

RESEARCH ARTICLE

Effect of Embelin on TRAIL Receptor 2 mAb-induced Apoptosis of TRAIL-resistant A549 Non-small Cell Lung Cancer Cells

Lei Jiang[&], Jin-Li Hao[&], Mu-Lan Jin^{*}, Yun-Gang Zhang, Ping Wei

Abstract

Introduction: Some non-small cell lung cancer (NSCLC) tumor cells are insensitive to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) -based therapy. This study was conducted to examine the effect of embelin on the sensitivity of the A549 NSCLC cell line to TRAIL receptor2 (TRAILR2) monoclonal antibodies and to investigate the potential mechanisms. **Materials and Methods:** A549 cells were treated with embelin, TRAILR2 mAb or a combination of both. Cell viability was measured using ATPlite assay and apoptosis rates were determined by flow cytometry with AnnexinV-FITC and propidium iodide staining, with the expression levels of proteins analyzed by Western blotting. **Results:** The cell survival rate of separate treatments with 100 ng/ml TRAILR2 antibody or 25 uM embelin were $81.5 \pm 1.57\%$ and $61.7 \pm 2.84\%$, respectively. Their combined use markedly decreased cell viability in A549 cells to $28.1 \pm 1.97\%$ ($P < 0.05$). The general caspase inhibitor Z-VAD-FMK could inhibit the embelin-enhanced sensitivity of A549 cells to TRAILR2 mAb ($75.97 \pm 3.17\%$) ($P < 0.05$). Both flow cytometry and cell morphological analysis showed that embelin was able to increase TRAIL-induced apoptosis in A549 cells. Combined treatment with embelin and TRAILR2 mAb augmented the activation of initiator caspases and effector caspase. In addition, A549 cells showed increasing levels of TRAILR2 protein and decreasing levels of Bcl-2, survivin and c-FLIP following the treatment with embelin+TRAILR2 mAb. **Conclusions:** Embelin could enhance TRAIL-induced apoptosis in A549 cells. The synergistic effect of the combination treatment might be due to modulation of multiple components in the TRAIL receptor-mediated apoptotic signaling pathway, including TRAILR2, XIAP, survivin, Bcl-2 and c-FLIP.

Keywords: TRAIL - embelin - lung cancer - apoptosis

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Introduction

Lung cancer is a highly malignant disease with an extremely poor prognosis (Field and Duffy, 2008), and 80%-85% of the total lung cancer cases are attributed to non-small cell lung cancer (NSCLC). Platinum-based chemotherapy has long been the standard treatment for advanced NSCLC (De Petris et al., 2006). However, NSCLC displays intrinsic or acquired resistance towards many conventional chemotherapeutic agents, and the median survival time for NSCLC remains poor (Ihde and Minna 1991; Stegehuis et al., 2009). Thus novel treatment strategies are badly needed to improve the outcomes of patients with this disease.

A promising candidate for cancer therapeutics might be the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Different studies have shown that TRAIL is capable of inducing apoptosis in a considerable number of cancer cells, while showing no significant adverse effects on normal cells and tissues (Mahalingam et al., 2009). TRAIL induces apoptosis upon binding to its death domain-containing receptors, TRAIL receptor 1

(TRAILR1/DR4) and TRAIL receptor 2 (TRAILR2/DR5). Upon binding to TRAILR1 or TRAILR2, it induces receptor trimerization to form a structure known as the death-inducing signaling complex, which activates caspase-8, ultimately resulting in activation of caspase-3 and apoptosis (Sprick et al., 2000). Agonistic TRAILR1 and TRAILR2 antibodies may have greater therapeutic potential than TRAIL antibodies due to a prolonged half-life in vivo and lack of interaction with so-called "decoy" TRAIL receptors (TRAIL-R3/DcR1, TRAIL-R4/DcR2, and OPG) (Duiker et al., 2006). But one problem for the use of TRAILR1 or TRAILR2 antibodies as anti-cancer agents is that not all cancers, including NSCLC, are sensitive to TRAIL-based therapy, possibly limiting its efficacy in the clinic (Koschny et al., 2007). Therefore, some groups are searching for strategies to sensitize cancer cells to TRAIL-mediated apoptosis efficiently.

The X-chromosome-linked inhibitor-of-apoptosis protein (XIAP) has been identified as one of the most potent inhibitors of caspase activity and apoptosis (Holcik et al., 2001). Embelin was identified as a fairly potent and cell-permeable, small molecular weight inhibitor

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of XIAP, acting by disrupting the binding of caspase 9 and Smac to XIAP (Nikolovska-Coleska et al., 2004). Embelin is an active component of a natural plant product. It has been shown to have anti-tumor, anti-inflammatory, and analgesic properties (Chitra et al., 1994). Through activating apoptosis machinery, it could exhibit cytotoxic effects in various cancer cell types, such as prostate cancer and colon cancer (Nikolovska-Coleska et al., 2004; Dai et al., 2009). Furthermore, embelin can increase the drug susceptibility and apoptosis of not only pancreatic cancer cells, but also of H460 lung cancer cells by combined treatment with cisplatin or other agents (Mori et al., 2007; Cheng et al., 2010). These studies suggest that embelin can be applied in combination with chemotherapeutic and other biological agents for cancer therapy.

Previous studies have demonstrated that embelin and TRAIL combination treatment synergistically induce apoptosis in pancreatic cancer cells and malignant glioma cells (Mori et al., 2007; Siegelin et al., 2009). The present study was conducted to examine the effects of treating TRAIL-resistant A549 NSCLC cells with TRAILR2 mAb in combination with embelin.

Materials and Methods

Cell Culture and Reagents

The human NSCLC cell line A549 was obtained from the American Type Culture Collection. The cell line was cultured in medium recommended by the supplier. The media was supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin during growth conditions. Cells were maintained at 37°C in a humidified atmosphere with 5% carbon dioxide. Embelin was obtained from Merck. The TRAILR2 mAb was kindly provided by Cotimes Biotech (SIPO of P.R.C application number is 200610109138.0). Dimethyl sulfoxide (DMSO) was purchased from Sigma. The general caspase inhibitor Z-VAD-FMK was obtained from Beyotime Institute of Biotechnology, while rabbit anti-human XIAP, anti-human survivin, anti-human Bcl-2, anti-human cleaved caspase-3, anti-human caspase-8 and mouse anti-human caspase-9 antibodies were purchased from Cell Signal Technology. Rabbit anti-human c-FLIP antibody from ABGENT and Goat anti-human TRAILR2 antibody from R&D Systems were used. Secondary horseradish peroxidase conjugated anti-rabbit, anti-mouse or anti-goat antibodies were purchased from Santa Cruz.

Cell Viability Assay

Cells were seeded into 96-well plates at a density of 5000 cells/well in 100 μ L tissue culture medium in triplicate. After incubation to allow cells to adhere, cells were exposed to various concentrations of embelin, TRAILR2 mAb, or combination treatment for 24 hours, as described in the manufacturer's instructions. Controls were treated with DMSO, and cellular viability was assessed using the ATPlite assay kit (Perkin-Elmer, USA) according to the manufacturer's instructions. In brief, 50 μ L of mammalian cell lysis solution was added directly to cells per well of a microplate. After shaking the plate for 5 minutes, 50 μ L substrate solution was added to the

wells and mixed for 10 minutes at room temperature. Luminescent signal was read using a microplate reader at wavelength of 550 nm. The cell survival rate was calculated according to the following formula: survival rate = (experimental group absorption value/control group absorption value) \times 100%. Cells were treated with test compounds as described above in the presence of 25 μ M Z-VAD-FMK, which is the general caspase inhibitor. The combination index (CI) method of Chou-Talalay was used to evaluate synergy (Chou 2008). In brief, $CI = AB / (A \times B)$. A and B represents cell survival rate of either group treated with A or B. AB represents cell survival rate of combined therapy group. It provides quantitative determination for synergism ($CI < 1$), additive effect ($CI = 1$) and antagonism ($CI > 1$).

Cell morphology and flow cytometry

A549 cells were cultured in 6-well plates at a density of 5×10^5 cells/ml overnight for determination of apoptosis. TRAILR2 mAb (100 ng/ml) or embelin (25 μ M) alone or combination of both were added to the cells, and DMSO was added in the control group. Morphological change was observed under inverted microscope, and cells were harvested for flow cytometry analysis after 12 h treatments to observe early apoptosis. The degree of apoptosis induced by 100 ng/ml TRAILR2 mAb with or without 25 μ M embelin was determined by Annexin V-FITC/PI apoptosis detection kit (KeyGEN Biotech, China), following the manufacturer's instructions and the data were analyzed by flow cytometry using WinMDI software. In flow cytometry analysis, Annexin V-FITC single positive cells in the bottom right quadrant represent early apoptotic cells, Annexin V and propidium iodide (PI) double positive staining cells in the top right quadrant represent late apoptotic cells. The positive cells for PI-staining only in the top left quadrant represents the necrotic population.

Western Blot Analysis

Fifty micrograms of protein were loaded onto 8%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred to polyvinylidene difluoride membranes. The blots were blocked for 1 hr at room temperature with 5% skim milk in TBST buffer. Subsequently, immunoblotting was done overnight at 4°C with an appropriate primary antibody. Dilution factor used for anti-XIAP and anti-caspase3 was 1:1000; for anti-Bcl-2, anti-survivin, anti-caspase 8, and anti-caspase 9 was 1: 500; for anti-TRAILR2 was 1:200; for anti-c-FLIP was 1: 100. Excess antibody was removed by washing the membrane with TBST. The membrane was then incubated with a horseradish peroxidase conjugated secondary antibody for 1 h at room temperature, followed by the addition of TBST as described above. Reaction products were visualized by using ECL Western Blotting Detection Reagent (Thermo scientific, USA) and β -actin was used as the loading control.

Statistical analysis

Each experiment was repeated independently three times and results were presented as mean \pm SD. SPSS

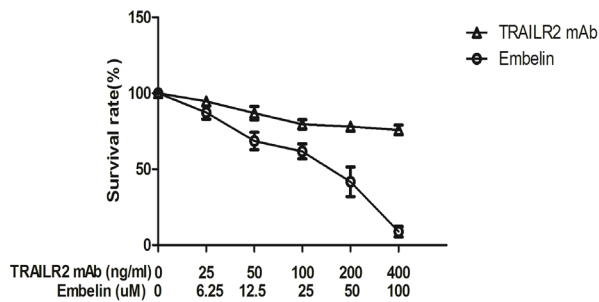


Figure 1. The Cell Survival Rate in A549 Cells with Increasing Concentrations of TRAILR2 mAb or Embelin for 24 h (%). Cell viability was determined by ATPlite assay. Cell viability is shown as the mean percentage compared with untreated cells, and bars represent the SD (n = 3)

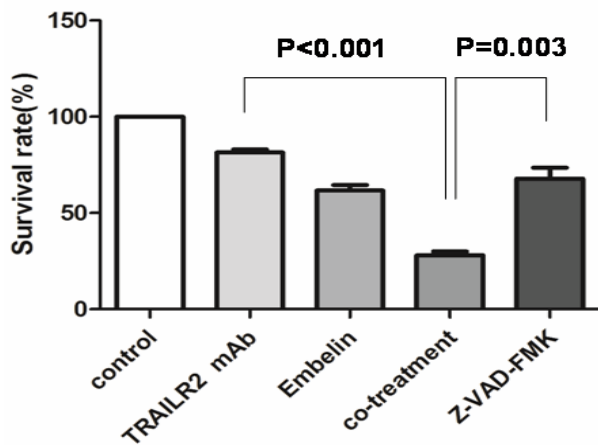


Figure 2. Embelin Enhanced Sensitivity of A549 Cells to TRAILR2 mAb. Cells were treated with TRAILR2 mAb (100 ng/ml) or embelin (25 uM) alone or in combination. The general caspase inhibitor Z-VAD-FMK was used to inhibit the embelin-enhanced sensitivity of A549 cells to TRAILR2 mAb. Cell viability was determined by ATPlite assay. Cell viability is shown as the mean percentage compared with untreated cells, and bars represent the SD (n = 3)

statistical software package v. 17.0 was used for all analyses. Data were analyzed by Student's t-test, and statistical significance was accepted at a *P*-value of less than 0.05.

Results

Embelin Enhances Sensitivity of A549 Cells to TRAILR2 mAb

A549 cells were treated with increasing concentrations of TRAILR2 mAb (0–400 ng/mL) or embelin (0–100 uM) for 24 hr. The results of cell viability assay showed that the cell line was refractory to the treatment with TRAILR2 mAb (Figure 1). Embelin caused concentration-dependent growth inhibition in A549 cells (Figure 1). We then treated A549 cells with 25uM embelin in the presence of different concentrations of TRAILR2 (50, 100, 200 and 400 ng/mL) and found that the combination index (CI) of each group was less than 1, with 0.66 ± 0.05 , 0.59 ± 0.07 , 0.73 ± 0.02 and 0.88 ± 0.09 , respectively, which indicated synergism of the two reagents. Therefore, we chose 25 uM embelin combined with 100 ng/ml TRAILR2 mAb to perform the following studies. The cell survival rate

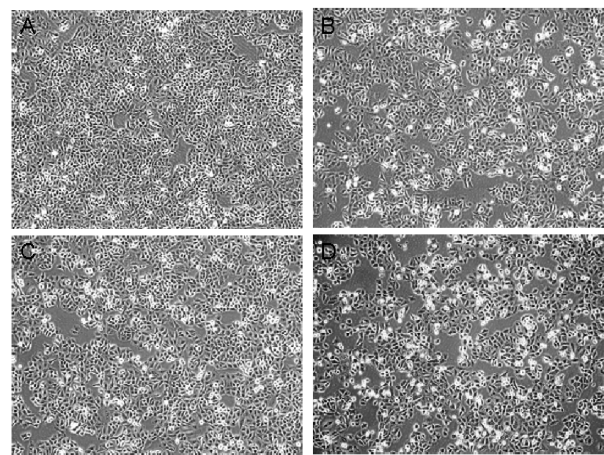


Figure 3. Embelin Enhanced TRAILR2 mAb-induced Apoptosis in A549 Cells as assessed by Cell Morphology (12 h). A. Control; B. TRAILR2 mAb (100 ng/ml); C. Embelin (25 uM); D. Embelin (25 uM)+TRAILR2 mAb (100 ng/ml)

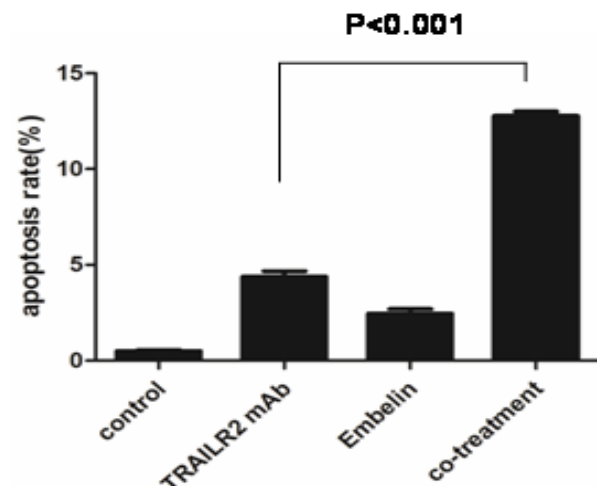


Figure 4. Embelin Increased TRAIL-induced Apoptosis in A549 Cells as Assessed by Flow Cytometry. A549 cells were treated with TRAILR2 monoclonal antibody (100 ng/ml) or embelin (25 uM) alone or in combination. DMSO was added in the control. Apoptosis rate (%) was determined by flow cytometry

of separate treatments with 100 ng/ml TRAILR2 mAb or 25 uM embelin were $(81.49 \pm 1.57)\%$ and $(61.70 \pm 2.84)\%$, respectively. However, their combined use markedly decreased cell viability in A549 cells ($P < 0.05$), and the survival rate was $(28.05 \pm 1.97)\%$ (Figure 2).

Morphological change was analyzed under an inverted microscope to examine characteristic of early apoptosis. We found that embelin could increase TRAIL-induced apoptosis after the combination treatment with 100 ng/ml TRAILR2 mAb and 25 uM embelin, with more significant membrane shrinkage, cell suspension, cell rounding, and light refraction alteration (Figure 3).

Combination treatment is caspase dependent and induces caspase activation

To determine whether caspase activation was involved in embelin-enhanced sensitivity to TRAILR2 mAb, A549 cells were treated with the combination of TRAILR2 mAb (100 ng/ml) and embelin (25 uM) in the presence of the general caspase inhibitor Z-VAD-FMK. The result

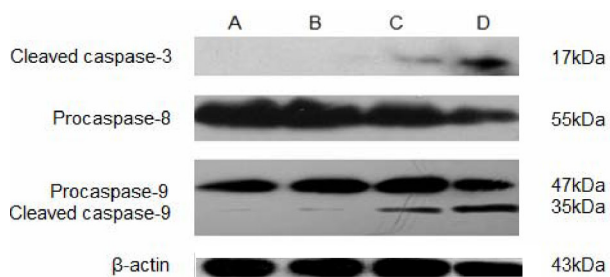


Figure 5. Immunoblots Showing the Effect of Embelin on TRAIL-induced Cleaved Caspase-3 and Caspase-8/-9 in A549 Cells. Immunoblots are representative of at least three independent experiments. A.control; B.TRAILR2 mAb (100 ng/ml); C.Embelin (25 uM); D.Embelin (25 uM)+TRAILR2 mAb (100 ng/ml). β -Actin was used to verify equal loading of proteins. Right, approximate molecular weights (kDa)

showed that Z-VAD-FMK significantly inhibited the embelin-enhanced sensitivity of A549 cells to TRAILR2 mAb ($P < 0.05$) (Figure 2).

The results of flow cytometry analysis showed that the apoptotic rates of A549 cells treated with TRAILR2 mAb (100 ng/ml), embelin (25 uM), or the combination treatment were $(4.39 \pm 0.50)\%$, $(2.79 \pm 0.47)\%$ and $(13.12 \pm 0.73)\%$, respectively (Figure 4). The combination treatment using TRAILR2 mAb and embelin resulted in a significant increase in apoptosis compared to each monotherapy ($P < 0.05$).

Since embelin augmented TRAIL-induced apoptosis, we next examined the activation of caspase-3/-8/-9, the initiator and effector-caspases of the intrinsic and extrinsic apoptotic pathways. A549 cells were treated with embelin, TRAILR2 mAb or the combination of both as indicated. Treatment with TRAILR2 mAb alone did not induce cleavage of caspase-3/-8/-9. Combining TRAILR2 mAb with embelin led to a significant increase in active cleaved caspase-3/-9, as well as a decrease in Procaspase-8 (Figure 5). However, we did not detect the procaspase-3 and cleaved caspase-8 after several repetitions.

Combination treatment alters TRAILR2, Bcl-2, Survivin and c-FLIP protein expression

Furthermore, we analyzed the expression of certain molecules in the TRAIL receptor-mediated apoptotic signaling pathway by western blot. A549 cells showed increasing levels of TRAILR2 protein and decreasing levels of Bcl-2, survivin and c-FLIP following the treatment with embelin + TRAILR2 mAb ($P < 0.05$). The expression level of XIAP showed no change in cells treated with embelin and/or TRAILR2 mAb ($P > 0.05$) (Figure 6).

Discussion

Activating the apoptotic machinery is the principle of many cancer therapies, such as chemotherapy (Fesik 2005). TRAIL-mediated apoptosis is very promising, as it induces apoptosis almost specifically in cancer cells (Mahalingam et al., 2009). But it has been reported that TRAIL may bind to nonfunctional decoy receptors, thus

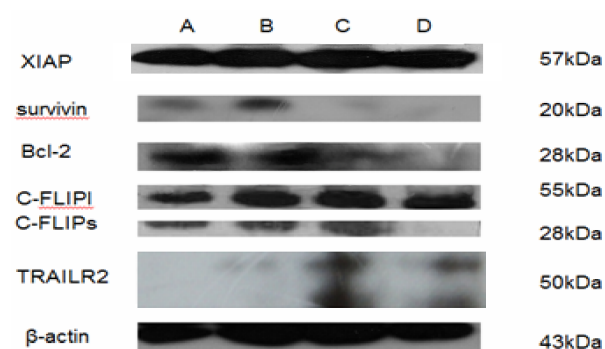


Figure 6. Immunoblots Showing Expression Levels of XIAP, Survivin, Bcl-2, c-FLIP, TRAILR2 after the Combination Treatment Using TRAIL and Embelin or Monotherapy. Immunoblots are representative of at least three independent experiments. A.control; B.TRAILR2 mAb (100 ng/ml); C.Embelin (25 uM); D.Embelin (25 uM)+TRAILR2 mAb (100 ng/ml). β -Actin was used to verify equal loading of proteins. Right, approximate molecular weights (kDa)

interfering with apoptosis (Jo et al., 2000). Targeting the transmembrane TRAIL death receptors, TRAILR1 and TRAILR2, with an agonistic antibody may be an attractive therapeutic approach. In addition, previous research has indicated that TRAIL death receptors are expressed in a large majority of tested NSCLC samples (Spierings et al., 2003). However, some cancer lines, including NSCLC cell lines, are refractory to these agents (Koschny et al., 2007; Luster et al., 2009). Fortunately, several studies have already shown that tumor cells resistant to agonistic TRAILR1 and TRAILR2 antibodies or TRAIL could be sensitized by treatment with other agents (Luster et al., 2009; Lan et al., 2013). Therefore, we investigated whether embelin, a natural benzoquinone product that was originally isolated from the Japanese herb *Ardisia*, could enhance a TRAILR2 mAb-induced apoptosis in NSCLC cells.

To address this question, we treated several NSCLC cell lines with TRAILR2 mAb (data not shown) and chose A549 cells as TRAIL-resistant cells. Firstly, we demonstrated that A549 cells were resistant to the apoptotic effect of TRAILR2 mAb, which confirmed the results in other previous reports (Voortman et al., 2007; Steghuis et al., 2009). However, combined treatment with embelin markedly increased the sensitivity of the NSCLC cell line to TRAILR2 mAb, and the combination index (CI) was less than 1, suggesting that they acted in synergy (Chou, 2008).

The combination of upregulation of TRAIL and downregulation of XIAP has recently been demonstrated an effective therapy for malignant tumors such as melanomas (Li et al., 2012). Embelin was identified as a small molecular weight inhibitor of XIAP. The antiapoptotic activity of XIAP is at least partially due to its ability to inhibit both mitochondrial-dependent and mitochondrial-independent apoptotic pathways by binding to and inhibiting the activation of initiator caspase-9, as well as the effector caspases (caspase-3 and -7), which are vital for the execution of apoptosis. Thus we demonstrated that the cytotoxicity induced by TRAILR2 mAb combined with embelin was caspase dependent. This was shown

by treating A549 cells with a combination of TRAILR2 mAb and embelin in the presence of the general caspase inhibitor Z-VAD-FMK, which significantly attenuated the cytotoxicity of the combination treatment. Western blot analysis indicated early activation of the initiator caspases, caspase-8/-9, as well as effector caspase-3 following the co-treatment. These results provide evidence that embelin could enhance TRAIL-induced apoptosis in NSCLC cells.

In addition, we employed Western blot to elucidate the potential mechanism of how embelin sensitized NSCLC cells to TRAIL-induced apoptosis. A large and growing body of literature has shown that resistance to TRAIL can occur at different levels in the signaling pathways of TRAIL-induced apoptosis, such as dysfunctions of the death receptors and overexpression of IAPs, Bcl-2 or cellular FADD-like interleukin-1 β -converting enzyme inhibitory protein (c-FLIP) (Zhang and Fang 2005; Pore et al., 2010; Kaminsky et al., 2013). Therefore, we first examined the expression level of TRAILR2 in A549 cells after embelin treatment, TRAILR2 mAb treatment, or combination treatment. We found that with the combination treatment of TRAILR2 mAb and embelin, the TRAILR2 protein expression was significantly increased in A549 cells. Different studies have shown that in NSCLC patients, high TRAILR2 expression levels were found in a majority of tumor biopsies (Spierings et al., 2003; Cooper et al., 2008). A recent study demonstrated that deregulation of TRAILR2, which mediates pro-apoptotic signals, played an important role in the early stages of NSCLC carcinogenesis (Cooper et al., 2008). All these findings suggest that the embelin-mediated enhancement of TRAIL-induced apoptosis in A549 cells was at least partially due to the up-regulation of TRAILR2 expression. However, the expression of XIAP did not change in A549 cells treated with embelin and/or TRAILR2 mAb, which is consistent with the idea that embelin does not affect XIAP protein expression (Nikolovska-Coleska et al., 2004). We also found decreasing levels of survivin in A549 cells when treated with embelin. This is consistent with other researches, which have demonstrated that down-regulation of survivin facilitated TRAIL-mediated apoptosis in glioma cells (Siegelin et al., 2009). Of the regulators analyzed in this study, the expression levels of c-FLIP and Bcl-2 proteins, which are involved in apoptosis inhibition (Gross et al., 1999; Kim et al., 2000), were decreased with the combination treatment of embelin and TRAILR2 mAb. These findings indicated that the co-treatment with embelin and TRAIL was able to regulate not only pro-apoptotic signals but also anti-apoptotic signals in NSCLC cells. Both expressional and post-translational mechanisms may be involved in the regulation and more work should be done in molecular level.

In summary, our results demonstrated that embelin could enhance TRAILR2 mAb-induced apoptosis in A549 cells. Furthermore, we provided novel evidence that the prominent synergistic effect of the combination treatment was due to modulation of multiple components in the TRAIL receptor-mediated apoptotic signaling pathway, including TRAILR2, XIAP, survivin, Bcl-2 and c-FLIP. These results obtained in NSCLC cells suggest that the combination of TRAILR2 mAb and embelin might be

used as a new strategy for the treatment of non-small cell lung cancer, and further studies are clearly required to explore this possibility.

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