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# Synergistic antitumor effect of TRAIL and adriamycin on the human breast cancer cell line MCF-7

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## Abstract

The aim of the present study was to determine the effect of the combination of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and adriamycin (ADM) on the human breast cancer cell line MCF-7 and to identify potential mechanisms of apoptosis. Cell viability was analyzed by the MTT assay and the synergistic effect was assessed by the Webb coefficient. Apoptosis was quantified using the annexin V-FITC and propidium iodide staining flow cytometry. The mRNA expression of TRAIL receptors was measured by RT-PCR. Changes in the quantities of Bax and caspase-9 proteins were determined by Western blot. MCF-7 cells were relatively resistant to TRAIL ( $IC_{50} > 10 \mu\text{g/mL}$ ), while MCF-7 cells were sensitive to ADM ( $IC_{50} < 10 \mu\text{g/mL}$ ). A subtoxic concentration of ADM ( $0.5 \mu\text{g/mL}$ ) combined with 0.1, 1, or  $10 \mu\text{g/mL}$  TRAIL had a synergistic cytotoxic effect on MCF-7 cells, which was more marked with the combination of TRAIL ( $0.1 \mu\text{g/mL}$ ) and ADM ( $0.5 \mu\text{g/mL}$ ). In addition, the combined treatment with TRAIL and ADM significantly increased cell apoptosis from 9.8% (TRAIL) or 17% (ADM) to 38.7%, resulting in a synergistic apoptotic effect, which is proposed to be mediated by up-regulation of DR4 and DR5 mRNA expression and increased expression of Bax and caspase-9 proteins. These results suggest that the combination of TRAIL and ADM might be a promising therapy for breast cancer.

Key words: TRAIL; TRAIL receptors; Breast cancer; Adriamycin; Apoptosis; Synergism

## Introduction

Worldwide, breast cancer is the most common form of malignancy in women, with high incidence and mortality rates (1). Adriamycin (ADM) is among the most effective antitumor drugs used for the management of breast cancer. Unfortunately, its clinical utility is limited by its toxic effects such as myelosuppression, nausea, vomiting, and cardiotoxicity (2). Thus, novel treatment strategies are urgently needed to improve the clinical management of breast cancer.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a tumor necrosis factor superfamily member, has been shown to induce apoptosis (3). TRAIL can bind to 5

different receptors: TRAIL-R1 (death receptor 4, DR4), TRAIL-R2 (DR5), TRAIL-R3 (decoy receptor, DcR1), TRAIL-R4 (DcR2), and osteoprotegerin (OPG). DR4 and DR5 are the death receptors that signal apoptosis, whereas DcR1, DcR2 and OPG are considered antagonistic because they are unable to induce such signaling due to the lack of an intracellular death domain or because they are secreted molecules (4).

TRAIL triggers apoptosis through binding to its receptors DR4 and/or DR5 in various cancer cell types but not in most normal cells (5,6). Thus, the selective killing of tumor

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cells by TRAIL has made TRAIL receptors attractive targets for cancer treatment. In preclinical models, recombinant soluble TRAIL has demonstrated impressive anticancer activity (7). Unfortunately, more than 50% of tumor cells are resistant to TRAIL (4). Thus, it is important to develop new strategies to overcome this resistance of tumor cells. Interestingly, several recent reports have described the ability of subtoxic concentrations of chemotherapeutic drugs to sensitize tumor cells that are resistant to TRAIL (8). The antitumor properties of TRAIL can be greatly enhanced when used in combination with chemotherapy, as demonstrated by many studies using different tumor cell lines and mouse models (7). These results suggested that the combination of an anticancer drug with TRAIL may enhance the TRAIL-based therapeutic activity (8). In the present study, we determined the effect of the combination of TRAIL with ADM on the human breast cancer cell line MCF-7 and explored the potential mechanisms of apoptosis induced by TRAIL alone or in combination with ADM.

## Material and Methods

### Material

The human breast cancer cell line MCF-7 and TRAIL were provided by Diao Pharmaceutical Group (China). ADM was supplied by Shenzhen Main Luck Pharmaceuticals Inc. (China). RPMI 1640 medium, dimethylsulfoxide and trypsin were purchased from Gibco (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (USA). Mouse monoclonal antibodies for  $\beta$ -actin and Bax were purchased from Santa Cruz Biotechnology (USA). Rabbit polyclonal antibody for caspase-9 was purchased from Lab Vision (USA). The LSAB kit was purchased from Dako (Japan). Goat anti-mouse IgG-HRP (horseradish peroxidase) was purchased from Bio-Rad (USA). Goat anti-rabbit IgG-HRP and the Trizol kit were purchased from Invitrogen (USA). A one-step RT-PCR kit was purchased from Takara Biotechnology (Dalian) Co., Ltd. (China). Polyvinylidene difluoride membranes were purchased from Millipore (USA). The annexin V-FITC apoptosis detection kit was purchased from Jingmei Biotech Co., Ltd. (China). The enhanced chemiluminescence detection kit and X-ray films were purchased from Roche (Switzerland). All other chemicals were of the highest grade available.

### Cell culture

The human breast cancer cell line MCF-7 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum plus ampicillin and streptomycin and incubated in 5% CO<sub>2</sub> at 37°C.

### Cell viability assay

Cell viability was analyzed by the MTT assay. A blank control group (nutritive medium only), a negative control group (untreated cells), a TRAIL alone group (cells treated

with TRAIL) and an ADM alone group (cells treated with ADM) were designed for this experiment. Briefly, 100  $\mu$ L exponentially growing tumor cell suspensions ( $1 \times 10^4$  cells) were seeded onto each well of a 96-well plate. TRAIL or ADM was then added to each well. The final concentration of TRAIL was 0 to 100  $\mu$ g/mL, while the final concentration of ADM was 0 to 50  $\mu$ g/mL. During incubation at 37°C for 24 h, 20  $\mu$ L MTT (5 mg/mL final concentration) was added to each well. The plates were then incubated at 37°C for an additional 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized in 150  $\mu$ L dimethylsulfoxide at 37°C for 10 min. The absorbance values of the solution in each well were measured at 570 nm using a microplate reader. Cell viability was determined by the formula: cell viability (%) = (absorbance of the treated wells - absorbance of the blank control wells) / (absorbance of the negative control wells - absorbance of the blank control wells)  $\times$  100%. All MTT experiments were performed in triplicate and repeated at least three times.

The 50% inhibitory concentrations (IC<sub>50</sub>) of a 24-h exposure, defined as the drug concentration resulting in 50% reduction of cell viability, were then determined from curves of reagent concentration versus cell viability at 24 h of incubation for the cell line analyzed. Cell sensitivity to the drug was evaluated by the IC<sub>50</sub> value. An IC<sub>50</sub> <10  $\mu$ g/mL indicated that the cells were sensitive to the drug, while an IC<sub>50</sub>  $\geq$ 10  $\mu$ g/mL indicated that cells were relatively resistant to the drug.

### Toxicity of the combination of TRAIL and ADM

The concentrations of the TRAIL and ADM combination were chosen as follows: TRAIL (0.01, 0.1, 1, and 10  $\mu$ g/mL) and ADM (0.05, 0.5, and 5  $\mu$ g/mL) on the basis of the effect of TRAIL and ADM treatment alone on cell viability. Cell viability after a 24-h exposure was then assessed by the MTT assay. A blank control group, negative control group, TRAIL alone group, ADM alone group, and a combination group (cells treated with TRAIL and ADM) were employed for this experiment.

### Evaluation of the synergistic effect

The synergistic effect of the TRAIL and ADM combination was analyzed by the Webb coefficient (9). Predicted cell viability (*c*) was calculated by the equation  $c = a \times b/100$ , where *a* and *b* indicate cell viability after the use of each agent. Synergism in drug interaction was indicated by a cell viability of  $\leq$ 70% of the predicted value. Based on the synergistic effect observed, the optimum concentrations of 0.1  $\mu$ g/mL TRAIL and 0.5  $\mu$ g/mL ADM were chosen for all later studies.

### Cell morphology

Experiments were randomly divided into four groups as follows: TRAIL alone group (cells treated with 0.1  $\mu$ g/

mL TRAIL), ADM alone group (cells treated with 0.5 µg/mL ADM), combination group (cells treated with 0.1 µg/mL TRAIL and 0.5 µg/mL ADM), and negative control group (untreated cells). Exponentially growing tumor cells were seeded onto a 96-well plate at a density of  $1 \times 10^5$ /mL cells/well. TRAIL and/or ADM was added to each well. After incubation at 37°C for 24 h, morphological cell changes were observed under an inverted microscope (100X; Olympus CK2, Japan).

### Flow cytometry analysis of apoptosis

The rate of apoptosis was measured by annexin V-FITC and propidium iodide staining flow cytometry. In brief, cells were collected, washed twice with cold phosphate-buffered solution (PBS), and permeabilized with 70% ethanol in PBS for 30 min. They were then resuspended in annexin V binding buffer at a concentration of  $1.5 \times 10^5$  cells/mL. FITC-conjugated annexin V (1 µg/mL) and propidium iodide (50 µg/mL) were added to the cells and incubated for 15 min at room temperature in the dark before flow cytometric analysis. The samples were detected using an Elite-ESP flow cytometer (Beckman-Coulter, USA). A minimum of 10,000 cells were analyzed in each sample. All experiments were repeated at least three times.

### Western blot analysis

Cells incubated with ADM (0.5 µg/mL) or TRAIL (0.1 µg/mL) alone or in combination for 24 h were lysed in lysis buffer. Protein concentrations were determined by the bicinchoninic acid method. Forty micrograms of cell lysate protein was subjected to 4–15% gradient SDS-PAGE using a Tris-glycine system and then gels were electroblotted onto polyvinylidene difluoride membranes for 45 min. The membranes were then incubated with 5% non-fat dry milk in PBS for 1 h in order to block nonspecific binding sites, and then incubated with the appropriate primary antibody concentration (1:200 dilution for caspase-9, 1:400 for Bax and 1:2000 for β-actin) for 2 h at 37°C in 5% non-fat dry milk. Membranes were subsequently rinsed in PBS and then incubated for 2 h at 37°C with a secondary antibody (HRP conjugated anti-rabbit or anti-mouse IgG) at 1:2000 dilution. After incubation, membranes were rinsed and blots were visualized by incubation with enhanced chemiluminescence detection reagents. Signal density was obtained by scanning exposed X-ray films on a Bio-Rad imaging system. Normalized density was obtained by dividing the rough density values of a sample band by those of the loading control band (β-actin). Immunoblotting data were quantitated from at least three experiments.

### Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cells using the Trisol reagent kit. RT-PCR was run using a one-step RT-PCR kit. β-actin was used as internal control. The upstream prim-

ers of DR4, DR5, DcR1, DcR2 and β-actin are 5'-CTGA GCAACGCAGACTCGCTGTCCAC-3', 5'-GCCTCATGGAC AATGAGATAAAGGTGGCT-3', 5'-GAAGAATTTGGTGCC AATGCCACTG-3', 5'-CTTTCCGGCGGCGTTCATGTCC TTC-3', and 5'-GTCAGAAGGATTCTATGTG-3', respectively. The downstream primers of DR4, DR5, DcR1, DcR2, and β-actin are 5'-TCCAAGGACACGGCAGAGCCTGTG CCAT-3', 5'-CCAAATCTCAAAGTACGCACAAACGG-3', 5'-CTCTTGGACTTGGCTGGGAGATGTG-3', 5'-GTTTCTT CCAGGCTGCTTCCCTTTGTAG-3', and 5'-ATCTCCTTC TGCATCCTGTC-3', respectively. The PCR products were a 506-bp fragment for DR4, a 502-bp fragment for DR5, a 612-bp fragment for DcR1, a 453-bp fragment for DcR2, and an 800-bp fragment for β-actin, respectively. PCR was performed in a 25-µL reaction volume. PCR cycles included a denaturation step at 94°C for 30 s, followed by an optimized annealing temperature of 62°C for 30 s and by an extension period at 72°C. The amplification cycles of DR4, DR5, DcR1, DcR2, and β-actin were repeated 25, 22, 25, 22, and 22 times, respectively. Amplified products were separated by 1.2% ethidium bromide-stained agarose gel electrophoresis and viewed under ultraviolet light. The electrophoresis photo was transferred to a computer, and the integrated absorbance values of the DR4, DR5 and β-actin bands were analyzed using the Bio-Rad image system. Semiquantitative analysis of DR4 and DR5 mRNA was performed by comparison to β-actin.

### Statistical analysis

Data are reported as means ± SD. Mean values were analyzed statistically by one-way ANOVA followed by the Student *t*-test. Linear correlation between cell viability and concentrations of ADM or TRAIL was analyzed using the SPSS 12.0 software. The synergistic effect was evaluated using the Webb coefficient (9). All *P* values were two-sided and the level of significance was set at *P* < 0.05.

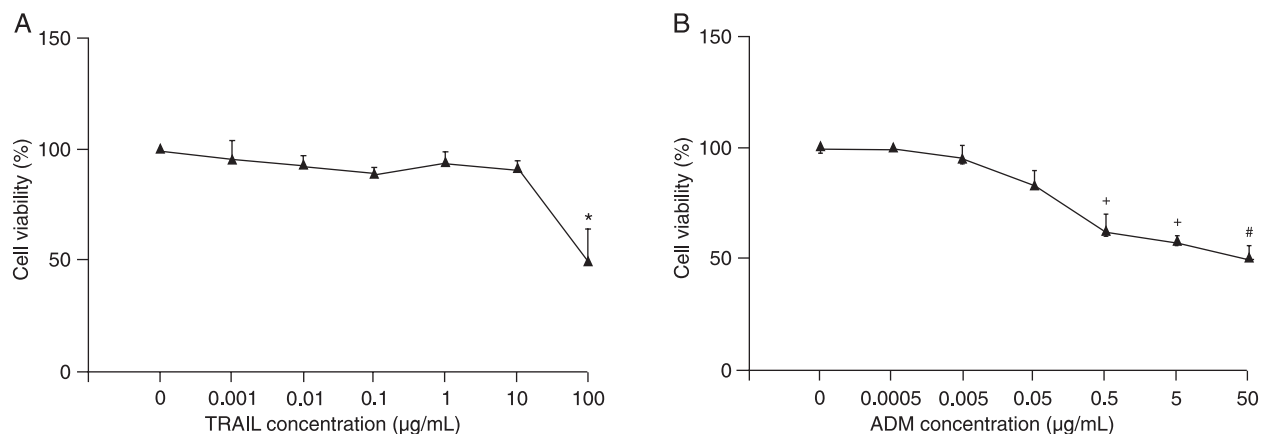
## Results

### Effect of TRAIL or ADM alone on MCF-7 cell viability

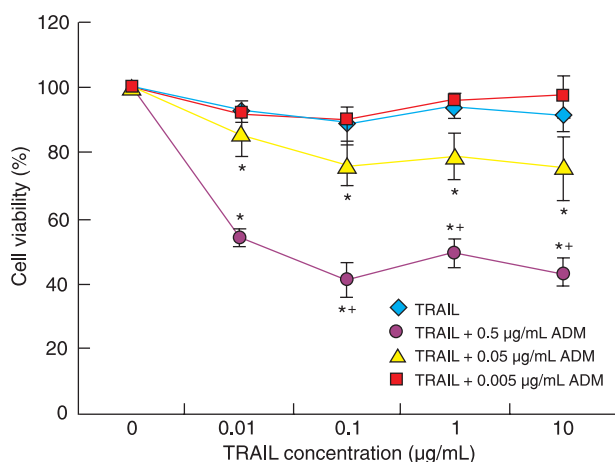
Twenty-four-hour exposure of MCF-7 cells to TRAIL at a range of concentrations from 0.001 to 10 µg/mL induced limited cell death, and there was no obvious correlation between cell viability and TRAIL concentration ( $r = -0.461$ ,  $P > 0.05$ ). IC<sub>50</sub> was 100 µg/mL (>10 µg/mL), suggesting that MCF-7 cells were relatively resistant to TRAIL (Figure 1A). Twenty-four-hour exposure of MCF-7 cells to ADM at a range of concentrations from 0.0005 to 50 µg/mL strongly induced cell death in a dose-dependent manner ( $r = -0.924$ ,  $P < 0.05$ ). IC<sub>50</sub> was 7.0 µg/mL, suggesting that MCF-7 cells were sensitive to ADM (Figure 1B).

### Effect of the combination of TRAIL and ADM on MCF-7 cell viability and analysis of the synergistic effect

Compared to the TRAIL alone group, cell viability



**Figure 1.** Effect of TRAIL or ADM alone on cell viability of human breast cancer MCF-7 cells. *Panel A*, TRAIL; *panel B*, ADM. MCF-7 cells were incubated with TRAIL (from 0 to 100 µg/mL) or ADM (from 0 to 50 µg/mL) for 24 h. Cell viability was evaluated by the MTT assay. TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; ADM = adriamycin; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Data are reported as means  $\pm$  SD of representative experiments. \* $P < 0.05$  compared to other concentrations of TRAIL; + $P < 0.05$  compared to 0, 0.0005, 0.005, 0.05 µg/mL ADM; # $P < 0.05$  compared to other concentrations of ADM (Student *t*-test).



**Figure 2.** Effect of the combination of TRAIL and ADM on cell viability of MCF-7 cells and analysis of its synergistic effect. MCF-7 cells were incubated with TRAIL (0.01, 0.1, 1, and 10 µg/mL) and ADM (0.005, 0.05, and 0.5 µg/mL) for 24 h. Cell viability was evaluated by the MTT assay. TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; ADM = adriamycin; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Data are reported as means  $\pm$  SD of representative experiments. The synergistic effect was analyzed by the Webb coefficient as described in Material and Methods. Synergism in drug interaction was indicated by observed cell viability of  $\leq 70\%$  of the predicted cell viability. \* $P < 0.05$  compared to the TRAIL alone group (Student *t*-test); +Synergistic effect.

showed no significant difference in the combination groups of 0.005 µg/mL ADM and 0.01, 0.1, 1, 10 µg/mL TRAIL, respectively ( $P > 0.05$ ). When 0.05 or 0.5 µg/mL ADM was combined with 0.01, 0.1, 1, and 10 µg/mL TRAIL, respectively, cell viability was significantly lower than that observed in TRAIL alone group ( $P < 0.05$ ). Webb coefficient analysis showed that 0.5 µg/mL ADM combined with 0.1, 1, 10 µg/mL TRAIL, respectively, had a synergistic cytotoxic effect ( $P < 0.05$ ), which was stronger in the group treated with the combination of 0.1 µg/mL TRAIL and 0.5 µg/mL ADM ( $P < 0.05$ ) (Figure 2).

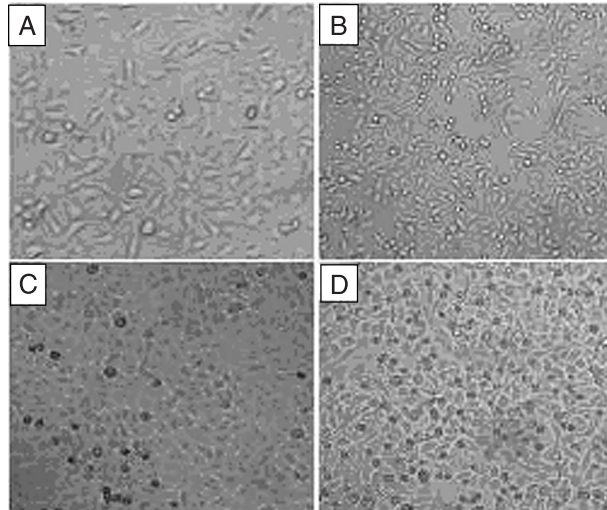
#### Morphologic changes of MCF-7 cells after treatment

After treatment with TRAIL for 24 h, some cells were floating and became rounder and smaller and refraction also

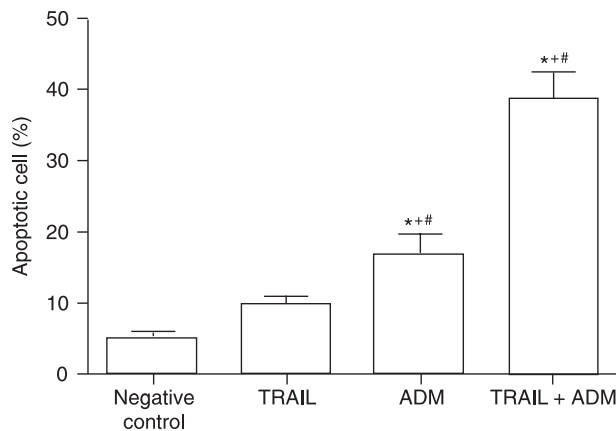
decreased; some cell debris was observed in the medium. The cells left on the wall became rounder and smaller (Figure 3B). In the ADM alone group, most cells were adhesive. The cells left on the wall underwent significant changes in morphology; the original shape was gone, cells became rounder and larger, and the cytoplasm became rougher (Figure 3C). In the combination group, the majority of cells were floating and had irregular cell walls and there were some cell debris in the medium (Figure 3D), whereas MCF-7 cells in the negative control group did not show obvious morphologic changes (Figure 3A).

#### Effect of TRAIL and ADM alone and of their combination on apoptosis in MCF-7 cells

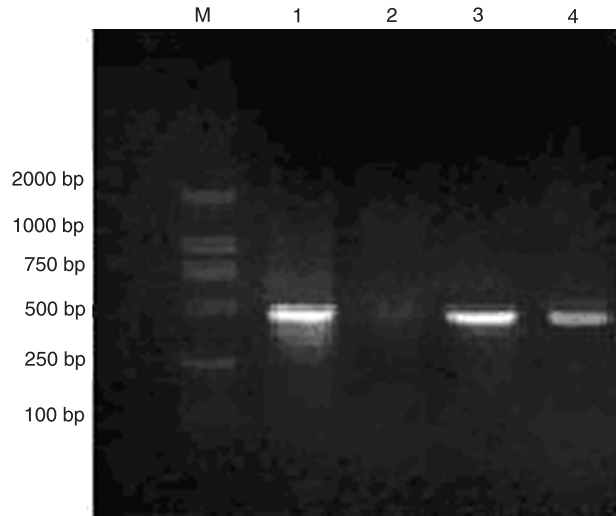
As shown in Figure 4, only 5.0% of untreated MCF-7 cells underwent apoptosis. Although the apoptotic cell ratio



**Figure 3.** Morphological changes of the human breast cancer cell line MCF-7 after treatment. MCF-7 cells were treated with TRAIL (0.1  $\mu\text{g}/\text{mL}$ ) and/or ADM (0.5  $\mu\text{g}/\text{mL}$ ) for 24 h and the morphological changes of cells were observed under an inverted microscope (200X). *Panel A*, Negative control group. *Panel B*, TRAIL alone group. Some cells were floating and became rounder and smaller and refraction also decreased. Some cell debris was observed in the medium. The cells left on the wall became rounder and smaller. *Panel C*, ADM alone group. The original shape was gone, cells became rounder and bigger, and the cytoplasm became rougher. *Panel D*, Combination group. The majority of cells were floating and had irregular cell walls and there was some cell debris in the medium. TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; ADM = adriamycin.



**Figure 4.** Effect of TRAIL or ADM alone and of their combination for 24 h on apoptosis of MCF-7 cells. Apoptosis was quantified using the annexin V-FITC and propidium iodide staining flow cytometry. The percentage of apoptotic cells was the average of three independent experiments. TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; ADM = adriamycin. \* $P < 0.05$  compared to negative control group; # $P < 0.05$  compared to the TRAIL alone group; ## $P < 0.05$  compared to the ADM alone group (Student *t*-test).



**Figure 5.** Expression of TRAIL mRNA receptors in MCF-7 cells. *Lane M*, DL2000; *lane 1*, DcR2 (453 bp); *lane 2*, DcR1; *lane 3*, DR5 (502 bp); *lane 4*, DR4 (506 bp). TRAIL = tumor necrosis factor-related apoptosis-inducing ligand.

was increased by TRAIL alone, it did not reach a significant level ( $P > 0.05$ ). ADM alone and the combination of TRAIL and ADM significantly increased the apoptotic rate ( $P < 0.05$ ), with the combined treatment having a stronger effect. In addition, the apoptotic rate in the combination group (38.7%) was greater than that induced by the sum of TRAIL and ADM alone (9.8 and 17%), suggesting that TRAIL combined with ADM had synergistic apoptotic effect on MCF-7 cells.

#### mRNA expression of TRAIL receptors in MCF-7 cells

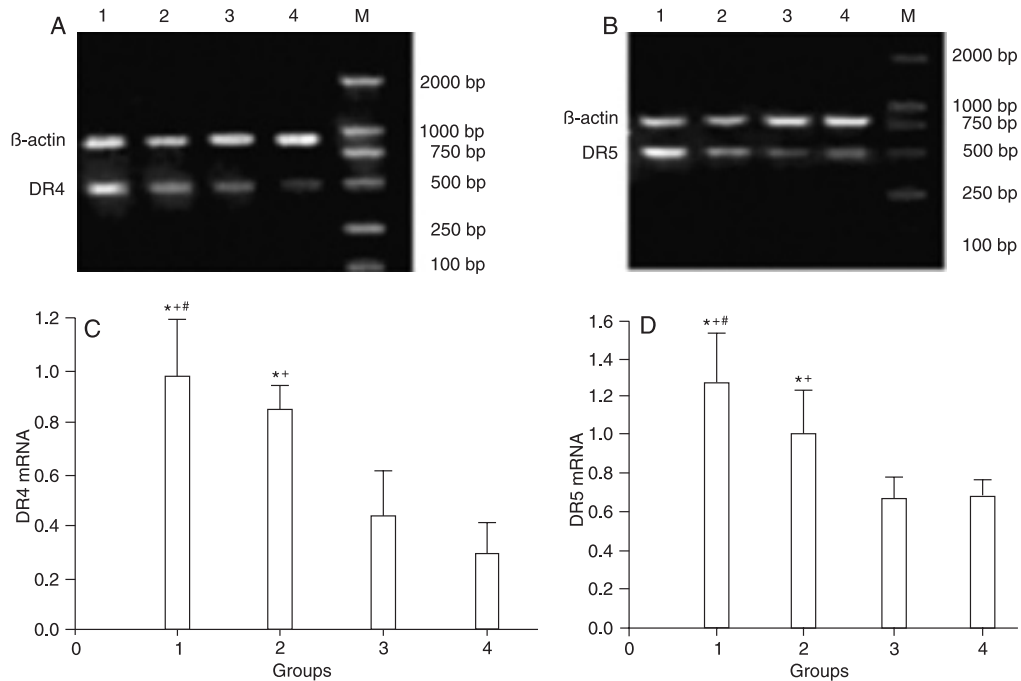
As shown in Figure 5, MCF-7 cells had higher DR5 and DcR2 mRNA expression but lower DR4 mRNA expression, whereas DcR1 mRNA could not be detected in MCF-7 cells.

#### Effect of TRAIL and/or ADM treatment on DR4 and DR5 mRNA in MCF-7 cells

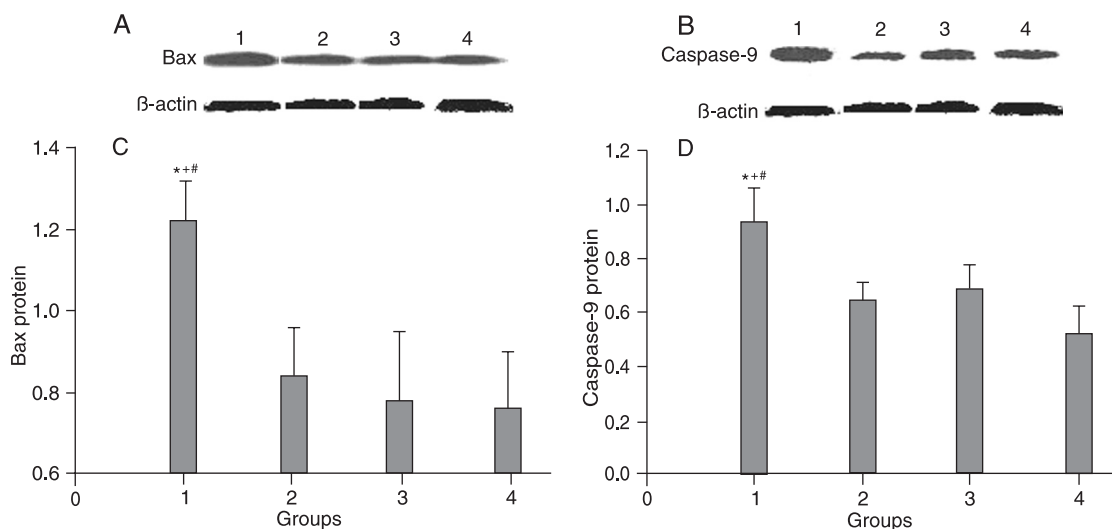
Compared with the other groups, the combination of TRAIL and ADM significantly up-regulated the expression of DR4 mRNA ( $P < 0.05$ ; Figure 6A and C) and DR5 mRNA ( $P < 0.05$ ; Figure 6B and D) in MCF-7 cells.

#### Effect of TRAIL and/or ADM treatment on Bax and caspase-9 protein levels in MCF-7 cells

Compared with the other groups, combined treatment with TRAIL and ADM significantly up-regulated the protein levels of Bax ( $P < 0.05$ ; Figure 7A and C) and caspase-9 ( $P < 0.05$ ; Figure 7B and D).



**Figure 6.** Effect of TRAIL and/or ADM on DR4 and DR5 mRNA expression in MCF-7 cells. *Panels A and C*, DR4 mRNA; *panels B and D*, DR5 mRNA. MCF-7 cells were treated with TRAIL (0.1  $\mu\text{g}/\text{mL}$ ) and/or ADM (0.5  $\mu\text{g}/\text{mL}$ ) for 24 h and the changes of DR4 and DR5 in mRNA levels were determined by RT-PCR.  $\beta$ -actin was used as internal control. *Lane M*, DL2000; *lane 1*, combination group; *lane 2*, ADM alone group; *lane 3*, TRAIL alone group; *lane 4*, negative control group. TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; ADM = adriamycin. \* $P < 0.05$  compared to negative control group; + $P < 0.05$  compared to the TRAIL alone group; # $P < 0.05$  compared to the ADM alone group (Student *t*-test).



**Figure 7.** Effect of TRAIL and/or ADM treatment on protein levels of Bax and caspase-9 in MCF-7 cells. *Panels A and C*, Bax; *panels B and D*, caspase-9. Cells were treated with TRAIL (0.1  $\mu\text{g}/\text{mL}$ ) or/and ADM (0.5  $\mu\text{g}/\text{mL}$ ) for 24 h and the expression of Bax and caspase-9 proteins was determined by Western blot analysis. The relative amount of each protein was compared to  $\beta$ -actin. TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; ADM = adriamycin. *Lane 1*, TRAIL plus ADM; *lane 2*, ADM alone; *lane 3*, TRAIL alone; *lane 4*, negative control. \* $P < 0.05$  compared to the negative control group; + $P < 0.05$  compared to the TRAIL alone group; # $P < 0.05$  compared to the ADM alone group (Student *t*-test).

## Discussion

TRAIL can induce apoptosis in a broad range of human cancer cell lines while sparing most normal cell types (3). Thus, TRAIL is a promising therapeutic agent against cancer because of its tumor selectivity (10,11). However, an increasing number of studies have demonstrated TRAIL resistance in primary human tumor cells, especially in those of solid tumor entities (12). In the present study, the data showed that human breast cancer MCF-7 cells were relatively resistant to TRAIL, but sensitive to ADM. The mechanism of TRAIL resistance remains unclear. Some results indicate that TRAIL resistance may be attributable to relatively low DR4 levels (13) or relatively high DcR2 expression (4). In this study, the results of RT-PCR showed that MCF-7 cells had higher DR5 and DcR2 expression but lower DR4 expression. In addition, DcR1 mRNA was absent in MCF-7 cells. We speculate that the mechanisms of MCF-7 cell resistance to TRAIL may be due to higher DcR2 expression and lower DR4 expression. However, the precise mechanism needs further study.

It has been demonstrated that subtoxic concentrations of chemotherapeutic drugs could restore the TRAIL-mediated pathway to death in cell lines that are resistant to TRAIL-induced cytotoxicity (8). In the present study, our data showed that the combination of TRAIL and ADM was superior to either therapy alone in inhibiting the survival of MCF cells. In addition, Webb coefficient analysis showed that a subtoxic concentration of ADM (0.5 µg/mL) combined with 0.1, 1, or 10 µg/mL TRAIL had a synergistic cytotoxic effect, which was stronger for the combination of 0.1 µg/mL TRAIL and 0.5 µg/mL ADM. Data reported by Ashkenazi et al. (14) showed that TRAIL cooperated synergistically with the chemotherapeutic drugs 5-fluorouracil or CPT-11, causing substantial tumor regression or complete tumor ablation, suggesting that the combination of an anticancer drug with TRAIL may enforce the TRAIL-based therapeutic strategy (8).

Apoptosis is one of the major processes that lead to cell death. Some studies have demonstrated that chemotherapeutic agents could induce apoptosis in malignant cells *in vitro* (15). Although TRAIL is capable of inducing apoptosis in tumor cells of diverse origin, unfortunately many tumors remain resistant to treatment with TRAIL (3,4,7,11,16,17). In the present study, the data showed that human breast cancer MCF-7 cells were relatively resistant to TRAIL. In addition, flow cytometry data showed that TRAIL alone could not induce significant apoptosis. Taken together, these results suggest that TRAIL alone may be ineffective for cancer therapy. Thus, it is important to develop strategies to overcome this resistance in tumor cells. Some recent reports have shown that subtoxic concentrations of chemotherapeutic drugs could sensitize tumor cells that are resistant to TRAIL (8). Several cytotoxic agents (cisplatin, paclitaxel, and ADM) administered in combina-

tion with TRAIL *in vitro* and *in vivo* enhanced apoptosis (18) and resulted in a synergistic apoptotic response in cancer cells (19). In the present study, the data showed that TRAIL or ADM alone could lead to 9.8 or 17% apoptosis, respectively, while their combination effectively increased cell apoptosis to 38.7%. These results suggest that TRAIL combined with subtoxic concentration of ADM had synergistic apoptotic effect on MCF-7 cells. In addition, these findings also demonstrated that synergy in cytotoxicity was paralleled by synergy in apoptosis. Therefore, we believe that the synergistic antitumor effect of the combination of TRAIL and ADM on MCF-7 cells is due to the induction of cellular apoptosis.

Apoptosis is controlled by multiple pathways that integrate both intra- and extracellular signals. TRAIL controls one such apoptotic pathway by binding to its two death receptors DR4 and DR5 (5,20). The extent of apoptosis induced by TRAIL is tightly regulated by the expression of these receptors and by downstream signaling. ADM, a DNA damaging agent, is accepted as a first-line chemotherapeutic agent for breast cancer. It has been demonstrated that DNA damaging chemotherapeutic drugs could sensitize tumor cells that are resistant to TRAIL by up-regulating the expressions of DR4 and DR5 in tumor cells (8,19,21). In this study, as determined by RT-PCR, the combined treatment with TRAIL and ADM increased both DR4 mRNA and DR5 mRNA in MCF-7 cells, suggesting that the combined treatment regulated DR4 and DR5 at the transcriptional level. In addition, some results also showed that combined treatment with DNA damaging agents and TRAIL could increase the levels of DR4 (17,19) and DR5 protein (19). Taken together, these findings indicate that the up-regulation of DR4 and DR5 enhanced the responsiveness of cells to TRAIL (22,23), an effect proposed to account for the synergistic apoptotic effect of TRAIL and chemotherapeutic drugs in several tumor cell lines (8,17).

It has been reported that the DR5 gene contains a p53 cis-element within the first intronic region (17). Similarly, an analogous p53 binding site has also been reported in the first intron of the DR4 gene (24). Some results have shown that the apoptotic effect of chemotherapeutic drugs and TRAIL may be p53 dependent (8). p53 promotes the expression of a number of genes that are involved in apoptosis, including those encoding death receptors and proapoptotic members of the Bcl-2 family such as Bax (25,26). Furthermore, Bax is essential for death receptor-mediated apoptosis in cancer cells (26). The results of LeBlanc et al. (27) have shown that HCT116 human colon carcinoma cells are completely dependent upon Bax for the induction of apoptosis by death receptor ligands. In most cases, p53-induced apoptosis proceeds through mitochondrial release of cytochrome c, which leads to caspase activation. Caspase-9, an essential downstream component of p53-induced apoptosis (27), is the major initiator caspase identified to date (28). Once activated, caspase-9 can in turn activate the effector



caspases (caspases-3, -6, -7) that finally dismantle the cell (23,26). Recently, some results have shown that TRAIL induces apoptosis in cancer cells in a caspase-dependent fashion (8,23,29) and anticancer drug-induced sensitization to TRAIL also depends on caspase activation (8). To explore the mechanisms of apoptosis induced by the combination of TRAIL and ADM, we assessed the protein expression of Bax and caspase-9. Consistent with its effect on apoptosis, the combined treatment with TRAIL and ADM significantly increased the protein levels of Bax and caspase-9. These data suggest that the apoptotic effect of combined treatment with TRAIL and ADM on MCF-7 cells may rely

on p53. Further investigation is required to determine the mechanisms of this combined therapy.

We showed here that human breast cancer MCF-7 cells were relatively resistant to TRAIL, and sensitive to ADM. Treatment with TRAIL plus subtoxic concentrations of ADM had a synergistic cytotoxic and apoptotic effect on MCF-7 cells. We propose that the synergistic effect was mediated by up-regulation of DR4 and DR5 mRNA expression and the increasing expression of Bax and caspase-9 proteins. These results raise the possibility that the combination of TRAIL and ADM might be a promising therapy for breast cancer.

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