

3,3'-diindolylmethane potentiates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of gastric cancer cells

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) specifically kills cancer cells without destroying the majority of healthy cells. However, numerous types of cancer cell, including gastric cancer cells, tend to be resistant to TRAIL. The bioactive product 3,3'-diindolylmethane (DIM), which is derived from cruciferous vegetables, is also currently recognized as a candidate anticancer agent. In the present study, a Cell Counting Kit 8 cell growth assay and an Annexin V-fluorescein isothiocyanate apoptosis assay were performed to investigate the potentiating effect of DIM on TRAIL-induced apoptosis in gastric cancer cells, and the possible mechanisms of this potentiation. The results obtained demonstrated that, compared with TRAIL or DIM treatment alone, co-treatment with TRAIL (25 or 50 ng/ml) and DIM (10 μ mol/l) induced cytotoxic and apoptotic effects in BGC-823 and SGC-7901 gastric cancer cells. Furthermore, western blot analysis revealed that the protein expression levels of death receptor 5 (DR5), CCAAT/enhancer binding protein homologous protein (CHOP) and glucose-regulated protein 78 (GRP78) were upregulated in the co-treated gastric cancer cells. To the best of our knowledge, the present study is the first to provide evidence that DIM sensitizes TRAIL-induced inhibition of proliferation and apoptosis in gastric cancer cells, accompanied by the upregulated expression of DR5, CHOP and GRP78 proteins, which may be involved in endoplasmic reticulum stress mechanisms.

Introduction

Despite advancements in the prevention and treatment through multimodal approaches, including targeted therapies, gastric cancer is one of the most common types of malignant tumor and remains the second leading cause of carcinoma-associated mortality worldwide (1).

Few agents exist that are truly cancer cell specific with regards to the induction of cell death. For example, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an agent that is preferentially cytotoxic to cancer cells over healthy cells (2). However, the effectiveness of TRAIL is significantly impeded by drug resistance, resulting in poor survival outcomes of patients with cancer (3,4). Emerging evidence provides support for the potential anticancer effect of bioactive products derived from cruciferous vegetables, such as brussels sprouts, broccoli, cabbage and cauliflower (5). Among these compounds, 3,3'-diindolylmethane (DIM) is generated in the acidic environment of the stomach via dimerization of the indole-3-carbinol monomers present in these vegetables (6).

A number of cellular stress conditions, including nutrient deprivation, hypoxia and treatment with a variety of pharmacological agents which inhibit glycosylation or deplete endoplasmic reticulum (ER) calcium stores, may lead to the accumulation and aggregation of unfolded and/or misfolded proteins in the ER lumen, which is termed ER stress. Notably, ER-induced apoptotic cell death has been identified as an important apoptotic pathway (7). Various mechanisms have been hypothesized to exhibit an important role in ER stress-induced apoptosis; the C/enhancer binding protein homologous protein (CHOP), glucose-regulated protein 78 (GRP78) and caspase-3, -9 and -12 are all considered to be involved in the apoptotic signaling pathway which occurs in response to ER stress (8). In addition, it has been demonstrated that effective TRAIL-based combination therapy can be achieved by upregulating death receptor 5 (DR5) expression (9). Furthermore, CHOP has been reported to directly regulate DR5 expression in human carcinoma cells (10).

The aim of the present study was to explore whether DIM potentiates TRAIL-induced apoptosis of gastric cancer cells and investigate the possible mechanisms of this process.

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Materials and methods

Cell culture. The human gastric cancer cell lines BGC-823 and SGC-7901 were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (Tianhang Biological Technology Co., Ltd., Hangzhou, China). All cells were maintained in a 5% CO₂ atmosphere at a temperature of 37°C.

Cell growth assay. To determine cell growth, a colorimetric water-soluble tetrazolium salt assay (Cell Counting Kit 8; Dojindo Laboratories, Kumamoto, Japan) was performed. This allowed the number of viable cells to be evaluated following treatment with various agent combinations.

Apoptosis assay. The detection of apoptotic cells was performed using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Beyotime Institute of Biotechnology, Nantong, China), according to the manufacturer's instructions. Cells were plated in 6-well plate (5 × 10⁵ cells/well) and allowed to grow to 75–80% confluence. Following treatment with DIM (10 µmol/l), TRAIL (25 or 50 ng/ml) or DIM (10 µmol/l) + TRAIL (25 ng/ml) for 24 h, the cells were collected, resuspended in 500 µl binding buffer, and 5 µl Annexin V-FITC and 5 µl propidium iodide were added. Untreated cells were washed twice with phosphate-buffered saline (Beyotime Institute of Biotechnology), collected, resuspended in 500 µl binding buffer, and 5 µl Annexin V-FITC and 5 µl propidium iodide were added, which served as the control. The data was quantified and analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. Western blot analysis was conducted as previously described (11). Briefly, cells were lysed by incubating in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS; pH 7.4; Beyotime Institute of Biotechnology) for 20 min at 4°C and the protein concentration was determined using the Beyotime assay system (Beyotime Institute of Biotechnology). Protein (60 µg) was run on 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK). Next, the membrane was blocked with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% nonfat milk for 2 h at room temperature and incubated overnight at 4°C with primary monoclonal antibodies against DR5 (cat. no. sc-166624), DR4 (cat. no. sc-8411), CCAAT/CHOP (cat. no. sc-7351), GRP78 (cat. no. sc-376768) and GAPDH (cat. no. sc-365062) purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The membrane was washed three times with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (cat. no. A0216, Beyotime Institute of Biotechnology) for 1 h at room temperature. Immunoreactive bands were subsequently identified using LumiGLO® chemiluminescent substrate (Cell Signaling Technology, Inc.). The light emitted by destabilized LumiGLO® reagent was subsequently captured on X-ray film, (Cell Signaling Technology, Inc.), normalized to the corresponding GAPDH level and analyzed

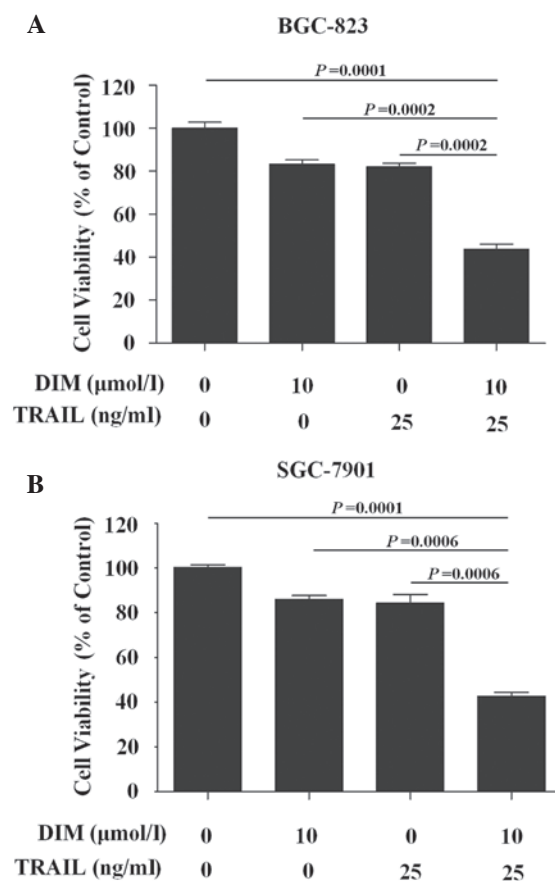


Figure 1. Effect of treatment with DIM or TRAIL alone or in combination, on the cytotoxicity of (A) BGC-823 and (B) SGC-7901 cells, as determined by a colorimetric water-soluble tetrazolium salt assay. DIM significantly sensitizes TRAIL-induced cytotoxicity of gastric cancer cells. Error bars indicate the standard deviation from the mean. DIM, 3,3'-diindolylmethane; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

with ImageJ software (version 1.44; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The SPSS software package (version 15.0; SPSS, Inc, Chicago, IL, USA) was used for data analysis, with data presented as the mean ± standard deviation (SD) and n indicating the number of independent experiments performed. After determining equal variance, comparisons among the means of multiple groups were performed using one-way analysis of variance and Fisher's least significant difference test. Additionally, two-tailed independent samples *t*-tests were used when appropriate. *P* < 0.05 was considered to indicate a statistically significant difference.

Results

DIM sensitizes gastric cancer cells to TRAIL-induced cytotoxicity. The present study examined the role of TRAIL and DIM treatment, alone or in combination, on the proliferation of human gastric cancer cell lines BGC-823 and SGC-7901. The treatment of gastric cancer cells with 10 µmol/l DIM for 24 h induced marginal cytotoxicity (<20%). Similarly, limited cytotoxicity (20%) was observed following treatment with TRAIL at concentrations of 25–50 ng/ml. However, compared to treatment with TRAIL or DIM alone, significant cytotoxic effects were induced

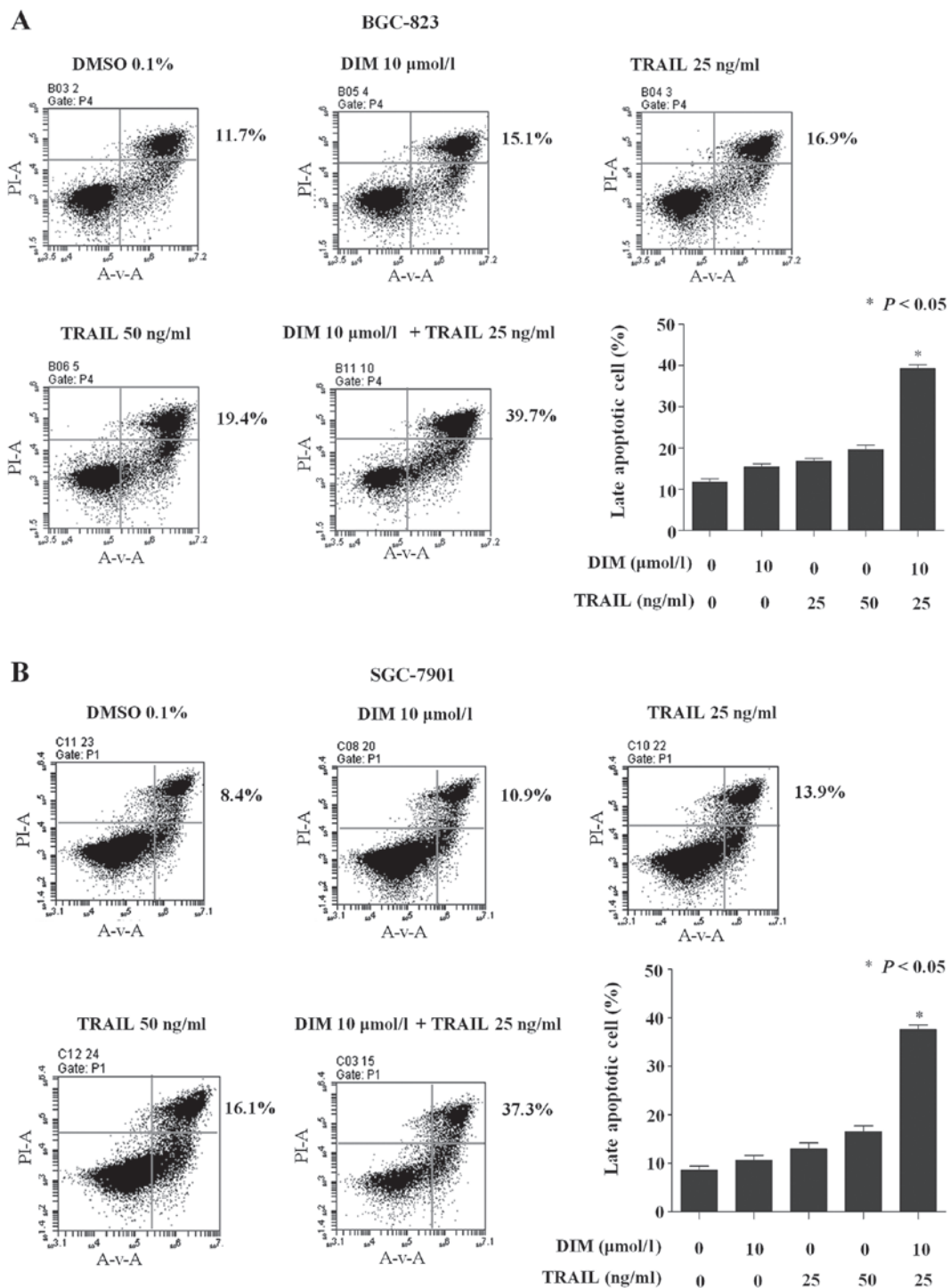


Figure 2. Effects of treatment with DIM or TRAIL alone or in combination, on the apoptosis of (A) BGC-823 and (B) SGC-7901 cells as determined by Annexin V-fluorescein isothiocyanate and PI staining. DIM significantly enhanced TRAIL-induced apoptosis of gastric cancer cells Error bars indicate the standard deviation from the mean. DMSO, dimethyl sulfoxide; DIM, 3,3'-diindolylmethane; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PI, propidium iodide.

following co-treatment of the two gastric cancer cell lines with TRAIL (25 ng/ml) and DIM (10 $\mu\text{mol/l}$), compared with control ($P=0.0001$ in BGC-823, $P=0.0001$ in SGC-7901), TRAIL ($P=0.0002$ in BGC-823, $P=0.0006$ in SGC-7901) or DIM alone ($P=0.0002$ in BGC-823, $P=0.0006$ in SGC-7901 (Fig. 1).

DIM enhances TRAIL-induced apoptosis in gastric cancer cells. To investigate whether apoptosis is correlated with the

inhibition of cell proliferation following DIM and TRAIL co-treatment, flow cytometric analysis was used to detect the increase in hypodiploid cell populations. The mean ($n=3$) proportions of late apoptotic BGC-823 cells were 15.1, 16.9, 19.4 and 39.7% for DIM (10 $\mu\text{mol/l}$), TRAIL (25 ng/ml), TRAIL (50 ng/ml), and TRAIL (25 ng/ml) plus DIM (10 $\mu\text{mol/l}$), respectively (Fig. 2A). Similarly, in SGC-7901 cells, the mean ($n=3$) proportions were 10.9, 13.9, 16.1 and 37.3% for

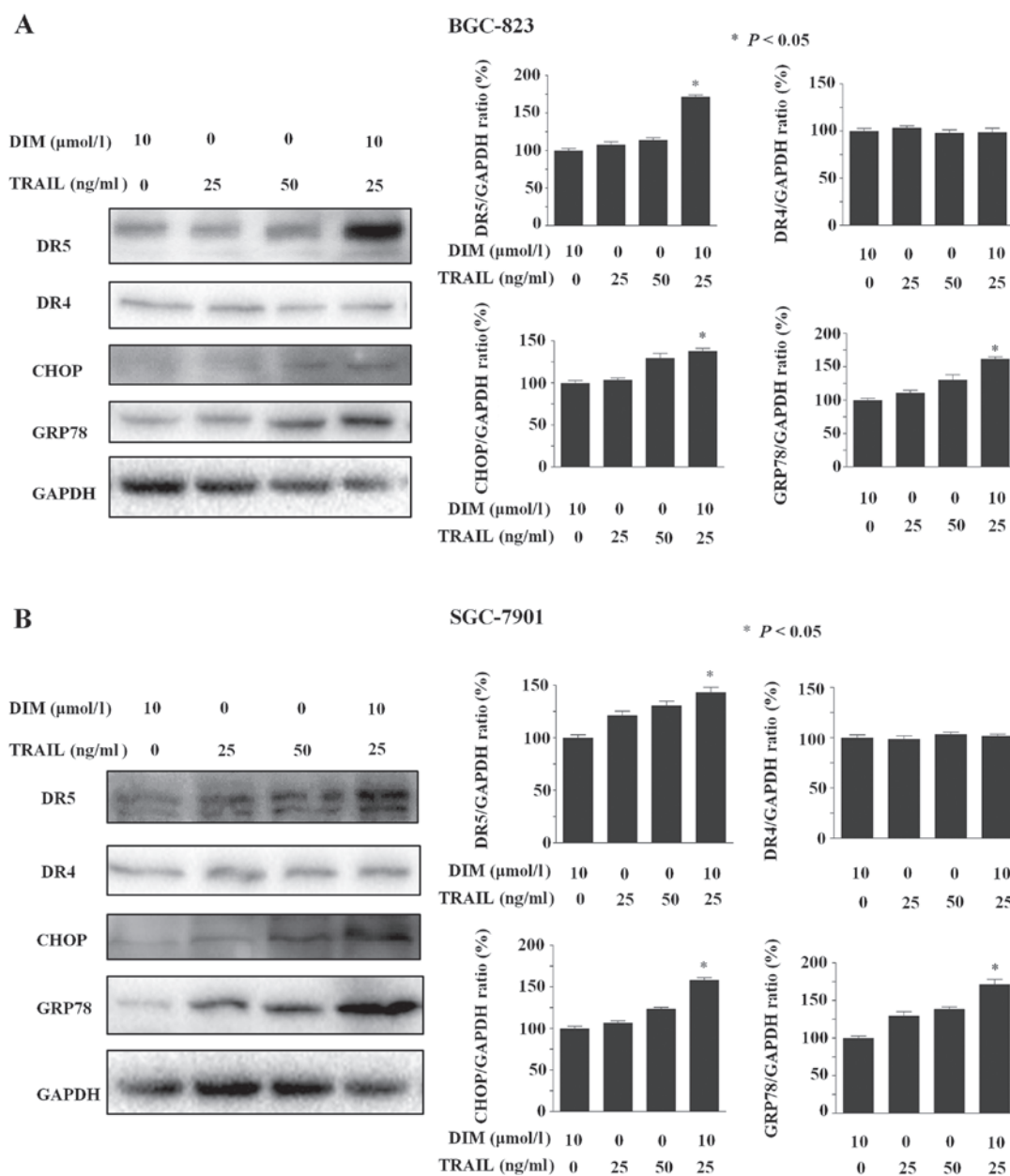


Figure 3. Effect of various DIM/TRAIL treatment strategies on the expression levels of DR5, DR4, CHOP and GRP78 in (A) BGC-823 and (B) SGC-7901 cells using western blot analysis. DIM plus TRAIL treatment significantly increased the protein expression levels of DR5, CHOP and GRP78 in gastric cancer cells. Error bars indicate the standard deviation from the mean. DIM, 3,3'-diindolylmethane; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR4/5, death receptor 4/5; CHOP, CCAAT/enhancer binding protein homologous protein; GRP78, glucose-regulated protein 78.

DIM (10 $\mu\text{mol/l}$), TRAIL (25 ng/ml), TRAIL (50 ng/ml), and TRAIL (25 ng/ml) plus DIM (10 $\mu\text{mol/l}$) (Fig. 2B). These results indicate that DIM sensitizes gastric cancer cells to TRAIL-induced apoptosis.

DIM plus TRAIL treatment induces the expression of DR5, CHOP and GRP78 proteins in gastric cancer cells. BGC-823 cells were treated with DIM for 24 h and subsequently analyzed by western blot to investigate the expression levels of various TRAIL receptor-associated proteins. The group of cells treated with TRAIL plus DIM were demonstrated to significantly induce the protein expression levels of DR5 ($P < 0.001$) but exhibited no effect on DR4 expression. This data indicates that DR5 may be involved in DIM enhancement of the apoptotic effects of TRAIL in BGC-823 cells. Significant upregulation of

DR5 by DIM plus TRAIL was also identified in SGC-7901 cells ($P < 0.05$). Furthermore, DIM and TRAIL co-treatment was observed to induce a significant increase in the protein expression levels of CHOP and GRP78 ($P > 0.05$; Fig. 3), two proteins that accumulate during endoplasmic reticulum (ER) stress (12).

Discussion

In the present study, the potential anticancer effect of DIM, a bioactive product derived from cruciferous vegetables, in regulating TRAIL signaling was explored in two gastric cancer cell lines. DIM was demonstrated to potentiate TRAIL-induced apoptosis in the gastric cancer cell lines BGC-823 and SGC-7901, as well as inducing increased DR5, CHOP and GRP78 protein expression levels.

Cytotoxic and apoptotic effects were observed when gastric cancer cells were treated with DIM or TRAIL alone; these results are consistent with a number of previous studies on various types of cancer, including gastric cancer (13-15). However, to the best of our knowledge, the present study is the first to demonstrate that DIM sensitizes TRAIL-induced cytotoxicity and apoptosis in gastric cancer cells. As the widespread application of TRAIL has been limited due to increasing drug resistance and cost (3,4), the results of the current study indicate that the use of low-cost DIM may enhance the clinical application of TRAIL-based therapy for various types of cancer in the future.

El-Deiry (9) previously reported that effective TRAIL-based combination therapy may be achieved by the upregulation of DR4 and DR5. Furthermore, DIM appears to induce apoptosis in pancreatic cancer cells via ER stress-dependent upregulation of DR5 (16). Based on this data, we hypothesized that DIM potentiates TRAIL-induced apoptosis of gastric cancer cells by upregulating DR5 expression. In the current study, DIM and TRAIL co-treatment appeared to induce DR5 expression, confirming this hypothesis.

CHOP has been reported to directly regulate DR5 expression in human carcinoma cells (10,17). For example, specific agents, such as dimethyl-celecoxib and 5,7-dimethoxyflavone, have been shown to induce the expression of DR5 via CHOP-dependent DR5 gene transactivation (18,19). Furthermore, previous studies have identified a close association between DR5 expression and ER stress (10,17). In the ER lumen, ER stress is activated when unfolded proteins accumulate (20). Through this response, specific apoptotic pathways may be activated to clear severely damaged cells in which the defects of protein folding cannot be resolved (12,21). Although the molecular mechanisms by which ER stress inducers regulate DR5 expression may vary between cell types, CHOP, an ER stress-inducible transcription factor, provides a common association between ER stress and DR5 expression. In the present study, treatment of gastric cancer cells with DIM plus TRAIL was demonstrated to induce the expression of CHOP and GRP78, two proteins that also accumulate during ER stress (12). The identification of CHOP induction by DIM indicates that DIM may trigger ER stress and increase the expression levels of GRP78, although this mechanism has yet to be fully clarified.

In conclusion, to the best of knowledge, the present study demonstrates for the first time that DIM sensitizes TRAIL-induced cytotoxicity and apoptosis in gastric cancer cells, accompanied by the upregulation of DR5, CHOP and GRP78 protein expression levels. We propose that this process of sensitization may involve ER stress mechanisms. The results of the current study highlight the possibility of producing less costly anticancer agents in the future, and the use of low-cost DIM may enhance the clinical application of TRAIL-based therapy for various types of cancer. Thus, the results of this study indicate that future studies, which investigate the anticancer effects of other active components derived from the diet or Traditional Chinese medicine may be of value.

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