

## RESEARCH COMMUNICATION

**Mechanisms of TRAIL and Gemcitabine Induction of Pancreatic Cancer Cell Apoptosis****Bin Zhao<sup>1</sup>, Lu Li<sup>2</sup>, Kai Cui<sup>3</sup>, Chang-Liang Wang<sup>3</sup>, Ai-Liang Wang<sup>4</sup>, Zi-Qiang Sun<sup>4</sup>, Bo Zhang<sup>1</sup>, Wu-Yuan Zhou<sup>3</sup>, Zuo-Xing Niu<sup>3</sup>, He Tian<sup>3</sup>, Yan Xue<sup>5</sup>, Sheng Li<sup>3\*</sup>****Abstract**

The aim of this study was to investigate induction of apoptosis by the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and gemcitabine in the pancreatic cancer cell line SW1990. The sensitivity of SW1990 cells to TRAIL and/or gemcitabine-induced apoptosis and the rate of apoptosis were assessed by MTT assay and flow cytometry, respectively. We used Hoechst 33342 staining to observe apoptotic morphology and expression levels of proteins were analyzed by Western blotting. Growth inhibition and apoptosis rates on treatment with the combination of TRAIL and gemcitabine were significantly higher than with each drug alone ( $p < 0.05$ ). Pancreatic cancer cells exhibited a typical apoptosis morphology after treatment with TRAIL or gemcitabine. The levels of cellular apoptosis-associated proteins such as Smac/DIABLO, Cyto C, and the activated fragment of caspase-3 (P17) increased, but the expression of XIAP was significantly decreased after 24 h ( $p < 0.05$ ). SW1990 cells responded to TRAIL and/or gemcitabine-induction of apoptosis in a time and concentration-dependent manner. The mechanism of the apoptosis-sensitization effect appeared associated with significant up-regulation of Smac/DIABLO and cytochrome C, down-regulation of XIAP, and activation of caspase-3.

**Keywords:** Pancreatic cancer cells - TRAIL - gemcitabine - apoptosis

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**Introduction**

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), a member of the tumor necrosis factor family, binds death receptors (DRs) to activate DR-mediated pathways leading to apoptosis. It is reported that full-length TRAIL is expressed on the cell membrane, and picomol levels can quickly induce apoptosis of many tumor cell lines, which suggests strong anti-tumor effects. However, more and more studies have revealed tolerance to the apoptosis-inducing effect of TRAIL and repeated use of TRAIL in sensitive cells will lead to the acquisition of resistance (Jin et al., 2004; Platzbecker et al., 2004; Zhang et al., 2005; Poulaki et al., 2005).

In this study, we used the various concentrations of TRAIL and/or gemcitabine to induce apoptosis in SW1990 cells, studying the relationship between the death receptor apoptosis pathway, represented by TRAIL, and the cytotoxic agent-mediated apoptosis pathway, represented by gemcitabine, to explore the possibility of synergizing these two for targeted treatment of pancreatic cancer.

**Materials and Methods***Materials*

The human pancreatic cancer cell line SW1990 was

provided and maintained by the Shandong Medical and Health Surgical Oncology Key Lab of the Shandong Cancer Research and Prevention Institute. Gemcitabine was obtained from LilyFrance S.A., TRAIL (50 µg; GF092) was purchased from Chemicon, Canada. Smac/DIABLO antibody was purchased from CALBIOCHEM. XIAP antibody was purchased from CST Corp., and β-actin, and caspase-3 antibodies were all from Santa Cruz, USA. Horseradish peroxidase (HRP)-labeled goat anti-rabbit and goat anti-mouse secondary antibodies were obtained from Beijing Zhong Shan Gold Bridge Corp. Ltd.

*MTT assay*

Experimental group was divided three groups, 200, 500, 1000, and 2500 ng/ml were used in the TRAIL group, and 10, 20, 40, and 60 µmol/L were used in the gemcitabine group. A mixture of 500 µg/ml TRAIL and 20 µmol/l gemcitabine was used in the TRAIL combined gemcitabine group. All samples were added in a total reaction volume of 200 µl each well, and the reactions were repeated five times for each concentration. No drug was added in the control group.

In all groups, cells were cultivated for 12, 24, 48, and 72 h. 20 µl MTT was added to each well and cultured for an additional four hours. The absorption value (A) of each well was detected by a microplate reader at

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wavelengths of 550 nm and 630 nm. The average A value was calculated, and the cell growth inhibition rate (CGIR) was obtained according to the following formula:  $(CGIR) = (1 - \text{experimental group A value}/\text{control group A value}) \times 100\%$ . The above procedure was repeated three times.

#### Detection of cell apoptosis rate by flow cytometry (FCM)

SW1990 cells were inoculated in 25-ml flasks at a concentration of  $5 \times 10^6$  cells/ml. TRAIL (500 ng/ml), gemcitabine (20  $\mu\text{mol/L}$ ) and TRAIL (500 ng/ml) with gemcitabine (20  $\mu\text{mol/L}$ ) were added to the cells separately; no drug was added in the control group. After 24 h treatment, cells were collected for FCM analysis. Data analysis criteria: the lower left and right quadrant of the FCM bi-parameter scatter diagram stands control AnnexinV+/PI cells and Annexin V+/PI+cells, respectively, and the upper right quadrant acts for necrotic Annexin V+/PI+ cells. The cell apoptosis rate was calculated according to the above standards.

#### Hoechst 33342 staining

SW1990 pancreatic cancer cells were seeded in 12-well culture plates in physiological saline. TRAIL (500 ng/ml), gemcitabine (20  $\mu\text{mol/L}$ ), and TRAIL (500 ng/ml) with gemcitabine (20  $\mu\text{mol/L}$ ) were added before culturing respectively. Hoechst 33342 dye was added after 24 h culture and the nucleus of cells were observed under fluorescent microscopy,

#### Detection of Smac/DIABLO, cytochrome C, XIAP, and caspase-3 by Western blotting

After 24 h treatment with different drugs, total protein was extracted from cells for SDS-PAGE, followed the transfer to nitrocellulose membrane by electrophoretic and staining. The nitrocellulose membrane was placed in a buffer with Diaminobenzidine (DAB), and proteins anchored on the membrane were detected using a digital gel imaging system. The data were analyzed using the software.

#### Statistical analysis

All data were given as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). SPSS for Windows 1.5 version was used for statistical analysis. A t-test was used for comparing the means of two samples. One-way analysis of variance (ANOVA) was used to compare the means of multiple samples, while the least considerable difference (LSD) was applied for one-to-one comparison.  $P < 0.05$  was considered statistically significant.

## Results

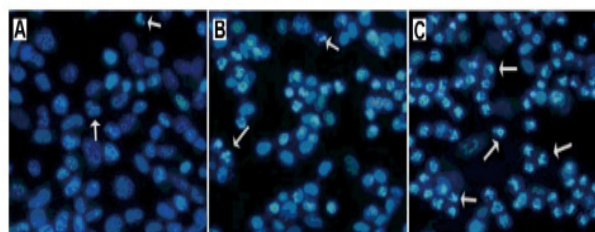
The CGIRs of various concentrations of TRAIL, gemcitabine, and TRAIL with gemcitabine with different treatment periods in SW1990 cells as determined by the MTT assay are shown in Table 1. The results showed that both TRAIL and gemcitabine could inhibit SW1990 cell growth in a time- and dose-dependent manner ( $P < 0.05$ ). The CGIR of TRAIL (500 ng/ml) with gemcitabine (2  $\mu\text{mol/L}$ ) in SW1990 cells treated for 24 h is 68.6%, suggesting that the growth inhibiting effect of the

**Table 1. The CGIR of TRAIL in SW1990 Cells Treated at Different Concentrations (ng/ml) for 12-72 h (%)**

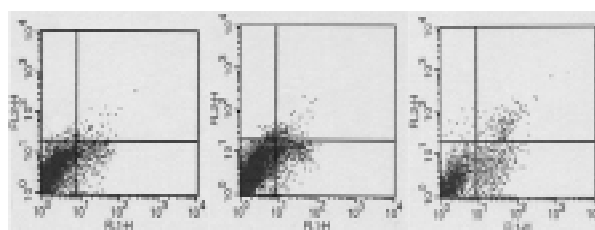
Time (h)	TRAIL concentration (ng/ml)( $\bar{x} \pm s$ )(%)			
	200	500	1000	2500
24	11.11 $\pm$ 0.96	46.03 $\pm$ 1.33	67.08 $\pm$ 1.26	76.19 $\pm$ 1.41
48	10.23 $\pm$ 1.11	48.35 $\pm$ 1.13	68.96 $\pm$ 1.14	78.55 $\pm$ 1.38
72	12.64 $\pm$ 0.80	48.21 $\pm$ 0.92	70.82 $\pm$ 1.33	77.38 $\pm$ 1.17

**Table 2. The GIR of Gemcitabine at Different Concentrations( $\mu\text{mol/l}$ ) in SW1990 Cells for 12-72 h (%)**

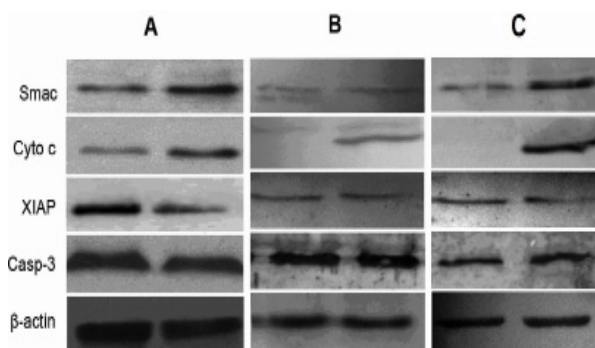
Time (h)	Gemcitabine concentration ( $\mu\text{mol/l}$ )( $\bar{x} \pm s$ )(%)			
	10	20	40	60
24	16.67 $\pm$ 0.61	19.44 $\pm$ 1.47	25.00 $\pm$ 2.50	41.66 $\pm$ 0.63
48	11.11 $\pm$ 1.33	18.06 $\pm$ 1.11	31.94 $\pm$ 0.27	36.11 $\pm$ 1.67
72	31.94 $\pm$ 2.11	43.06 $\pm$ 0.95	50.00 $\pm$ 0.81	50.00 $\pm$ 1.41



**Figure 1. SW1990 Cells Apoptotic Morphology after Treatment with Different Concentrations of TRAIL and/or Gemcitabine. A: TRAIL (500 ng/ml); B: gemcitabine (20  $\mu\text{mol/L}$ ); C: TRAIL(500ng/ml)+gemcitabine(20 $\mu\text{mol/L}$ )**



**Figure 2. The Apoptotic Rates of SW1990 Cells after Treatment with Different Concentrations of TRAIL and/or Gemcitabine for 24 h. A: TRAIL (500ng/ml); B: gemcitabine (20 $\mu\text{mol/L}$ ); C: TRAIL(500ng/ml)+gemcitabine(20 $\mu\text{mol/L}$ )**



**Figure 3. The Expression Levels of Smac/DIABLO, Cytochrome c, and the Active Cleaved Fragment p17 of Caspase-3, XIAP of SW1990 cells after Treatment with Different Concentrations of TRAIL and/or Gemcitabine for 24 h. A: treated with TRAIL (500 ng/ml). B: with gemcitabine (20  $\mu\text{mol/L}$ ); C: TRAIL(500ng/ml)+gemcitabine(20 $\mu\text{mol/L}$ )**

**Table 3. The CGIR of the Combination of TRAIL and Gemcitabine in SW1990 Cells Treated for 24 h (x±s) (%)**

Cell type	Time(h)	TRAIL (500ng/ml)	gemcitabine (10µmol/L)	gemcitabine (20µmol/L)	TRAIL(500ng/ml)+ gemcitabine20µmol/L)
SW1990	24	46.03±1.33	16.67 ± 0.61	19.44 ± 1.47	68.60 ± 0.85

combination of TRAIL and gemcitabine is obviously higher than the single agent treatment ( $P < 0.05$ ).

SW1990 cells showed typical apoptotic morphology after treatment with TRAIL (500 ng/ml) and/or gemcitabine (20 µmol/L) or TRAIL (500ng/ml)+gemcitabine(20µmol/L) (Figure 1). The apoptotic rates of SW1990 cells treated with TRAIL (500ng/ml), gemcitabine (20µmol/L) or TRAIL(500ng/ml)+gemcitabine(20µmol/L) for 24 h were 5.64%, 15.30% and 27.27% (Figure 2).

The expression levels of Smac/DIABLO, cytochrome c, and the active cleaved fragment p17 of caspase-3 of SW1990 cells was increased after treated with TRAIL (500 ng/ml) for 24 h, while the XIAP protein was reduced (Figure 3A). SW1990 cells treated with gemcitabine (20 µmol/L) and the combination of TRAIL (500 ng/ml) with gemcitabine (20 µmol/L) for 24 h exhibited the similar pattern for the intracellular proteins listed above (Figure 3B, C), respectively.

## Discussion

TRAIL can activate intracellular signal transduction pathways by binding its corresponding receptors, which induce apoptosis of multiple tumor cells and transfected cells, while remaining non-toxic to normal cells. The affinity of TRAIL for tumor cells makes it a promising agent for use in tumor treatment (Pitti et al., 1992). Many studies have revealed that TRAIL regulates cell apoptosis through two different signal transduction pathways, and one is the caspase mediated pathway (Lin et al., 2006). TRAIL binds death receptors of the cell membrane to activate apoptotic pathways. DR4 or DR5 binds TRAIL to form a ligand-receptor trimer that induces the binding of the cytoplasmic death domain of DR and Fas-associated death domain (FADD). The N-terminal death effect domain (DED) of FADD then binds procaspase-8 to form DR4/DR5-FADD-procaspase-8, a death inducing signal complex (DISC) that self-catalyzes the conversion of procaspase-8 into active caspase-8. After the activation of caspase-8, the following two pathways are activated for apoptosis: (1) Mitochondria-independent pathway: activated caspase-8 directly activates downstream effectors such as caspase-3, caspase-6, or caspase-7 to induce apoptosis.

(2) Mitochondria-dependent pathway: activated caspase-8 induces the cleavage of Bid, a Bcl-2 family member, to form a truncated Bid (tBid), which is located in the mitochondrial membrane and disrupts or lowers the mitochondrial transmembrane potential leading to the release of cytochrome C (cyto c) and Smac/DIABLO. Cyto c, Apaf-1, and dATP synergize to induce the self-catalytic activity of procaspase-9 to form effectively caspase-9 and further activate effector proteins leading to cell apoptosis.

Moreover, TRAIL can exert an anti-tumor effect by

activating the Akt pathway, nuclear factor, PKC, and MAPK family members. These activated pathways or factors can regulate the apoptosis-inducing effect of TRAIL (Wachter et al., 2004; Zhang et al., 2005; Kim and Lee, 2005), leading to varying degrees of resistance of cells to TRAIL. Furthermore, the expression of tumor cells DR4 and DR5 and the intracellular expression level of caspase-8 can affect the sensitivity of cells to TRAIL.

Smac/DIABLO only induces apoptosis in injured cells (Verhagen et al., 2000). Under normal conditions, Smac/DIABLO is located in mitochondria and released into the cytoplasm to interact with repressive apoptotic proteins (IAPs) that can inhibit caspase activity when cells are subjected to different kinds of apoptotic stimuli, such as anti-cancer drugs, chemical or physical apoptotic signals. This interaction gives rise to the disruption of the inhibitory effect of IAPs on caspase leading to apoptosis (Verhagen et al., 2000; Du et al., 2000). This mechanism reveals the importance of Smac/DIABLO in TRAIL-induced apoptosis (Ng and Bonavida, 2002).

The results showed higher GIR and apoptosis rate appeared in SW1990 cells when treated with TRAIL combined gemcitabine than single agent treatment, suggesting that the combination of TRAIL and gemcitabine has a synergistic effect on growth inhibition and the induction of apoptosis in SW1990 cells.

## Acknowledgements

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