Tumori, 97: 762-770, 2011

Simultaneously targeting Bcl-2 and Akt pathways reverses resistance of nasopharyngeal carcinoma to TRAIL synergistically

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ABSTRACT

Aims and background. Despite progress in treatment techniques, the five-year survival rate of nasopharyngeal carcinoma (NPC) is disappointing. Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) can selectively induce apoptosis in most tumor cells while sparing normal cells. Given the antiapoptotic functions of Bcl-2 and Akt, we examined the effects of targeting these pathways alone or simultaneously on TRAIL apoptosis in NPC cell lines.

Methods and study design. We first tested the cytotoxic effect of TRAIL and the expression of death receptors, Bcl-2, Akt, and p-Akt on four NPC cell lines by MTT and Western blotting, respectively. Small interfering RNAs (siRNAs) targeting Bcl-2 and PI3-K inhibitor (LY294002) were used alone or combined with TRAIL in the cell lines and cytotoxicity was examined by MTT. Apoptosis rates, mitochondrial transmembrane potential, and apoptotic pathway signals were detected by flow cytometric analysis, DiOC6(3) assays, and Western blotting after the various combination treatments on CNE-2, the cell line that was most resistant to TRAIL.

Results. Although no direct correlation between the sensitivity to TRAIL and the relative expression levels of Bcl-2 and activated Akt was found in the NPC cell lines examined, siRNA mediated the downregulation of Bcl-2 and LY294002-induced inactivation of Akt, increasing the sensitivity of all examined NPC cell lines to TRAIL. Synergistic enhancement of TRAIL-mediated cytotoxicity was observed in combination treatment of Bcl-2 siRNA and LY294002 compared to cells treated with each treatment alone. The synergistic effects were mediated through increased apoptotic signaling of the mitochondrial pathway, as was evident from the more increased mitochondrial depolarization, activation of caspase-9 and caspase-3, and suppression of XIAP.

Conclusions. This study provides proof of principle that TRAIL combined with simultaneously targeting the Bcl-2 and Akt signaling pathways may have potential as a novel future treatment strategy for NPC.

Key words: nasopharyngeal carcinoma, TRAIL, resistance, Bcl-2, Akt.

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Received November 25, 2010;
accepted July 8, 2011.

Introduction

Although rare in most parts of world, nasopharyngeal carcinoma (NPC) is endemic in certain regions, especially in southeast Asia. The incidence is approximately 30 to 80×10^5 population per year in southern China[1]. Radiotherapy alone was the standard treatment for almost all stages of NPC until 1990[2]. Patients presenting with early-stage disease are a minority and may be effectively treated with radiotherapy alone[3]. A 5-year local control rate ranging from 80% to 95% may be achieved in these good-prognosis patients. However, the vast majority of patients with NPC present with locally advanced disease. Despite progress in treatment techniques, the outcome with radiotherapy alone is disappointing, with 5-year survival rates between 34% and 52%[4]. Thus, new therapies which focus on special targets are being studied.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) superfamily. TRAIL has been shown to induce apoptosis in various tumor cell lines *in vitro* and to suppress the growth of tumors *in vivo*[5,6]. TRAIL-induced apoptosis is initiated by TRAIL binding to the death receptor (DR). The Fas-associated death domain (FADD) and caspase-8 are aggregated and recruited to the DR-TRAIL complex to form the death-inducing signaling complex (DISC)[7]. Then the apoptosis signal is transmitted through 2 types of pathway: extrinsic and intrinsic. The extrinsic pathway is activated in a mitochondria-independent manner. Upon recruitment, caspase-8 is activated and induces direct cleavage of downstream effector caspases such as caspase-3 and caspase-7 to induce apoptosis[5]. The intrinsic pathway is activated in a mitochondria-dependent manner[8]. Signals originating from the DISC may be linked to mitochondria by Bid, which contains a homology domain protein of the Bcl-2 family. Once cleaved by caspase-8, Bid translocates to mitochondria and activates the Bcl-2 family members Bax and Bak, leading to mitochondrial depolarization[9]. Apoptogenic factors such as cytochrome c, apoptosis-inducing factor (AIF), caspase-2 or caspase-9, downstream of the mitochondria and the apoptosome, are released from mitochondria into the cytosol to trigger activation of caspase-3 through formation of the apoptosome complex[9]. Unlike the other death ligands such as CD95 or TNF-α, TRAIL does not cause systemic toxicity in mice or nonhuman primates, although further studies may be necessary to evaluate the possible cytotoxicity of TRAIL in normal human tissues, e.g., hepatocytes[10]. DR expression was detected in head and neck tumor specimens but not in any surrounding normal tissues. These findings supported the idea that the presence of DRs in some head and neck cancers may make them more susceptible to TRAIL-mediated apoptosis[11]. Thus, TRAIL may be a promising novel therapeutic agent for human head and neck cancer including NPC. However, to date there has been limited clinical application of TRAIL. Griffith *et al.*[12] proposed TRAIL gene therapy with viral vector delivery. Most of the clinical studies carried out to date used humanized monoclonal agonistic antibodies to DR4 or DR5, in part for their longer half-life. In a phase I study there was limited toxicity of mapatumumab, a fully human monoclonal antibody with agonist activity to DR[13].

However, recent studies have shown that some cancer cells are resistant to TRAIL-mediated apoptosis[14]. The etiology of cancer cell resistance to TRAIL apoptosis is complex and attributable to a number of factors, including high expression of antiapoptotic molecules and activation of prosurvival signaling pathways. These factors are potential candidates for therapeutic intervention. Bcl-2 and Akt are 2 factors that are implicated in cancer cell resistance to TRAIL. Bcl-2 overexpression is prognostic in NPC[15] and blocks TRAIL apoptosis in some cancer cells[16]. Among the cellular signaling pathways that promote cell survival, Akt is one of the important survival factors that contributes to the resistance to apoptotic signals. Activation of the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway plays an important role in survival when cells are exposed to various kinds of apoptotic stimuli including TRAIL[17]. Previous studies have shown that inhibition of Bcl-2 expression and suppression of the PI3-K/Akt pathway sensitized some cancer cells to TRAIL-mediated apoptosis[18,19].

TRAIL has shown difficulty in inducing apoptosis in some NPC cell lines[20] and the potential reasons remain unknown. Some research demonstrated that targeting the Bcl-2 or Akt pathway enhanced the chemosensitivity of NPC cells[21,22], so we hypothesized that targeting the Bcl-2 and Akt signaling pathways could enhance the sensitivity to TRAIL of NPC cells and their combination would be a more efficient approach to reactivate TRAIL apoptosis. This study was designed to investigate if treatment of NPC cells with Bcl-2 small interfering RNA (siRNA) transfection and PI3-K inhibitors that inactivate Akt simultaneously would be an effective way to induce TRAIL-mediated apoptosis. We also examined factors that mediate activation of the extrinsic and intrinsic apoptotic pathways.

Materials and methods

Cell lines and reagents

Four human NPC cell lines (CNE-1, CNE-2, 5-8F, 6-10B) were used in this study. CNE-1 is a well-differentiated and CNE-2 a poorly differentiated NPC cell line. 5-8F is a highly metastasized and 6-10B a poorly metastasized cell line. All the cells were kindly provided by the Department of Otolaryngology and Head and Neck Surgery, Xiangya Second Hospital of Central South University. They were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and penicillin/streptomycin, and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. TRAIL and PI3-K inhibitor (LY294002) were purchased from Pharmacia Corporation/Pfizer, Inc. and G. D. Searle & Co, respectively. The drugs were dissolved in dimethylsulfoxide (DMSO) at the appropriate concentration and stored at -20 °C until used.

Cell viability assay

Cells were plated into 96-well plates (3×10^3 cells/well) in 100 μL of medium. Twenty-four hours later the cells were treated with drugs or not, and incubated for 24 hours. Cell viability was assessed by MTT assay. 10 μL of 5 mg/mL MTT solution (Sigma) was added to each well. After 3 hours of incubation, 200 μL of DMSO was added to each well. The optical density (OD) of

each well was measured at 570 nm with a microplate reader (viability = $OD_{treat}/OD_{control}$).

Flow cytometric analysis

Cells were incubated with TRAIL at a concentration of 100 ng/mL. After 24 hours, the cells were harvested. The cell pellets were suspended in 500 μL binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 2.5mM $CaCl_2$) at a density of approximately 1 × 10^6/mL. Samples were incubated with 1 μL annexin V-FITC and 5 μL PI for exactly 5 minutes at room temperature in the dark and then measured on a FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA). Annexin V-FITC and PI fluorescence were detected in the FL-1 (green) and FL-2 (red) channels, respectively, after correction for the spectral overlap between the 2 channels. Data were analyzed by using CellQuest software (Becton Dickinson).

Western blotting

Cells were washed with PBS and lysed in lysis buffer (20 mM Na_2PO_4, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 μM phenylmethylsulfonyl fluoride, 100 μM NaF, and 2 μM Na_3VO_4). Proteins were separated by polyacrylamide gel electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Membranes were incubated with primary antibodies specific for Bcl-2 (1:500), total Akt (recognize Akt1, Akt2, Akt3 and phosphorylation site) (1:500), p-Akt (phosphorylated Akt on Ser473) (1:500), caspase-8 (1:200), caspase-9 (1:200), caspase-3 (1:200), DR4 (1:500), DR5 (1:500), Bid (1:500), truncated form of Bid (tBid) (1:1000), FADD (1:500), Bax (1:500), Bak (1:500), the short form of the cellular FLICE-inhibitory protein (FLIPs) (1:500), XIAP (1:500), and β-actin (1:1000). The membranes were incubated with horseradish peroxidase-conjugated goat antirabbit secondary antibodies. (All the antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA.). Lastly, the proteins were detected by enhanced chemiluminescence.

Bcl-2-siRNA transfection

pSilencer 3.1-H1 linear vector was purchased from Invitrogen Corporation, USA. The Bcl-2-siRNA insert sequence with the following sense and antisense sequences was: for Bcl-2 sense 5'-AGT ACA TCC ATT ATA AGCT-3' and antisense 5'-AGCTTATAATGGATGTACT-3'. A negative control vector that expresses a hairpin siRNA with limited homology to any known sequences in humans was provided with the vector kit. Plasmid DNA was purified with CsCl-ethidium bromide gradient centrifugation. The purified DNA was diluted to 1 g/L and frozen at -20 °C. Transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In brief, the cells were seeded into wells of a 6-well plate at 10^5 cells/cm^2 and cultured for 24 hours until 60-70% confluence. Then Bcl-2 siRNA or negative siRNA plasmid (4 μL) was diluted with 100 μL OPTI-MEM (Invitrogen) for 5 minutes at room temperature. During this incubation period, 5 μL Lipofectamine 2000 was diluted in 100 μL OPTI-MEM. These two mixtures were combined, mixed gently, and incubated for 20 minutes at room temperature for complex formation. The 200-μL siRNA-Lipofectamine 2000 mixture was then added into the cells. The transfected cells were cultured 48 hours and then assayed.

Assessment of mitochondrial transmembrane potential

Assessment of mitochondrial transmembrane potential ($\Delta\psi m$) by the cationic lipophilic fluorochrome 3,3'-dihexyloxacarbo-cyanide iodide ($DiOC_6(3)$) (460 ng/mL, Molecular Probes, Eugene, OR, USA) was used to measure the mitochondrial transmembrane potential. Cells were treated for 24 hours with TRAIL (100 ng/mL), then incubated for 30 minutes at 37.8 ºC in complete media with $DiOC_6$, washed twice with PBS, and analyzed in a FACS Vantage cytofluorometer (Becton Dickinson).

Statistical analysis

All assays were repeated in 3 independent experiments, and only representative blots are presented. Immunoblots were scanned by HP PrecisionScan Pro 3.13. Statistical comparisons of mean values were performed using 1-way ANOVA. Correlation of the variables was assessed by bivariate correlation analysis. All P values were determined by 2-sided tests. A significance criterion of $P<0.05$ was applied. Statistical analysis was done with SPSS 12.0 software.

Results

Sensitivity to TRAIL varied among NPC cells

We first tested the cytotoxic effect of TRAIL on 4 NPC cell lines by MTT. NPC cells showed differential responses, and CNE-2 cells were more refractory than other NPC cell lines (Figure 1A). To examine the relative effect of Bcl-2, Akt and DR on the TRAIL response, cell extracts from untreated cell lines were tested by Western blotting. Western blot analysis revealed highest levels of Bcl-2 and p-Akt protein expression in CNE-2 cells, which were most resistant, and lowest levels in CNE-1 cells, which were most sensitive, but there was no clear correlation between Akt activation levels, Bcl-2 protein expression levels, and sensitivity to TRAIL in the other NPC cell lines (Figure 1B). Total Akt, DR4, and DR5 protein levels were almost equal among these cell lines (Figure 1B).

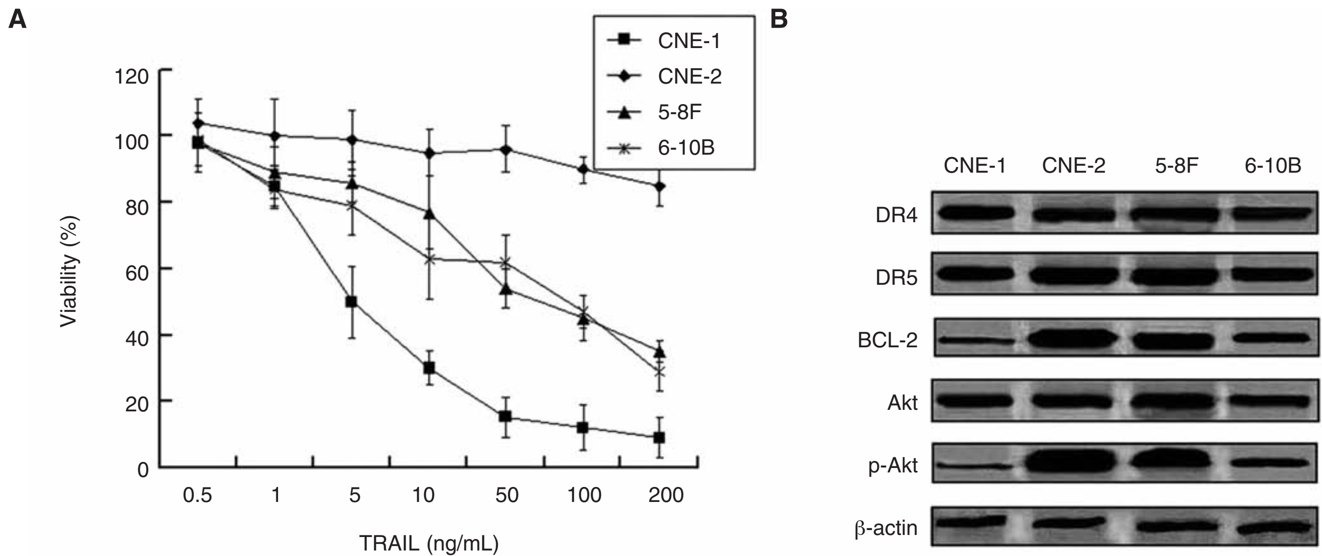

Figure 1 - The sensitivity of NPC cells to TRAIL. A) Viability of 4 NPC cell lines responding to various TRAIL concentrations as assessed by MTT assay. B) Expression of DR4, DR5, Akt, p-Akt, and Bcl-2 in 4 NPC cell lines detected by Western blotting.

Combination of Bcl-2 siRNA and LY294002 sensitized NPC cell lines to TRAIL

To test the roles of Bcl-2 protein and Akt activation in the response of NPC cells to TRAIL, we silenced Bcl-2 in CNE-2 cells by siRNA and inactivated Akt by treatment with LY294002 (1 M) for 8 hours. Bcl-2 siRNA significantly decreased Bcl-2 expression within 24 hours of transfection in the 4 NPC cell lines. LY294002 significantly decreased Akt phosphorylation in these cell lines but had no effect on Bcl-2 expression (Figure 2). Aside from these observations we found no evidence of functional synergy with combined therapy of Bcl-2 siRNA and LY294002, because the magnitude of the combined therapeutic effects was similar to that of single thera-

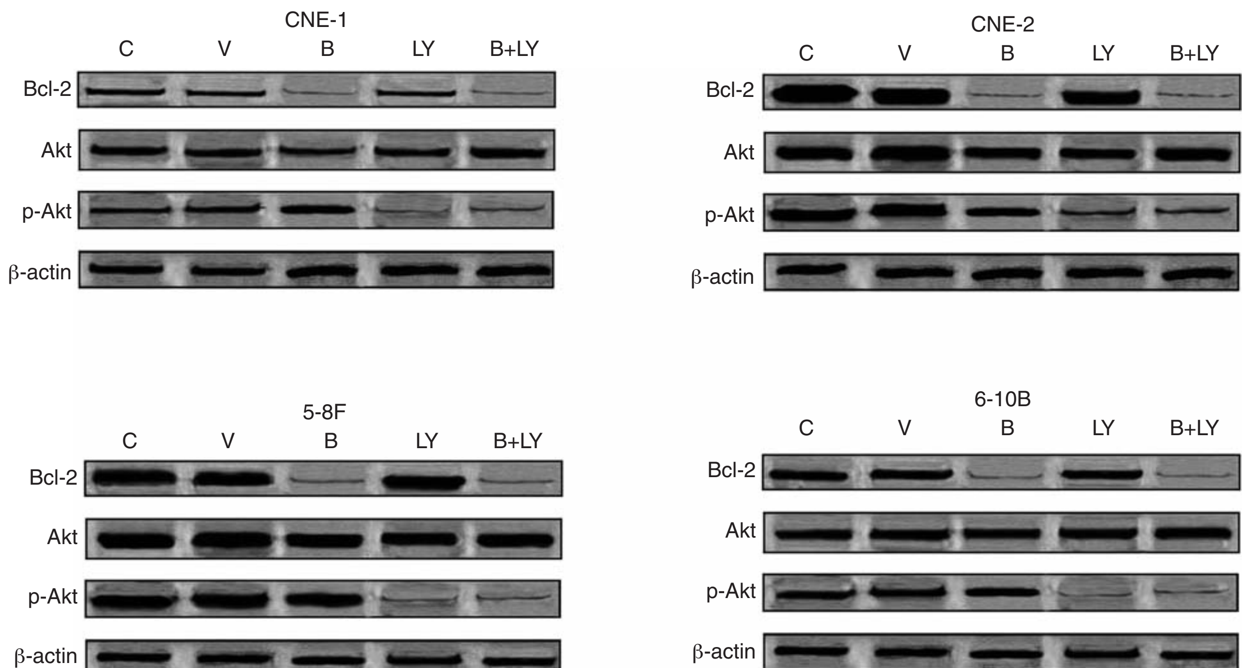

Figure 2 - Expression of Akt, p-Akt, and Bcl-2 in 4 NPC cell lines detected by Western blotting following treatment with Bcl-2 siRNA and LY294002. C, control; V, vector control; B, Bcl-2-siRNA treatment; LY, LY294002 treatment.

peutic effects. The expression of the DRs was not affected in this experiment (Figure 2). Then we tested the cytotoxic effect of Bcl-2 siRNA and LY294002 on the 4 NPC cell lines by MTT. Neither single nor combined treatment had a cytotoxic effect on NPC cells in the absence of TRAIL (Figure 3A). After we established the effects of Bcl-2 and p-Akt reduction, we examined whether this modification was capable of acting in a synergistic manner with TRAIL to induce cell death in NPC cells. TRAIL (100 ng/mL) was added to cells of the 4 NPC cell lines that were treated with Bcl-2 siRNA and pretreated with LY294002 (1 μM) for 8 hours simultaneously or singly. MTT assay was performed 24 hours later. Bcl-2 siRNA or LY294002 alone significantly enhanced TRAIL-induced death in all 4 NPC cell lines (Figure 3B). Compared with single treatments, combination treatment amplified TRAIL-induced death only in CNE-2, 5-8F, and 6-10B (Figure 3B).

Combination of Bcl-2-siRNA transfection and LY294002 did not activate the extrinsic apoptotic pathway

We selected CNE-2, which was most resistant to TRAIL, for the next experiments and used flow cytometric analysis to ascertain whether increased cell death was due to apoptosis. The result further showed that combination treatment exerted a synergistic effect on TRAIL-induced apoptosis (Figure 4A). In order to understand the molecular mechanism underlying the effects of the combination of Bcl-2-siRNA transfection and LY294002 on TRAIL-induced apoptosis, we studied apoptotic pathway signals. As shown in Figure 4B, single treatments and combined treatment did not affect the expression of DR4 and DR5. There are 2 major isoforms of FLIP, the long isoform ($FLIP_L$) and the short isoform ($FLIP_S$). While $FLIP_S$ inhibits apoptosis by preventing the recruitment of caspase-8 into the DISC, the role of $FLIP_L$ is controversial. Indeed, $FLIP_L$ has been reported to be both an inhibitor and inducer of apoptosis signals, possibly depending on its expression levels[23]. We observed a significant inhibition of $FLIP_S$ protein expression in cells treated with LY294002 but not in cells treated with Bcl-2 siRNA. The protein expression of FADD was unaffected in the present experiments (Figure 4B). Cleavage of caspase-8 is the most important event in the extrinsic apoptotic pathway, and we found that TRAIL-induced cleavage of caspase-8 was enhanced by LY294002 but not by Bcl-2 siRNA. Cleavage of caspase-8 induced by TRAIL in combination treatment of Bcl-2 siRNA and LY294002 was similar to that observed after LY294002 treatment alone. The results showed that combination treatment of Bcl-2 siRNA and LY294002 did not synergistically enhance the TRAIL-induced extrinsic apoptotic pathway.

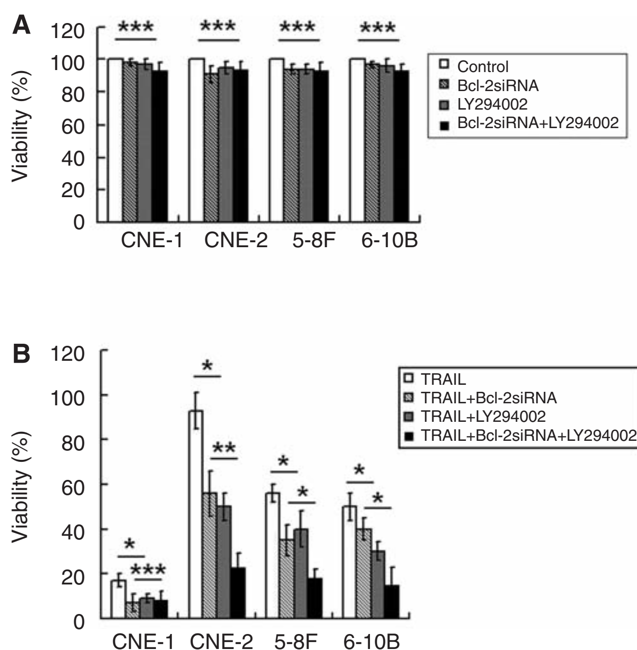

Figure 3 - Combination treatment of Bcl-2-siRNA transfection and LY294002 sensitized NPC cells to TRAIL-induced apoptosis. The cell viability of 4 NPC cell lines treated by single treatment or combination treatment with or without TRAIL was assessed by MTT. A) NPC cells were treated with Bcl-2 siRNA and LY294002 alone or together. B) Bcl-2 siRNA and LY294002 were added to TRAIL alone or together. (TRAIL: 100 ng/mL TRAIL treatment; LY294002: pretreatment with 1 μM LY294002 for 8 hours.) $^*P<0.05$, $^{**}P<0.01$, $^{***}P>0.05$.

Combination of Bcl-2-siRNA transfection and LY294002 activated TRAIL-induced mitochondrial events

We subsequently investigated whether the Bcl-2 protein level and Akt activation regulate TRAIL-induced apoptosis by inducing dissipation of $\triangle\psi m$ and cytochrome c release from mitochondria. Mitochondrial depolarization induced by TRAIL as measured by $DiOC_6(3)$ fluorescence was increased by Bcl-2 siRNA or LY294002 and more perceptibly by their combination (Figure 5A). We have also investigated processing of Bid, the Bcl-2 family member that has been described as being cleaved by caspase-8 and then triggering cytochrome c release. As shown in Figure 5B, Bid processing was increased by LY294002 and occurred at similar levels in combination treatment. It appears that combination treatment acts synergistically downstream of Bid. It was interesting that LY294002 increased the cleavage of Bid in the absence of TRAIL.

Combination of Bcl-2-siRNA transfection and LY294002 activated the extrinsic apoptotic pathway

We found that combination treatment activated TRAIL-induced mitochondrial events synergistically without any synergistic effect on Bid cleavage, suggesting that the combined treatment exerts synergistic effects downstream of Bid. In order to confirm our hypothesis, we studied the facts downstream of Bid. Once cleaved by caspase-8, Bid translocates to mitochondria and acti-

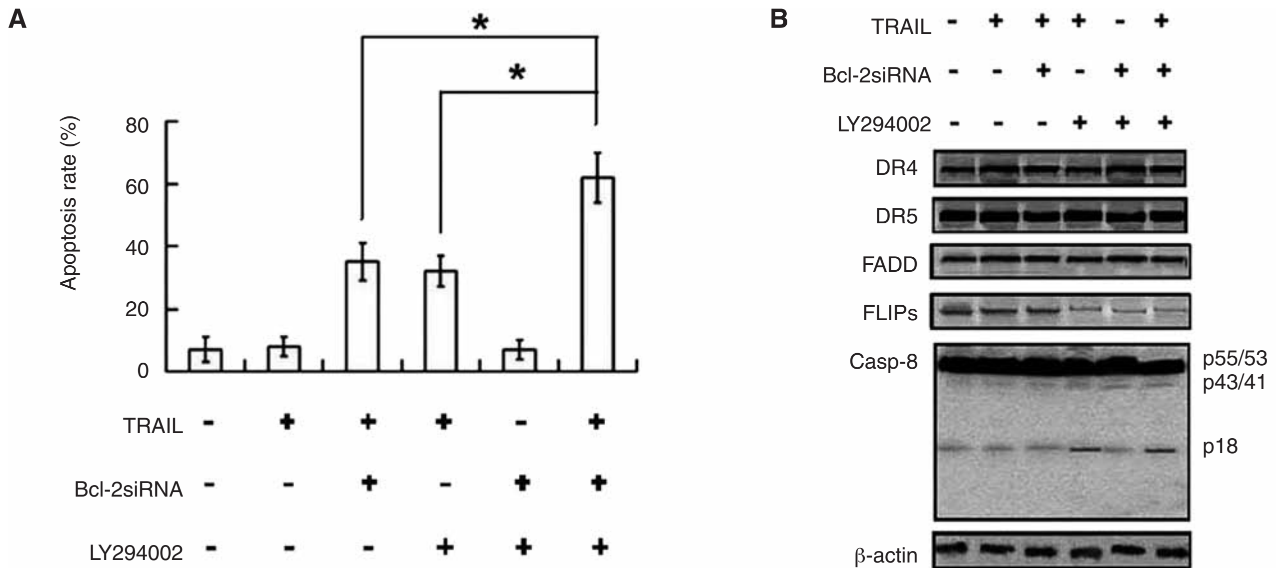

Figure 4 - A) The cell apoptosis rate of CNE-2 cells undergoing single or combination treatment was estimated by flow cytometry. B) Protein expression of extrinsic apoptotic pathway modulators affected by combination of Bcl-2-siRNA transfection and LY294002. (TRAIL: 100 ng/mL TRAIL treatment; LY294002: pretreatment with 1 μM LY294002 for 8 hours.) *P <0.05

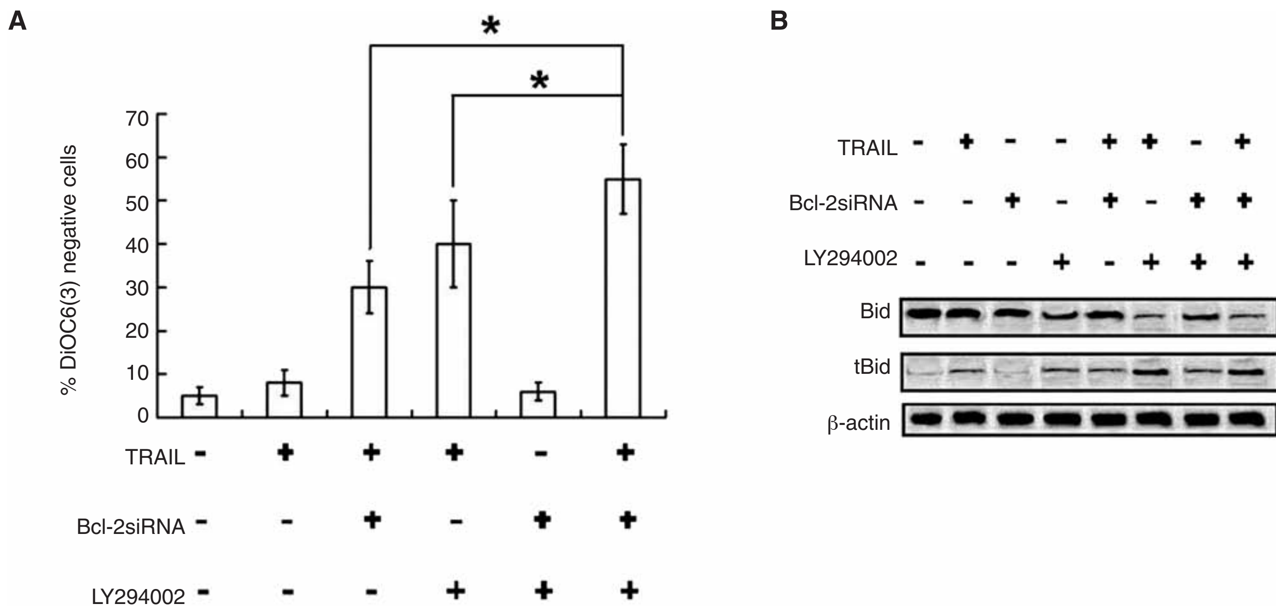

Figure 5 - Combination of Bcl-2-siRNA transfection and LY294002 synergistically activated the intrinsic apoptotic pathway. A) The $\triangle\psi m$ was determined by staining cells with the potential-sensitive fluorochrome $DiOC_6(3)$ and analyzed by flow cytometry (*P <0.05). B) Bid cleavage was detected by Western blotting.

vates the Bcl-2 family members Bax and Bak, leading to mitochondrial depolarization[9]. Levels of proapoptotic Bak and Bax were found to be increased by Bcl-2 siRNA and LY294002, but this increase was not augmented when the 2 agents were combined (Figure 6A). The inhibitor of apoptosis proteins (IAPs) are a family of antiapoptotic proteins that block cell death in part by inhibiting the downstream caspase activation pathways and thus play a critical role in determining the cell fate[24]. As shown in Figure 6B, we observed a synergistic inhibition of the IAP XIAP. This inhibition was more pronounced in the groups treated with combination treatment compared with each agent alone (Figure 6B). Synergistic activation of effector caspase-3 and caspase-9 induced by TRAIL was observed in the combination-treated cells (Figure 6C).

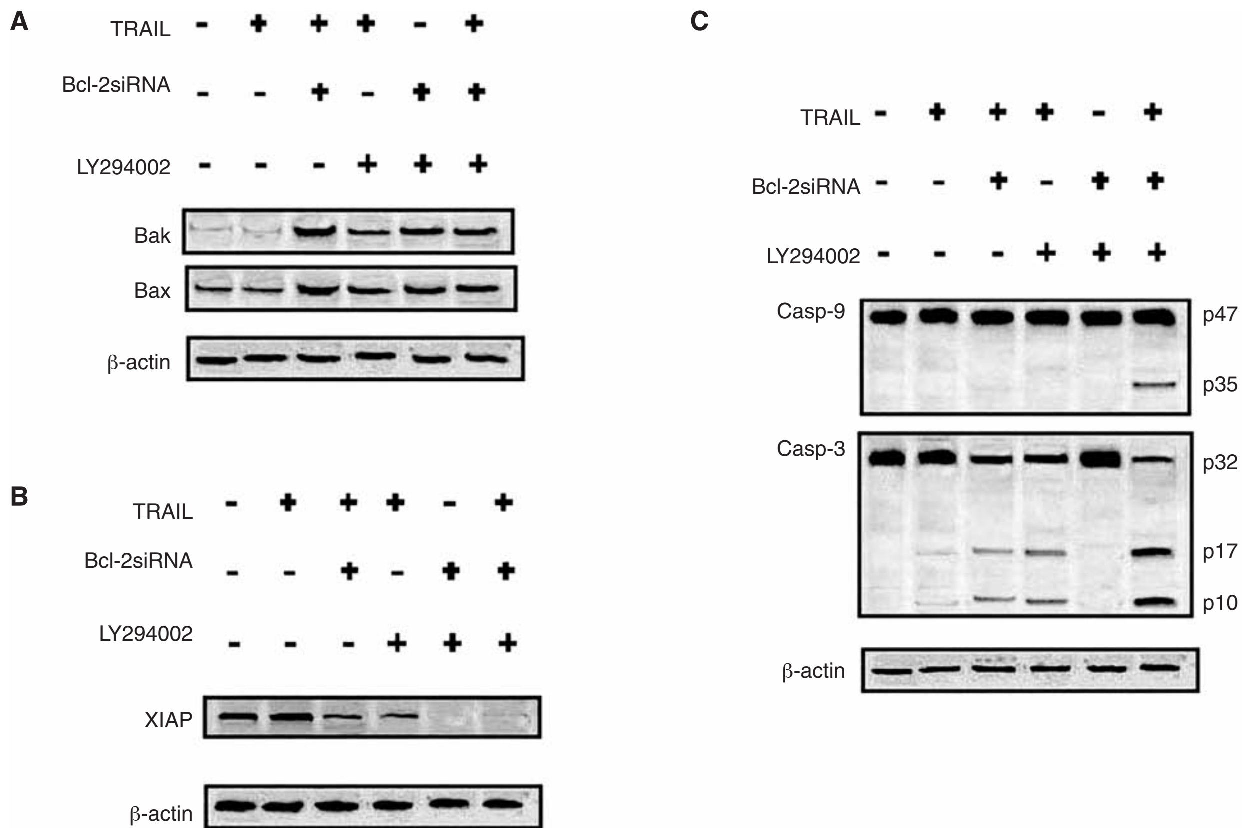

Figure 6 - Protein expression downstream of Bid detected by Western blotting. A) Bax and Bid; B) XIAP; C) Caspases 9 and 3.

Discussion

Despite aggressive therapies, resistance to current treatment protocols has been a major obstacle in clinical oncology. Most anticancer agents generate cytotoxicity through induction of apoptosis in cancer cells[5]. Apoptosis is mediated through the DR and the mitochondrial pathway[25]. TRAIL is a promising candidate for cancer therapy, but the emergence of drug resistance limits its potential use. Prior studies showed that TRAIL could not induce apoptosis effectively in some NPC cell lines[20]. Some resistant cancer cells can be sensitized by combination of TRAIL with different chemotherapeutic drugs, indicating that combination therapy can be used to reverse the resistance of cancer cells to TRAIL[14].

Bcl-2 is overexpressed in many tumors. Its overexpression in NPC correlates with superficial NPC recurrence and progression and with poor prognosis of invasive cancer[15], and it is a factor in patient responses to chemotherapy and radiotherapy[26,27]. Previous studies have demonstrated a role for Bcl-2 in protecting cells from TRAIL-mediated apoptosis[28]. In addition, inhibitors of Bcl-2 are among the novel molecules that have recently been tested as reactivators of the mitochondrial apoptotic pathway in many cancer cells[29], and inhibition of the expression of Bcl-2 appears to be a mandatory novel therapy for future cancer treatment. In our study, we suppressed Bcl-2 expression to activate the TRAIL-induced mitochondria-dependent apoptotic pathway in CNE-2 cells via the activation of Bax/Bak with no effects on the extrinsic apoptotic pathway. This enhancement of the TRAIL effect was associated with increases in activated caspase-9 and caspase-3, consistent with apoptotic signaling. In addition, the expression of XIAP, which inhibits caspase-9 and caspase-3 through binding to their intermediate and fully cleaved forms[24], was suppressed by Bcl-2 inhibition. This led us to assume that suppression of Bcl-2 augmented TRAIL-induced apoptosis via effects downstream of Bid cleavage.

Studies have found that the Akt pathway causes resistance to chemotherapy-induced apoptosis[30]. It has been demonstrated that Akt might inhibit a variety of apoptotic stimuli in multiple ways[31]. These include direct phosphorylation and modulation of the proapoptotic proteins Bad[31] and caspase-9[32], and activation of antiapoptotic NF-κB-mediated transcriptional pathways[33], preventing them from inducing the transcription of proapoptotic genes. However, once expressed, active Akt is under tight regulation by PI3-K and other kinases of the signaling pathway that promote cell sur-

vival[34]. Because PI3-K targets Akt for survival, we have modulated the activation of Akt by the PI3-K inhibitor LY294002. While NPC cell lines showed differential responses to treatment with TRAIL alone, increased cell death of all 4 cell lines was observed when TRIAL was combined with LY294002. This suggests that active Akt in NPC cells is responsible for TRAIL resistance. Although ineffective alone, TRAIL in combination with LY294002 induced a drop in mitochondrial transmembrane potential and subsequently activated caspases 8, 9, 3, Bad and Bax in CNE-2 cells. Previous studies[35,36] have shown that the reduction of Akt activity by PI3-K inhibitors reduces the expression of FLIP and XIAP, resulting in reversion of the cell line resistance to TRAIL. Consistent with these studies, LY294002 sensitized the CNE-2 cells to TRAIL by reducing FLIP and XIAP. We have also shown that LY294002 enhanced the sensitivity to TRAIL-induced apoptosis in NPC cells, an effect that was mediated by increasing the Bid cleavage induced by TRAIL. Bid cleavage is usually the result of TRAIL treatment and occurs upstream of the mitochondria[37]. Interestingly, we found that LY294002 treatment induced Bid cleavage in the absence of TRAIL, suggesting that Akt inactivation induced Bid cleavage alone. Majewski *et al.*[38] showed that strong expression of Akt inhibited Bid cleavage in the absence of serum and TRAIL. We speculated that LY294002 enhanced Bid cleavage in the environment of serum deficiency in our experiment.

We observed that Bcl-2 siRNA and PI3-K inhibitors sensitized NPC cells to TRAIL each by affecting different modulators, providing a further rationale for their combination. When Bcl-2 siRNA and LY294002 were combined, the expected synergy in sensitizing NPC cells to TRAIL was observed. In this study, we report for the first time the synergistic sensitization of NPC cells to TRAIL-mediated apoptosis by simultaneously targeting the Bcl-2 and Akt signaling pathways. This new treatment increased the dissipation of the mitochondrial transmembrane potential and cleavage of caspases 9 and 3 synergistically. In other words, the synergistic effects were observed downstream of Bax and Bad. In addition, XIAP expression was decreased by combination treatment more than by single treatments. We considered that XIAP was downregulated by targeting the Bcl-2 and Akt signaling pathways, resulting in synergistic cleavage of caspases 9 and 3. Altogether, the main functions of this combination were enhancement of TRAIL-induced mitochondrial depolarization and suppression of XIAP in a synergistic manner.

In conclusion, we examined the role of the Bcl-2 and Akt pathways in TRAIL resistance of NPC cells and explored the potential mechanisms. Combined targeting of the Bcl-2 and Akt signaling pathways synergistically sensitized NPC cells to TRAIL. Our results provide a potential novel strategy for NPC treatment. The combination definitely deserves additional *in vivo* and clinical studies.

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