

# TRAIL mutant membrane penetrating peptide alike (TMPPA) TRAIL-Mu3 enhances the antitumor effects of TRAIL *in vitro* and *in vivo*

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**Abstract.** The aim of the present study was to prepare a tumor necrosis factor ligand superfamily member 10 (TRAIL) mutant membrane penetrating peptide alike (TMPPA), TRAIL-Mu3, and to investigate its antitumor effects in colorectal cancer *in vitro* and *in vivo*. The pMD19/TRAIL plasmid with designed primers was amplified to construct the target gene; it was ligated with an expression plasmid and the expression was confirmed. Subsequently, TRAIL-Mu3 was purified and further confirmed by western blot analysis. Immunofluorescence analysis was used to detect the distribution of TRAIL-Mu3 in colorectal cancer cells. In addition, the present study investigated the antitumor effects of TRAIL-Mu3 on colorectal cancer *in vitro* and *in vivo*. A novel TMPPA, TRAIL-Mu3, was synthesized in the present study. Following a series of detection experiments, it was confirmed that the TRAIL-Mu3 gene was obtained and was able to express TRAIL-Mu3 successfully. The immunofluorescence analysis demonstrated that TRAIL-Mu3 exhibited a markedly enhanced affinity to the colorectal cancer cell surface. In addition, TRAIL-Mu3 exerted stronger antitumor effects, compared with TRAIL, on colorectal cancer *in vitro* and *in vivo*.

## Introduction

Tumor necrosis factor (TNF) ligand superfamily member 10 (TRAIL), a member of the TNF superfamily, is considered to

be an optimal candidate for cancer therapy due to its tumor cell specificity and negligible cytotoxicity to normal cells *in vitro* and *in vivo* (1). TRAIL has antitumor activity in a variety of tumor cell lines (2,3). However, a large number of cancer cells, particularly in highly malignant tumors, are resistant to apoptosis induction by TRAIL, and cancer cells that were originally sensitive to TRAIL-induced apoptosis may become resistant following repeated exposure (4). Therefore, it is of importance to develop a more efficient form of TRAIL for cancer therapy.

Crossing biological barriers represents a principal limitation for the clinical application of biomolecules including nucleic acids, peptides or proteins (5). Previous studies have used cell-penetrating peptides (CPPs) as an efficient method for delivering therapeutic targets into cells (6,7). CPPs, additionally termed protein transduction domains, comprise short and usually basic amino acid-rich peptides originating from proteins which are able to cross biological barriers. Typically, these peptides are cationic, rich in lysine or arginine residues, or amphipathic in nature (8,9). CPPs have emerged as a novel class of non-viral vectors allowing for the delivery of various biomolecules across biological barriers from low molecular weight drugs to nano-sized particles (10).

In the present study, a number of amino acids of the N-terminal in soluble fragments (114-281aa) of the TRAIL protein were selectively altered to form penetrating peptide-like amino acid sequences. A total of >10 TRAIL penetrating peptide like mutants were synthesized. Following further detection and screening, one TRAIL penetrating peptide like mutant with eight consecutive Arg sequences, which approximately maintained the conformation of the TRAIL protein and had a penetrating peptide like structure, was identified. This TRAIL mutant membrane penetrating peptide alike (TMPPA) was termed TRAIL-Mu3. The present study examined the design, synthesis, expression, identification and purification of TRAIL-Mu3. In addition, the antitumor effects of TRAIL-Mu3 in colorectal cancer was detected *in vitro* and *in vivo*.

## Materials and methods

**Materials.** The high-efficiency prokaryotic expression vector pET32a plasmid and the BL21 (DE3) competent

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**Key words:** tumor necrosis factor ligand superfamily member 10, trail mutant membrane penetrating peptide alike, colorectal cancer, antitumor effects



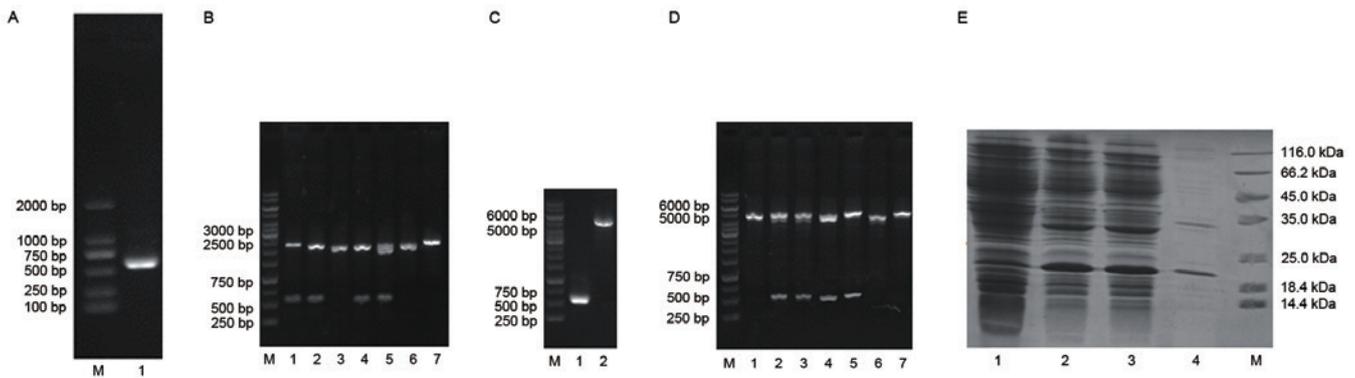


Figure 1. Results of electrophoresis identification during TRAIL-Mu3 synthesis. (A) Polymerase chain reaction products of the TRAIL-Mu3 gene. M, DNA marker; lane 1, TRAIL-Mu3. (B) Digestion of pMD19/TRAIL-Mu3 and electrophoresis products. M, DNA marker; lanes 1, 2, 4 and 5, four colonies transformed with pMD19/TRAIL-Mu3; lanes 3, 6 and 7, three colonies transformed with pMD19-T vector. (C) Digestion of the pET32a plasmid and TRAIL-Mu3 DNA. M, marker; lane 1, TRAIL-Mu3; lane 2, pET32a plasmid. (D) Digestion of pET32a/TRAIL-Mu3 and electrophoresis products. Lane M, marker; lanes 2, 3, 4 and 5, four colonies transformed with pET32a/TRAIL-Mu3; lanes 1, 6 and 7, three colonies transformed with the pET32a plasmid. (E) pET32a/TRAIL-Mu3 expression electrophoresis. Line M, marker; lane 1, induction electrophoresis sample; lane 2, post-electrophoresis sample; lane 3, supernatant; lane 4, precipitate. TRAIL, tumor necrosis factor ligand superfamily member 10.

12/12 light/dark cycle with free access to food and water. The vehicle, TRAIL and TRAIL-Mu3 were injected through the tail vein five times in 5 days. The length, width, and weight of the tumor was measured using a slide caliper every 3 or 4 days. Tumor volume (TV) was estimated using the formula:  $TV (mm^3) = (width^2 \times length)/2$ . The growth inhibition rate of the tumor was calculated using the formula: Inhibition rate of tumor (%) =  $(1 - \text{average weight in treated group}/\text{average weight in control}) \times 100$  (11,12).

**Side effects.** During the experimental period, side effects, including weight loss, mental state, appetite, behavior change and reactions, were observed.

**Statistical analysis.** The experiments were repeated at least three times. All data are expressed as the mean  $\pm$  standard error of the mean unless otherwise stated. Comparisons were analyzed using one-way analysis of variance and Least Significant Difference post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**PCR amplification of TRAIL-Mu3 gene.** The amplified products of the TRAIL-Mu3 gene were detected by 3% agarose gel electrophoresis, and the results demonstrated that a specific segment of  $\sim 500$  bp (Fig. 1A) was obtained.

**Identification of the enzyme-digested products of pMD19/TRAIL-Mu3.** Following ligation of the pMD19-T vector and TRAIL-Mu3 genes, they were transformed into bacteria and cultured. A total of seven single colonies were isolated and the plasmids were extracted. Subsequently, the plasmids were digested and detected by agarose gel electrophoresis. The results demonstrated that there were two segments of  $\sim 500$  bp and  $\sim 2.7$  kb in lines 1, 2, 4 and 5 (Fig. 1B), which were equal to the size of the TRAIL-Mu3 gene and pMD19-T vector plasmid, respectively. Therefore, these four colonies were transformed with pMD19/TRAIL-Mu3, and were further assayed by sequencing.

**Identification of enzyme-digested products of pET32a/TRAIL-Mu3.** The pET32a plasmid and TRAIL-Mu3 DNA were digested by *NdeI* and *EcoRI* enzymes and detected by electrophoresis. The results demonstrated that there were two segments of  $\sim 500$  bp and  $\sim 5.4$  kb (Fig. 1C), which were equal to the size of the TRAIL-Mu3 gene and pET32a plasmid, respectively. Subsequently, the plasmids were ligated, transformed into bacteria and cultured. A total of seven single colonies were isolated and the plasmids were extracted. The plasmids were digested and detected by agarose gel electrophoresis. The results demonstrated that there were two segments of  $\sim 500$  bp and  $\sim 5.4$  kb in lines 2, 3, 4 and 5 (Fig. 1D), which were equal to the size of the TRAIL-Mu3 gene and pET32a plasmid, respectively. Therefore, these four colonies were transformed with pET32a/TRAIL-Mu3, and they were further analyzed by sequencing.

**pET32a/TRAIL-Mu3 expression.** As presented in Fig. 1E, there was strong expression in the prior-to-induction electrophoresis sample, post-electrophoresis sample, supernatant and precipitate, and that this was most apparent in the supernatant.

**Purification of TRAIL-Mu3 protein.** The results of this analysis are presented in Fig. 2. Following cation exchange purification (Fig. 2A), 15 ml eluent with a concentration of 2.273 mg/ml was obtained. The purity of TRAIL-Mu3 was thus demonstrated to be high. Via hydroxyapatite purification (Fig. 2B), 12 ml hydroxyapatite eluent with a concentration of 2.080 mg/ml was obtained. The purpose of this step was to attempt to remove contaminating proteins and pyrogen. Following anion exchange purification (Fig. 2C) 20 ml flow through fluid with a concentration of 0.846 mg/ml was obtained. In this step, the pyrogen was further removed. Following repeated purification, sufficient protein was obtained to evaluate its bioactivity *in vitro* and *in vivo*.

**Western blot analysis of TRAIL-Mu3 protein.** The results of the western blotting (Fig. 3) demonstrated that there was a positive band of  $\sim 19.6$  kDa for TRAIL-Mu3 and the TRAIL

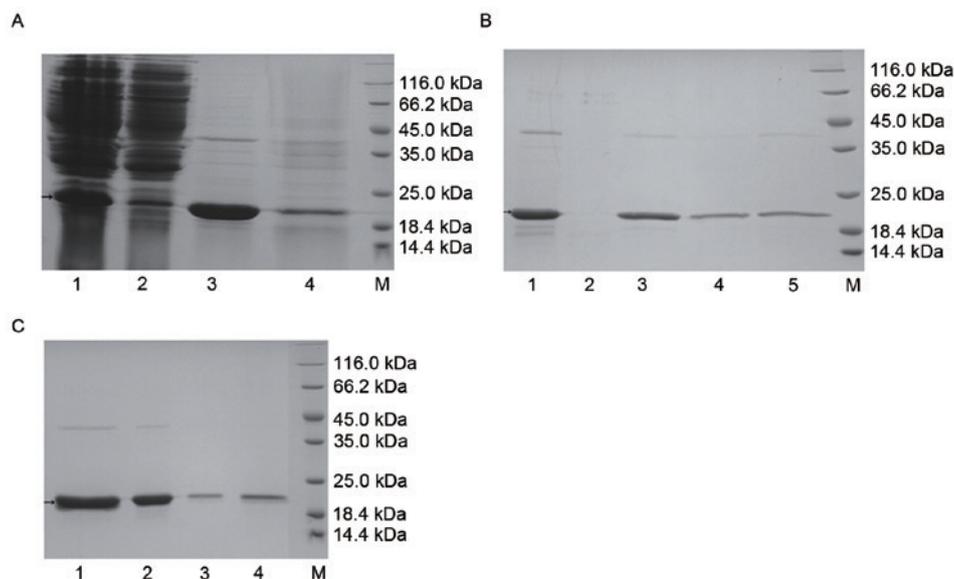


Figure 2. Purification of TRAIL-Mu3 protein. (A) Cation exchange purification. Lane 1, cation exchange liquid; lane 2, cation exchange flow-through liquid; lane 3, cation exchange eluate; lane 4, cation exchange sodium hydroxide eluent; M, unstained protein molecular weight marker. (B) Hydroxyapatite purification. Lane 1, hydroxyapatite sample solution; lane 2, hydroxyapatite flow-through liquid; lane 3, hydroxyapatite sodium chloride eluent; lane 4, hydroxyapatite phosphate eluent; M, unstained protein molecular weight marker. (C) Anion exchange purification. Lane 1, anion exchange liquid; lane 2, anion exchange flow-through liquid; lane 3, sodium chloride eluent; lane 4, sodium hydroxide eluent; M, unstained protein molecular weight marker. TRAIL, tumor necrosis factor ligand superfamily member 10.

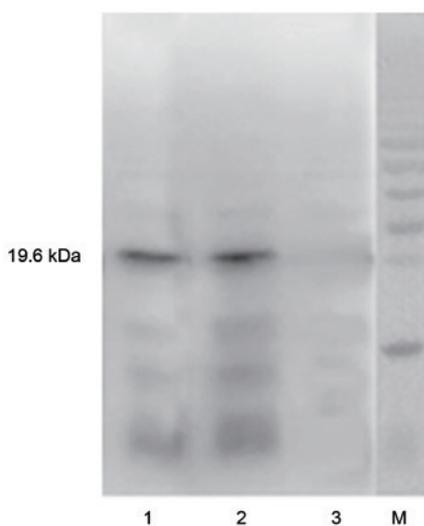


Figure 3. Western blot detection of TRAIL-Mu3 protein. Lane 1, TRAIL-Mu3; lane 2, TRAIL; lane 3, negative control; M: Thermo Fisher Scientific, Inc., page ruler pre-stained protein ladder. TRAIL, tumor necrosis factor ligand superfamily member 10.

protein. However, the same band was not present for the supernatant of lysed BL21 (DE3) *E. coli*.

**Immunofluorescence analysis.** The results demonstrated that there was little TRAIL aggregated on the cell membrane of SW620 cells (Fig. 4A); however, extensive TRAIL-Mu3 was observed around the cell membrane of the cells (Fig. 4B).

**TRAIL-Mu3 mediated toxicity on colorectal cancer cell lines.** The  $IC_{50}$  values of TRAIL and TRAIL-Mu3 in various colorectal cancer cell lines are presented in Table I. The results demonstrated that TRAIL-Mu3 was able to enhance

the antitumor effects of TRAIL in all five colorectal cancer cell lines. In addition, TRAIL-Mu3 was able to reverse the resistance of TRAIL-resistant HT-29 colorectal cancer cells.

**Antitumor effects in HT-29 xenograft nude mice.** The treatment commenced on day 10 following the injection of tumor cells. The tumor growth curves and inhibitive rates are presented in Fig. 5. The results demonstrated that the tumor growth inhibition rate increased with the increase in TRAIL-Mu3 concentration. In addition, the tumor growth inhibition rate of TRAIL-Mu3 was significantly increased compared with TRAIL at the same concentration of 45 mg/kg.

**Side effects, survival quality and analysis.** All the mice in the TRAIL and TRAIL-Mu3 groups began to manifest slight syndromes such a stunt responses, bad appetite, and little activity on the 8th day after the treatment. However, there were no significant differences between TRAIL and TRAIL-Mu3 groups in mental status, weight, appetite and so on.

## Discussion

TRAIL, which is able to selectively induce apoptosis in cancer cells, is a potential targeted drug for cancer therapy (20). However, its clinical use is limited by cellular resistance to cell death which occurs in ~50% of cancer cells (21). Various methods have been developed to overcome TRAIL resistance (4,22).

In recent years, CPPs have become one of the most popular techniques for intracellular access (6). CPPs are typically short cationic sequences and may be derived from natural sources or be synthetically designed constructs. It was previously demonstrated that this sequence was able to be shortened to a few amino acids, without altering its translocation capacity (23). In

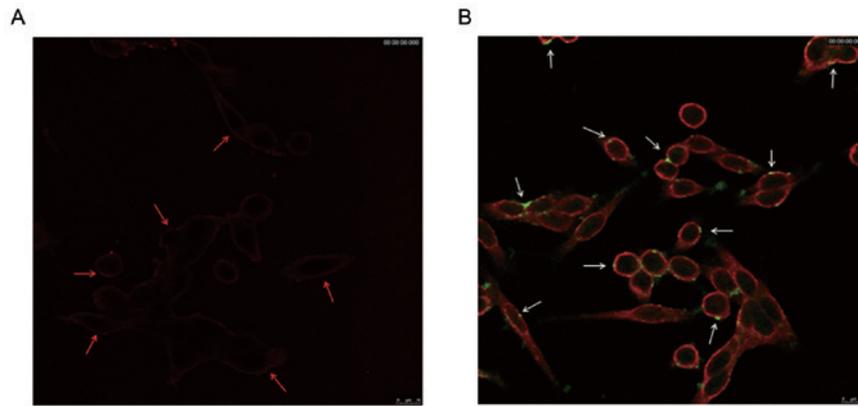


Figure 4. Immunofluorescence analysis (magnification x400). (A) SW620 cells treated with TRAIL (double staining of TRAIL and cell membrane; red arrows indicate cell membranes). (B) SW620 cells treated with TRAIL-Mu3 (double staining of TRAIL-Mu3 and cell membrane; white arrows indicate that stained TRAIL-Mu3 was aggregated around the cell surface). TRAIL, tumor necrosis factor ligand superfamily member 10.

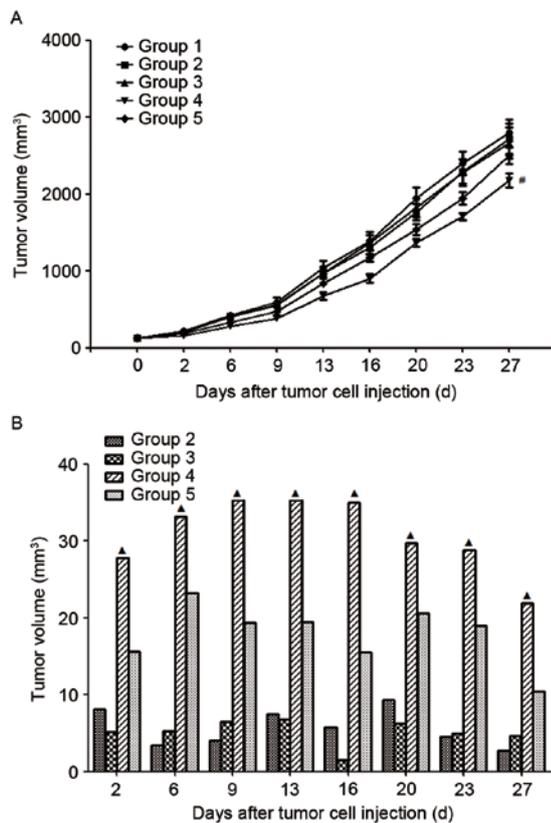


Figure 5. (A) Tumor growth curve and (B) inhibition rates. Group 1, treated with vehicle; Group 2, treated with TRAIL-Mu3 at a concentration of 5 mg/kg; group 3, treated with TRAIL-Mu3 at a concentration of 15 mg/kg; group 4, treated with TRAIL-Mu3 at a concentration of 45 mg/kg; and group 5, treated with TRAIL at a concentration of 45 mg/kg. #P<0.05 vs. group 1, 2, 3, 5; ▲P<0.05 vs. group 2, 3, 5. TRAIL, tumor necrosis factor ligand superfamily member 10.

addition, this highly efficient translocation capacity has been observed in a variety of cell lines with minimal toxicity, overcoming challenges frequently associated with other delivery methods (24,25).

In the present study, 5 amino acids of the extracellular region (114-281aa) of wild-type TRAIL were selectively altered to form a continuous sequence of 8 Arg residues

Table I. The IC<sub>50</sub> values of TRAIL and TRAIL-Mu3 in various colorectal cancer cell lines.

Cell line	IC <sub>50</sub> , μg/ml	
	TRAIL	TRAIL-Mu3
HCT-15	0.008	0.001
COLO 205	0.008	0.002
SW620	0.009	0.002
HT-29	>100	0.030
HCT 116	0.015	0.002

IC<sub>50</sub>, half-maximal inhibitory concentration; TRAIL, tumor necrosis factor ligand superfamily member 10.

which approximately maintained the conformation of TRAIL protein, and additionally had a penetrating peptide-like structure. This TMPPA was termed TRAIL-Mu3.

Following PCR amplification with designed primers, a target gene of ~500 bp was obtained. The gene was ligated with pMD19-T vector and pET32a plasmid, successively. Following transformation into bacteria, culturing, enzyme digestion, electrophoresis and sequencing, it was confirmed that TRAIL-Mu3 was successfully synthesized. Positively-transformed bacteria with plasmid pET32a/TRAIL-Mu3 were obtained. The following SDS-PAGE electrophoresis demonstrated that the positively-transformed bacteria with plasmid pET32a/TRAIL-Mu3 were able to express TRAIL-Mu3 successfully. Subsequently, cation exchange purification, hydroxyapatite purification and anion exchange purification methods were used to purify the TRAIL-Mu3 proteins. The results demonstrated that the purity of TRAIL-Mu3 protein was high. Western blot analysis further confirmed that the TRAIL-Mu3 protein was successfully obtained.

TRAIL-Mu3 has a penetrating peptide-like structure; therefore, the affinity of TRAIL-Mu3 to the cancer cell membrane was detected. In the immunofluorescence analysis, it was observed that TRAIL-Mu3 exhibited significantly stronger affinity to the SW620 colorectal cancer cell

membrane compared with TRAIL. It was hypothesized that TRAIL-Mu3 may enhance the affinity to the cancer cell membrane. TRAIL-Mu3 was able to aggregate on the cancer cell membrane, and exerted increased signal transduction and antitumor effects.

The present study aimed to investigate the antitumor effects of TRAIL-Mu3 on tumor cells *in vitro* and *in vivo*. The antitumor effects of TRAIL-Mu3 and TRAIL were detected in 32 different tumor cell lines through CCK-8 method. It was observed that TRAIL-Mu3 exhibited markedly stronger antitumor effects compared with TRAIL in these 32 tumor cell lines (data not shown), including 5 colorectal tumor cell lines. Additionally, TRAIL-Mu3 was able to reverse the resistance of TRAIL-resistant tumor cell lines (data not shown) including HT-29 tumor cell line. The antitumor effects of TRAIL-Mu3 were further detected in HT-29 tumor-bearing mice. Similarly, TRAIL-Mu3 at the same concentration exhibited significantly better antitumor effects compared with TRAIL on HT-29 tumor-bearing nude mice.

To the best of our knowledge, the present study was the first to introduce the concept of mutations in TRAIL and to obtain the novel drug TRAIL-Mu3. In addition, TRAIL-Mu3 exerted stronger antitumor effects compared with TRAIL in colorectal cancer *in vitro* and *in vivo*. However, there remain certain limitations to the present study. The detailed mechanisms underlying the antitumor effects of TRAIL-Mu3 were not completely elucidated. Future studies are required to complete the investigation into the mechanism.

The present study constructed a novel drug TMPPA termed TRAIL-Mu3. TRAIL-Mu3 exerted significantly stronger antitumor effects compared with TRAIL on colorectal cancer *in vitro* and *in vivo*. TRAIL-Mu3 was able to reverse the resistance of TRAIL-resistant HT-29 colorectal cancer cells successfully.

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