AY4, an agonistic anti-death receptor 4 MAB, induces apoptotic cell death in anaplastic thyroid cancer cells via downregulation of Bcl-xL with reactive oxygen species generation

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Abstract
Anaplastic thyroid carcinoma (ATC) is an aggressive human tumor with a median survival of 6 months. We previously developed an agonistic anti-death receptor 4 MAB, AY4, and demonstrated the antitumor effects of AY4 in head and neck cancer cells. Presently, we show that ATC cells are sensitive to AY4 and that the sensitivity correlates with the reduced expression level of Bcl-xL and reactive oxygen species (ROS) generation. AY4 induced death of C-643, U-HTH 7, HTH83, and SW1736 cells. To elucidate the role of ROS generation in AY4-induced apoptosis of ATC cells, U-HTH 7 and SW1736 cells were pretreated with an antioxidant (N-acetyl cysteine, NAC) followed by AY4 treatment. The cell death was blocked by NAC. AY4-induced cell death was accompanied by the downregulation of the anti-apoptotic protein, Bcl-xL (BCL2L1). To examine the link between the apoptotic response and Bcl-xL protein expression, U-HTH 7 cells were transfected with Bcl-xL plasmid. The consequence of the overexpression of Bcl-xL appeared to decrease AY4-mediated cell death by blocking ROS generation in U-HTH 7 cells. By contrast, Bcl-xL knockdown using small interfering RNA of Bcl-xL enhanced AY4 sensitivity in HTH83 and C-643 cells and rendered the cells sensitive to AY4-induced cell death. The results support the conclusion that the expression level of Bcl-xL is important in the AY4-induced apoptosis of ATC cells through ROS generation. AY4 may be a promising tool for ATC therapy.

Key Words
- death receptor 4
- agonistic antibody
- apoptosis
- ROS
- anaplastic thyroid carcinoma (ATC)

Introduction
Anaplastic thyroid carcinoma (ATC) is one of the most aggressive human malignancies and is mainly responsible for thyroid cancer-related death (Ain et al. 2000). ATC is highly metastatic and has a poor outcome when treated with therapies including surgery, radiation, and combined chemotherapy (Veness et al. 2004). Therefore, the development of effective treatment approaches for patients with ATC is essential.

The death receptor ligand tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL) is an attractive
anticancer agent because it can selectively kill tumor cells, but not normal cells (Ashkenazi & Herbst 2008). There are two functional receptors for TRAIL, death receptor 4 (DR4) and 5 (DR5). Each contains a death domain, which triggers cell death upon TRAIL binding. Binding of TRAIL to DR4 and DR5 leads to oligomerization of the receptors and forms the death-inducing signaling complex (DISC) with Fas (TNFRSF6) associated via death domain (FADD), caspase 8, and ribosome inactivating protein. The formation of DISC activates caspase 8 and subsequently induces a signaling pathway for apoptotic cell death (Ashkenazi 2002).

Many types of cancer cells are resistant to the apoptosis-inducing activity of TRAIL. In response, researchers have developed a death receptor agonist capable of overcoming TRAIL resistance in cancer cells. The resistance mechanisms that inhibit the DR4- and DR5-mediated apoptotic pathway include loss and/or mutation of pro-apoptotic receptors; upregulation of Bcl-2, Bcl-xL, and/or Mcl-1; and upregulation of inhibitors of apoptosis proteins (IAPs), such as X-linked IAP (XIAP) and survivin (Ashkenazi & Herbst 2008). Conticello et al. (2007) have reported that proteasome inhibitor, bortezomib, sensitizes ATC cells to TRAIL-induced apoptosis through upregulation of TRAIL receptors, p21 and pro-apoptotic molecules and downregulation of Bcl-2, Bcl-xL and inhibitor of apoptosis-1. Festa et al. (2009) have also reported that R-roscovitine sensitizes ATC cells to TRAIL-induced apoptosis via caspase activation and inhibition of the IKK/NF-kB pathway. Borbone et al. (2010) have reported that HDAC inhibitors such as SAHA and MS-275 induced apoptosis in thyroid cancer cells through proteasome-dependent inhibition of TRAIL degradation.

Bcl-xL (BCL2L1) is an anti-apoptotic protein localized to the outer mitochondrial membrane (Chao et al. 1995, Huang 2000). Thyroid cancer cells can display pronounced production of Bcl-xL (Stassi et al. 2003) and a negative correlation has been described between the Bcl-xL levels and sensitivity to TRAIL-induced cell death (Petrella et al. 2006).

Intracellular reactive oxygen species (ROS) usually include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (•OH) that are able to cause progressive modification or degradation of cellular molecules such as nucleic acids, proteins, and lipids (Finkel 2003). ATC FRO cells can be induced to undergo apoptotic cell death by _Pteris semipinnata_ L.SF (PsL5F) through ROS generation as a secondary messenger (Liu et al. 2009). However, TRAIL-induced apoptosis in thyroid cancer and the involvement of ROS generation have not yet been reported.

Our previous study documented the selective binding of MAB AY4 to DR4 and the activation of a specific DR4 without cross-reactivity with decoy receptors (Sung et al. 2009). We also recently reported that AY4 effectively induces apoptosis in head and neck cancer cells via mitochondrial ROS generation (Lee et al. 2012).

This study identifies a role of the anti-apoptotic protein Bcl-xL in the regulation of AY4-induced apoptotic cell death in thyroid cancer cells. In addition, data are presented to demonstrate that Bcl-xL regulates ROS-mediated apoptosis and contributes to AY4-induced apoptotic cell death. The clarified understanding of the mechanisms of AY4-induced apoptotic cell death in thyroid cancer cells may prelude the development of effective therapeutic strategies.

### Materials and methods

#### Cell culture and reagents

Human ATC cell lines HTH83, C-643, U-HTH 7, and SW1736 were provided by Dr Yoon Woo Koh (Department of Otorhinolaryngology, Yonsei University College of Medicine, South Korea). U-HTH 7 cells were cultured in high glucose DMEM (Gibco/Invitrogen). HTH83, C-643, U-HTH 7, and SW1736 cells were cultured in RPMI-1640. All cell lines were maintained at 37 °C and 5% CO$_2$ in media supplemented with 10% fetal bovine serum (Gibco/Invitrogen). AY4, murine MAB, and human recombinant TRAIL were provided by Dr Yong-Sung Kim (Sung et al. 2009, 2010). N-acetyl cysteine (NAC) was purchased from Sigma–Aldrich.

#### Small interfering RNA or plasmid transfection

Transfection with small interfering RNA (siRNA) was done as described previously (Lee et al. 2012). HTH83 and C-643 cells were transfected with siRNA (100 nM) using Lipofectamine 2000 (Gibco/Invitrogen). pSFFV-neo Bcl-xL plasmid (plasmid 8749) was obtained from Addgene (Cambridge, MA, USA) (Chao et al. 1995). Bcl-xL-transfected U-HTH 7 cells were selected 48 h after transfection using 1200 μg/ml G418 (Gibco/Invitrogen). After 2 weeks, the surviving colonies were pooled to avoid clonal variation.

#### Determination of cell viability

Cells were seeded at a density of 5×10$^3$ cells per well into 96-well plates and cultured for 24 h and then treated with AY4 (0.5, 1, 5, and 10 μg/ml) or TRAIL (0.5 μg/ml) for 24 h. After the incubation, each well received 40 μl...
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma–Aldrich) for 4 h. The insoluble formazan precipitate was dissolved in dimethyl sulfoxide and quantified by spectrophotometry at 570 nm. The data are presented as a percentage relative to untreated control cells.

**Annexin V-FITC and propidium iodide staining**

Cells (1 × 10^5) were seeded into six-well plates and pretreated for 1 h with NAC (5 mM) followed by 24 h of treatment with AY4 at a concentration of 1 μg/ml and harvesting. The cells were incubated with Annexin V-FITC and propidium iodide (PI) according to the manufacturer’s protocol (BD Biosciences, San Jose, CA, USA). The stained cells were analyzed using flow cytometry (BD Biosciences).

**Western blot analysis**

Cell lysates were prepared and western blot analyses were performed as recently described (Lee et al. 2012). Antibodies to Bcl-xL, XIAP, cleaved caspase 3, cleaved caspase 8, Bid, poly (ADP-ribose) polymerase (PARP), c-Jun N-terminal kinase (JNK), phospho-JNK (p-JNK), p38, and p-p38 were acquired from Cell Signaling Technology (Beverly, MA, USA). Anti-DR4 and DR5 antibodies were acquired from KOMA Biotech (Seoul, Korea). α-Tubulin antibody was purchased from Calbiochem (San Diego, CA, USA).

![Figure 1](image-url)

**Figure 1**

Effect of AY4 on anaplastic thyroid cancer cell lines and expression of DR4 and DR5 in ATC cell lines. (A) HTH83, C-643, U-HTH7, and SW1736 cells were treated with AY4 (0.5, 1, 5, and 10 μg/ml) for 24 h. Relative cell viabilities were quantified with the MTT assay. The scale bars represent the mean ± s.d. (***P < 0.01; ****P < 0.001). (B) Western blot analysis was performed using antibodies against DR4 or DR5 antibody. α-Tubulin was used as loading control.
Measurement of ROS production

Measurement of ROS production and mitochondrial superoxide was prepared and performed as described previously (Lee et al. 2012). AY4-treated cells were incubated with 10 μM 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) dye (Molecular Probes, Eugene, OR, USA). For the measurement of mitochondrial superoxide, AY4-treated cells were incubated with MitoSOX Red (Molecular Probes). The fluorescent-stained cells (1×10⁶) were analyzed by flow cytometry.

Statistical analysis

The results are presented as mean±s.d., with P<0.05 being considered significant using Student’s t-test.

Results

Induction of cytotoxicity in ATC cell lines by AY4 and expression of DR4 and DR5

To investigate the effect of AY4 on the HTH83, C-643, U-HTH 7, and SW1736 ATC cells, we initially treated the cells with AY4 (0.5, 1, 5, and 10 μg/ml) or TRAIL (0.5 μg/ml) for 24 h (Fig. 1A). The viability of U-HTH 7 cells was highly decreased by 0.5 μg/ml AY4 compared with TRAIL-treated cells. Moreover, the viability of SW1736 cells was decreased by 20–30% after the treatment with AY4. And the viabilities of HTH83 and C-643 cells were decreased by 10% after treatment with high dose of AY4. The levels of DR4 and DR5 were ascertained in the four selected ATC cell lines. DR4 was expressed in U-HTH 7, SW1736, HTH83, and C-643 cells. DR5 was expressed in HTH83 or SW1736 cells, while cells from the remaining cell lines displayed low expression of DR5 (Fig. 1B).

AY4-induced apoptotic protein expression and ROS generation in U-HTH 7 cells

To investigate the activation of apoptotic protein in U-HTH 7 cells treated with 1 μg/ml AY4 for various times, we performed western blot analysis using antibodies against cleaved PARP, cleaved caspase 3, cleaved caspase 8, Bcl-xL, XIAP, Bid, p-JNK, and JNK in AY4-treated U-HTH 7 cells. α-Tubulin was used as a loading control (Fig. 2A). Increased expressions of cleaved caspase 3, cleaved caspase 8, and PARP were detected from 3 h after AY4 treatment, and expression of Bcl-xL, XIAP, and Bid began to decrease from 3 h. However, p-JNK was detected at 1 h after treatment with AY4 and then decreased. In a previous study, we demonstrated that ROS generation contributes to AY4-induced apoptotic cell death in head and neck cancer cell lines (Lee et al. 2012). Given this prior finding, we wondered whether AY4-induced apoptosis correlated with ROS generation in ATC cells, so we performed fluorescence-activated cell sorting (FACS) analysis after DCFDA staining in AY4-treated U-HTH 7 cells (Fig. 2B). Fluorescence signals were evidently shown starting from 3 h after AY4 treatment.
NAC protects from AY4-induced apoptotic cell death

U-HTH 7 and SW1736 cells were treated with NAC for 1 h before 24 h of treatment with AY4. Blockage of AY4-induced cell death by NAC was observed using light microscopy (Fig. 3A) as we stained the apoptotic cells with Annexin V-FITC and PI and analyzed them using FACS (Fig. 3B). NAC inhibited the increase of ROS in AY4-treated cells (Fig. 3C). To examine the relationship between ROS and the apoptotic signaling pathway of cell death induced by AY4, western blot analysis was done to determine whether the AY4-induced apoptotic pathway would be decreased by NAC in U-HTH 7 and SW1736 cells (Fig. 3D). The increased expressions of cleaved caspase 3, caspase 8, and PARP that were induced by AY4 were attenuated by the pretreatment of NAC. Furthermore, the pretreatment of NAC before AY4 treatment kept the expression level of Bcl-xL, XIAP, and Bid from decreasing in AY4-treated cells. Taken together, the results are consistent with the view that ROS generation contributes directly to AY4-induced apoptotic cell death.

Overexpression of Bcl-xL protein decreased AY4-induced apoptotic cell death via inhibition of mitochondrial ROS generation

Decreased Bcl-xL was evident from 3 h after AY4 treatment in U-HTH 7 cells (Fig. 2A). To demonstrate the link between the decreased expression level of Bcl-xL and ROS production, Bcl-xL or control plasmid was stably transfected in U-HTH 7 cells, and the effect on AY4-induced apoptotic cell death was determined by western blot analysis. As shown in Fig. 4A, the decreases in level of the protein expressions of the cleaved caspase 3 and cleaved PARP were clearly observed after AY4 treatment in Bcl-xL-transfected cells compared with mock-transfected cells. Flow cytometric analysis of ROS generation stained by DCFDA or mitochondrial superoxide by MitoSox also showed more substantial reduction in AY4-induced fluorescence in Bcl-xL-transfected cells than in mock-transfected cells (Fig. 4B and C). This finding suggests that overexpression of Bcl-xL causes the decrease in AY4-induced cell death with inhibition of mitochondrial ROS generation.

Figure 3

NAC prevents AY4-induced apoptotic cell death. U-HTH 7 or SW1736 cells were pretreated with NAC for 1 h and then treated with 1 μg/ml AY4 for 24 h. (A) AY4-induced cell death was ascertained with light microscopy. (B) To measure cell death, cells were stained with Annexin V-FITC and propidium iodide and analyzed using FACS. (C) U-HTH 7 or SW1736 cells were pretreated with NAC for 1 h before the 24-h AY4 treatment and then stained with DCFDA dye and analyzed using FACS (**P < 0.01). (D) At the same time, western blot analysis was performed with antibodies against cleaved caspase 3, PARP, Bcl-xL, XIAP, Bid cleaved caspase 8, and α-tubulin.
Knockdown of Bcl-xL in HTH83 or C-643 cells sensitizes cells to AY4-induced apoptotic cell death

Figure 1A showed that HTH83 or C-643 cells were less sensitive to AY4-induced cell death than U-HTH 7 or SW1736 cells. To demonstrate the role of decreased expression levels of Bcl-xL in AY4-induced apoptotic cell death with ROS generation, we specifically knocked down Bcl-xL in HTH83 and C-643 cells. Cell death was observed after AY4 treatment in HTH83 and C-643 cells lacking Bcl-xL using light microscopy (Fig. 5A). At the same time, we stained the apoptotic cells with Annexin V-FITC and PI and analyzed using FACS. The result showed the finding that knockdown of Bcl-xL in HTH83 and C-643 cells become sensitive to AY4-induced apoptotic cell death (Fig. 5B). Also, western blot analysis was performed using antibodies against cleaved caspase 3, PARP, Bcl-xL, and α-tubulin (Fig. 5C). Increased expressions of cleaved caspase 3 and PARP were evident in Bcl-xL siRNA-transfected cells compared with control siRNA-transfected cells. To examine the relationship between apoptotic response and ROS production, we performed FACS analysis after DCFDA staining in siRNA-transfected cells. Interestingly enough, detection of fluorescence signals in Bcl-xL siRNA-transfected cells treated with AY4 was significant, compared with control siRNA-transfected cells (Fig. 5D). Taken together, these findings demonstrate that Bcl-xL is responsible for AY4-induced apoptotic cell death via regulating of ROS production (Fig. 6).

Discussion

Previously, AY4 as a single agent was shown to be capable of inducing caspase-dependent apoptotic cell death of several tumor types without cytotoxicity to normal human hepatocytes (Sung et al. 2009). Synergistic effects of AY4 with histone deacetylase inhibitors enhanced the cell death in the T-cell acute lymphoblastic leukemia cells that were intrinsically resistant to AY4 (Sung et al. 2010). In our recent paper, we were able to clearly show that ROS generation plays a critical role in caspase-dependent cell death that is induced by AY4 in head and neck cancer cells. Also, we provided evidence of the importance of Bcl-xL expression level in cell death by showing cleaved caspase 3 and cleaved PARP signaling that lead to cell death in AY4-resistant head and neck cancer cells with Bcl-xL knocked down using Bcl-xL siRNA (Lee et al. 2012). Unfortunately, we were not able to identify the phenotype of cell death in previous study. However, our finding in this paper is that NAC (ROS scavenger) treatment inhibits AY4-induced cell death as well as it affects Bcl-xL expression, which therefore indicated that ROS generation causes cell death through AY4-induced apoptotic cell death by down-regulating Bcl-xL in ATC cell lines. As shown in Fig. 4, when Bcl-xL is overexpressed, AY4-induced ROS generation is inhibited, and cleaved caspase 3 and cleaved PARP are decreased compared with the control. And when we knocked down Bcl-xL protein in HTH83 and C643 in which 5–20% of cell death is induced by AY4 (Fig. 1), about 50% of cell death and the increase in ROS were observed (Fig. 5). Therefore, this paper demonstrates the clear correlation between ROS generation and Bcl-xL in AY4-induced cell death, that is, ROS generation decreases Bcl-xL, which leads to AY4-induced cell death in ATC cells.
When the four selected ATC cell types were treated with TRAIL or AY4, as shown in Fig. 1, AY4-treated ATC cells were more sensitive to cell death compared with TRAIL-treated cells. Furthermore, there is no significant correlation between the expression of DR4 and level of AY4-induced cell death in thyroid cancer (Fig. 1B). In another study, DR4 or DR5 expression level was not correlated with sensitivity to stimulation of apoptotic cell death (Ashkenazi 2002).

We have reported that ROS is a mediator in AY4-induced cell death in head and neck cancer cell lines. As shown in Fig. 3, the antioxidant NAC inhibited AY4-induced cell death in U-HTH 7 or SW1736 thyroid cancer cells, indicating that ROS generation has an important role in AY4-mediated apoptotic cell death. Other studies related to thyroid cancer have reported that apoptotic cell death of ATC FRO cells by PsL5F was mediated via upregulated ROS (Liu et al. 2009). The production of ROS has also been associated with bortezomib-induced apoptosis in thyroid cancer cells (Du et al. 2009). Moreover, 15-deoxy-delta12,14-prostaglandin J2 is cytotoxic via intracellular ROS in cells of the CG3 thyroid papillary cancer cell line (Chen et al. 2002). The collective data implicate ROS as an important mediator of the death of thyroid cancer cells.

The Bcl-2 family consists of both pro-apoptotic (Bax, Bak, and Bad) and anti-apoptotic (Bcl-2 and Bcl-xL) proteins, which respectively promote and inhibit the execution of the apoptotic cell death (Kelekar & Thompson 1998). Bcl-xL can inhibit the apoptosis with BH3-containing death agonists such as Bax (Minn et al. 1999). The upregulation of Bcl-xL expression protects thyroid cancer cells from iodine-induced apoptosis (Liu et al. 2010). These observations prompted us to wonder whether high expression of Bcl-xL in thyroid cancer cells is less sensitive to AY4-induced cell death. As shown in Fig. 4, when we transfected U-HTH 7 cells with Bcl-xL plasmid, decreased cell death by AY4 was
evident compared with mock-transfected U-HTH 7 cells. Interestingly, we also observed that Bcl-xL overexpressing cells displayed the inhibited productions of ROS as well as mitochondrial superoxide. Thus, Bcl-xL overexpression may be a consequence of the inhibition of ROS generation by AY4. Next, we used HTH83 and C-643 cells; however, as shown in Fig. 1, HTH83 and C-643 cells displayed less sensitivity to AY4 or TRAIL alone at the tested concentrations than U-HTH 7 and SW1736 cells. To examine the link of Bcl-xL protein and sensitivity of AY4-induced cell death in more detail, we transfected HTH83 or C-643 cells with Bcl-xL siRNA. Treatment of AY4 of Bcl-xL knockdown HTH83 or C-643 cells caused the generation of ROS and subsequently induced apoptotic cell death. On the basis of these results, the expression level of Bcl-xL seems to be critical for AY4-induced cell death in ATC.

Taken together, our results demonstrate an apoptotic effect of AY4 on ATC cell lines. The most important mechanism of cell death by AY4 in ATC cells involved the decreased Bcl-xL protein during cell death and the production of ROS as Bcl-xL and ROS were negatively associated with cell death.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This study was supported by a grant from the Korean Health Technology R&D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (A101800) and the National Research Foundation of Korea (NRF) grant (2012R1A2A2A01013982).

Figure 6
Proposed signaling pathway for AY4-induced cell death in anaplastic thyroid cancer.
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Received in final form 19 February 2013
Accepted 20 February 2013

Made available online as an Accepted Preprint 21 February 2013