

# Tunicamycin enhances TRAIL-induced apoptosis by inhibition of cyclin D1 and the subsequent downregulation of survivin

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Abbreviations: DcR, decoy receptor; DISC, death-inducing signaling complex; DR, death receptor; TRAIL, TNF-related apoptosis-inducing ligand

## Abstract

TNF-related apoptosis-inducing ligand (TRAIL) has been proposed as a promising cancer therapy that preferentially induces apoptosis in cancer cells, but not most normal tissues. However, many cancers are resistant to TRAIL by mechanisms that are poorly understood. In this study, we showed that tunicamycin, a naturally occurring antibiotic, was a potent enhancer of TRAIL-induced apoptosis through downregulation of survivin. The tunicamycin-mediated sensitization to TRAIL was efficiently reduced by forced expression of survivin, suggesting that the sensitization was mediated at least in part through inhibition of survivin expression. Tunicamycin also repressed expression of cyclin D1, a cell cycle regulator commonly overexpressed in thyroid carcinoma. Furthermore, silencing cyclin D1 by RNA interference reduced survivin expression and sensitized thyroid cancer cells to TRAIL; in contrast, forced expression of cyclin D1 attenuated tunicamycin-potentiated TRAIL-induced apoptosis via over-riding downregulation of survivin. Collectively, our results demonstrated that tunicamycin promoted TRAIL-induced apoptosis, at least in part, by inhibiting the expression of cyclin D1 and subsequent survivin. Of note, tunicamycin did not sensitize the differentiated thyroid epithelial cells to

TRAIL-induced apoptosis. Thus, combined treatment with tunicamycin and TRAIL may offer an attractive strategy for safely treating resistant thyroid cancers.

**Keywords:** apoptosis; BIRC5 protein, human; cyclin D1; thyroid neoplasms; TNF-related apoptosis-inducing ligand; tunicamycin

## Introduction

Undifferentiated thyroid carcinomas are refractory to most traditional chemotherapy and radiotherapeutic methods (Ain, 1998). TNF-related apoptosis-inducing ligand (TRAIL) initiates apoptosis by binding to its cell surface death receptors 4 and 5 (DR4 and DR5), which contain a cytoplasmic death domain that serves as a protein interaction module to recruit the death domain-containing adaptor protein FADD to the death-inducing signaling complex (DISC) (LeBlanc *et al.*, 2003). The signal is propagated through caspases 8 and 10, finally leading to activation of effector caspases such as caspase 3 (Sprick *et al.*, 2000; Wang *et al.*, 2001). TRAIL also has three decoy receptors (DcR1, DcR2 and osteoprotegerin), which inhibit apoptosis by sequestering TRAIL from DR4 and DR5 death receptors (LeBlanc *et al.*, 2003).

Recent studies showed that TRAIL may be considered as a promising anticancer agent due to its ability to induce apoptosis in a variety of tumor cell types with only negligible effects on normal cells (Pan *et al.*, 1997a, b). However, malignant thyroid cancer cells exhibit considerable heterogeneity in their sensitivity to TRAIL, even among those expressing the TRAIL-responsive cognate death receptors, DR4 and DR5 (Bretz *et al.*, 1999a, b; Mitsiades *et al.*, 2000; Wang *et al.*, 2004). Clearly, the identification of drugs that sensitize tumors to TRAIL-induced apoptosis, without compromising its tumor selectivity, could substantially broaden the therapeutic impact of TRAIL.

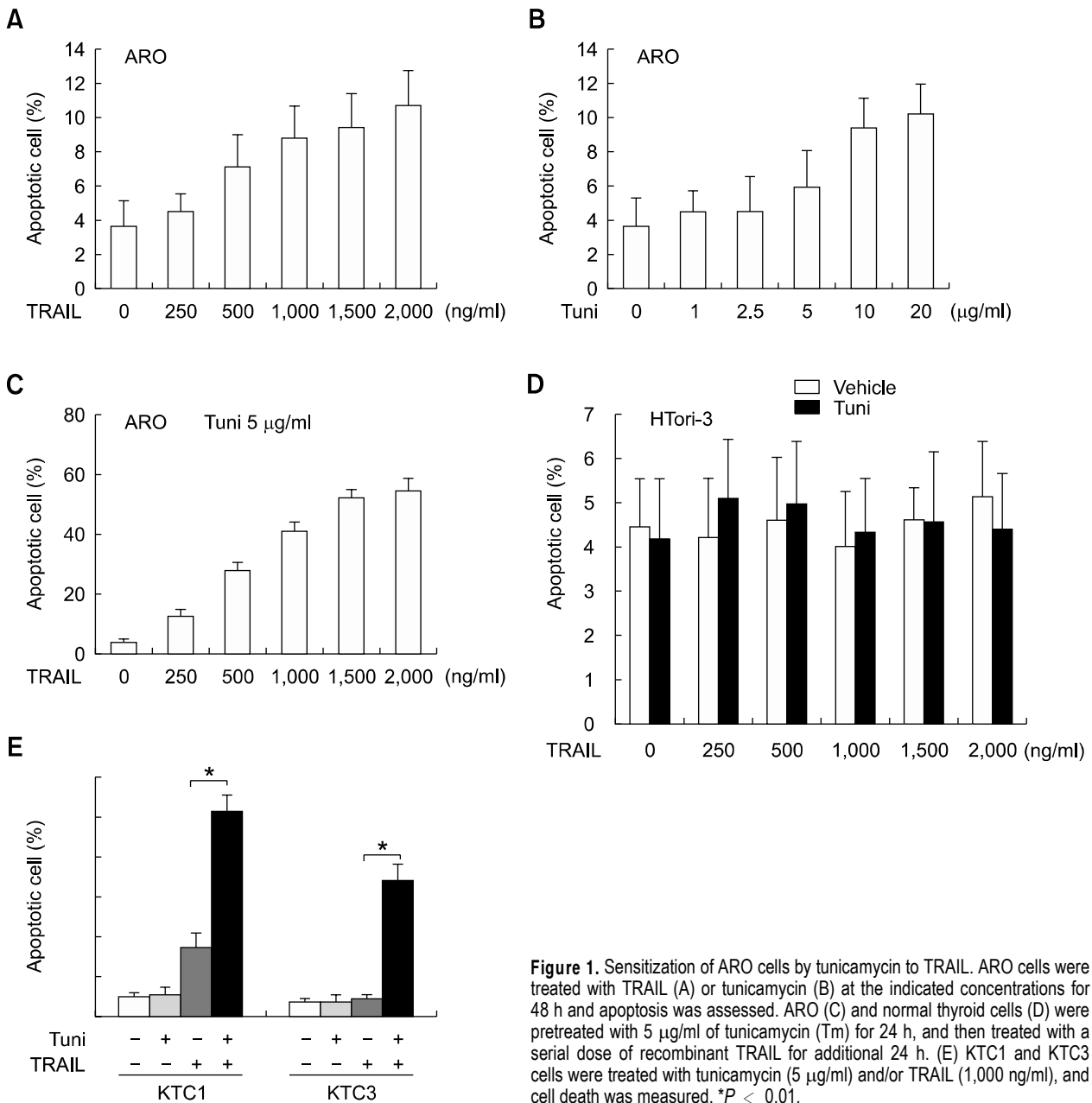
Tunicamycin, a naturally occurring antibiotic, blocks the first step in the biosynthesis of *N*-linked oligosaccharides in cells. Our present experiments demonstrate that tunicamycin significantly increases the sensitivity of human thyroid cancer ARO cells to apoptosis induced by TRAIL by selectively repressing cyclin D1, a cell cycle regulator commonly overexpressed in thyroid carcinoma (Lloyd *et*

*al.*, 1997; Erickson *et al.*, 1998, 2000; Wang *et al.*, 1998). Its overexpression has also been reported to be more frequent in poorly differentiated thyroid tumors. Cyclin D1 repression, in turn, induces G1 cell cycle arrest and reduces the levels of survivin, an anti-apoptotic protein whose expression is cell cycle-regulated (Li *et al.*, 1998). Our results provide a novel mechanism underlying the sensitizing effects of tunicamycin on TRAIL-induced apoptosis by repression of cyclin D1 and subsequent down-regulation of survivin.

**Results**

**Subtoxic doses of tunicamycin significantly sensitize human thyroid cancer cells, but not normal human thyroid epithelial cells, to TRAIL-induced apoptosis**

The cytotoxic activity of human recombinant soluble TRAIL was tested in ARO undifferentiated thyroid carcinoma cells (Figure 1A). As previously reported, ARO cells demonstrated a limited cell death (< 10%) upon treatment with up to 2 µg/ml TRAIL



**Figure 1.** Sensitization of ARO cells by tunicamycin to TRAIL. ARO cells were treated with TRAIL (A) or tunicamycin (B) at the indicated concentrations for 48 h and apoptosis was assessed. ARO (C) and normal thyroid cells (D) were pretreated with 5 µg/ml of tunicamycin (Tm) for 24 h, and then treated with a serial dose of recombinant TRAIL for additional 24 h. (E) KTC1 and KTC3 cells were treated with tunicamycin (5 µg/ml) and/or TRAIL (1,000 ng/ml), and cell death was measured. \* $P < 0.01$ .

for 48 h, suggesting that these cells are resistant to the apoptotic effects of TRAIL. To examine whether tunicamycin might restore the sensitivity of TRAIL-resistant ARO cells to TRAIL-induced apoptosis, we tested the cytotoxic effect of tunicamycin alone or in combination with TRAIL. Tunicamycin alone did not induce any morphological signs of cell death up to 20 µg/ml (Figure 1B). However, pre-treatment with 5 µg/ml tunicamycin for 24 h then stimulation with TRAIL (0-2 µg/ml) for an additional 24 h dramatically induced cell death, with >50% apoptosis induction in cells treated with 5 µg/ml tunicamycin and 1.5 µg/ml TRAIL (Figure 1C).

Next we investigated whether the combined treatment undermines the tumor selectivity of TRAIL. Human differentiated thyroid epithelial HTori-3 cells were resistant to TRAIL and tunicamycin, moreover, their viability was not significantly affected by the combined treatment with tunicamycin and TRAIL for 24 h (Figure 1D). These results suggest that sensitizing regimens using tunicamycin with TRAIL may be preferentially toxic for undifferentiated thyroid carcinoma cells over normal thyroid epithelial cells.

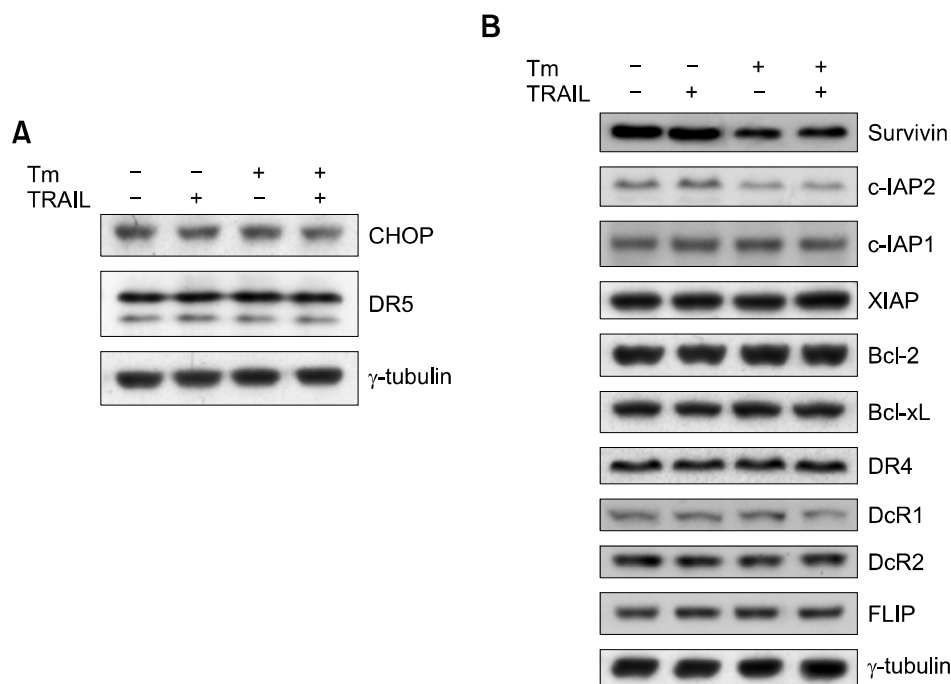
Tunicamycin also sensitized otherwise insensitive thyroid cancer KTC1 and KTC3 cells (Figure 1E), suggesting that it might generally enhance the antitumoral effects of TRAIL in thyroid cancer cells.

### Tunicamycin reduces expression of survivin in thyroid cancer cells

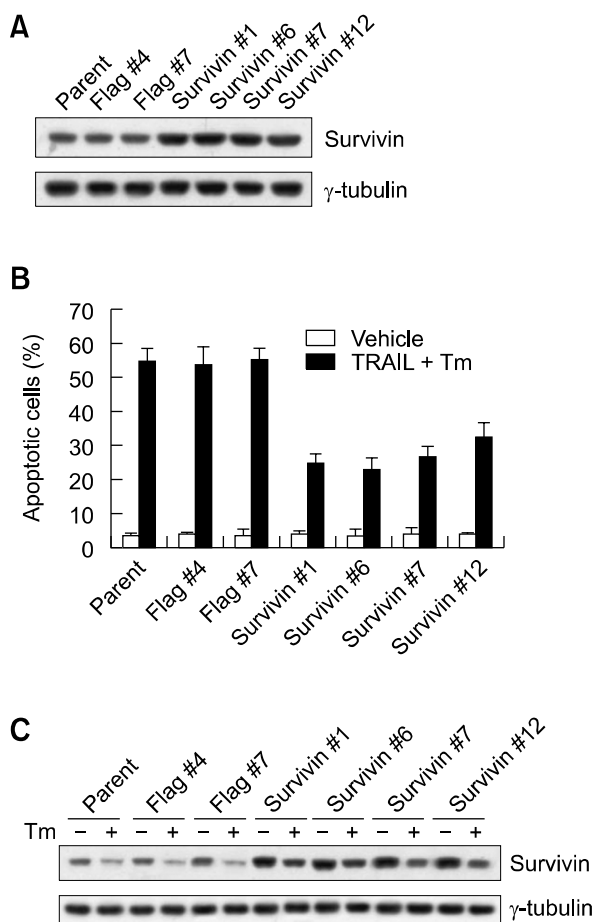
A previous report showed that tunicamycin increased the sensitivity of prostate cancer cells to TRAIL via induction of CHOP and subsequent upregulation of DR5 (Shiraishi *et al.*, 2005). In tunicamycin-treated ARO cells, only minor induction of CHOP was demonstrated, furthermore, no induction of DR5 was observed (Figure 2A). This suggested that other unidentified molecules might mediate the promoting effect of tunicamycin in ARO cells. To explore the underlying mechanisms by which tunicamycin sensitizes TRAIL-induced apoptosis in ARO cells, we analyzed the expression of several apoptosis-related proteins following treatment with tunicamycin and TRAIL. Treatment with tunicamycin alone led to a significant decrease in survivin and cIAP-2 protein levels, whereas XIAP, cIAP-1, Bcl-2 and Bcl-xL remained unaltered (Figure 2B). The combined treatment did neither change the expression levels of DR4, DcR1, DcR2 nor FLICE-inhibitory protein (FLIP) (Figure 2B).

### Overexpression of survivin compromises the sensitizing effect of tunicamycin on TRAIL in thyroid cancer cells

To examine whether this downregulation of survivin by tunicamycin is critical to trigger TRAIL-induced apoptosis, we established stable cell lines over-



**Figure 2.** Tunicamycin enhances TRAIL-induced apoptosis by downregulation of survivin. (A) ARO cells were treated with tunicamycin and/or TRAIL for 24 h, and CHOP and DR5 expression was determined by immunoblotting. (B) ARO cells were treated as (A) and western blotting was performed as antibodies indicated.



**Figure 3.** Forced expression of survivin reduces the sensitizing effect of tunicamycin on TRAIL. (A) ARO thyroid cancer cells were stably transfected with plasmids containing empty vector or survivin. (B) ARO cells were pre-treated with tunicamycin for 24 h and then treated with TRAIL for an additional 24 h, and apoptotic cells were assessed. (C) Parental ARO cells and sublines overexpressing survivin were treated with tunicamycin for 24 h and protein levels of survivin were evaluated with Western blotting.

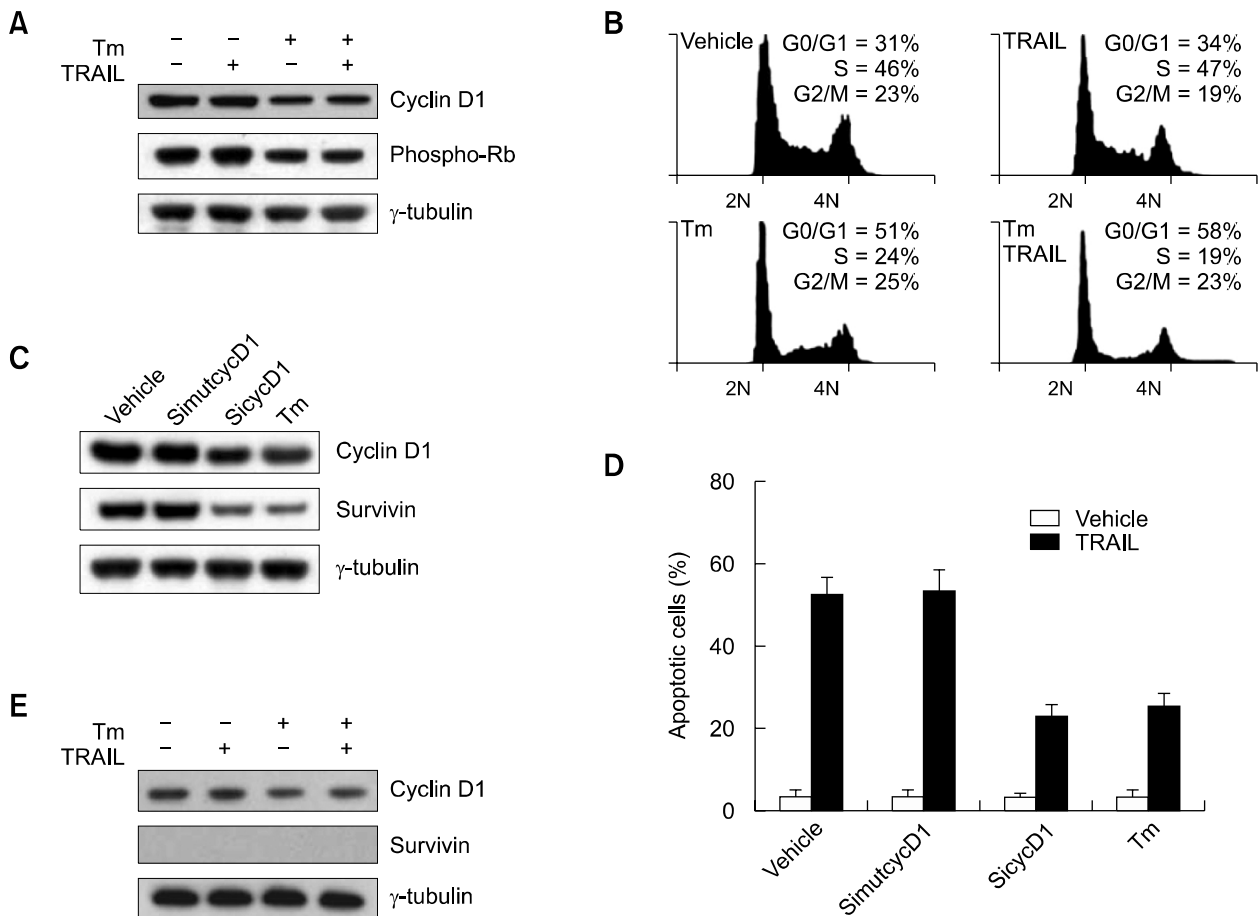
expressing survivin (Figure 3A). Ectopic expression of survivin significantly attenuated tunicamycin-facilitated TRAIL-induced apoptosis (Figure 3B). To further confirm whether the difference in survivin expression triggered by tunicamycin accounted for its effect on TRAIL sensitivity, we analyzed the changes in the total protein levels of survivin. The total protein levels of survivin in the tunicamycin-treated survivin stable cell lines still remained as high as those in untreated empty vector stable cells, despite the tunicamycin-mediated downregulation of endogenous survivin (Figure 3C). Taken together, these results support our hypothesis that downregulation of survivin, at least partially contributes to sensitization of TRAIL.

### Tunicamycin reduces survivin levels through downregulation of cyclin D1

The expression of the IAP family member survivin is cell cycle-dependent with lowest levels at G1 (Li *et al.*, 1998). Tunicamycin has been reported to block G1/S cell cycle progression (Brewer *et al.*, 1999; Hoozemans *et al.*, 2006), and previous studies suggested that agents that can arrest cells in the G1 phase of the cell cycle may increase TRAIL-induced cytotoxicity (Kim *et al.*, 2005a, b; Lu *et al.*, 2005). We therefore postulated that the reduction in survivin levels secondary to the tunicamycin-induced G1 arrest might account for the TRAIL-sensitizing effects of these drugs. In deed, tunicamycin treatment of ARO thyroid cancer cells potently reduced cyclin D1 protein levels (Figure 4A). Consistent with the established function of cyclin D in promoting cell cycle progression through G1 to S phase, the reduction in cyclin D1 levels by tunicamycin led to a dramatic reduction in phosphorylated Rb (Figure 4A). Cell cycle distribution confirmed that tunicamycin dramatically increased G0/G1 cells (Figure 4B). To examine the specific contribution of cyclin D1 to the decreased survivin level and TRAIL-sensitizing actions of tunicamycin, we selectively suppressed the expression of cyclin D1 by siRNA (Figure 4C). Silencing cyclin D1 inhibited survivin expression to a comparable degree as tunicamycin treatment (Figure 4C). In addition, knockdown of cyclin D1 significantly enhanced TRAIL-induced apoptosis (Figure 4D). To confirm the involvement of survivin and cyclin D1 in sensitizing effect of tunicamycin, we investigated their expression in HTori-3 cells. No survivin was observed and only little cyclin D1 was expressed in HTori-3 (Figure 4E). Collectively, these results suggested that the sensitizing effects of tunicamycin on TRAIL-induced apoptosis mediated at least in part by cyclin D1 downregulation and subsequent suppression of survivin expression.

### Ectopic expression of cyclin D1 attenuates the sensitizing effects of tunicamycin on TRAIL-induced apoptosis by preventing downregulation of survivin

Next, we examined whether forced expression of cyclin D1 could recover tunicamycin-mediated downregulation of survivin. ARO cells were stably transfected with expression vectors encoding cyclin D1 (Figure 5A). Tunicamycin-induced downregulation of survivin was alleviated by overexpression of cyclin D1 (Figure 5B), suggesting that cyclin D1 may be important for regulating survivin expression in these cells. We further investigated



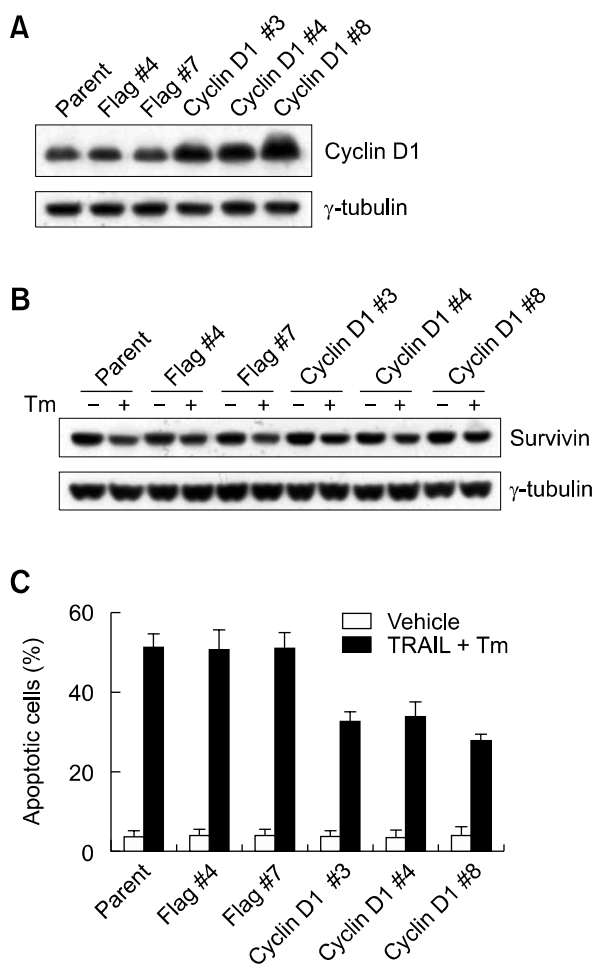
**Figure 4.** Tunicamycin reduces survivin levels through downregulation of cyclin D1. (A) ARO cells were treated with tunicamycin and/or TRAIL for 24 h, and cyclin D1 and phosphorylated Rb expression was determined by immunoblotting. (B) ARO cells were treated as in (A) and fixed for flow cytometric analysis of DNA content. G0/G1 phase is indicated by the 2N peak, S phase is indicated by the cross-hatched peak, and G2/M phase are indicated by the 4N peak. (C) ARO cells were transfected with indicated siRNAs or treated with tunicamycin for 24 h, and protein levels of survivin were determined. (D) ARO cells were treated as in (C), and then treated with TRAIL for additional 24 h, and apoptosis was assessed. (E) HTori-3 cells were treated as in (A) and western blot analysis was performed using the indicated antibodies.

whether forced enhancement of cyclin D1 could over-ride the sensitizing effects of tunicamycin on TRAIL-induced apoptosis. Apoptosis induced by combined treatment with tunicamycin and TRAIL was significantly attenuated by forced expression of cyclin D1 (Figure 5C). Taken together, our results demonstrate that subsequent downregulation of survivin by repression of cyclin D1 may provide one mechanism by which tunicamycin overcomes barriers blocking TRAIL-induced apoptosis of ARO cells.

## Discussion

The efficiency of TRAIL-induced apoptosis in malignant thyroid cancers is variable and many human tumors are resistant to TRAIL-induced

apoptosis or acquired resistance to TRAIL during treatment, thereby potentially limiting its therapeutic utility. Hence, it is imperative to identify drugs or agents, which overcome the *de novo* or acquired resistance of neoplasms to TRAIL-induced apoptosis. Tunicamycin has been used to sensitize spontaneously resistant prostate cancer cells and acquired resistant colon cancer cells to TRAIL-induced apoptosis (Shiraishi *et al.*, 2005). However, the mechanism responsible for the promoting effect of tunicamycin on TRAIL-induced apoptosis is not completely identified. Although previous studies suggested that tunicamycin sensitize prostate cancer cells to TRAIL-induced apoptosis via upregulation of CHOP and subsequent DR5, no increase of DR5 was observed in tunicamycin-treated ARO cells. Here we showed that TRAIL-resistant ARO cells could be converted



**Figure 5.** Forced expression of cyclin D1 suppressed the sensitizing effect of tunicamycin on TRAIL by downregulation of survivin. (A) ARO cells were stably transfected with plasmids containing empty vector or cyclin D1. (B) Parental cells and sublines overexpressing cyclin D1 were treated with tunicamycin for 24 h and protein levels of survivin were evaluated with Western blotting. (C) Cells were pre-treated with tunicamycin for 24 h and then treated with TRAIL for an additional 24 h, and apoptotic cells were assessed.

to TRAIL-sensitive cells by cotreatment with tunicamycin by suppression of cyclin D1 and subsequent downregulation of survivin.

Survivin is a member of the IAP family that is selectively expressed in many human neoplasms, but not normal adult tissues, and plays a dual role in apoptosis suppression and cell cycle regulation. Previous reports have indicated that survivin is overexpressed in thyroid carcinomas and its high expression level correlated with unfavorable prognosis and resistant to therapy. Indeed, survivin overexpression has been shown to confer protection against many apoptotic stimuli, while inhibition of survivin by a variety of strategies sensitizes cancer cells to apoptosis (Li *et al.*, 1998, 1999; Griffith *et al.*, 2002; Chawla-Sarkar *et al.*, 2004;

Fulda *et al.*, 2004). Since survivin block apoptosis at the effector phase, a point where multiple signaling pathways converge, strategies for removing the inhibitory effects of survivin seem to be potentially useful for overcoming the resistance of cancer cells to anticancer drugs. We found that in parallel with the downregulation of cyclin D1 following treatment with tunicamycin, survivin was dramatically reduced. The functional significance of downregulation of cyclin D1 and the subsequent suppression of survivin expression in tunicamycin-enhanced TRAIL-induced apoptosis was confirmed by our observations that silencing cyclin D1 downregulates survivin and promotes TRAIL-induced apoptosis, on the contrary, forced overexpression of cyclin D1 could attenuate tunicamycin-potentiated TRAIL-induced apoptosis, over-riding tunicamycin-mediated downregulation of survivin. Furthermore, we have shown that ectopic expression of survivin partially inhibits apoptosis induced by tunicamycin and TRAIL. Thereby, survivin may be a key mediator of TRAIL resistance in thyroid cancer cells and that the sensitizing effect of tunicamycin on TRAIL-induced apoptosis may at least partially, result from the downregulation of survivin protein levels *via* tunicamycin-mediated suppression of cyclin D1, although we cannot exclude the possibility that tunicamycin may have a direct effect on survivin expression as well. Because both cyclin D1 and survivin are amplified and/or overexpressed in a number of human thyroid carcinomas, these tumors may be particularly vulnerable to the combination of tunicamycin and TRAIL.

In our study, combined treatment with tunicamycin and TRAIL was cytotoxic to ARO cells, while differentiated HTori-3 thyroid epithelial cells were spared. Therefore, treatment with TRAIL in combination with subtoxic doses of tunicamycin suggests a potential applicability for treating resistant thyroid cancer cells, although extensive animal studies and preclinical trials using this regimen will be required.

## Methods

### Plasmids, antibodies and reagents

Tunicamycin and recombinant TRAIL were obtained from Sigma (St. Louis, MO) and R&D systems (Minneapolis, MN), respectively. The following antibodies were used for immunoblotting: DR4, DR5, DcR2 (Stressgen, Victoria, Canada), tubulin (Sigma-Aldrich), DcR1 (BD Biosciences, San Jose, CA), XIAP and survivin (R&D Systems, Minneapolis, MN), phospho S249 Rb and cIAP-2 (Abcam, Cambridge, MA), cyclin D1, cIAP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and FLIP (Novus Biologicals,

Littleton, CO).

### Cell culture

Human ARO thyroid carcinoma cells and immortalized, differentiated thyroid epithelial HTori-3 cells were obtained from the American Type Culture Collection (ATCC) and European Tissue Culture Collection (ETCC), respectively. KTC1 and KTC3 thyroid carcinoma cells were generously provided by Dr Junichi Kurebayashi (Kawasaki University of Medical Science, Japan). Cells were maintained in RPMI 1640 supplemented with 10% FBS (Invitrogen, Carlsbad, CA).

### Western blot analysis

Cell lysates were prepared in modified RIPA buffer (50 mM Tris, 150 mM NaCl and 1% Triton X-100) with complete protease inhibitors cocktail. Cell lysate was analyzed by western blot analysis with ECL detection reagents (Amersham Bioscience, Buckinghamshire, UK).

### Construction of survivin or cyclin D1 plasmid and generation of ARO cells stably overexpressing survivin or cyclin D1

A cDNA encoding human survivin or cyclin D1 was generated by PCR from human brain cDNA library (Invitrogen, Carlsbad, CA) and subcloned into the eukaryotic expression plasmid pcDNA3. The constructs were verified by DNA sequencing. Cells were transfected with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) as instructed by the supplier. Control cells were transfected with the pcDNA3-Flag. Transfected cells were selected in medium containing G418 (800 µg/ml) and resistant colonies were expanded. Western analyses employing the antibodies to survivin, cyclin D1 or Flag were used to identify positively expressing cells.

### Small interfering RNA (siRNA)

The siRNA sequences used here were as follows: siRNA against cyclin D1 (sicycD1), GUUCAUUUCCAAUCC-GCCC. The scramble nonsense siRNA (scramble; CCGU-AUCGUAAGCAGUACU) that has no homology to any known genes was used as control. In addition, position mismatched (sequence underlined) siRNA against cyclin D1 (simutycD1; GUUCAUUUGGAAUCCGCC) was also used to confirm the specificity of sicycD1. Transfection of siRNA oligonucleotide was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

### Detection of cell death

Cell death was measured by Annexin-PE Apoptosis Detection Kit I according to the manufacturer's protocol (BD Bioscience), samples were analyzed by fluorescence-activated cell scanner (FACScan) flow cytometer (Becton Dickinson, Franklin Lakes, NJ). At least 500 nuclei were scored per experiment and all experiments were performed

three times.

### Flow cytometry

After fixation with 70% ethanol, cells were rehydrated in PBS, stained with propidium iodide (Sigma) and analyzed for DNA content by flow cytometry (FACScan, Becton Dickinson). Cell cycle distribution was modeled using ModFit software (Verity Software House).

### Data analysis

Statistical difference was evaluated using the two-tailed Student's *t* test; *P* < 0.05 was considered significant.

### Acknowledgements

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