

Down-regulation of c-FLIP contributes to the sensitization effect of 3,3'-diindolylmethane on TRAIL-induced apoptosis in cancer cells

Siyuan Zhang, Han-Ming Shen,
and Choon Nam Ong

Department of Community, Occupational and Family Medicine,
Faculty of Medicine, National University of Singapore, Singapore,
Republic of Singapore

Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor superfamily, which has been shown to preferentially induce apoptosis in cancer cells without adverse effects on normal cells. However, there are still some cancer cells, especially those with high malignancy, resistant to TRAIL-induced apoptosis, impeding the clinical anticancer efficiency of TRAIL. In this report, we showed that 3,3'-diindolylmethane, an indole compound derived from cruciferous vegetables, is capable of overcoming TRAIL resistance by sensitizing TRAIL-induced apoptosis in human cancer cells. Noncytotoxic concentrations of 3,3'-diindolylmethane significantly enhanced TRAIL-resistant cancer cells to TRAIL-induced apoptosis via promoting the caspase cascade, a process independent of nuclear factor- κ B activation and cell surface TRAIL receptor expression. In the search of the molecular mechanisms involved in the sensitization activity of 3,3'-diindolylmethane, we found that combined treatment of 3,3'-diindolylmethane and TRAIL led to significant down-regulation of the cellular FLICE inhibitory protein expression (c-FLIP). Furthermore, we provided evidence showing that the reduced c-FLIP level is predominately mediated by the ubiquitin-proteasome degradation system. These findings reveal a novel anticancer property of 3,3'-diindolylmethane and suggest that this compound could have potential use in cancer therapy to overcome TRAIL resistance. [Mol Cancer Ther 2005;4(12):1972–81]

Introduction

Apoptosis is a highly regulated cell death event involved in many physiologic and pathologic processes. Aberrant regulation of the apoptosis process has been implied in many human diseases, including cancer. Failure of triggering apoptosis process results in not only malignancies but also resistance of cancer cells to chemotherapy (1). Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a recently identified cytokine belonging to the TNF superfamily. Four transmembrane receptors for TRAIL have been identified, including TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), and TRAIL-R4 (DcR2). DR4 and DR5 are typical cell death receptors with death effector domain on cytoplasmic tail of the receptors. On the ligation with TRAIL protein, the trimerized TRAIL cell death receptors (DR4 or DR5) recruit Fas-associated death domain protein and caspase-8 via their death domains and form a death-inducing signaling complex, which results in the autoactivation of caspase-8 (2–4). DcR1 and DcR2 are decoy receptors lacking death domains and competitively bind to TRAIL and antagonize the apoptosis-inducing capability of TRAIL (3, 4). Recently, TRAIL has triggered a great deal of interest and has been considered as a promising candidate for cancer chemotherapy. Compared with other apoptosis-inducing factors, such as TNF and Fas ligand, compelling evidence has suggested that recombinant TRAIL protein preferentially induces apoptosis in malignant cancer cells without adverse effects to normal tissues or cells, making TRAIL a safer alternative for TNF and Fas ligand (5, 6). However, not all the cancer cells respond well to TRAIL and some cancer cells are TRAIL resistant (7). The mechanisms of TRAIL resistance may include (a) unfavorable ratio of death receptors (DR4/DR5) to decoy receptors (DcR1/DcR2), (b) activation of anti-apoptotic signaling [nuclear factor- κ B (NF- κ B), Akt, etc.], and (c) high expression of inhibitors of apoptosis (IAP), including cellular FLICE inhibitory protein (c-FLIP), cellular IAP proteins (c-IAP), X-linked IAP (XIAP), etc. (8).

3,3'-Diindolylmethane is an acid-catalyzed self-condensation metabolite of indole-3-carbinol (I3C), an indole compound found in many cruciferous vegetables, which has been found to possess strong anticancer property (9). Although 3,3'-diindolylmethane has been reported to induce apoptosis in breast and prostate cancer cells (10, 11), the anticancer potential of this compound is relatively less studied. Recently, I3C, the parent compound of 3,3'-diindolylmethane, has been reported to sensitize TRAIL-induced apoptosis in breast cancer cells via up-regulation of membrane cell death receptors DR4 and DR5 (12). In the present study, we found that 3,3'-diindolylmethane is highly efficient in sensitizing TRAIL-induced apoptosis in

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Requests for reprints: Choon Nam Ong, Department of Community, Occupational and Family Medicine, Faculty of Medicine, National University of Singapore, 16 Medical Drive, Singapore 117597, Singapore. Phone: 65-6874-4982; Fax: 65-6779-1489. E-mail: cofongcn@nus.edu.sg

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TRAIL-resistant cancer cells. Different from the effect of I3C, 3,3'-diindolylmethane promotes TRAIL-induced apoptosis independent of TRAIL receptors expression but rather via enhancement of ubiquitination and consequent proteasome degradation of apoptosis inhibitory protein c-FLIP. These findings thus reveal a novel anticancer property of 3,3'-diindolylmethane and support the synergistic application of 3,3'-diindolylmethane in cancer therapy to overcome TRAIL resistance.

Materials and Methods

Chemicals and Reagents

3,3'-Diindolylmethane was purchased from LKT Laboratories (St. Paul, MN). Human recombinant TRAIL (carrier free) was from R&D Systems (Minneapolis, MN) and was dissolved in 0.1% bovine serum albumin as stock solution (50 $\mu\text{g}/\text{mL}$). 4',6-Diamidino-2-phenylindole (DAPI) was from Molecular Probes (Eugene, OR). The pancaspase inhibitor Z-VAD-FMK, the caspase-8 inhibitor Z-IETD-CHO, and the caspase-3 inhibitor Z-DEVD-CHO were from Biomol (Plymouth Meeting, PA). The proteasome inhibitors (MG132, ALLN, and epoxomicin) were all from Calbiochem (San Diego, CA). Trizol RNA extraction kit and LipofectAMINE 2000 transfection reagent were from Invitrogen (Carlsbad, CA). Anti-caspase-3, anti-Bcl-2, anti-Bcl-xL, and anti-Mcl-1 antibodies were from Cell Signaling (Beverly, MA). Anti-caspase-8 and anti-c-FLIP-L antibodies were from Calbiochem. The anti-XIAP and anti-poly(ADP-ribose) polymerase antibody were from BD PharMingen (Los Angeles, CA). Anti-DR4, anti-DcR1, anti-DcR2, anti-c-IAP-1, anti-c-IAP-2, and anti-ubiquitin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-DR5 antibody was from Chemicon (Temecula, CA). Anti-hemagglutinin (HA)-peroxidase high-affinity (3F10) antibody was from Roche Diagnostics (Indianapolis, IN). Anti-FLAG antibody and anti-FLAG M2 agarose were purchased from Sigma-Aldrich (Singapore). The secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG and rabbit anti-goat IgG) and the enhanced chemiluminescence substrate were from Pierce (Rockford, IL). All the common chemicals were from Sigma-Aldrich.

Cell Culture and Treatments

Human liver cancer cells (HepG2), human cervical cancer cells (HeLa), and human colorectal cancer cells (HT29 and HCT116) were from the American Type Culture Collection (Manassas, VA). HeLa and HepG2 were maintained in DMEM (Sigma-Aldrich) with 10% fetal bovine serum (Hyclone, Logan, UT). HT29 and HCT116 cells were cultured in the McCoy's 5A medium with 10% fetal bovine serum. 3,3'-Diindolylmethane was dissolved in DMSO at 100 mmol/L as stock solution, and human recombinant TRAIL was prepared in PBS (contain 0.1% bovine serum albumin) at 50 $\mu\text{g}/\text{mL}$ and then further diluted with 0.1% bovine serum albumin-PBS for specific treatment.

Detection of Cytotoxicity and Apoptosis

The general cytotoxicity of 3,3'-diindolylmethane and TRAIL on different human cancer cells was detected by

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test as described previously (13) and the results were presented as the relative cell viability compared with the control group. Apoptotic cells with typical morphologic changes and chromatin condensation were examined by DAPI staining (14). Briefly, at the end of designated experiments, the cells were fixed with 70% ethanol at room temperature for 10 minutes and stained with 300 $\mu\text{g}/\text{mL}$ DAPI (in PBS) at room temperature for another 10 minutes. The cells with condensed nucleus were visualized and counted under an inverted fluorescent microscope (Nikon ECLIPSE TE2000-S, Nikon Instruments, Tokyo, Japan).

Plasmids, Transient Transfection, and Reporter Assay

pNF- κ B-luc and pTAL-luc control vectors were from Clontech (Palo Alto, CA). The synthetic *Renilla* luciferase reporter vector (phRL-TK) was from Promega (Madison, WI). CrmA expression vector was kindly provided by Dr. Z.G. Liu (National Cancer Institute, NIH). FLAG-FLIP-L expression vector was provided by Dr. Jurg Tschopp (University of Lausanne, Lausanne, Switzerland). HA-ubiquitin vector was kindly provided by Dr. Takahashi (RIKEN Brain Science Institute, Saitama, Japan).

The transient transfection of pNF- κ B-luc and pTAL-luc was done in HepG2 cells using LipofectAMINE 2000 transfection reagent according to the manufacturer's protocols. The *Renilla* luciferase vector, which acts as an internal control, was also cotransfected with the above vectors. The luciferase activity was measured in the cellular extracts using a Dual-Luciferase Reporter Assay System (Promega) based on the protocol provided by the manufacturer. Briefly, following the treatments, the cell lysate was collected from each well after the addition of $1\times$ cell lysis reagent (50 $\mu\text{L}/\text{well}$ in 24-well plate). After adding the luciferase assay substrate, the firefly luciferase activity (relative light units) was determined using a luminometer (Lumi-One, Trans Orchid, Tampa, FL) for a total period of 10 seconds after a 5-second delay time. The *Renilla* luciferase activity was then measured for another 10 seconds by adding in 100 μL Stop&Glo substrate.

For antiapoptotic protein overexpression experiments, cells were transiently transfected with pcDNA, FLAG-FLIP-L, FLAG-XIAP, or CrmA expression vectors, respectively, using the LipofectAMINE 2000 transfection reagent. A red fluorescence protein expression vector (pDsRed, Clontech) was cotransfected as a transfection marker. After 24 hours of transfection, the cells were pretreated with 3,3'-diindolylmethane (20 $\mu\text{mol}/\text{L} \times 1$ hour) followed by TRAIL (5 ng/mL $\times 12$ hours). Cell death was determined by morphologic changes examined under an inverted fluorescent microscope.

RNA Extraction and Reverse Transcription-PCR

RNA extraction was carried out using a total RNA extraction kit (Trizol) following the instructions from the manufacturer. Total RNA (5 μg) from each sample was subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase (Promega). For PCR, the amplification reaction was carried with 200 pmol of each primer, 200 $\mu\text{mol}/\text{L}$ of each deoxynucleotide triphosphate,

and 0.5 units Taq DNA polymerase II (Promega). The PCR conditions were optimized to achieve exponential amplification in which the PCR product formation is proportional to the starting cDNA. The reverse transcription-PCR primers used for DR5, DR4, DcR1, and DcR2 were as reported previously (15). Other primers used were (a) c-FLIP-L sense 5'-CTTGGCCAATTTGCCTGTAT-3' and antisense 5'-GG-CAGAAACTCTGCTGTTCC-3', (b) XIAP sense 5'-GAA-GACCTTGGGAACAACA-3' and antisense 5'-CGCCTTA-GCTGCTCTTCAGT-3', and (c) GAPDH sense 5'-AAGGT-GAAGGTCGGAGTCAA-3' and antisense 5'-AAGCAGTT-GGTGGTCAGGA-3'. PCR products were size fractionated using 1.0% agarose gel and visualized by ethidium bromide staining.

Immunoprecipitation and Western Blotting

For the assay of protein ubiquitination, cells were first lysed with a buffer containing 1% Triton X-100, 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, and protease inhibitor cocktail for 1 hour followed by centrifugation. Equal amount of proteins were immunoprecipitated with anti-FLAG M2 coupled agarose beads (Sigma-Aldrich) for at least 4 hours at 4°C. The beads were washed with cell lysis buffer four times and then subjected to Western blot. For this, cells were lysed by M2 cell lysis buffer [20 mmol/L Tris (pH 7.0), 0.5% NP40, 250 mmol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA, 2 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L β -glycerophosphate, 1 mmol/L sodium vanadate, and protease inhibitor cocktail] or Western cell lysis buffer [1% Triton X-100, 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, and protease inhibitor cocktail]. Equal amounts of protein were loaded with SDS sample buffer and separated using SDS-polyacrylamide gel (Bio-Rad PROTEAN III System) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). After blocking with 5% nonfat milk in TBST [10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween 20], the membrane was probed with designated first and second antibodies, developed with enhanced chemiluminescence method (Pierce), and visualized by Kodak Image Station 440CF (Rochester, NY).

Statistical Analysis

The data are presented as means \pm SD with at least three sets of independent experiments. The differences among different groups were examined using a Student's *t* test and *P* < 0.05 was considered statistically significant.

Results

3,3'-Diindolylmethane Sensitizes Cancer Cells to TRAIL-Induced Apoptosis

Previous studies have reported that 3,3'-diindolylmethane can directly induce apoptosis in human cancer cells at relative high concentrations (10, 11, 16). Here, we first examined the cytotoxicity of 3,3'-diindolylmethane in different cancer cell lines, including human hepatocarcinoma cells (HepG2), human cervical cancer cells (HeLa), and human colorectal cancer cells (HT29 and HCT116). As shown in Fig. 1A, after treating the cells with different

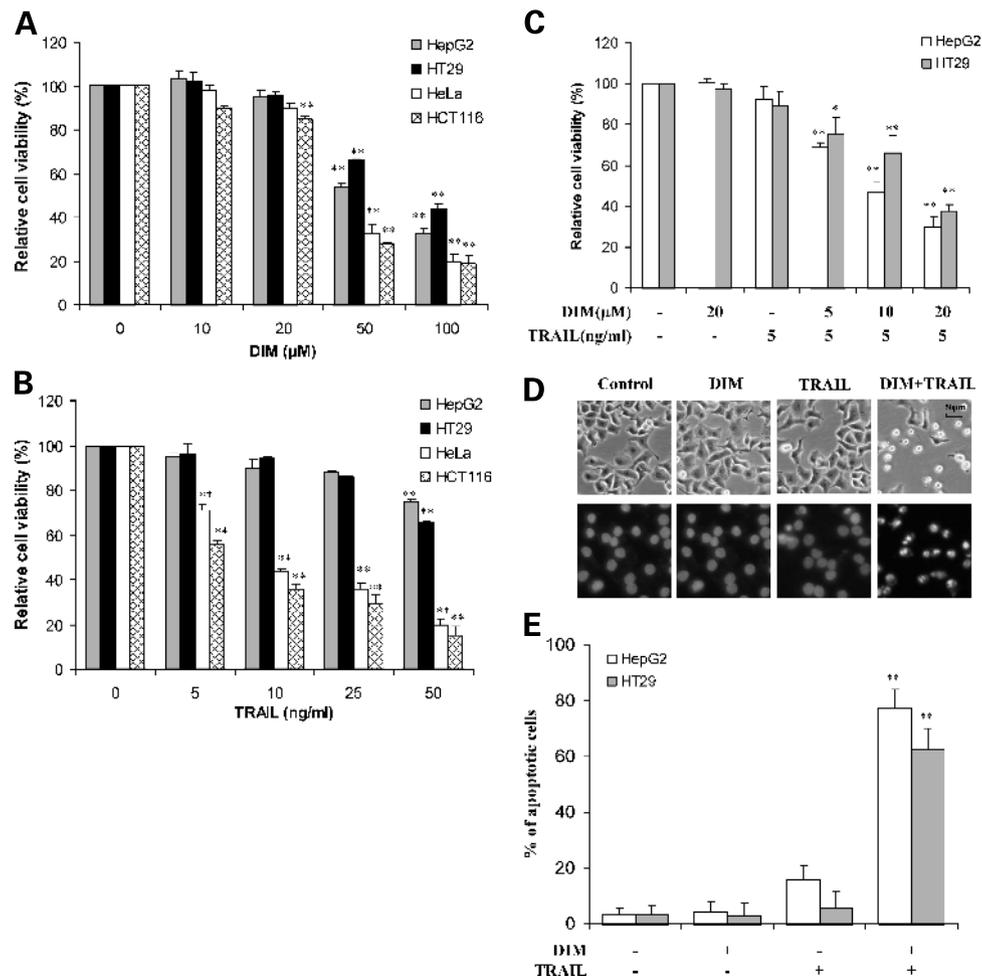
concentrations of 3,3'-diindolylmethane (5–100 μ mol/L) for 24 hours, inhibition of cell viability was only observed in the cells treated with relative high concentrations of 3,3'-diindolylmethane (\geq 50 μ mol/L). Treatment with 3,3'-diindolylmethane at \leq 20 μ mol/L concentration showed no or minor cytotoxicity to all the four cell lines tested. Next, we evaluated the cytotoxicity of human recombinant TRAIL on different cancer cell lines. As shown in Fig. 1B, the four cell lines tested showed different sensitivity to TRAIL-induced cell death. HeLa and HCT116 cells were more sensitive, as TRAIL (5 ng/mL) significantly reduced cell viability after 12 hours of treatment, whereas only a limited cell death was observed in HepG2 and HT29 cells even with the highest concentration of TRAIL (50 ng/mL). To test the synergistic effect of 3,3'-diindolylmethane and TRAIL, the two TRAIL-resistant cell lines (HepG2 and HT29) were pretreated with low and moderate concentrations of 3,3'-diindolylmethane (up to 20 μ mol/L) for 1 hour followed by a low concentration of TRAIL (5 ng/mL). As shown in Fig. 1C, 3,3'-diindolylmethane pretreatment markedly enhanced TRAIL-induced cytotoxicity in a concentration-dependent manner.

The nature of cell death induced by combined treatment of 3,3'-diindolylmethane and TRAIL was further examined by various methods for detection of apoptosis. Here, we used DAPI staining for evaluating the characteristic nuclear condensation of apoptotic cell death. As shown in Fig. 1D, after combined treatment of 3,3'-diindolylmethane (20 μ mol/L) and TRAIL (5 ng/mL), HepG2 cells rapidly underwent apoptosis as evidenced by significant morphologic changes and chromatin condensation. Similar sensitization activity of 3,3'-diindolylmethane was also observed in HT29 cells (data not shown). The quantification of apoptotic cells was summarized in Fig. 1E. Data suggested that 3,3'-diindolylmethane pretreatment greatly sensitized TRAIL-induced apoptosis in two TRAIL-resistant cell lines (HepG2 and HT29). Other apoptosis detection methods, such as terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay and measurement of sub-G₁ cells, revealed similar results (data not shown).

3,3'-Diindolylmethane Promotes Caspase Activation in TRAIL-Treated Cells

To explore the involvement of caspases in 3,3'-diindolylmethane-sensitized apoptosis, we first measured the caspase-3/7 activity by using the fluorometric caspase substrates. As shown in Fig. 2A, either 3,3'-diindolylmethane or TRAIL alone caused a relatively minor increase of caspase-3/7 activity (<2-fold compared with the control), whereas significant augment of caspase-3/7 activity (>5-fold increase) was observed with combined treatment of 3,3'-diindolylmethane and TRAIL (Fig. 2A). Moreover, the increase of caspase activation was completely abolished by both caspase-3-specific inhibitor DEVD-CHO and pancaspase inhibitor Z-VAD-FMK (Fig. 2A). We next examined the cleavage and activation of caspases after 3,3'-diindolylmethane and TRAIL treatments by Western blot. As shown in Fig. 2B, 3,3'-diindolylmethane treatment (20 μ mol/L \times 12 hours) of HepG2 cells did not cause any significant

Figure 1. 3,3'-Diindolylmethane sensitizes TRAIL-induced apoptosis. **A**, effects of 3,3'-diindolylmethane (DIM) on cancer cell viability. Four different human cancer cells (HepG2, HT29, HeLa, and HCT116) were treated with various concentrations of 3,3'-diindolylmethane (0–100 μM /L) for 24 h. The cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and expressed by relative cell viability compared with untreated control group (considered as 100%). **B**, effects of TRAIL on cell viability. Four cancer cell lines were treated with various concentrations of TRAIL (0–50 ng/mL) for 24 h and cell viability was measured as described in **A**. **C**, sensitization of TRAIL-resistant cancer cells to TRAIL-induced cytotoxicity by 3,3'-diindolylmethane. In combined treatments, cells were always pre-treated with 3,3'-diindolylmethane for 1 h followed by TRAIL treatment. Two TRAIL-resistant cancer cell lines HepG2 and HT29 were incubated with different concentrations of 3,3'-diindolylmethane and/or TRAIL (5 ng/mL) for 24 h. **D**, morphologic changes of apoptotic cells. HepG2 cells were treated as described in **C** for 12 h. Then, cells were visualized with a normal light microscope (*top*) and with an inverted fluorescence microscope after DAPI staining (*bottom*). **E**, quantification of apoptotic cells with evident nuclear condensation after DAPI staining. *Columns*, mean of three independent experiments; *bars*, SD. *, $P < 0.05$; **, $P < 0.01$ compared with control group (Student's *t* test).



caspase-8 or caspase-3 cleavage, whereas TRAIL (5 ng/mL) treatment led to a gradual cleavage of precursor of caspase-8 and a slight cleavage of caspase-3 at 12 hours. Notably, the combined treatment of 3,3'-diindolylmethane and TRAIL significantly enhanced the cleavage of both caspase-8 and caspase-3 as evidenced by the decrease of procaspase-8 and increase of the active forms of caspase-3 (p21 and p17 fragments). A concentration- and time-dependent enhancement of poly(ADP-ribose) polymerase cleavage was also detected in HepG2 cells treated with 3,3'-diindolylmethane and TRAIL (Fig. 2C). Finally, various caspase inhibitors, including pancaspase inhibitor (Z-VAD-FMK), caspase-8 inhibitor (ITED-CHO), and caspase-3 inhibitor (DEVD-CHO), were able to almost completely prevent apoptotic cell death in both HepG2 and HT29 cells treated with 3,3'-diindolylmethane and TRAIL (Fig. 2D). Thus, data from this part of study suggested that 3,3'-diindolylmethane is able to sensitize TRAIL-induced apoptosis in TRAIL-resistant cancer cells via promoting the caspase cascade.

Sensitization Effect of 3,3'-Diindolylmethane Is Independent of Cell Death Receptors and NF- κ B Pathway

It has been reported that the sensitivity of cancer cells to TRAIL-induced apoptosis is regulated by the different

expression levels of cell death receptors and decoy receptors of TRAIL (17). The overexpressed decoy receptor may be responsible for TRAIL resistance in certain cancer cells (18). A recent report also suggested that I3C, precursor of 3,3'-diindolylmethane, could sensitize prostate cancer cells to TRAIL-induced apoptosis via up-regulation of TRAIL death receptors (12). These observations led us to examine the possible effects of 3,3'-diindolylmethane on the expression of TRAIL receptors. In our study, 3,3'-diindolylmethane, TRAIL, or combined treatment did not change the mRNA level of DR5 and DcR2, although the combined treatment of 3,3'-diindolylmethane and TRAIL tended to enhance the gene transcription of both DR4 and DcR1 to certain degree (Fig. 3A). On the other hand, no significant changes for all the receptors were observed at the protein level with various treatments (Fig. 3B). It is thus believed that 3,3'-diindolylmethane sensitizes TRAIL-induced apoptosis independent of TRAIL death receptor expression regulation.

NF- κ B is an antiapoptotic signaling pathway that regulates the expression of a variety of antiapoptotic proteins, such as c-FLIP (19), c-IAPs (20), and antiapoptotic Bcl-2 family members (21). It has been reported that TRAIL also triggers NF- κ B activation via death receptors

(DR4 and DR5) and may inhibit TRAIL-induced apoptosis (22, 23). Recent evidence also showed that 3,3'-diindolylmethane could inhibit NF-κB activation (24). Thus, we examined the effects of TRAIL and 3,3'-diindolylmethane

on NF-κB activation by using the NF-κB luciferase assay (Fig. 3C). The results suggest that the well-known NF-κB activator TNF-α significantly stimulates the NF-κB activity in HepG2 cells. In contrast, TRAIL failed to activate NF-κB pathway and 3,3'-diindolylmethane did not significantly alter NF-κB activity in cells treated with either TNF-α or TRAIL, indicating that the NF-κB signaling pathway is not directly involved in the sensitization activity of 3,3'-diindolylmethane to TRAIL-induced apoptosis.

c-FLIP Down-Regulation Contributes to the 3,3'-Diindolylmethane-Sensitized Apoptosis

In view of unchanged death receptor protein expression on 3,3'-diindolylmethane and TRAIL treatments, we focused on some important apoptosis regulatory proteins that are involved in TRAIL-induced apoptosis. The changes of major apoptotic regulators in TRAIL signaling were evaluated by Western blot after 3,3'-diindolylmethane and/or TRAIL treatment. As shown in Fig. 4, the protein levels of major apoptosis regulators examined were not affected by 3,3'-diindolylmethane and TRAIL treatments, including (a) proteins for death-inducing signaling complex formation [TNF receptor activation factor-2 (TRAF2) and Fas-associated death domain protein], (b) antiapoptotic Bcl-2 members (Bcl-2, Bcl-xL, and Mcl-1), and (c) IAP proteins (c-IAP-1 and c-IAP-2; Fig. 4). As indicated in Fig. 2B, the expedition of caspase-8 cleavage induced by pretreatment of 3,3'-diindolylmethane strongly suggests that the upstream events above caspase-8 activation may be involved in this sensitization process. It is well known that the recruitment of procaspase-8 and formation of death-inducing signaling complex are essential for the full activation of caspase-8 (1, 8), and this process is competitively inhibited by c-FLIP proteins (25). Here, we observed a significantly higher expression level of c-FLIP in TRAIL-resistant cancer cells compared with TRAIL-sensitive

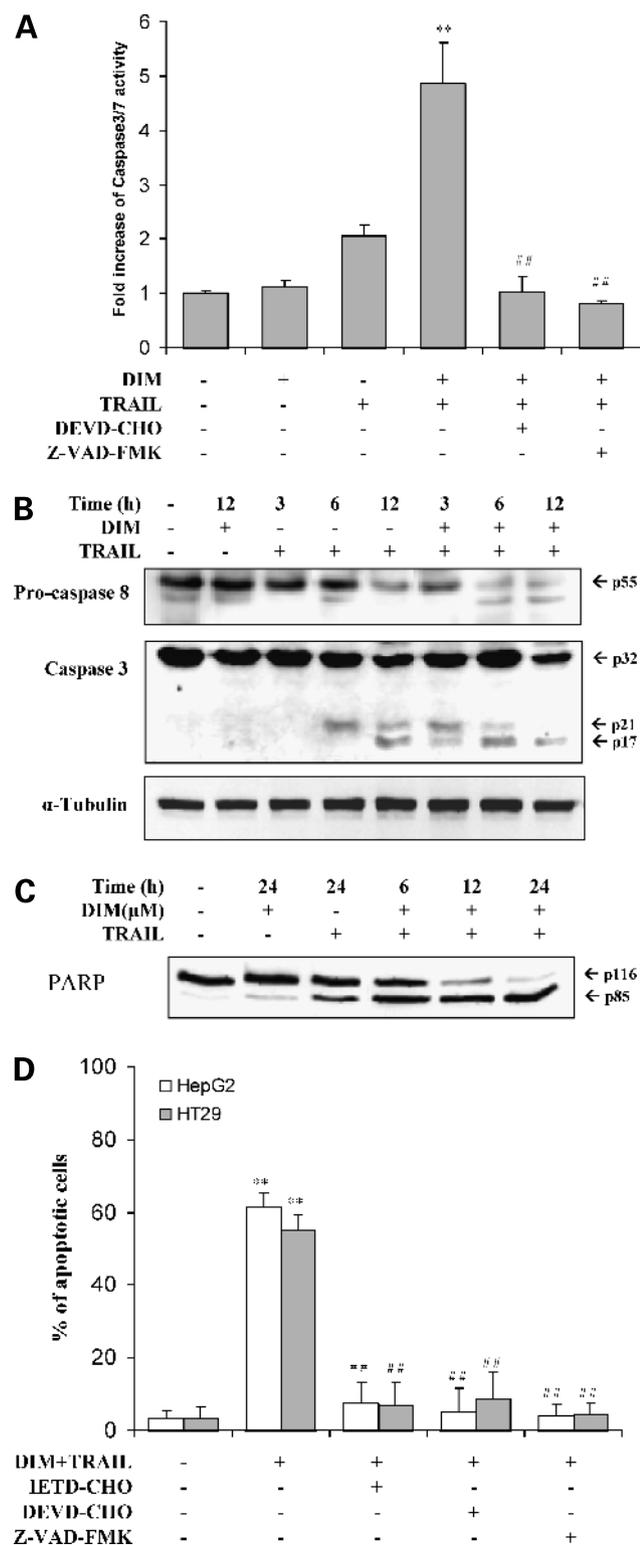


Figure 2. 3,3'-Diindolylmethane facilitates a caspase-dependent apoptosis triggered by TRAIL. **A**, increase of caspase-3/7 activity by combined treatment with 3,3'-diindolylmethane and TRAIL in HepG2 cells. HepG2 cells were pretreated with 3,3'-diindolylmethane (20 μmol/L) alone or together with caspase-3 (DEVD-CHO, 50 μmol/L) and pancaspase inhibitor (Z-VAD-FMK, 50 μmol/L) for 1 h. Then, the cells were incubated with TRAIL (5 ng/mL) for another 6 h. Cell lysates were subjected to caspase activity assay using Apo-ONE fluorescence caspase activity kit. The increase of caspase-3/7 activity was presented as fold increase compared with control. **B**, enhanced caspase cleavage triggered by 3,3'-diindolylmethane and TRAIL. HepG2 cells were treated with 3,3'-diindolylmethane (20 μmol/L) and/or TRAIL (5 ng/mL) at the indicated times. **C**, promotion of poly(ADP-ribose) polymerase (PARP) cleavage by 3,3'-diindolylmethane and TRAIL. HepG2 cells were treated with either 3,3'-diindolylmethane (20 μmol/L) and/or TRAIL (5 ng/mL) as indicated. **D**, caspase inhibitors blocked apoptosis induced by 3,3'-diindolylmethane and TRAIL. Both HepG2 and HT29 cells were pretreated with 3,3'-diindolylmethane (20 μmol/L) alone or together with caspase-8 (IETD-CHO, 50 μmol/L), caspase-3 (DEVD-CHO, 50 μmol/L), or pancaspase inhibitor (Z-VAD-FMK, 50 μmol/L) for 1 h followed by TRAIL (5 ng/mL) for another 12 h. The percentage of the apoptotic cells was measured with DAPI staining. Columns, mean of at least three independent experiments; bars, SD. **, *P* < 0.01 compared with control group; ##, *P* < 0.01 compared with 3,3'-diindolylmethane and TRAIL combined treatment group (Student's *t* test).

cells (Supplementary Fig. S1),¹ suggesting the important role of c-FLIP in TRAIL resistance. More importantly, the combined treatment of 3,3'-diindolylmethane and TRAIL markedly reduced the protein level of the large form of c-FLIP (c-FLIP-L) in the TRAIL-resistant cancer cell line HepG2 (Fig. 4). Similar effects were also observed in HT29 cells (data not shown). In addition to c-FLIP, the protein level of another apoptosis inhibitory protein, XIAP, which inhibits downstream effector caspases, was also greatly down-regulated time-dependently after the combined treatments. This observation implied that the down-regulation of these antiapoptotic proteins (FLIP and XIAP) may contribute to 3,3'-diindolylmethane-sensitized cell death.

To further explore the mechanisms involved in the down-regulation of c-FLIP and XIAP, we first did semi-quantitative reverse transcription-PCR. It is interesting to note that neither 3,3'-diindolylmethane nor TRAIL treatments inhibited c-FLIP and XIAP gene transcription (Fig. 5A). This observation suggested that the down-regulation of c-FLIP and XIAP protein level induced by 3,3'-diindolylmethane and TRAIL is unlikely to be regulated at the transcriptional level. We thus examined whether the reduced protein level is due to enhanced protein degradation via either proteolytic cleavage by other proteins/enzymes or by the ubiquitin-proteasome pathway (26, 27). As shown in Fig. 5B, inhibition of proteasome activity by MG132 significantly reversed c-FLIP down-regulation induced by 3,3'-diindolylmethane and TRAIL, whereas the pancaspase inhibitor Z-VAD-FMK failed to reverse this trend. In addition, the pretreatment with lysosome inhibitor calpeptin also had no protective effect on the down-regulation of c-FLIP protein level (data not shown). These observations strongly suggested the potential role of ubiquitin-proteasome pathway in regulating the c-FLIP protein. On the other hand, it is interesting to note that the decrease of XIAP protein level was partially recovered by caspase inhibitor, suggesting that the reduction of XIAP protein level is due to cleavage by activated caspases (28). Therefore, it seems that down-regulation XIAP is the result, but not the cause, of apoptosis under the combined treatment of 3,3'-diindolylmethane and TRAIL.

To further validate the role of c-FLIP and XIAP in 3,3'-diindolylmethane-sensitized apoptosis, expression vectors of c-FLIP and XIAP were transfected into HepG2 cells. The expression vector of CrmA, a specific inhibitor of caspase-8, was used as a positive control (Fig. 6A and B). As expected, the CrmA-transfected cells were largely resistant to 3,3'-diindolylmethane and TRAIL-induced apoptosis. Similar to CrmA, the cells with successful transfection of FLAG-tagged c-FLIP-L expression vector (FLAG-FLIP-L) also showed a significant resistance to apoptosis induced by the combined treatment. In contrast, the overexpression of XIAP protein only provided a

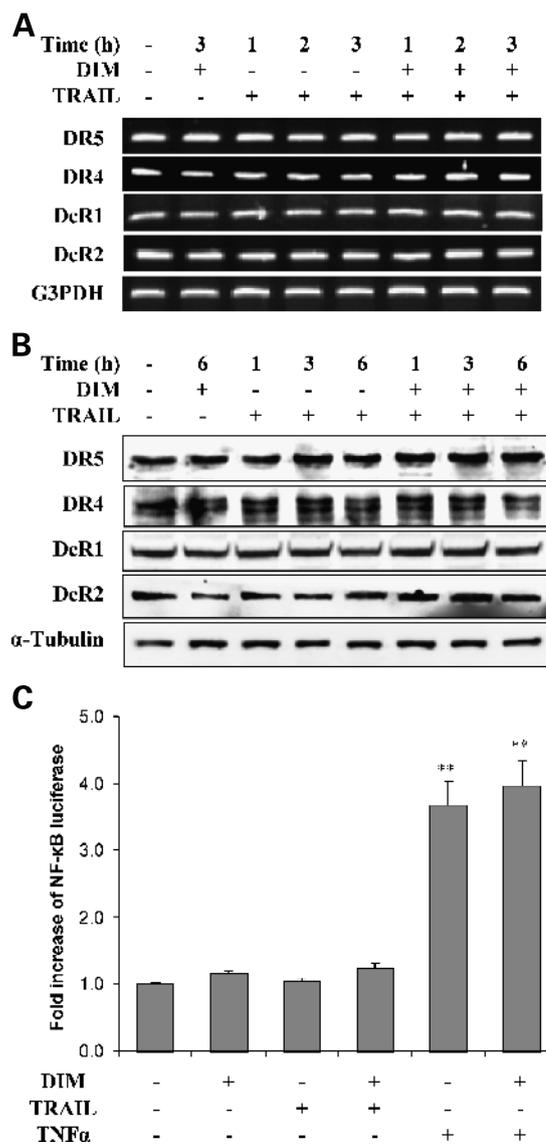


Figure 3. Effect on TRAIL receptors and NF- κ B activation induced by 3,3'-diindolylmethane and TRAIL. **A**, effects of 3,3'-diindolylmethane and TRAIL on the mRNA level of TRAIL receptors. HepG2 cells were treated 3,3'-diindolylmethane (20 μ mol/L) and/or TRAIL (5 ng/mL) and the mRNA level was measured by reverse transcription-PCR. **B**, protein level of TRAIL receptors detected by Western blot after cells were treated with 3,3'-diindolylmethane and/or TRAIL as indicated. **C**, effects of 3,3'-diindolylmethane and TRAIL on NF- κ B activation. Cells were transiently transfected with a NF- κ B luciferase vector together with *Renilla* luciferase vector as a transfection control. Twenty-four hours after transfection, cells were treated with 3,3'-diindolylmethane (20 μ mol/L), TNF- α (25 ng/mL), or TRAIL (5 ng/mL) individually or in combination for 6 h. Columns, mean of three independent transfection experiments; bars, SD. **, $P < 0.01$ compared with the nontreated control group (Student's t test).

marginal protection against apoptotic cell death induced by 3,3'-diindolylmethane and TRAIL. Taken together with the data from Fig. 5C, it is believed that XIAP does not play a critical role in the sensitization of 3,3'-diindolylmethane on TRAIL-induced apoptosis.

¹ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

c-FLIP Protein Levels Are Mediated via the Ubiquitin-Proteasome Pathway

The ubiquitin-proteasome pathway is a highly regulated system responsible for degradation of majority of short-lived regulatory proteins in eukaryotic cells (26). The turnover of both c-FLIP and XIAP has been reported to be actively regulated by the ubiquitin-proteasome system (29, 30). Results in Fig. 5 have already indicated the possibility that the down-regulation of c-FLIP protein level is mediated via the ubiquitin-proteasome system. To further confirm the importance of ubiquitin-proteasome pathway in 3,3'-diindolylmethane-sensitized cell death, we first examined the effect of proteasome inhibitors on cell death induced by 3,3'-diindolylmethane and TRAIL. As shown in Fig. 7A and B, three different proteasome inhibitors effectively suppressed the apoptosis triggered by 3,3'-diindolylmethane and TRAIL. We next explored whether c-FLIP protein is polyubiquitylated on combined treatment with 3,3'-diindolylmethane and TRAIL. HepG2 cells were transiently transfected with FLAG-FLIP-L and HA-tagged ubiquitin expression vector followed by 3,3'-diindolylmethane and TRAIL combined treatment. As shown in Fig. 7C, the slight and gradual increase in the total cell lysate smears suggested a time-dependent enhancement of overall ubiquitylated proteins after the combined treatment. More importantly, in the immunoprecipitation experiments, the significant enhancement of polyubiquitin smear could be detected after the specific pull-down of c-FLIP protein (Fig. 7C), whereas ubiquitination of FLIP was not significantly increased under 3,3'-diindolylmethane or TRAIL treatment alone (Supplementary Fig. S2),¹ suggesting an inducible ubiquitination of c-FLIP protein in cells treated with 3,3'-diindolylmethane and TRAIL. These data clearly showed that c-FLIP down-regulation by the ubiquitin-proteasome pathway is an important mechanism contributing to the sensitization of 3,3'-diindolylmethane to TRAIL-induced apoptosis.

Discussion

TRAIL is a TNF superfamily member that has an exceptional capability of inducing apoptosis in a broad spectrum of human cancer cells from different origins, such as colon, lung, and breast (31). With proper preparation, the recombinant TRAIL selectively targets cancer cells but has no adverse effects on normal cells (31, 32). This characteristic makes TRAIL a promising anticancer agent. However, some cancer cells, especially highly malignant cancer, are resistant to TRAIL-induced apoptosis, which impedes the clinical anticancer efficiency of TRAIL (8). In this report, we showed a unique anticancer activity of 3,3'-diindolylmethane, an indole compound derived from cruciferous vegetables. 3,3'-Diindolylmethane significantly sensitizes cancer cells to TRAIL-induced apoptosis. This sensitization seems to be independent of TRAIL death receptor expression regulation, or the NF- κ B signaling pathway, but is achieved through enhanced ubiquitination-proteasomal degradation of antiapoptotic protein c-FLIP.

It is well known that TRAIL-induced apoptosis is mainly executed through the cell death receptor pathway and subject to regulation at multiple levels (7, 33). Current knowledge suggests that the resistance of cancer cells to TRAIL-induced apoptosis could be determined by the following mechanisms: (a) the expression and distribution of cell membrane TRAIL receptors (death receptors versus decoy receptors), (b) activation of major antiapoptotic NF- κ B pathway, and (c) expression and regulation of antiapoptotic proteins, including c-FLIPs, Bcl-2 family members, and IAPs. In the search of the molecular mechanisms involved in the sensitization of 3,3'-diindolylmethane, we first examined the expression level of TRAIL receptors after 3,3'-diindolylmethane and TRAIL combined treatment. A recent report suggested that I3C, the parent compound of 3,3'-diindolylmethane, promotes TRAIL-induced apoptosis via up-regulation of DR4 and DR5 (12). However, in our system, the protein levels of both death receptors (DR4 and DR5) and decoy receptors (DcR1 and DcR2) were not affected by the combined treatment of 3,3'-diindolylmethane and TRAIL (Fig. 3A and B). Because the up-regulation of TRAIL death receptor (DR4 and DR5) protein level requires considerable period, it thus explains the

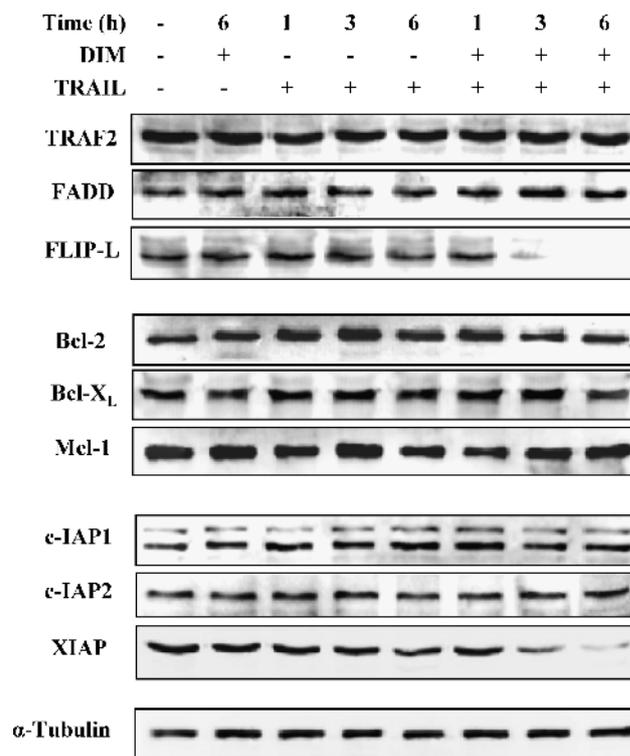


Figure 4. 3,3'-Diindolylmethane and TRAIL combined treatment leads to down-regulation of c-FLIP and XIAP. HepG2 cells were treated with 3,3'-diindolylmethane (20 μ mol/L) and/or TRAIL (5 ng/mL) at the indicated times. Cell lysates were collected and a series of Western blots were done for detection of protein expression levels of major apoptosis regulators, including death-inducing signaling complex components [TRAF2, Fas-associated death domain (FADD), and FLIP], antiapoptotic Bcl-2 family members (Bcl-2, Bcl-xL, and Mcl-1), and IAPs (c-IAP-1, c-IAP, and XIAP). α -Tubulin was used as a loading control.

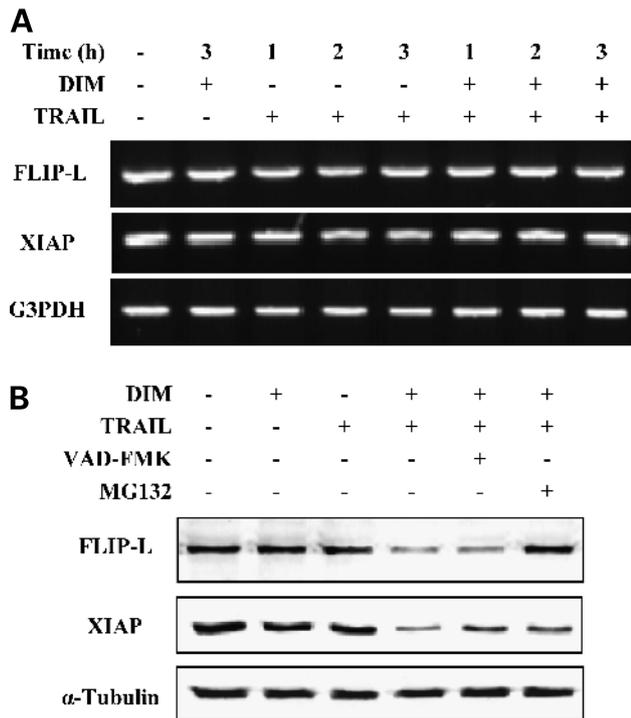


Figure 5. Down-regulation of c-FLIP involves proteasomal degradation. **A**, effects of 3,3'-diindolylmethane and TRAIL on c-FLIP and XIAP gene transcription. HepG2 cells were treated with treated 3,3'-diindolylmethane (20 $\mu\text{mol/L}$) and/or TRAIL (5 ng/mL) at the indicated times. The mRNA levels were monitored by reverse transcription-PCR. **B**, effects of a proteasome or caspase inhibitor on the down-regulation of c-FLIP and XIAP. HepG2 cells were treated 3,3'-diindolylmethane (20 $\mu\text{mol/L}$) and/or TRAIL (5 ng/mL) for 3 h. The pancaspase inhibitor Z-VAD-FMK (50 $\mu\text{mol/L}$) or proteasome inhibitor MG132 (5 $\mu\text{mol/L}$) were added together with 3,3'-diindolylmethane. At the end of experiments, cell lysates were collected and subjected to Western blot.

relatively slow induction of apoptosis by I3C and TRAIL (~48 hours) shown in the earlier report (12). However, in this study, evident apoptosis was observed after 6 and 12 hours in cells treated with 3,3'-diindolylmethane and TRAIL (Figs. 1 and 2), suggesting that 3,3'-diindolylmethane not only sensitizes but also expedites the cell death process triggered by TRAIL via a different and fast-acting mechanism.

One important finding from our study is that treatment with 3,3'-diindolylmethane and TRAIL leads to down-regulation of c-FLIP and XIAP, two critical apoptosis regulatory proteins in cell death receptor-mediated apoptosis (34). Furthermore, we provided evidence showing that the reduced level of c-FLIP protein (but not XIAP) contributes to the enhanced apoptotic cell death in the combined treatment (Figs. 5 and 6).

c-FLIP protein is a potent negative inhibitor of the TNF-related death receptor-initialized apoptosis, and two isoforms, c-FLIP-L and c-FLIP-s, have been identified (17). They are structurally similar to caspase-8, containing two death effector domains in their NH_2 terminus. However, COOH-terminal caspase domain presented in c-FLIP-L is enzymatically inactivated, whereas the c-FLIP-s lacks the

entire COOH-terminal caspase domain. The c-FLIP proteins competitively bind to Fas-associated death domain via their death effector domain within death-inducing signaling complex, which in turn prevents the caspase-8 recruitment and activation (17, 25, 35). High-level expression of c-FLIP is found in many cancer cells (35).

The expression level of c-FLIP has also been well shown to be critical in TRAIL-induced apoptosis. For instance, the tumor with aberrant high expression of FLIPs renders a strong TRAIL resistance (36). Several studies indicated that the down-regulation of c-FLIP by antisense oligonucleotide or small interfering RNA approach can sufficiently and effectively overcome the TRAIL resistance in many types of cancer cells (29, 36–38). At present, the regulation of FLIP protein level is not well understood. At transcriptional level, FLIP mRNA may be up-regulated by mitogen-activated protein kinase, Akt pathway, or $\text{NF-}\kappa\text{B}$ activation (17). At post-translational level, the down-regulation of the FLIP protein has been reported to be due to proteolytic cleavage by caspase (39, 40) or

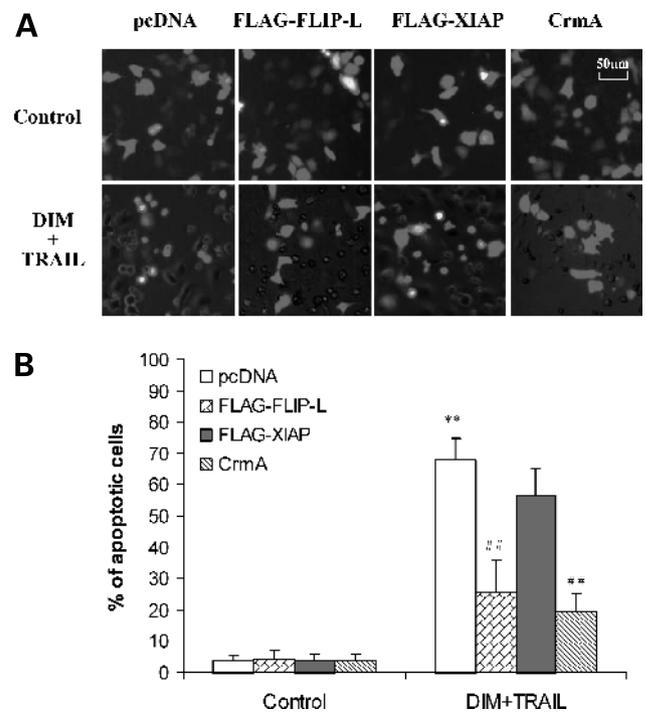


Figure 6. Overexpression of c-FLIP protects cell death induced by 3,3'-diindolylmethane and TRAIL. **A**, suppression of cell death by overexpression of c-FLIP protein. HepG2 cells were transiently transfected with pcDNA (vector control), FLAG-FLIP-L, FLAG-XIAP, or CrmA expression vectors together with pDsRed vector as a transfection indicator. Twenty-four hours after transfection, cells were treated with 3,3'-diindolylmethane (20 $\mu\text{mol/L}$) and TRAIL (5 ng/mL) for another 12 h. Under a fluorescent microscope, the successfully transfected cells were in bright red and the morphologic changes of apoptotic cells were evaluated. **B**, quantification of the data obtained from **A**. Quantification of cell death was done by counting the percentage of dead cells from a total of 200 randomly selected transfected cells. Columns, mean of three independent transfection experiments; bars, SD. **, $P < 0.01$ compared with the control group; #, $P < 0.01$ compared with pcDNA-transfected group treated with 3,3'-diindolylmethane and TRAIL (Student's t test).

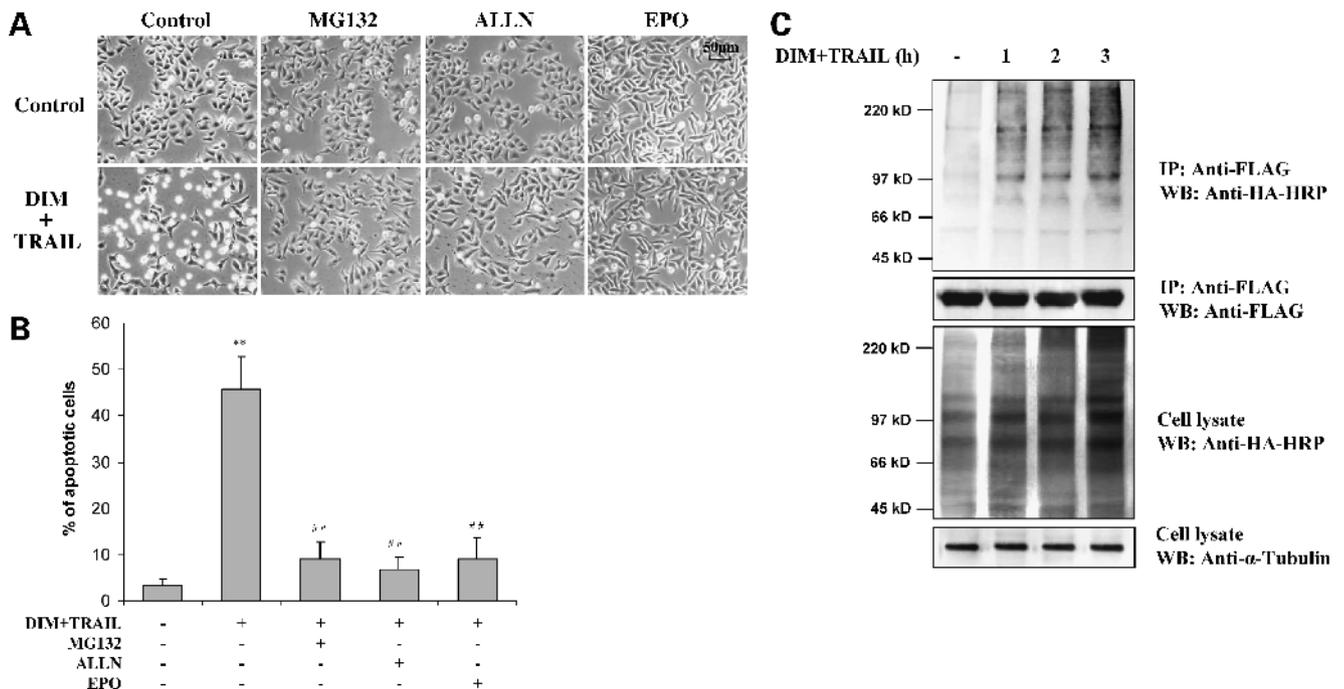


Figure 7. 3,3'-Diindolylmethane and TRAIL enhances c-FLIP ubiquitination and proteasomal degradation. **A**, proteasome inhibitors protect against cell death induced by 3,3'-diindolylmethane and TRAIL. HepG2 cells were pretreated with MG132 (20 μ mol/L), ALLN (40 μ mol/L), and epoxomicin (EPO; 400 nmol/L) for 1 h before combined treatments of 3,3'-diindolylmethane and TRAIL for 6 h. The morphologic changes of cells were examined and recorded using an inverted microscope. **B**, quantification of apoptotic cell death using DAPI staining. Cells were treated as described in **A**. Columns, mean of three independent experiments; bars, SD. **, $P < 0.01$ compared with control group; ##, $P < 0.01$ compared with combined treatment group (Student's t test). **C**, combined treatment promotes protein ubiquitination of c-FLIP. HepG2 cells were transiently transfected with FLAG-FLIP-L and HA-ubiquitin expression vectors. Twenty-four hours after transfection, cells were treated with 3,3'-diindolylmethane (20 μ mol/L \times 1 h) followed by TRAIL (5 ng/mL) at the indicated times. Cell lysates were collected and immunoprecipitated with anti-FLAG M2 agarose beads for 4 h and then subjected to Western blot analysis with anti-HA-peroxidase antibody. The cell lysates were run in parallel and probed with anti-HA-peroxidase antibody. α -Tubulin was served as a loading control for the total cell lysate.

ubiquitin-dependent proteasomal degradation (29, 41). Here, we systematically evaluated the mechanisms for c-FLIP down-regulation in cells treated with 3,3'-diindolylmethane and TRAIL. Although c-FLIP is one of the target genes of NF- κ B (19), in our study, neither TRAIL nor 3,3'-diindolylmethane has any effect on the NF- κ B signaling pathway (Fig. 3C). Furthermore, there is no evident change of FLIP mRNA level on treatment with 3,3'-diindolylmethane and TRAIL (Fig. 5A), thus excluding the possibility of c-FLIP down-regulation via gene transcription. Moreover, the reduced c-FLIP protein level is unlikely the result of caspase-mediated proteolytic cleavage because the pancaspase inhibitor Z-VAD-FMK failed to reverse the trend (Fig. 5B). On the other hand, we provide convincing evidence showing the involvement of the ubiquitin-proteasome pathway in controlling of c-FLIP protein level (Figs. 5, 6, and 7).

It has been reported that the ubiquitination of c-FLIP can be triggered under various conditions, including treatment with peroxisome proliferator-activator receptor γ modulating drugs and adenoviral infection (29, 42). At present, the molecular mechanism controlling FLIP ubiquitination is still largely unknown. It has been proposed that TRAF2, which contains a RING finger domain with E3 ligase activity, can interact with FLIP (39, 43).

However, whether this potential interaction between TRAF2 and FLIP is responsible for FLIP ubiquitination and degradation is not clear. As the TRAF2 expression level remains constant with 3,3'-diindolylmethane or TRAIL treatment (Fig. 4), it remains to be determined whether the combined treatment with 3,3'-diindolylmethane and TRAIL is able to promote the interaction of between TRAF2 and FLIP and subsequently enhance the FLIP ubiquitination.

In summary, this study reveals a novel anticancer function of 3,3'-diindolylmethane. This metabolite of an indole compound, I3C, which is unique to cruciferous vegetables, could effectively sensitize the TRAIL-resistant cancer cells to TRAIL-induced apoptosis via enhancing c-FLIP ubiquitination and proteasome-dependent degradation. The efficiency of 3,3'-diindolylmethane as a sensitizer and the potential ability of synergistic usage of 3,3'-diindolylmethane and TRAIL to overcome TRAIL resistance deserve to be evaluated in cancer therapy.

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Siyuan Zhang, Han-Ming Shen and Choon Nam Ong

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