

# Tunicamycin sensitizes human prostate cells to TRAIL-induced apoptosis by upregulation of TRAIL receptors and downregulation of cIAP2

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**Abstract.** The addition of tunicamycin to prostate cancer cells enhances cell death mediated by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). In this study, we investigated whether tunicamycin, an endoplasmic reticulum stress inducer, can potentiate TRAIL-induced apoptosis in human prostate cancer cells. We evaluated the combination of tunicamycin and TRAIL and found synergistic promotion of apoptosis in prostate cancer cells. The combined treatment with tunicamycin and TRAIL significantly induced apoptosis, and stimulated caspase-3, -8 and -9 activity, as well as the cleavage of poly (ADP-ribose) polymerase. We found that tunicamycin promoted TRAIL-induced apoptosis by the upregulation of death receptor (DR)4 and DR5 and the downregulation of cellular inhibitor of apoptosis 2 (cIAP2). In addition, downregulation of cIAP2 expression using small interfering RNA significantly attenuated the apoptosis induced by TRAIL. Taken together, our results demonstrate that the combination of tunicamycin and TRAIL may provide a novel strategy for treating prostate cancer by overcoming critical mechanisms of apoptosis resistance.

## Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis selectively in cancer cells *in vitro* and *in vivo* and has little or no cytotoxicity to normal cells (1-5). The potential significance of TRAIL as an anticancer agent is supported by its selective toxicity to human tumor xenografts

but not normal tissues in animal models (1,2). TRAIL is therefore a promising agent for cancer therapeutics. However, many tumors remain resistant to treatment with TRAIL.

One of the hallmark features of cancer is the ability to evade apoptosis, particularly by the upregulation of anti-apoptotic genes, such as certain members of the Bcl-2 (6) and inhibitor of apoptosis (IAP) protein families (7). IAPs, particularly cellular IAP1 (cIAP1), cIAP2 and X-linked IAP (XIAP), function to evade cell death by preventing the activation of caspase-8 or inhibiting the activity of caspase-9, -3 and -7 (8-10). cIAP1 and cIAP2 possess an E3 ubiquitin ligase domain that promotes their proteasome-dependent degradation, together with other targets (11,12). In prostate cancer cells, the targeted inhibition of survivin increases sensitivity to flutamide (13) and paclitaxel (14). XIAP inhibition has been reported to increase sensitivity to cisplatin (15), while an antisense oligonucleotide targeting XIAP has shown promise in preclinical studies (16). However, little is known about the effect of specifically targeting cIAP1 or cIAP2 in prostate cancer cells. In addition, while a number of studies have investigated the effects of the knockdown of individual IAPs on cell survival (17-21), few studies have examined the effect of the knockdown of multiple IAPs.

In this study, we investigated whether IAP knockdown can sensitize prostate cancer cells to apoptosis, and determined the relative contribution of cIAP2 to this process. We investigated both the effect on cell death, and the impact of the specific inhibition of cIAP2 on the survival of prostate cancer cells. Our results demonstrate that the combined knockdown of cIAP2 and TRAIL is required to significantly enhance cytotoxicity in DU-145 cells. This effect was caspase-dependent and specific for TRAIL treatment. This study therefore demonstrates the importance of targeting cIAP2 to enhance sensitivity to TRAIL-induced apoptosis, and suggests that tumor cell survival may be inhibited by specific targeting, including that of cIAP2.

## Materials and methods

**Cell culture and survival assay.** DU-145 prostate cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) containing

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10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), penicillin (100 U/ml), streptomycin (100 U/ml) and 26  $\mu$ M sodium bicarbonate for monolayers. Cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO<sub>2</sub>. One day prior to the experiment, cells were plated into 60-mm dishes. Tunicamycin (Sigma-Aldrich) was added directly to the cell cultures at the indicated concentrations. For the trypan blue exclusion assay, trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution, and 0.3 ml of phosphate-buffered saline (PBS). Samples were mixed thoroughly, incubated at room temperature for 15 min and examined under a light microscope. At least 300 cells were counted for each survival determination.

**Reagents.** Recombinant human TRAIL ligand was obtained from Koma Biotech Inc. (Seoul, Korea). Tunicamycin was obtained from Sigma Chemical Co. (St Louis, MO, USA). Z-VAD-fmk was purchased from Calbiochem (San Diego, CA, USA) and a stock solution was prepared in DMSO. Polyclonal anti-poly (ADP-ribose) polymerase (PARP), anti-Bcl-X<sub>L</sub>, anti-Mcl-1, anti-death receptor (DR)5, anti-DR4, anti-DcR2 and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-XIAP, anti-cIAP-1 and anti-cIAP-2 from R&D Systems (Minneapolis, MN, USA); anti-caspase-3 and anti-caspase-9 from Cell Signaling Technology; and anti-FLIP from Calbiochem. Monoclonal antibodies were purchased from the following companies: anti-caspase-8 from Upstate Biotechnology and anti-Bcl-2 from Santa Cruz.

**Western blot analysis.** Cells were lysed with 1X Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M SDS, 0.3 mM bromophenol blue) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). The samples were diluted with 1X lysis buffer containing 1.28 M  $\beta$ -mercaptoethanol, and equal amounts of protein were loaded on 10% SDS-polyacrylamide gels. SDS-PAGE analysis was performed according to the Laemmli method using a Bio-Rad gel apparatus.

**Flow cytometry analysis for DNA fragmentation and DAPI staining.** Cell counts were performed using a hemocytometer. Approximately 5x10<sup>5</sup>/ml DU-145 cells were suspended in 100  $\mu$ l of PBS and supplemented with 200  $\mu$ l 99% ethanol while being gently vortexed. The cells were incubated at 4°C for at least 1 h. After fixing, the cells were washed with PBS with 2% fetal bovine serum (FBS) and suspended in 250  $\mu$ l 1.12% sodium citrate buffer together with 12.5  $\mu$ g of RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250  $\mu$ l propidium iodide (PI) for 30 min. Fluorescence emitted from the PI-DNA complex was analyzed at 488 nm. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on a FC 500 (Beckman Coulter) flow cytometer for relative DNA content based on red fluorescence. DU-145 cells were treated with TRAIL (50 ng/ml) in the presence or absence of tunicamycin (2  $\mu$ g/ml). The cells were fixed with 4% paraformaldehyde on a glass slide for 30 min at room temperature. Cells were then washed with PBS, incubated with 300 nM DAPI (MicroProbe, San Jose, CA, USA) for 10 min, and examined by fluorescence microscopy. Apoptotic cells were

identified by condensation and fragmentation of nuclei. DAPI staining experiments were performed in duplicate.

**Transfection with small interfering RNA (siRNA), stable transfection and luciferase assay in DU-145 cells.** DU-145 cells were plated in 6-well plates and allowed to adhere for 24 h. On the day of transfection, 10  $\mu$ l Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were added to 50 nmol/l siRNA in a final volume of 100  $\mu$ l OPTI-MEM medium. After 48 h of transfection, cells were treated with TRAIL for 2 h. The DU-145 cells were stably transfected with the pcDNA 3.1-cIAP2 plasmid or control plasmid pcDNA 3.1 vector using Lipofectamine as instructed by the manufacturer (Invitrogen). After 48 h of incubation, transfected cells were selected in primary cell culture medium containing 700  $\mu$ g/ml G418 (Invitrogen). After 2 or 3 weeks, to rule out the possibility of clonal differences between the generated stable cell lines, the pooled DU-145/pcDNA 3.1 and DU-145/cIAP2 clones were tested for cIAP2 expression by immunoblotting before being used in the study. The luciferase cIAP2 and NF- $\kappa$ B plasmids were a gift from Dr T.H. Lee (Yeonsei University). Cells were seeded in 6-well plates at 2.5x10<sup>5</sup> cells/plate and grown for 24 h before transfection. Cells were co-transfected with 1  $\mu$ g of plasmid and 1  $\mu$ g of the pCMV galactosidase normalization plasmid by 4-h incubation with Lipofectamine reagent (Invitrogen). Luciferase and galactosidase activities were determined according to the manufacturer's instructions (Promega, Madison, WI, USA).

**Reverse transcription (RT)-PCR analysis.** Total RNA was extracted from DU-145 cells using the TRIzol reagent (Invitrogen). A cDNA was synthesized from 2  $\mu$ g of total RNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (Takara Co. Ltd., Japan). Human DR5 mRNA was amplified using the sense primer 5'-AAG ACC CTT GTG CTC GTT GT-3' and the antisense primer 5'-GAC ACA TTC GAT GTC ACT CCA-3'. The PCR cycling conditions (35 cycles) were chosen as follows: 30 sec at 94°C, 45 sec at 60°C, 30 sec at 72°C for DR5 with a subsequent 5-min extension at 72°C. Reaction products were analyzed on 1.0% agarose gels and bands were visualized by ethidium bromide (EtBr).

**Statistical analyses.** The difference between the treated and control cells were analyzed by the Student's t-test, and a probability of p<0.05 was considered significant.

## Results

**Tunicamycin sensitizes prostate cancer cells to TRAIL-induced apoptosis.** To investigate the effect of tunicamycin on TRAIL-induced cytotoxicity, DU-145 human prostatic adenocarcinoma cells were treated with TRAIL in the presence or absence of tunicamycin. DU-145 cells were pre-treated with various concentrations of tunicamycin for 20 h and then exposed to TRAIL for 4 h. Cells were moderately sensitive to either tunicamycin or TRAIL alone. However, pre-treatment with tunicamycin enhanced TRAIL-induced cytotoxicity (Fig. 1A). When apoptosis was examined by PI staining, we found that apoptosis was induced by 8.5% with tunicamycin, by 12.5% with TRAIL, and by 45% with the combination of both (Fig. 1B). Cells were then pre-treated with tunicamycin (2  $\mu$ g/ml) for 20 h, followed by

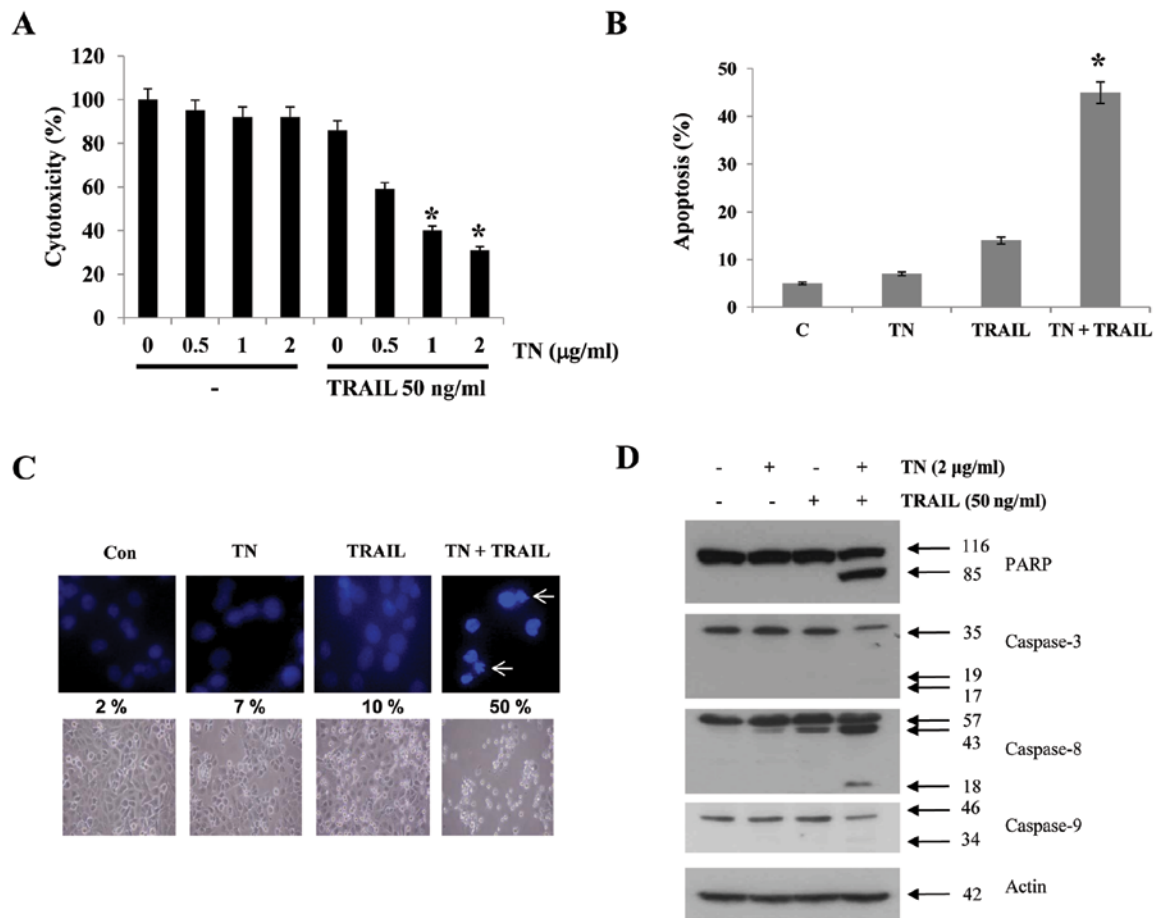


Figure 1. Tunicamycin enhances TRAIL-induced DU-145 cell death. (A) Cells were pre-treated with various concentrations of tunicamycin for 20 h before exposure to TRAIL (50 ng/ml) for 4 h. Cell viability was then analyzed by trypan blue exclusion assay. Data represent the means  $\pm$  standard error of the mean (SEM) of 3 samples. \* $p < 0.05$  compared to tunicamycin plus TRAIL-treated cells. (B) Cells were treated for 4 h with TRAIL (50 ng/ml) in the presence or absence of 2  $\mu$ g/ml tunicamycin for 20 h. After treatment, apoptosis was detected by FACS analysis. C, control, TN, tunicamycin, TN + TRAIL, TRAIL 50 ng/ml plus tunicamycin 2  $\mu$ g/ml. Data represent the means  $\pm$  SEM of 3 samples. \* $p < 0.05$  compared to tunicamycin plus TRAIL-treated cells. (C) Cells were treated with 2  $\mu$ g/ml tunicamycin for 20 h and were then treated with TRAIL (50 ng/ml) for 4 h. Cell death was determined by DAPI staining. (D) Cells were pre-treated with tunicamycin for 20 h and then treated with TRAIL for 4 h. Whole-cell extracts were prepared and analyzed by western blotting using antibodies against PARP, caspase-3, caspase-8 and caspase-9. Error bars represent SEM of 3 separate experiments.

exposure to TRAIL (50 ng/ml) for 4 h. The results indicated that tunicamycin and TRAIL treatment alone induced 7% and 10% apoptosis, respectively. Combination treatment enhanced apoptosis by 50% (Fig. 1C). We then examined the effect of tunicamycin, TRAIL, and their combination on the activation of caspase-3, caspase-8, caspase-9 and PARP cleavage. We found that although tunicamycin and TRAIL had little effect on the caspases and PARP, combination treatment was highly effective (Fig. 1D). Taken together, our results indicate that tunicamycin enhances TRAIL-induced apoptosis.

**Tunicamycin induces the expression of TRAIL receptors, DR4 and DR5, in cancer cell lines.** To determine how tunicamycin potentiates TRAIL-induced apoptosis, we investigated its effects on the TRAIL receptors, DR4 and DR5. DU-145 cells were treated with various concentrations of tunicamycin for 24 h, and whole-cell extracts were prepared and examined for expression of DR4 and DR5 proteins. Tunicamycin induced the expression of DR4 and DR5 (Fig. 2A, left panel) in a dose-dependent manner, with optimum induction occurring at around

1 to 2  $\mu$ g/ml. The time-dependence of DR induction was also examined. Tunicamycin induced the expression of DR4 and DR5 (Fig. 2A, right panel) in a time-dependent manner. To determine whether the induction of TRAIL receptors by tunicamycin occurred at the transcriptional level, we examined mRNA for DR4 and DR5 expression after the cells were treated with various concentrations of tunicamycin for different time periods. A DR5 transcript was induced in a dose- and time-dependent manner by tunicamycin (Fig. 2B), suggesting that tunicamycin acts at the transcriptional level. We also investigated whether the upregulation of DR5 and DR4 by tunicamycin is specific to PC3 or whether it also occurs in other cell types. Prostate cancer cells (PC3 and LNCaP), colon cancer cells (HCT116 and HT29), cervical cancer cells (HeLa and Caski), breast cancer cells (MDA231), and lung cancer cells (A549 cells) were exposed to tunicamycin (2  $\mu$ g/ml) for 24 h and then examined for DR5 and DR4 protein expression. Tunicamycin induced the expression of DR5 (Fig. 2C, top panel) in the LNCaP, HCT116, HeLa, Caski and MDA231 cells. Tunicamycin induced the expression of DR4 (Fig. 2C, middle panel) in the PC3 HCT116,

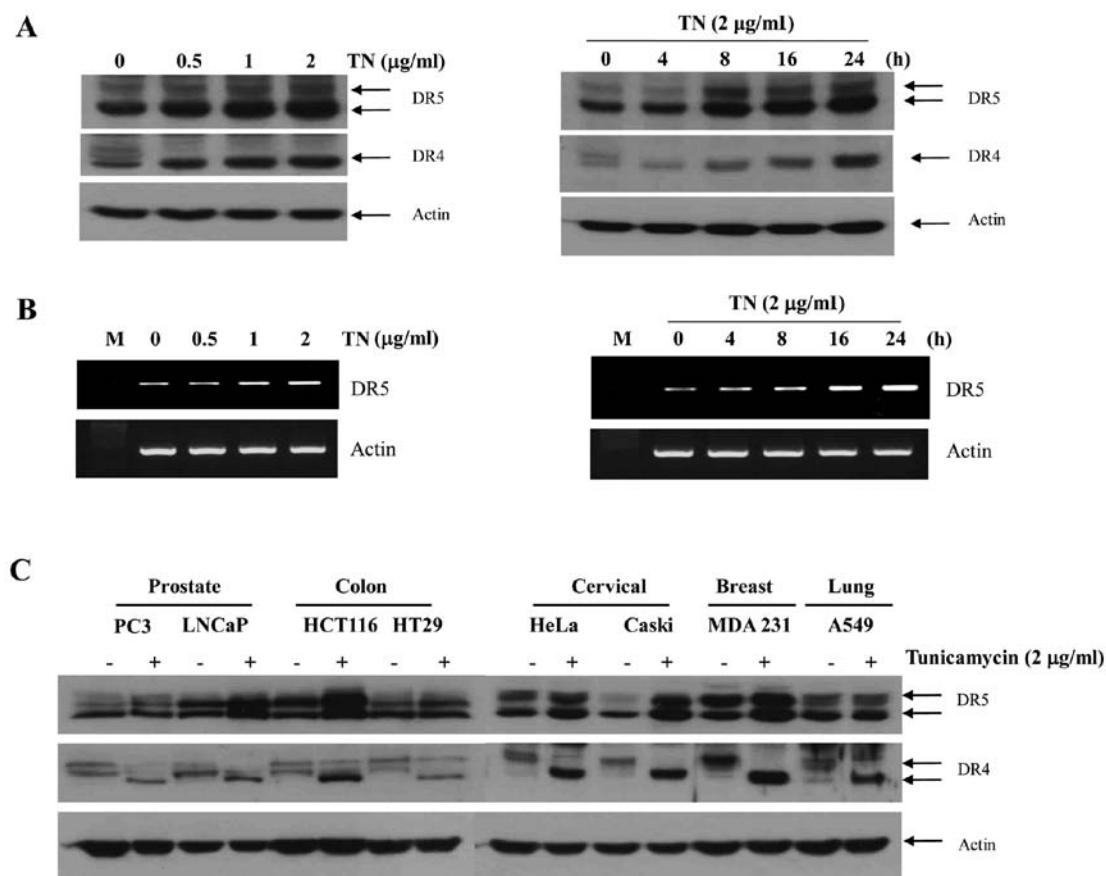


Figure 2. Tunicamycin-induced DR5 and DR4 expression. (A) DU-145 cells were treated with the indicated doses of tunicamycin at the indicated times. Whole cell extracts were prepared and analyzed for DR4 and DR5 expression by western blotting. (B) Tunicamycin-induced DR5 gene expression. DU-145 cells were treated with the indicated doses of tunicamycin at the indicated times, and total RNA was extracted and examined for expression of DR5 by RT-PCR. Actin was used as the internal control to show equal RNA loading. (C) Cells were treated with 2 µg/ml tunicamycin for 24 h, and whole cell extracts were prepared and analyzed by western blotting. Equal amounts of protein (20 µg) were separated by SDS-PAGE and immunoblotted. Equal protein loading was evaluated by actin. TN, tunicamycin.

HeLa, Caski, MDA231 and A549 cells. No significant induction of DR5 expression was noted in the PC3 and A549 cells. The induction of either DR4 or DR5 expression was minimal in PC3 prostate cancer and HT29 colon cancer cells after exposure to tunicamycin. These findings suggest that the upregulation of DR5 and DR4 by tunicamycin is not cell type-specific.

*DR induction by tunicamycin is required for TRAIL-induced apoptosis.* To determine the role of DR5 and DR4 in TRAIL-induced apoptosis, we used siRNAs specific to DR5 and DR4 to downregulate their expression. The transfection of cells with siRNA for DR5 but not with the control siRNA reduced tunicamycin-induced DR5 expression (Fig. 3A). Similarly, the transfection of cells with siRNA for DR4 reduced the tunicamycin-induced DR4 expression (Fig. 3B). However, DR4 siRNA and DR5 siRNA had minimal effects on the tunicamycin-induced upregulation of DR4 and DR5. We then examined whether the suppression of DR5 and/or DR4 by siRNA can abrogate the sensitizing effects of tunicamycin on TRAIL-induced apoptosis using immunoblotting (Fig. 3C) and DAPI staining assays (Fig. 3D). The results revealed that the effect of tunicamycin on TRAIL-induced apoptosis was effectively abolished in cells transfected with both DR5 and DR4 siRNAs, whereas treatment with only DR4 siRNA or only DR5

siRNA had no effect. The silencing of both receptors abolished apoptosis to the same degree as the silencing of either DR4 or DR5, suggesting that both DR4 and DR5 are major players in TRAIL-induced apoptosis.

*Tunicamycin downregulates the expression of cIAP2 but has no effect on XIAP, survivin and Bcl-2 family proteins.* We then examined whether tunicamycin can affect the expression of any of the anti-apoptotic proteins. Cells were exposed to various concentrations of tunicamycin for 24 h and were then examined for the expression of Bcl-x<sub>L</sub>, Mcl-1, XIAP and survivin (Fig. 4A). Tunicamycin downregulated the transcript for cIAP2 in a dose- and time-dependent manner, suggesting that it acts at the transcriptional level (Fig. 4B). Tunicamycin may thus affect TRAIL-induced apoptosis not only through induction of DR4 and DR5 but also through the downregulation of cIAP2. We examined whether tunicamycin can affect cIAP2 promoter activity or related NF-κB promoter activity, as we expected that the cIAP2 promoter is regulated by the NF-κB transcription factor (data not shown).

*Silencing of cIAP2 enhances TRAIL sensitization by tunicamycin which blocks cIAP2 expression.* We investigated whether the downregulation of cIAP2 by tunicamycin is specific to

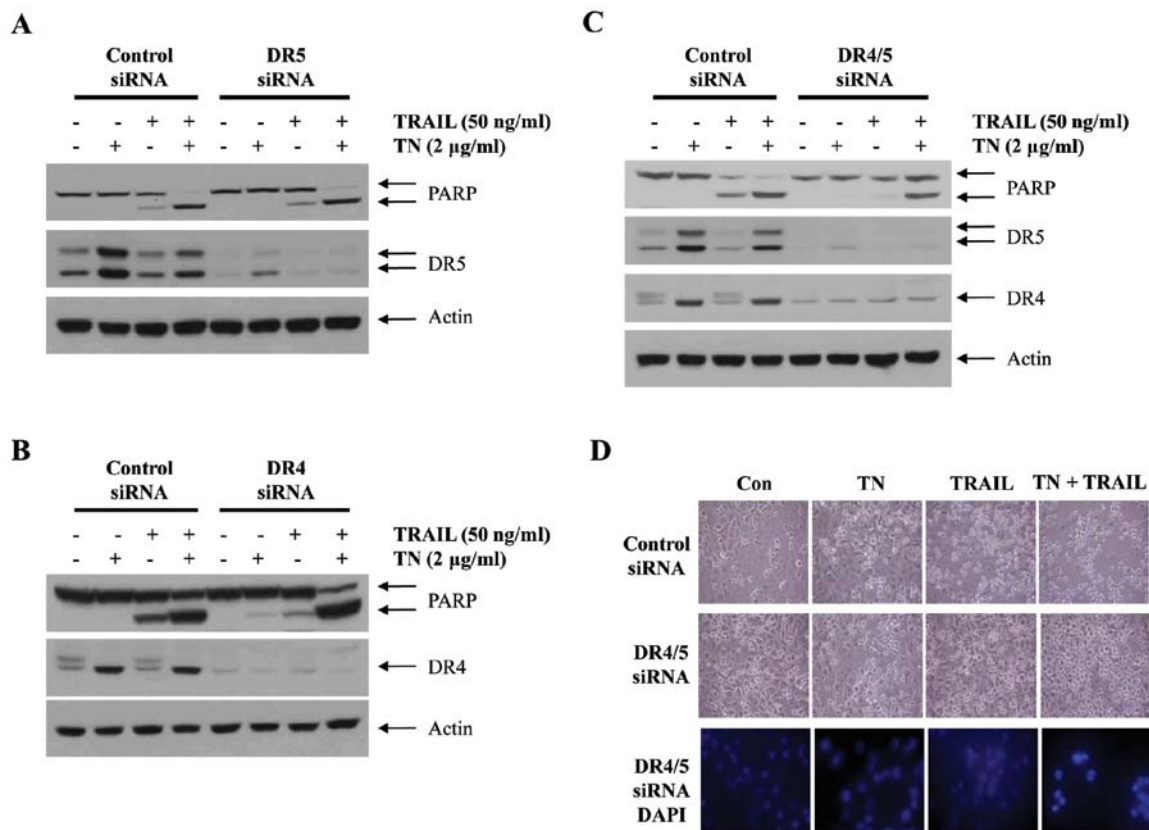


Figure 3. Effect of knockdown of death receptors (DRs) on tunicamycin-induced sensitization of TRAIL. DU-145 cells were transfected with (A) DR5 siRNA, (B) DR4 siRNA, and (C) combined DR4 and DR5 siRNA. After 48 h, cells were pre-treated with tunicamycin for 20 h and then treated with TRAIL for 4 h. Whole cell extracts were prepared and analyzed by western blotting using antibodies against PARP, DR4 and DR5. (D) Cells were treated with 2 µg/ml tunicamycin for 20 h and then treated with TRAIL (50 ng/ml) for 4 h. Cell death was determined by DAPI staining. TN, tunicamycin.

DU-145 cells or whether it also occurs in other cell types (Fig. 5A). The induction of cIAP2 was minimal in HeLa cervical cancer and A549 lung cancer cells after exposure to tunicamycin. These findings suggest that the downregulation of cIAP2 by tunicamycin is not cell type-specific. We examined the functional significance of cIAP2 downregulation in the enhancement of TRAIL-induced apoptosis by tunicamycin using stable cell lines overexpressing cIAP2 (#10, #16) (Fig. 5B, top panel). The apoptotic morphology observed in the control cells treated with tunicamycin plus TRAIL was not observed in cIAP2 #10 and cIAP2 #16 cells (data not shown). Furthermore, we found that the increase in the sub-G1 cell population by tunicamycin plus TRAIL was effectively blocked in cIAP2-overexpressing cells (Fig. 5B, bottom panel). We then investigated whether the silencing of cIAP2 or the overexpression of cIAP2 can block TRAIL-induced cell death and inhibit TRAIL sensitization in prostate cancer cells. DU-145 cells were successfully transfected with control or specific siRNAs against cIAP2. After 48 h, cells were exposed to TRAIL for 4 h, harvested, and lysates subjected to western blotting. The expression of cIAP2 was shown to be reduced in cells treated with specific siRNA compared to control siRNA (Fig. 5C, bottom panel). The reduction in the expression of cIAP2 significantly enhanced TRAIL-induced cell death (Fig. 5C, top panel). These results indicate that apoptosis by tunicamycin plus TRAIL is associated with cIAP2 expression in DU-145 cells.

## Discussion

In this study, we show that the antibiotic component, tunicamycin, can enhance the apoptotic effect of TRAIL against prostate cancer cells. The mechanism by which tunicamycin mediates its effects on TRAIL-induced apoptosis involve the induction of TRAIL receptors and the downregulation of cIAP2. We found that the induction of TRAIL receptors by tunicamycin was not cell type-specific but was observed in a wide variety of cell types, including colon, cervical and breast cancer cells (Fig. 2C).

The induction of TRAIL receptor expression by tunicamycin treatment resulted in the sensitization of DU-145 cells to TRAIL-induced apoptosis. Furthermore, caspase-3, -8 and -9 were activated only when tunicamycin and TRAIL were provided together (Fig. 1D), and the activation of these caspases was blocked in the presence of z-VAD, a pan caspase inhibitor (data not shown). Caspase-8 is known to act directly downstream of the TRAIL receptors and Bid is a known mediator connecting the DR pathway to the mitochondrial apoptotic pathway (22-24). Importantly, the downregulation of DR4 and DR5 expression using DR4 and DR5 siRNAs efficiently reduced the tunicamycin-dependent sensitization to TRAIL-induced apoptosis (Fig. 3). The upregulation of DR4 and DR5 by tunicamycin thus sensitizes prostate cancer cells to TRAIL-induced apoptosis. Moreover, the combined treatment

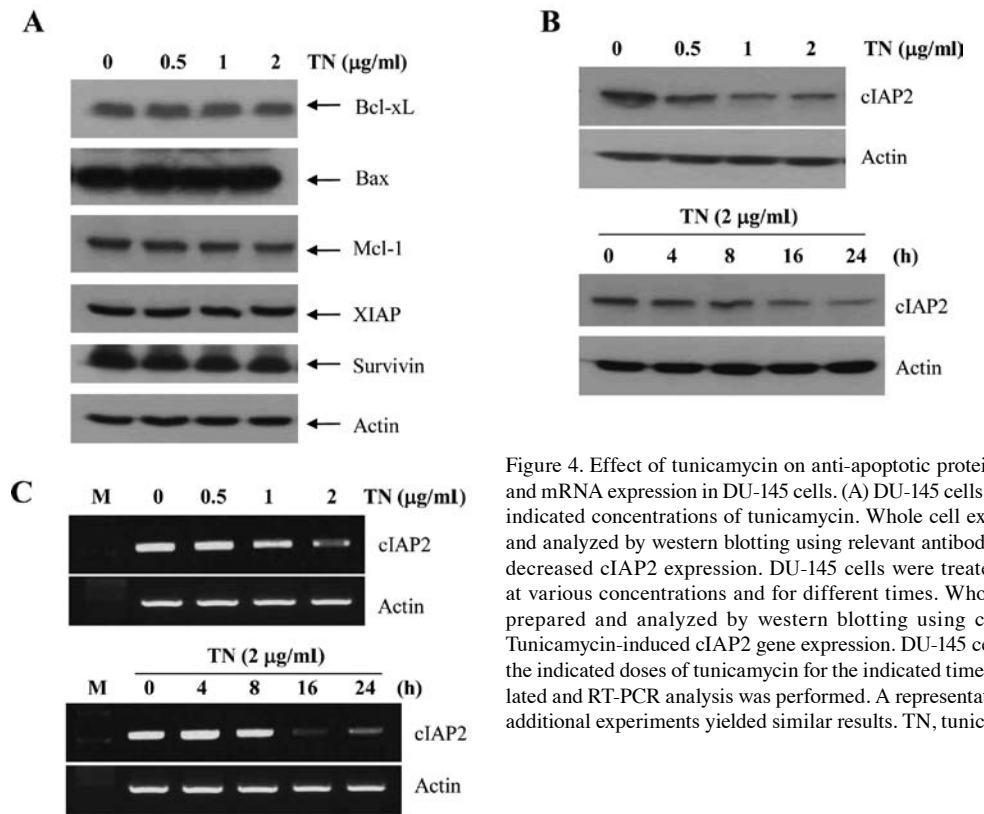


Figure 4. Effect of tunicamycin on anti-apoptotic protein and cIAP2 protein and mRNA expression in DU-145 cells. (A) DU-145 cells were treated with the indicated concentrations of tunicamycin. Whole cell extracts were prepared and analyzed by western blotting using relevant antibodies. (B) Tunicamycin decreased cIAP2 expression. DU-145 cells were treated with tunicamycin at various concentrations and for different times. Whole cell extracts were prepared and analyzed by western blotting using cIAP2 antibody. (C) Tunicamycin-induced cIAP2 gene expression. DU-145 cells were treated with the indicated doses of tunicamycin for the indicated times, total RNA was isolated and RT-PCR analysis was performed. A representative study is shown; 2 additional experiments yielded similar results. TN, tunicamycin.

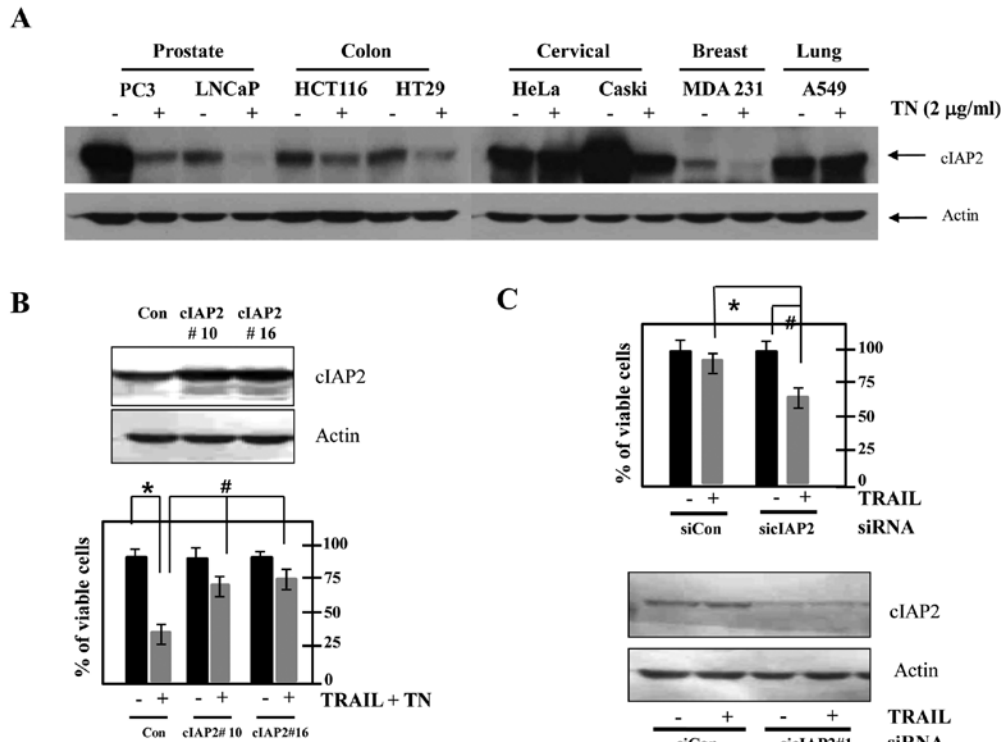


Figure 5. Tunicamycin-induced cIAP2 downregulation critically contributes to tunicamycin-stimulated TRAIL-induced apoptosis. (A) DU-145 cells were treated with tunicamycin for 24 h and western blotting of cIAP2 and actin was performed. (B) The protein levels of cIAP2 were examined by western blotting using anti-cIAP2 antibody in DU-145 cells stably transfected with control vector or plasmid cIAP2. Control cells (Con) and the cells stably overexpressing cIAP2 were treated with tunicamycin and TRAIL for 24 h. Cell viability was assessed using a sub-G1 fraction by flow cytometry. Data represent the means  $\pm$  SEM of 3 samples; \* $p < 0.05$  compared with untreated cells and # $p < 0.05$  compared with the control cells treated with tunicamycin plus TRAIL. (C) To examine the effect of cIAP2 downregulation on TRAIL-induced apoptosis, DU-145 cells were transfected with the control siRNA or cIAP2 siRNA, incubated for 24 h and further treated with or without 50 ng/ml TRAIL for 4 h. Cellular viability was determined using a sub-G1 fraction by flow cytometry. Data represent the means  $\pm$  SEM of 3 samples; \* $p < 0.05$  compared with transfection with control siRNA or cIAP2 siRNA with TRAIL; # $p < 0.05$  compared with the cells transfected with cIAP2 siRNA but untreated with TRAIL. Western blotting of cIAP2 was done to confirm the downregulation of cIAP2 by siRNA transfection. Actin levels were assessed to show equal gel loading. TN, tunicamycin.

with both DR4 and DR5 siRNAs blocked this sensitization (Fig. 3C); however, either DR4 or DR5 siRNA alone did not block the sensitizing effect of tunicamycin on TRAIL-induced apoptosis (Fig. 3A and B). Both TRAIL receptors therefore play a functional role. Our findings indicate that DR4 and DR5 upregulation accounts, at least in part, for the sensitizing effect of tunicamycin on TRAIL-induced apoptosis. Our findings are in agreement with those from other studies reporting that tunicamycin upregulates DR5 expression and sensitizes TRAIL-induced apoptosis in a p53-independent manner (25). Combined treatment with tunicamycin plus TRAIL may thus be useful for p53-deficient cancer cells. A number of studies have demonstrated that DR5 upregulation may be a promising strategy for sensitizing cancer cells to TRAIL-induced apoptosis (26,27). Chen *et al* (28) reported tunicamycin-induced upregulation of CHOP. CHOP protein was first identified as a member of the CCAAT/enhancer binding proteins (C/EBPs) that dimerize with transcription factor C/EBP and LAP (29).

Besides the induction of TRAIL receptors, the downregulation of cIAP2 by tunicamycin may also lead to the enhancement of TRAIL-induced apoptosis. Tunicamycin was found to have little effect on other anti-apoptotic proteins, including Mcl-1 and survivin (Fig. 4A). Several apoptotic pathways are induced in prostate cancer cells by tunicamycin. We previously provided evidence that tunicamycin promotes the induction of the TRAIL receptor not only in melanoma but also in prostate cancer cells (25,30). In this study, we show that the reduction of cIAP2 by tunicamycin in the prostate cancer cell line, DU-145, correlates with an increase in the number of apoptotic cells. In certain prostate cancer cells, tunicamycin induces the concomitant upregulation of TRAIL receptors and the activation of a gene program of apparent opposite function, characterized by the induction of the anti-apoptotic IAP family member, cIAP2, a NF- $\kappa$ B target gene. cIAP2 expression was significantly modulated at both the mRNA and protein levels by tunicamycin in a cell context-dependent manner (Fig. 5). In addition, cIAP2 protein levels can be regulated by alternative signaling pathways either in parallel with or in addition to NF- $\kappa$ B signaling. cIAP2 and XIAP have been reported to be upregulated in a Ras-dependent manner through receptor tyrosine kinase activation (31), as well as through other signaling pathways including PI3K (32,33), protein kinase C  $\Delta$  (34) and cAMP (35). In DU-145 cells, tunicamycin induces the NF- $\kappa$ B-mediated downregulation of cIAP2. This finding presents the possibility of being able to bypass NF- $\kappa$ B inhibition to induce sensitivity and eliminate the inherent difficulty in trying to inhibit NF- $\kappa$ B.

In conclusion, in this study we investigated the molecular mechanisms by which tunicamycin enhances TRAIL-induced apoptosis in DU-145 cells. We found that tunicamycin effectively sensitizes DU-145 prostate cancer cells to TRAIL-induced apoptosis through the downregulation of cIAP2 and upregulation of DR5. Tunicamycin as a potential TRAIL sensitizer may enhance therapeutic outcome by either lowering TRAIL resistance or increasing the damage of cancer cells.

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