Abstract. Non-small cell lung cancer (NSCLC) is relatively insensitive to chemotherapy. NP [vinorelbine (NVB) + cisplatin] is the standard chemotherapy regimen in clinical treatment; however, its side-effects are intolerable for most patients. In some reports, the TNF-related apoptosis-inducing ligand (TRAIL) can enhance the sensitivity of chemotherapy drugs by inducing apoptosis without toxicity to normal cells. In the present study, we evaluated the antitumor effects of the two drugs (TRAIL and NVB alone or in combination) by inducing apoptosis in vitro and in vivo. Using the human NSCLC cell line (A549) and a BALB/c nude mice model, we observed the cell viability (MTT assay), cell apoptosis [Hoechst staining, Annexin V/propidium iodide (PI) staining assay, immunohistochemical staining, RT-PCR and western blotting] and cell proliferation (soft agar colony formation and cell cycle assay). The results showed that TRAIL and NVB alone inhibited tumor growth both in vivo and in vitro. However, the combination of the two drugs produced a more potent antitumor effect (P<0.05) and caused more severe apoptosis in tumor tissue (P<0.05). The key molecular protein level of the mitochondrial apoptotic pathway (Bax and caspase-3) was further upregulated by the combination of the two drugs (P<0.01) and either drug alone (P<0.05). The mRNA level of Bcl-2 and Bax in the combination group was downregulated and upregulated respectively, when compared with the control group (P<0.01). The combination group of A549 cells also showed lower viability compared with the one drug alone group (P<0.05). Moreover, the Hoechst staining assay and Annexin V/PI staining assay showed that the combination of the two drugs induced a more potent apoptosis than either drug alone (P<0.05, early apoptosis P<0.01, respectively). In addition, the cell colony numbers were deduced after treatment with TRAIL or NVB alone (P<0.05) and the two drug combination (P<0.01). Cell cycle analysis showed that TRAIL and NVB alone markedly increased the percentage of cells in G1 phase (P<0.05) and the combination of the two drugs decreased the percentage of cells in G2 and S phase (P<0.05). Thus, the combination of TRAIL and NVB can inhibit lung cancer cell growth by affecting the level of key signaling protein expression of the apoptosis pathway.

Introduction

Lung cancer is the leading cause of cancer-related mortality in the USA. In 2007, an estimated 213,380 new cases and 160,390 deaths were calculated in the USA (1,2). The 5-year relative survival rate from 1995 to 2001 for patients with lung cancer was 15.7%. Lung cancer represents 15% of all new cancer cases diagnosed and 31 and 26% of all cancer deaths in men and women, respectively. The two types of lung cancer are the small cell lung cancers and the non-small cell lung cancers (NSCLCs). Approximately 80% of all lung cancers are NSCLC, and adenocarcinoma is the most frequently occurring type worldwide (3). The general prognosis of lung cancer is poor since diagnosis is often made when the disease is already at an advanced stage. The 5-year relative survival rate varies markedly depending on the stage at diagnosis, from 49 to 16 to 2% for patients with local, regional and distant stage disease, respectively. Five-year survival is ~40-50% for early stage lung cancer, but only 1-5% in advanced, inoperable lung cancer. The current standard of care to treat solid cancers includes surgery to remove the bulk of the tumor and subsequent radiotherapy and/or chemotherapy to kill residual cancerous cells (4). NP [vinorelbine (NVB) + cisplatin] is the standard chemotherapy regimen in clinical treatment but its side-effects are intolerable for most patients and NSCLC is relatively insensitive to chemotherapy. During the last decade, the Eastern Cooperative Oncology Group (ECOG) has studied a series of combination chemotherapy regimens in metastatic
NSCLC. Despite the development of new chemotherapy combinations, a large ECOG trial evaluating the most common NSCLC chemotherapy doublets showed no survival advantage for any regimen (5,6). New effective agents of combination chemotherapy are required for the treatment of patients with advanced NSCLC.

In recent years, the development of novel cancer therapeutics capable of overcoming these difficulties has become a new field of interest in cancer research. TRAIL, with its property of inducing apoptosis in a wide variety of tumor cells and xenografts without toxicity to normal cells, appears to be a promising anticancer agent. TRAIL is a member of the TNF superfamily that has emerged, since its discovery in 1995, as a prominent biologically-targeted antitumor protein (7). TRAIL-induced apoptosis is mediated by the extrinsic pathway, but when a cell is additionally stressed it can be enhanced by the intrinsic pathway, resulting in expedition of apoptosis. Several studies have combined chemotherapeutic drugs and TRAIL as a means to enhance the sensitivity of chemotherapy drugs by inducing apoptosis (8). For example, the use of gemcitabine, oxaliplatin and irinotecan in combination with TRAIL treated gastroenterological tumors more effectively than any of these agents alone (9,10). In the present study, we used the NSCLC cell line A549 and a nude mice model to detect the antitumor effect of NVB and TRAIL alone or combination and its mechanisms.

Materials and methods

Reagents. Recombinant human TRAIL. Apo II ligand was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA). NVB was purchased from Shanghai Amino Acids Company (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and mouse antibodies of Bax and caspase-3 were from Maixin Bio (Fuzhou, China); iblot western detection stack/iblot dry blotting system was purchased from Invitrogen (Carlsbad, CA, USA). Annexin V-FITC apoptosis detection kit and Cycle Test™ Plus DNA Reagent kit were purchased from Becton-Dickinson (San Jose, CA, USA). All other chemicals, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Cell culture. A human NSCLC cell line, A549 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM containing 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a 37°C humidified incubator with 5% CO₂.

Animals. BALB/c nude male mice with an initial body weight of 20-22 g were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed under pathogen-free conditions with a 12-h light/dark cycle. Food and water were provided ad libitum throughout the experiment. All animal treatments were strictly in accordance with international ethics guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, and the experiments were approved by the Institutional Animal Care and Use Committee of Fujian Provincial Cancer Hospital.

Tumor xenograft. Forty mice were injected with A549 cell suspension at the right flank. After 7 days, mice were randomly divided into four groups (control, TRAIL 0.08 mg/kg, NVB 5 mg/kg, and TRAIL 0.08 mg/kg + NVB 5 mg/kg). Drugs were administered once every day for three consecutive cycles of 5 days apart while the same volume of NS was administered for the control group. The primary tumor size was measured using a caliper square along the long (a) and the short (b) axis, and the tumor volume was calculated by the following formula: Tumor volume (mm³) = ab²/2.

Cell viability. Cell viability was assessed by MTT colorimetric assay. A549 cells were seeded into 96-well plates at a density of 1x10⁵ cells/well in 0.1 ml medium. The cells were treated with various concentrations of TRAIL and NVB for different periods of time. At the end of the treatment, 100 µl MTT [0.5 mg/ml in phosphate-buffered saline (PBS)] were added to each well, and the samples were incubated for an additional 4 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 µl DMSO. The absorbance was measured at 570 nm using an ELISA reader (BioTek, Model ELx800, USA). The rate of cell growth inhibition was calculated according to the formula: Cell growth inhibition rate = (OD value of the control group - OD value of treated group)/(OD value of the control group - a blank group OD value). Half inhibitory concentration (IC₅₀) of drugs in A549 cells was further calculated by modified Kou-type method: lgIC₅₀ = Xm-I [ΣP-(3-Pm-Pn)/4], in which Xm, lg maximum dose; I, lg (maximum dose/adjacent dose); ΣP, sum of positive response rate; Pm, the largest positive response rate; Pn, the smallest positive response rate.

Hoechst 33342 staining. Adherent cells were incubated in DMEM medium plus 5% FBS for 6 h. The cells were then washed, fixed with 4% paraformaldehyde, and stained with Hoechst 33342 (5 µg/ml) for 20 min at 25°C. The slides were then examined by fluorescence microscopy and photographed. Cells with signs of apoptosis (fragmented nuclei) were enclosed within circles.

Soft agar colony formation assay. After A549 cells were treated with drugs for 24 h, cells were harvested and pipetted well to become single-cell suspension in DMEM with 10% FBS at a concentration of 5x10⁴ cells/ml. Normal melting point agar (0.4 ml of 0.3% agar in 1.6 ml DMEM containing 10% FBS) was placed into each well of a 6-well plate. After solidification of the bottom agar, 1 ml of cell mixture consisting of 0.02 ml of cell suspension (5x10⁴ cells/ml) and 0.98 ml of 0.8% lower melting point agar in DMEM containing 10% FBS were poured over the bottom agar. After solidification of the upper agar, wells were incubated at 37°C in a humidified 5% CO₂ atmosphere. Colony formation in the agar was then photographed and counted under a phase-contrast microscope.

Flow cytometry analysis with Annexin V/propidium iodide (PI) staining. After incubation with various concentrations of drugs, apoptosis of A549 cells was determined by flow cytometry analysis using a fluorescence-activated cell sorting (FACS) caliber (Becton-Dickinson) and apoptotic cells were identified by the Annexin V-FITC/PI apoptosis detection kit.
The ratios of Annexin V+/PI- and Annexin V+/PI+ region were calculated with CellQuest software (BD Biosciences, San Jose, CA, USA), which indicate early- and late-stage apoptosis rate, respectively.

Flow cytometry analysis with cell cycle. The cell cycle analysis was carried out by flow cytometry using a fluorescence-activated cell sorting caliber (Becton-Dickinson) and PI staining. After treatment with TRAIL and NVB for 24 h, A549 cells were harvested and adjusted to a concentration of 1x10^6 cells/ml, and fixed in 70% ethanol at 4˚C overnight. The fixed cells were washed twice with cold PBS, and then incubated for 30 min with RNase (8 µg/ml) and PI (10 µg/ml). The fluorescent signal was detected through the FL2 channel and the proportion of DNA in different phases was analyzed using ModfitLT version 3.0 (Verity Software House, Topsham, MA, USA).

Immunohistochemistry. In situ apoptosis detection by TUNEL staining. The 4-µm-thick sections of tumor samples were analyzed by TUNEL staining using TumorTACS In Situ Apoptosis kit (R&D Systems). Apoptotic cells were counted as DAB-positive cells (brown stained) at five arbitrarily selected microscopic fields at a magnification of x400. TUNEL-positive cells were counted as a percentage of the total cells.

Preparation of tumor homogenate and western blotting. Three tumors were randomly selected from the control or the treatment group, homogenized in non-denaturing lysis buffer using homogenizer and centrifuged at 15,000 x g for 15 min followed by determination of protein concentration in supernatants. Equal protein per lysate was resolved on Tris-glycine gel, transferred onto PVDF membranes, and blocked for 2 h with 5% non-fat dry milk. Membranes were incubated with desired primary antibody Bax, caspase-3 and β-actin (at a dilution of 1:1,000) overnight at 4˚C and then with appropriate HRP-conjugated secondary antibody followed by enhanced chemiluminescence detection.

RNA extraction and RT-PCR analysis. Total RNA was isolated from fresh tumor with TRizol reagent. Oligo(dT)-primed RNA (1 µg) was reverse transcribed with SuperScript II reverse transcriptase according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of B-cell leukemia/lymphoma 2 (Bcl-2) and Bax by PCR with Taq DNA polymerase (Fermentas).

Statistical analyses. All data analyses were performed using statistical software SPSS 11.0. Data are expressed as means ± standard deviation. A t-test was performed for comparison between two groups, and one-way ANOVA was used for multiple group comparison, with post-hoc comparisons by LSD test if the variances were equal, or Tamhane's T2 method if the variances were unequal. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Cell viability. The relative cell viability was decreased after treatment with TRAIL for 24 or 48 h (P<0.01, vs. control group). The IC_{50} of TRAIL was calculated to be 250.5 mg/ml after 24 h and 101.2 mg/ml after 48 h (Fig. 1, P<0.01). With the increase of NVB dose, the inhibition rate became increasingly higher. The IC_{50} of NVB was 23.2 and 7.8 mg/ml, after 24 or

Figure 1. A549 cell viability after treatment with TRAIL. (A) A549 cells were treated with the indicated concentrations of TRAIL for 24 and 48 h. (B) The half inhibitory concentration (IC_{50}) of drugs on A549 cells after 24 h was calculated according to the modified Kou-type method: lgIC_{50} = Xm-I [∑P-(3-Pm-Pn)/4], in which Xm, lg maximum dose; I, lg (maximum dose/adjacent dose); ∑P, sum of positive response rate; Pm, the largest positive response rate; Pn, the smallest positive response rate. (C) The IC_{50} value of drugs on A549 cells after 48 h. TRAIL, TNF-related apoptosis-inducing ligand.
Figure 2. A549 cell viability after treatment with NVB. (A) A549 cells were treated with the indicated concentrations of NVB for 24 and 48 h. (B) The IC$_{50}$ value of drugs on A549 cells after 24 h, calculated according to the modified Kou-type method: $\lg IC_{50} = X_m - 1 \left( \sum P - (3P_m - P_n)/4 \right)$, in which $X_m$, lg maximum dose; $I$, lg (maximum dose/adjacent dose); $\sum P$, sum of positive response rate; $P_m$, the largest positive response rate; $P_n$, the smallest positive response rate. (C) The IC$_{50}$ value of drugs on A549 cells after 48 h. NVB, vinorelbine.

Figure 3. A549 cell viability after treatment with TRAIL and NVB alone or in combination. A representative image of control (A), TRAIL (B), NVB (C) and combination group (D). (E) Graphs represent averages of three independent experiments; data are means ± SD. *P<0.01, vs. control. TRAIL, TNF-related apoptosis-inducing ligand; NVB, vinorelbine.
The concentration of TRAIL and NVB in the following experiments was 30 and 0.05 mg/ml. The inhibition rate of the two drug combination was significantly higher than the single drug and the control group (Fig. 3, P<0.01, compared with control).

Hoechst 33342 staining to detect the apoptosis rate. To confirm the apoptosis-inducing effects of the two drugs, A549 lung cancer cells were stained with Hoechst 33342 assay to evaluate for DNA fragmentation. The treated A549 lung cancer cells by the two drug combination displayed many apoptotic bodies. The results showed that the A549 lung cancer cell apoptotic rates were ~17% after TRAIL or NVB alone treatment for 24 h and the combination of the two drugs induced a cell death rate ~30% for 24 h (Fig. 4, P<0.05 and P<0.01, compared with control).

Flow cytometry to detect the early and late apoptosis rate. In Fig. 5, the profiles of Annexin V-positive percentages are shown for the treatments with drugs for 24 h. After 24-h treatment, the Annexin V-positive percentages of A549 cancer cells were significantly increased compared to the control group. The late apoptosis rate and early apoptosis rate of the combination of the two drugs was higher compared to the control (P<0.01).

Soft agar colony to detect proliferation. We examined the effect of drugs on A549 cell survival using a colony formation assay. Colony number reduction could be caused by cell cycle arrest or cell death. As shown in Fig. 6, the colony number of the control group was ~16. The number was decreased significantly to ~10 and 5 after treatment with either drug alone (TRAIL or NVB) (P<0.05) or the two drug combination (P<0.01).

Flow cytometry to detect the cell cycle. Subsequently, we detected the cell cycle after A549 cells were treated with TRAIL or NVB alone or in combination for 24 h. The results showed that the two drug combination markedly increased the percentage of cells in G1 phase (Table I, P<0.01) and decreased the percentage of cells in G2 and S phase (P<0.05). These results demonstrated that the drugs arrested A549 cell cycle in the G0/G1 phase.

The antitumor effect of TRAIL or NVB. The antitumor effect of the drugs in vivo was evaluated by measuring tumor
volume and weight in lung xenograft mice. As shown in Fig. 7, administration of TRAIL or NVB decreased tumor volume and weight by 25 and 26% respectively, compared with control (P<0.05). The combination of TRAIL and NVB decreased tumor volume and weight ~40% (P<0.01).

The apoptotic effect of TRAIL or NVB in mice. The apoptosis rate of the drugs in the mice was detected by immunohistochemistry. The results showed that TRAIL or NVB induced significant apoptosis effects on the tumor and the combination of the two drugs increased the apoptosis rate more than either drug alone (Table II, P<0.05 and P<0.01, compared with control). The combination of TRAIL and NVB decreased tumor volume and weight ~40% (P<0.01).

Discussion

Chemotherapy often causes unpleasant side-effects including bone marrow inhibition, nausea and vomiting. It has been reported that failure to control side-effects can lead to 25-50% of patients delaying or refusing possible life-saving therapy. To enhance the sensitivity of NSCLC to chemotherapy and to decrease the side-effects of chemotherapy drugs, chemotherapy combinations have been used in clinical trials such as ECOG. NSCLC chemotherapy doublets showed no survival advantage for any regimen over traditional cisplatin/paclitaxel (4). Thus, novel treatment strategies are urgently needed to improve the clinical management including NSCLC treatment. Several studies have combined chemotherapeutic drugs and TRAIL as a means to treat tumors. For example, the use of gemcitabine, oxaliplatin and irinotecan in combination with TRAIL treated gastroenterological tumors more effectively than any of these agents alone (11). Cisplatin, in combination with TRAIL-encoding retrovirus, resulted in greater anticancer activity in ovarian carcinoma cells in vitro and in xenografts (12).

NVB is a semi-synthetic vinca-alkaloid approved for the treatment of NSCLC, which has also demonstrated activity against breast and ovarian cancer, Hodgkin lymphoma and nasopharyngeal carcinoma (13-15). NVB has been evaluated in NSCLC in the adjuvant and advanced settings as a single agent and in combination with other agents (typically a platinum or gemcitabine) with modest success (16). In the present

Table I. Percentage of G0/G1, G2/M and S phase of A549 cells after treatment with TRAIL and NVB alone or in combination.

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1</th>
<th>G2/M</th>
<th>S</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>35.67±4.21</td>
<td>40.23±4.78</td>
<td>24.01±3.10</td>
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<tr>
<td>TRAIL</td>
<td>55.57±4.85</td>
<td>23.54±3.21</td>
<td>20.89±2.16</td>
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<tr>
<td>NVB</td>
<td>56.01±6.17</td>
<td>24.12±3.05</td>
<td>19.81±3.20</td>
</tr>
<tr>
<td>TRAIL+NVB</td>
<td>52.37±6.25</td>
<td>32.01±4.50</td>
<td>15.62±2.45</td>
</tr>
</tbody>
</table>

Data represent the mean of three independent measurements ± SD. *P<0.01 and "P<0.05 compared with control. TRAIL, TNF-related apoptosis-inducing ligand; NVB, vinorelbine.

Table II. Apoptosis rate of tumor in mice after treatment with TRAIL and NVB alone or in combination.

<table>
<thead>
<tr>
<th>Group</th>
<th>Apoptosis rate (%)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.51±4.25</td>
</tr>
<tr>
<td>TRAIL</td>
<td>15.25±6.51</td>
</tr>
<tr>
<td>NVB</td>
<td>17.20±4.31</td>
</tr>
<tr>
<td>TRAIL+NVB</td>
<td>21.24±6.52</td>
</tr>
</tbody>
</table>

Data represent the means of three independent measurements ± SD. *P<0.01 and "P<0.05 compared with control. TRAIL, TNF-related apoptosis-inducing ligand; NVB, vinorelbine.

The apoptotic effect of TRAIL or NVB in mice. The apoptosis rate of the drugs in the mice was detected by immunohistochemistry. The results showed that TRAIL or NVB induced significant apoptosis effects on the tumor and the combination of the two drugs increased the apoptosis rate more than either drug alone (Table II, P<0.05 and P<0.01, compared with control). The combination of TRAIL and NVB decreased tumor volume and weight ~40% (P<0.01). The mRNA level of Bcl-2 was deduced after the two drug combination treatment (Fig. 8, P<0.01).
study, we detected the effects of combination of TRAIL and NVB on A549 cells and lung carcinoma-xenograft nude mice. The results showed that the combination of the two drugs can inhibit the cell growth and proliferation through blocking the cells in the G0/G1 phase. At the same time, we also observed that the combination group significantly induced apoptosis of the cell line and tumor. The MTT, Hoechst staining and soft agar colony formation results also showed that TRAIL or NVB
alone demonstrated a certain degree of apoptosis-inducing effects and antiproliferation on the A549 cell line and lung carcinoma-xenograft. These results led us to detect further different treatment results of drugs alone or in combination. We focused on the apoptosis of the cell line and xenograft treated by different drugs.

Apoptosis can be initiated by two distinct, but interconnected, molecular signaling pathways. There are two pathways through which apoptosis may occur. The extrinsic pathway is mediated by death receptors belonging to the TNF receptor superfamily such as TRAIL-R1/R2 (17,18). The intrinsic, or mitochondrial pathway, is triggered in response to cellular stress and DNA damage and involves activation of p53 and the release of pro-apoptotic and anti-apoptotic members of the Bcl-2 protein family that can cause release of apoptosis-inducing factors from mitochondria (19). To maintain control of the apoptotic machinery, both the extrinsic and intrinsic pathways are highly regulated at multiple levels by pro-apoptotic and anti-apoptotic modulators. Administration of TRAIL to mice transplanted with human tumor xenografts derived from colon carcinoma, breast cancer, mammary adenocarcinoma, multiple myeloma or malignant glioma, exerted marked antitumor activity without systemic toxicity (20). The majority of the studies published since the initial report describing TRAIL (8) have focused on the in vitro and in vivo tumoricidal activity of TRAIL. In these preclinical trials, recombinant human TRAIL induced apoptosis in multiple malignant cell lines, derived from both solid and hematologic malignancies, either alone or in combination with various chemotherapy agents or radiation (21,22). Some reports showed that TRAIL regulated the signal molecules such as cFLIP, Bcl-2 family members, IAP proteins, the extracellular signal regulated kinases survival pathway, Akt and Toso to induce apoptosis.

Flow cytometry and immunohistochemistry showed that the combination of TRAIL and NVB can induce more apoptosis of A549 cells and xenograft than one drug alone. In order to clarify the effect of key signaling molecules, the protein and mRNA of apoptosis-related molecules on the apoptosis pathway was detected by western blotting and PCR. The results showed that caspase-3, Bcl-2 and Bax level, the key signal molecules of mitochondrial apoptosis pathway, was apparently regulated by TRAIL and NVB. The mitochondrial apoptosis pathway included the intrinsic pathway. Intrinsic stresses such as oncogenes, direct DNA damage, hypoxia and survival factor deprivation can activate the intrinsic apoptotic pathway. Studies have clarified that p53 protein initiates apoptosis by transcriptionally activating pro-apoptotic Bcl-2 family members and suppressing anti-apoptotic Bcl-2 proteins and cellular inhibitor of apoptosis (CIAPs). Other p53 targets include BCL2 associated X-protein (BAX), Noxa, p53-upregulated modulator of apoptosis (PUMA) and the more recently identified BID (23-26). The downstream target gene of that signaling pathway will lead to increases in reactive oxygen species and generalize oxidative damage to all mitochondrial components. Our next study will focus on the whole pathway of mitochondrial apoptosis.

Acknowledgements

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22. Gazitt Y: TRAIL is a potent inducer of apoptosis in myeloma cells derived from multiple myeloma patients and is not cytotoxic to hematopoietic stem cells. Leukemia 13: 1817-1824, 1999.


