did not (Fig. 1C). Flow cytometric analyses also revealed an enhanced apoptosis, but not necrosis, of A549 cells induced by co-treatment with 100 μM naringenin and 100 ng/mL TRAIL for 12 h (Fig. 1D and F). In addition, we examined the question of whether or not naringenin plus TRAIL displayed the antiproliferative effects in human normal lung fibroblast WI-38 cells. Compared with untreated cells, decreased cell proliferation was not observed in normal lung WI-38 cells treated with TRAIL alone, naringenin alone or naringenin plus TRAIL (Fig. 1E). These results indicate a selective apoptosis induction of the combined treatment with naringenin and TRAIL against TRAIL-resistant A549 cells.

3.2 Sensitization to TRAIL-induced apoptosis by naringenin is dependent on caspases

To confirm the activation of apoptotic signals, the cells were treated for defined periods of time with 100 ng/mL TRAIL and 100 μM naringenin, alone or in combination. Co-treatment with naringenin and TRAIL effectively decreased procaspase-3 and procaspase-8 in a time-dependent manner. However, treatment with 100 μM naringenin alone for 3–12 h did not induce proteolytic processing of caspases. In response to TRAIL, procaspase-3 and procaspase-8 were either not cleaved or were only partially cleaved at 12 h. Poly(ADP-ribose) polymerase (PARP) was progressively degraded from 6–12 h after the combined treatment, whereas they were not degraded following treatment with TRAIL or naringenin alone (Fig. 2A). In contrast, WI-38 cells did not induce proteolytic processing upon treatment with the same agents (Fig. 2A). Similar activation patterns of caspases were observed in response to naringenin plus TRAIL (Fig. 2B). To further determine whether this synergistically increased activation of caspases was a result of apoptosis, we inhibited caspases by the addition of 50 μM of the pan-caspase inhibitor z-VAD-fmk. This treatment effectively reduced the sub-G1 population by co-treatment with 100 μM naringenin and 100 ng/mL TRAIL in A549 cells (Fig. 2C). These results indicate that apoptosis induced by combined application of naringenin and TRAIL is associated with activation of caspases in TRAIL-resistant A549 cells. Several intracellular proteins, including inhibitor of apoptosis proteins (IAP), are capable of inhibiting caspase-3- and caspase-7-mediated apoptosis [27, 28]. To explore the underlying mechanisms by which naringenin enhances TRAIL-induced apoptosis in TRAIL-resistant A549 cells, we examined the possibility that naringenin might down-regulate expression levels of these anti-apoptotic proteins. We observed no significant differences in protein levels of

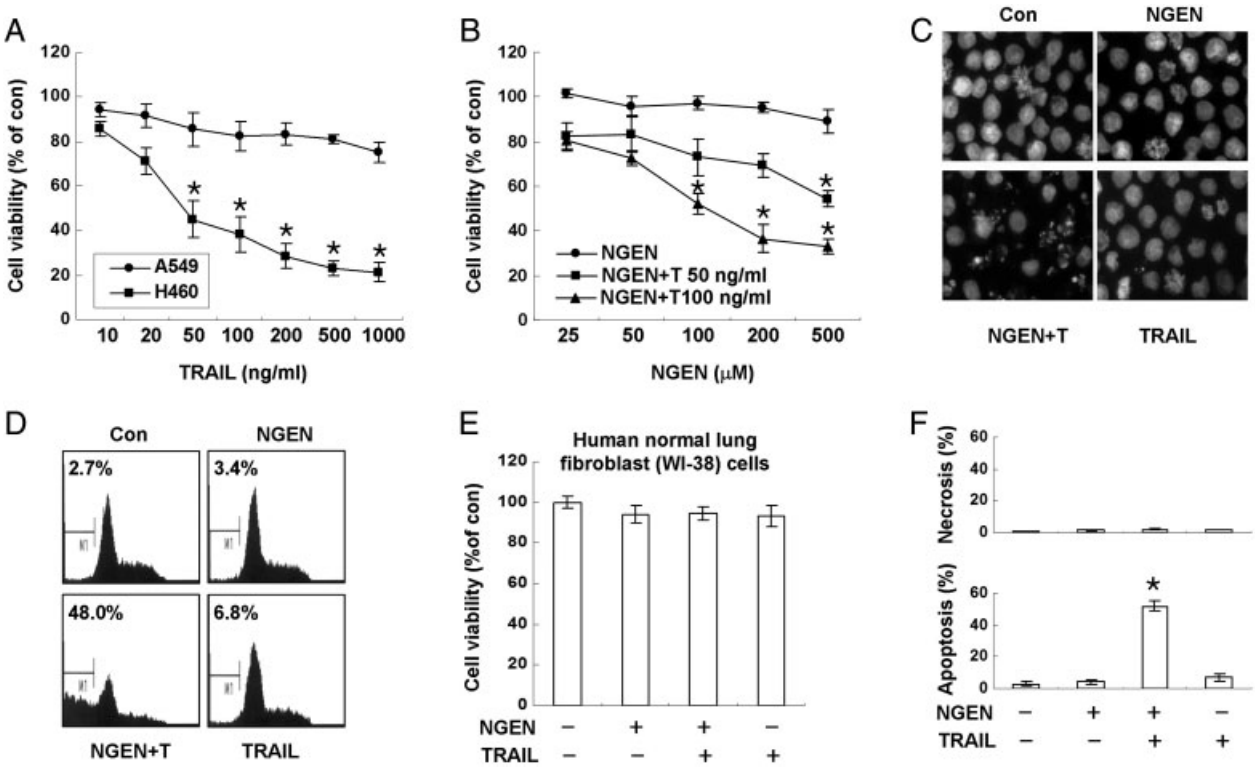

Figure 1. Effects of naringenin and TRAIL on cell proliferation and apoptosis. (A and B) The indicated cell lines were seeded in 96-well plates. On the second day, the cells were treated with different concentrations of TRAIL for 24 h (A) and the cells were pre-treated with different concentrations of naringenin (NGEN) for 30 min, and subsequently incubated with TRAIL (50 or 100 ng/mL) for 12 h, prior to the determination of cell viability using the MTT assay (B). Significant differences among groups were determined using the unpaired Student's *t*-test. A $^{*}p<0.05$, significantly different from following TRAIL in A549 cells. (C) A549 cells were seeded in 24-well plates and treated with NGEN (100 μM), TRAIL (T, 100 ng/mL), or in combination for 12 h. NGEN was added 30 min before TRAIL; after fixing, the cells were stained with DAPI solution. (D) The cells were treated as in (C); after 12 h, the cells were harvested for flow cytometry analysis of the sub-G1 population. (E) Human normal lung fibroblast WI-38 cells were seeded in 96-well plates. On the second day, the cells were treated with NGEN (100 μM) and TRAIL (100 ng/mL), or in combination. NGEN was added 30 min before TRAIL. After 24 h, cell viability was determined by MTT assay. (F) The cells grown under the same conditions as D were assessed for apoptosis *versus* necrosis by staining with Annexin V-FITC and PI. Each point represents the mean ± SD of three independent experiments. The significance was determined by a Student's *t*-test ($^{*}p<0.05$, compared with control).

the tested IAP protein (XIAP, c-IAP1 and c-IAP2) following treatment with naringenin and TRAIL, either alone or in combination (Fig. 2D).

3.3 Naringenin sensitization requires Bid cleavage and, thus, amplification at the mitochondria

To decide on the fate of the cells, proapoptotic and anti-apoptotic members of the Bcl-2 protein families must interpret a wide array of diverse upstream survival and distress signals [29]. Therefore, we investigated the question of whether combined treatment induces apoptosis by modulating expression of Bcl-2 family members. Treatment with 100 μM naringenin and 100 ng/mL TRAIL, alone or in combination, did not affect expression levels of Bcl-2, Bcl-XL and Bax proteins in A549 cells (Fig. 3A). In contrast, the expression of pro-apoptotic Bim_{EL}, a BH3-only Bcl2 protein, was slightly upregulated by naringenin and TRAIL co-treatment, but not upon treatment with naringenin and TRAIL alone (Fig. 3A). We next examined the effects of naringenin and TRAIL on Bid cleavage in A549 cells. Bid cleavage was assessed as a reduction in whole Bid protein because the antibody recognized only the whole Bid molecule, but not the cleavage product (Fig. 3A). Treatment of A549 cells with 100 ng/mL TRAIL resulted in a slight reduction in whole Bid, indicating marginal Bid cleavage. Treatment with 50–200 μM of naringenin did not induce cleavage of Bid. However, reduction in Bid was markedly increased by combined treatment with naringenin and TRAIL (Fig. 5A). Next, the role of the mitochondria in naringenin and TRAIL-induced apoptosis of cells was further investigated by the examination of the effect of naringenin and TRAIL on MMP. Treatment of cells with 50–200 μM naringenin for 12 h had no effect on loss of MMP, and little effect was observed in the presence of 100 ng/mL TRAIL. In contrast, co-treatment with naringenin and TRAIL caused a significant

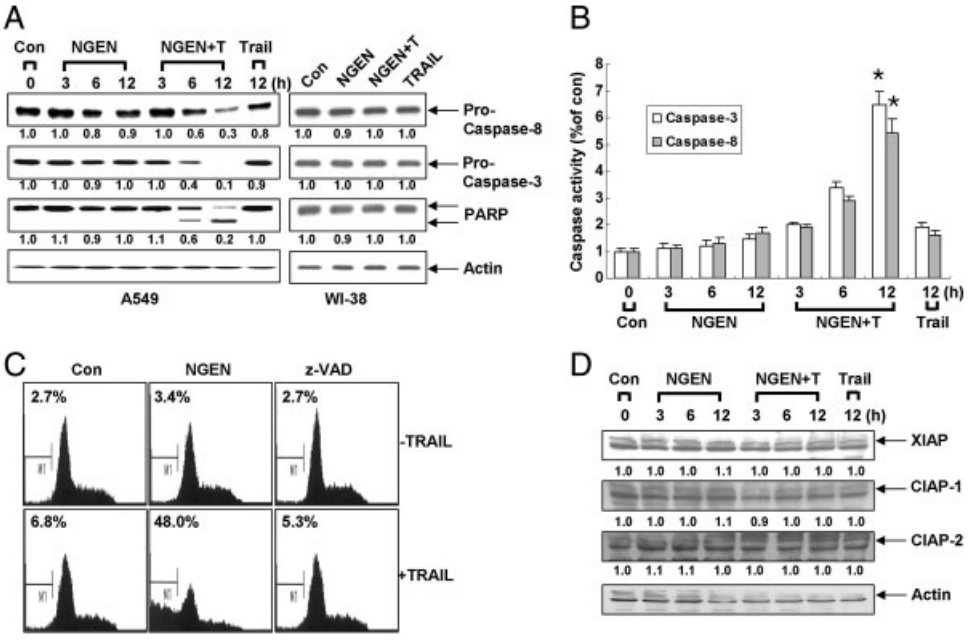

Figure 2. Effects of naringenin and TRAIL on caspases, PARP and anti-apoptotic IAP proteins. (A) A549 and WI-38 cells were incubated in the absence or presence of NGEN (100 μM) for 30 min, and subsequently treated with or without TRAIL (100 ng/mL) for the indicated times. Caspase-3, caspase-8 and PARP processing were determined by Western blot analysis. (B) The cells were treated as in (A) and caspase-3 and caspase-8 activity was determined using caspase assay kits. The significance was determined by a Student's *t*-test ($^*p<0.05$, compared with control). (C) The cells were treated with or without z-VAD-fmk (50 μM) for 1 h. The cells were then incubated, as in Fig. 1D. After 12 h, the cells were harvested for the analysis of the sub-G1 population. (D) The cells were treated as in (A) and XIAP, CIAP-1 and CIAP-2 processing were determined by Western blot analysis. Actin was used as an internal control. The numbers represent the average densitometric analyses as compared with actin in, at a minimum, two or three different experiments.

and concentration-dependent increase in loss of MMP in A549 cells (Fig. 3B). These results suggest a direct role for mitochondria in naringenin/TRAIL-induced apoptosis in TRAIL-resistant A549 cells. To further confirm the role of Bid in cell death induced by naringenin and TRAIL, we examined the question of whether or not siRNA-mediated ablated Bid expression could provide protection against cell death. As shown in Fig. 3C, Bid knockdown significantly reduced induction of apoptosis (Fig. 3C) and MMP loss (Fig. 3D) in the presence of 100 ng/mL TRAIL, alone or in combination with 100 μM naringenin, demonstrating that naringenin effectively acts to lower the threshold at which TRAIL-induced tBid cleavage triggers the mitochondrial apoptosis program.

3.4 Naringenin enhances TRAIL-induced apoptosis through upregulation of DR5

To further understand the mechanism by which naringenin induces apoptosis, the effects of naringenin on expression of DR4 and DR5 in A549 cells and WI-38 cells were studied. Naringenin increased DR5 protein levels in a time- and concentration-dependent manner, and a weak increase was observed in DR4 levels post-naringenin exposure in A549 cells (Fig. 4A and B). In contrast, DR4 and DR5 protein levels were not altered in WI-38 cells treated with 50–200 μM naringenin (Fig. 4C). To determine whether or not naringenin enhances TRAIL-induced apoptosis through DR5 upregulation, we examined the effects of the naringenin and TRAIL combination on loss of MMP and apoptosis induction in cells, where DR5 expression was silenced with DR5 siRNA. Naringenin increased DR5 levels in control siRNA-transfected cells (Fig. 4D, lane 2). In DR5 siRNA-transfected cells, basal levels of DR5 were reduced (Fig. 4D, lane 3), and were not increased further by treatment with 100 μM naringenin for 12 h (Fig. 4D, lane 4). These results indicate successful silencing of DR5 expression. Fluorescence-activated cell sorter analysis of the MMP assay detected up to 70% loss of MMP in control siRNA-transfected cells, but only 35% loss of MMP in DR5 siRNA-transfected cells upon co-treatment with 100 μM naringenin and 100 ng/mL TRAIL (Fig. 4E). Furthermore, silencing of DR5 expression significantly inhibited cleavage of PARP and decreased procaspase-3 by co-treatment with 100 μM naringenin and 100 ng/mL TRAIL in control siRNA-transfected cells. These results suggest that differential regulations of naringenin-induced DR5 expression may be responsible for the selective apoptosis induction of the combined treatment against A549 cells. Collectively, these results indicate that upregulation of DR5 is a key event that mediates mitochondria dependent augmentation of apoptosis induced by the combination of naringenin and TRAIL.

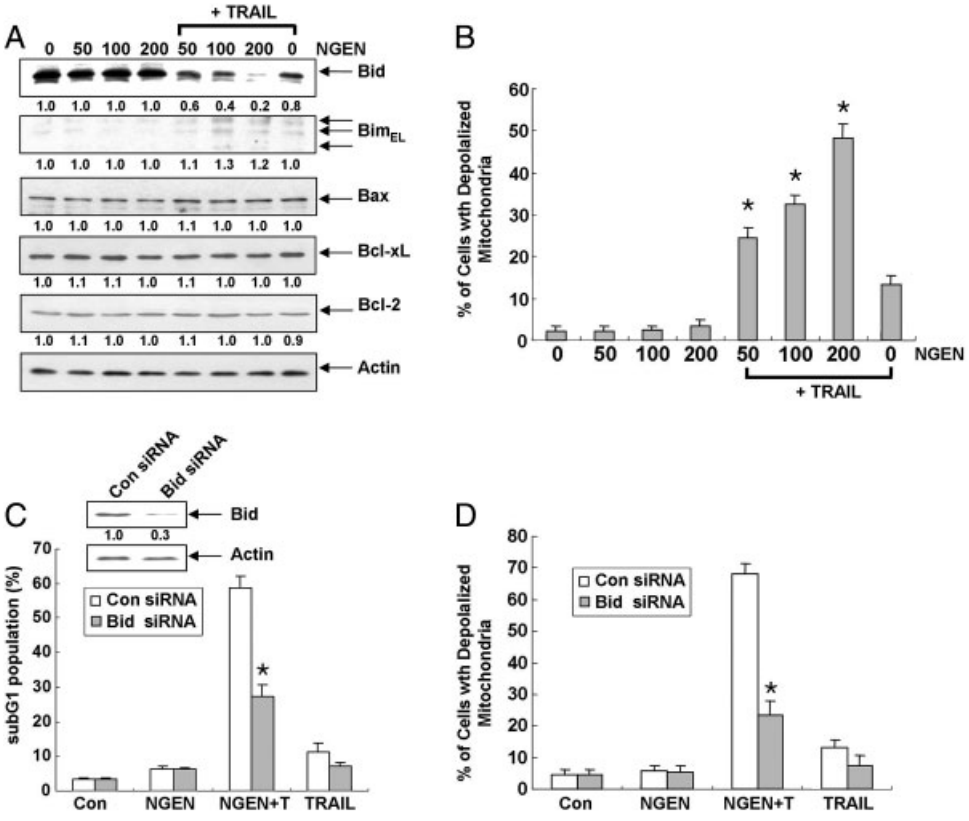

Figure 3. Effects of naringenin and TRAIL on loss of MMP in A549 cells. (A) The cells were incubated in the absence or presence of different concentrations of NGEN for 30 min, and subsequently treated with or without TRAIL (100 ng/mL) for 12 h. Indicated Bcl-2 member protein processing was determined by Western blot analysis. The numbers represent the average densitometric analyses as compared to actin in, at a minimum, two or three different experiments. (B) The cells were treated as in (A) for 12 h, after which mitochondrial dysfunction was assessed by flow cytometry. The significance was determined by a Student's *t*-test ($^*p<0.05$, compared with control and NEGN alone). (C) A549 cells were transiently transfected with a siRNA construct specific for Bid or a control construct for 24 h. The cells were then incubated for 12 h, as in Fig. 1D, followed by the analysis of the (C) sub-G1 population or (D) mitochondrial dysfunction by flow cytometry. The significance was determined by a Student's *t*-test ($^*p<0.05$, compared with control siRNA).

3.5 Clonogenic survival is reduced by naringenin/TRAIL co-treatment

To investigate the clonogenic capacity of TRAIL-resistant NSCLC cells after naringenin and TRAIL co-treatment, A549 cells were incubated with 100 μM naringenin and 100 ng/mL TRAIL, either alone or in combination. After 24 h, dead cells were removed and cell viability assays of re-grown tumor cell colonies were performed at days 1, 2, 3, 5, 10 and 15 after treatment (Fig. 5A and B). Incubation with 100 μM naringenin alone resulted in a clear reduction of viable cells after 48 h. However, by day 5, tumor cell re-growth had already more than compensated for the initial cell loss (Fig. 5A). Treatment with 100 μM of naringenin and 100 ng/mL of TRAIL resulted in reduced survival of clonogenic tumors, with sustained *in vitro* eradication of tumor cells (Fig. 5B).

Knowledge of whether or not combined treatment with naringenin and TRAIL selects for resistant tumor cells, which could re-establish the tumor with a sensitization-resistant phenotype, is of clinical relevance. Therefore, we treated A549 cells overnight with 100 μM of naringenin and 100 ng/mL of TRAIL, and expanded the sporadically surviving cells for 2 additional months. However, these pulse-selected cells could still be re-sensitized for 100 ng/mL TRAIL-induced apoptosis by 100 or 100–200 μM naringenin treatment (Fig. 5C and D). Thus, pulse selection does not select tumor cell variants that are resistant to co-treatment with naringenin and TRAIL.

4 Discussion

Data from previous studies have suggested naringenin induction of apoptosis in several cancer cells [30–33] however, the exact molecular mechanisms and pathways by which these drugs achieve their synergistic effects remain to be fully elucidated. In this study, we investigated the ability of naringenin to sensitize TRAIL-resistant A549 cells. Our observations revealed that naringenin enhances apoptosis to TRAIL-induced apoptosis by upregulation of DR5 without inhibition of cell growth in human normal lung fibroblast WI-38 cells.

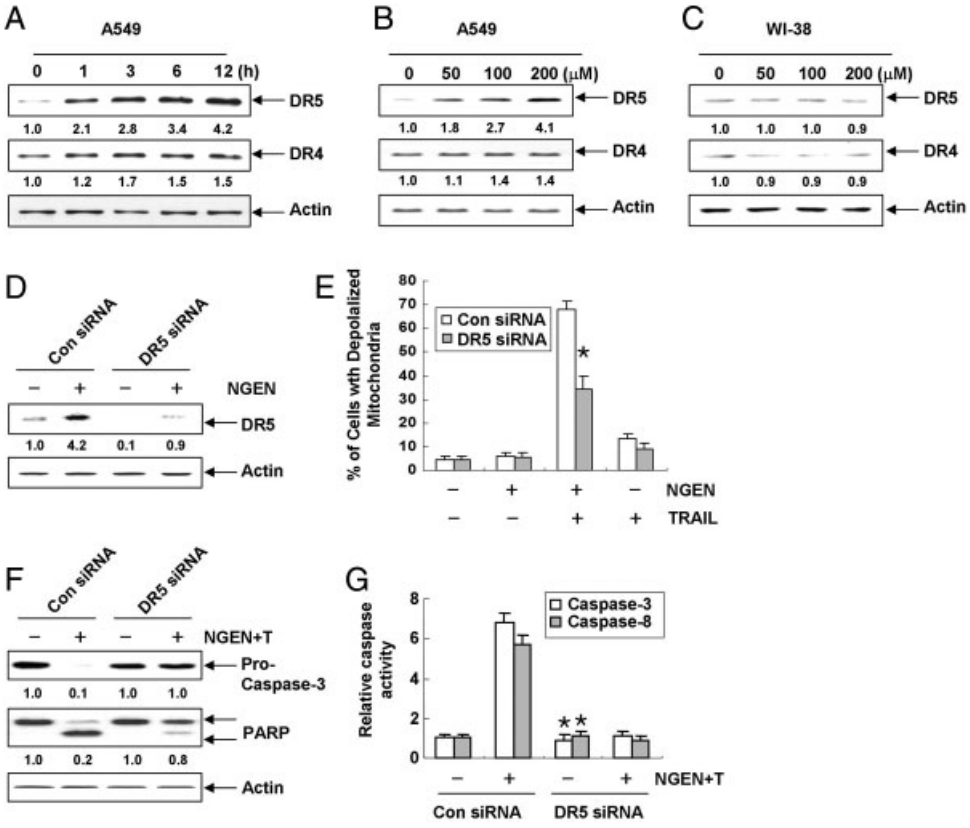

Figure 4. Effect of DR5 expression by naringenin in A549 cell-induced apoptosis. (A) A549 cells were treated with NGEN (100 μM) for the indicated lengths of time. (B and C) The indicated cell lines were treated with the given concentrations of NGEN for 12 h. (A–C) Expression of DR4 and DR5 protein was determined by Western blot analysis. (D and F) A549 cells were seeded in a 24-well cell culture plate and transfected on the second day with control or DR5 siRNA. Forty hours later, the cells were treated with NGEN (100 μM) and, after 12 h, the cells were harvested and examined by Western blot. The numbers represent the average densitometric analyses as compared with actin in, at a minimum, two or three different experiments. (E and G) After transfection with control or DR5 siRNA, the cells were incubated in the absence or presence of NGEN (100 μM) for 30 min, and subsequently treated with or without TRAIL (100 ng/mL) for 12 h, followed by analyses of mitochondrial dysfunction by flow cytometry (E) or caspase-3 and caspase-8 activity (F). The significance was determined by a Student's *t*-test (*$p<0.05$, compared with control siRNA).

Expression levels of death receptors may play a critical role in determination of the intensity and/or duration of death receptor-mediated apoptotic signaling in response to death ligands. TRAIL is known to trigger apoptosis through binding to the death receptors DR4 and DR5 [34, 35], which contain cytoplasmic death domains responsible for recruitment of adaptor molecules involved in caspase activation [36]. Under physiologic conditions, the binding of TRAIL shows a higher affinity for DR5 than DR4 [37]. Moreover, a recent study using a phage display of death receptor-selective TRAIL variants showed that DR5 may play a more prominent role than DR4 in mediation of apoptotic signals emanating from TRAIL in cells expressing both death receptors [38]. Upregulation of DR5 expression and abrogation of TRAIL resistance in cancer cells has been attributed to various agents, including naturally occurring flavonoids such as baicalein, quercetin, silibinin and luteolin [39–42]. This indicates that the combination of TRAIL and flavonoids capable of DR5 upregulation may be a promising strategy for sensitization of tumors to TRAIL-induced apoptosis. It has been proposed that c-Jun NH2-terminal kinase (JNK)-mediated upregulation of DR5 contributes to induction of apoptosis [43], and that use of phosphoinositide-3-kinase inhibitors wortmannin or LY-294002 downregulates active Akt and reverses cellular resistance to TRAIL [44]. In contrast, phosphor-Akt or JNK was not presently modulated by naringenin (data not shown), suggesting that naringenin-sensitized, TRAIL-induced apoptosis is not associated with JNK or Akt. This finding differs from the previous demonstration of a linkage between MAPK, PI3/Akt and cell death in TRAIL-resistant A549 cells [45].

It has been reported that the perturbation of the mitochondrial membrane results in the release of mitochondrial proteins, activation of caspases and loss of normal mitochondrial respiratory function [46]. Members of the Bcl-2 family of proteins are involved in both negative and positive regulation of apoptosis induced by this

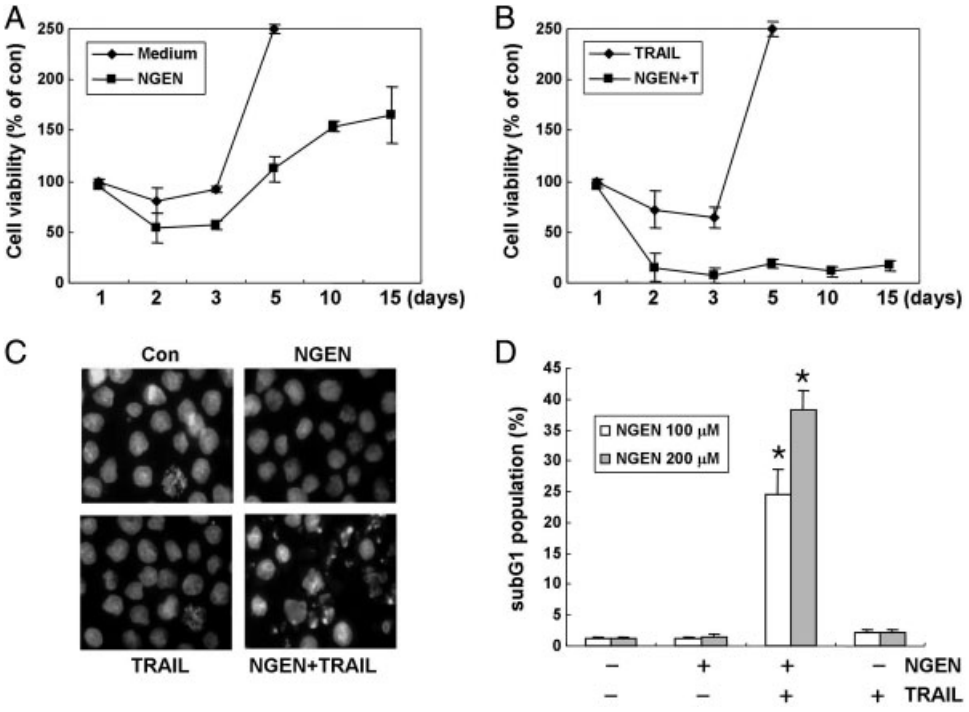

Figure 5. Clonogenic survival of A549 cells is reduced by naringenin and TRAIL co-treatment. (A and B) A549 cells were treated with medium, NGEN (100 μM) and TRAIL (100 ng/mL) alone, or with a combination of NGEN and TRAIL for 12 h. NGEN was added 30 min before TRAIL. Dead cells were washed, and fresh medium was added twice *per* week. Re-growth of tumor cells was quantified by MTT assay at the indicated time points after initial treatment. One of three independent experiments is shown. (C and D) A549 cells were treated with (C) NGEN (100 μM) or (D) NGEN (100–200 μM) and TRAIL (100 ng/mL) for 12 h. NGEN was added 30 min before TRAIL. Dead cells were washed, and the surviving cells were cultured for 2 months and re-challenged with NGEN and TRAIL for 12 h prior to the analysis of (C) apoptotic bodies by staining with DAPI solution or (D) sub-G1 population by flow cytometry. The significance was determined by a Student's *t*-test ($^*p<0.05$, compared with NEGN or TRAIL alone).

pathway, and cleavage of Bid, a BH3-only Bcl-2 protein, by caspase-8 serves as a link between the death receptor and mitochondrial death pathways [47]. We identified degradation of the whole Bid as a key event upstream of naringenin and TRAIL-induced mitochondrial membrane disruption. Moreover, loss of MMP and apoptosis were blocked by knockdown of Bid expression by siRNA. These findings demonstrate that Bid cleavage is an important process in naringenin-mediated cell death by TRAIL. However, neither Bid nor DR5 silencing completely blocked co-treatment with naringenin and TRAIL-induced apoptosis indicating that other mechanisms are at the root of naringenin effects.

In conclusion, the present study demonstrates that naringenin significantly enhances TRAIL-induced apoptosis through the induction of DR5 expression in human A549 cells, but not in non-malignant cells. Although further study of chemotherapeutic effects *in vivo* will be necessary, these results raise the possibility that the combination of flavonoid and TRAIL might show promise as an agent of therapy for lung cancer.

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The authors have declared no conflict of interest.

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