

Generation of a novel TRAIL mutant by proline to arginine substitution based on codon bias and its antitumor effects

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Abstract. TNF ligand superfamily member 10 (*TRAIL*) is a member of the tumor necrosis factor superfamily. The present study was performed in an effort to increase the expression of soluble (*sTRAIL*) by rebuilding the gene sequence of *TRAIL*. Three principles based on the codon bias of *Escherichia coli* were put forward to design the rebuild strategy. Relying on these three principles, a P7R mutation near the N-terminal region of *sTRAIL*, named *TRAIL-Mu*, was designed. *TRAIL-Mu* was subsequently cloned into the *PTWIN1* plasmid and expressed in *E. coli* BL21 (DE3). Using a high-level expression system and a three-step purification method, soluble *TRAIL-Mu* protein reached ~90% of total cellular protein and purity was >95%, demonstrating success in overcoming inclusion body formation. The cytotoxic effect of *TRAIL-Mu* was evaluated by sulforhodamine B assay in the MD-MB-231, A549, NCI-H460 and L02 cell lines. The results demonstrated that *TRAIL-Mu* exerted stronger antitumor effects on *TRAIL*-sensitive tumor cell lines, and was able to partially reverse the resistance of a *TRAIL*-resistant tumor cell line. In addition, *TRAIL-Mu* exhibited no notable biological effects in a normal liver cell line. The novel *TRAIL* variant generated in the present study may be useful for the mass production of this important protein for therapeutic purposes.

Introduction

TNF ligand superfamily member 10 (*TRAIL*) was cloned in 1995, and is characterized by rapidly inducing apoptosis in a

wide variety of tumor cell lines at picomolar concentrations (1). Preclinical cytotoxicity studies confirmed that *TRAIL* exerted potent tumoricidal activity while lacking severe toxicity towards normal tissues *in vitro* (2) and *vivo* (3,4), making it a potentially useful cancer therapeutic agent (5).

Among the numerous expression systems for *TRAIL*, including *Escherichia coli* (6), *Pichia pastoris* (7), Sf9 insect cells (8), Chinese hamster ovary cells (9) and *Agrobacterium tumefaciens* (10), *E. coli* is generally considered the best option due to its known genetic properties, high density fermentation, rapid biomass accumulation, inexpensive cultivation costs and simple processing conditions.

Although a number of positive reports on the expression of *TRAIL* in *E. coli* have been presented, certain problems remain to be solved (6,11-13). One of the biggest problems associated with *TRAIL* protein expression in *E. coli* is inclusion body formation. During the high-level production of heterologous proteins, the macromolecule concentration environment presents a challenge to the obtainment of the correct and bioactive conformation; when this fails, folded proteins deposit to form insoluble aggregates known as inclusion bodies (14). Considering the difficulty refolding of inclusion body proteins into bioactive forms, and that this results in high cost and poor recovery, a number of alternative solutions have been considered (15).

Gopal and Kumar (16) and Makrides (17) summarized five target strategies to increase the expression quantity and solubility of heterologous proteins: gene sequences, vector, host, culture parameters of host strain, and co-expression of other genes. The present study proposed a strategy of altering the human *TRAIL* gene sequence in the N-terminal region according to the codon bias of *E. coli*, in order to increase the expression quantity, solubility and cytotoxic activity of *sTRAIL*, with three principles: i) Multiple (≥ 3) continuous or discontinuous (adjacent) Arg sequences may be effective; ii) two consecutive and identical bases, for example GG, TT, CC, AA, may not be used in the last two sites of the amino acid triplet code; and iii) adjusting the total % of G and C from 40 to 70% in the first ten codons of the *sTRAIL* gene.

Relying on these three principles, a P7R mutation near the N-terminus of *sTRAIL* was generated, with the quantity of G and C increased and the last two repeated base sequences of

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the triplet code avoided. The novel mutant, named *TRAIL-Mu*, was cloned into the *PTWIN1* plasmid and expressed in *E. coli* BL21 (DE3). Purified by a three-step purification method, the effect of the mutant protein on the viability of MD-MB-231, A549, NCI-H460, and L02 was examined.

Materials and methods

Construction of expression vector. The generated mutant *sTRAIL* gene, termed *TRAIL-Mu*, and the wild-type *sTRAIL*, termed *TRAIL-Wi*, were cloned by two cycles of polymerase chain reaction (PCR). The extracellular portion of human *TRAIL* (114-281 aa; gene ID 8743) was used as the template for the first run of the PCR, which was the laboratory preservation, and the first PCR product was used as the template for the second run. Two forward primers and one reverse primer were designed using the primer design principles for PCR (Table I). *KOD-Plus-Neo* (Toyobo Co., Ltd., Osaka, Japan) was used in the first cycle for high fidelity and *Ex Taq* (Takara Bio, Inc., Otsu, Japan) was used in the second cycle to link the first cycle production to the *T vector* (Takara Bio, Inc.). The PCR was performed under the following conditions: In the first run, pre-denaturation at 94°C for 2 min, 25 cycles of 98°C for 30 sec (denaturation), 68°C for 1 min (annealing and extension), and post-extension at 68°C for 5 min; in the second run, pre-denaturation at 94°C for 2 min, 25 cycles of 98°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and post-extension at 72°C for 3 min.

The second PCR products were subcloned into *pMD19-T vector* (Takara Bio, Inc.) and sequenced (BGI, Shenzhen, China), and the correct plasmids were purified and digested with *NdeI* and *PstI* (Takara Bio, Inc.), and linked to the corresponding sites of the expression vector *pTWIN1* using *T4 DNA ligase* (Takara Bio, Inc.). The resulting plasmids were termed *pTWIN1/TRAIL-Wi* and *pTWIN1/TRAIL-Mu*, respectively. Successful cloning was confirmed by gel electrophoresis and DNA sequencing (BGI). The first ten codons of the gene sequences of *sTRAIL* (114-281 aa; gene ID 8743), *TRAIL-Wi* and *TRAIL-Mu* are presented in Table II.

Expression of *TRAIL-Wi* and *TRAIL-Mu*. The recombinant plasmids *pTWIN1/TRAIL-Wi* and *pTWIN1/TRAIL-Mu* was separately transformed into the *E. coli* BL21 (DE3) strain by treatment with an ice bath for 30 min, heat shock at 42°C for 90 sec and a second ice bath for 3 min. The transformed bacteria was cultured in lysogeny broth medium (0.5% yeast extract, 1% Peptone, and 1% NaCl; pH 7.0) on a rotary shaker (250 rpm) at 37°C for 12 h, and subsequently inoculated into Terrific Broth medium (1.2% peptone, 2.4% yeast extract, 72 mM K₂HPO₄, 17 mM KH₂PO₄, and 0.4% glycerol; pH 7.2) on a rotary shaker (250 rpm) at 37°C for 2 h and 20°C for 3 h, and the proportion of inoculation was 2%. Isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 M) was added to induce the expression of the protein of interest at 20°C for 12 h. Subsequently, the cultures were centrifuged at 7,850 x g for 2 min at 4°C. A total of 2 g (wet weight) of bacteria was resuspended in 8 ml 50 mM Na₂HPO₄, and sonication (2 sec at a time for 10 min at 4°C) was performed on ice using an ultra-sonicator (SCIENTZ-IIID; Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) until the cells were lysed. The supernatant was collected by centrifugation at 12,000 x g for 10 min at 4°C.

Purification of *TRAIL-Wi* and *TRAIL-Mu*. Following centrifugation, *TRAIL-Wi* and *TRAIL-Mu* proteins were purified separately with different flow rates using Ni²⁺ affinity chromatography (Chelating Sepharose FF; 23.5 ml; 1.6x11 cm; GE Healthcare, Chicago, IL, USA), cation exchange chromatography (SP Sepharose Fast Flow; 22 ml; 1.6x10.5 cm; GE Healthcare) and anion exchange chromatography (Q Sepharose Fast Flow; 20.5 ml; 1.6x10 cm; GE Healthcare).

TRAIL-Wi protein (40 ml) was injected onto the Ni²⁺ Affinity Chelating Sepharose FF column (23.5 ml; 1.6x11 cm; GE Healthcare, Chicago, IL, USA) which was connected to the Akta purifier (GE Healthcare). The Ni²⁺ Affinity Chelating Sepharose FF column was previously equilibrated with 5 column volumes of a 20 mM imidazole buffer containing 20 mM Na₂HPO₄-NaH₂PO₄, 0.5 M NaCl and 20 mM imidazole (pH 7.4). The unbound proteins were washed with imidazole buffer until the absorbance at 280 nm reached zero, and the bound protein was eluted by the column with a gradient of 120 to 500 mM imidazole buffer (pH 7.0). *TRAIL-Wi* was at the rate of 5 ml/min and *TRAIL-Mu* was at the rate of 13 ml/min. Ion-exchange chromatography was performed at 13 ml/min for *TRAIL-Wi* and 5 ml/min for *TRAIL-Mu* with the corresponding buffer. Absorbance at 280 nm was monitored with an ultraviolet detector connected to the data acquisition package. Absorption peaks were identified by 15% agarose gel analysis followed by staining with Coomassie brilliant blue R-250 (Beijing Biodee Biotechnology, Co., Ltd., Beijing, China) at room temperature for 1 h. Purification efficiency was determined by gel image gray scale analysis (Image J software version 1.48 u; National Institutes of Health, Bethesda, MD, USA). Protein concentration was determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Biological activity of *TRAIL*. Human breast cancer MD-MB-231 cells, A549 cells, NCI-H460 cells and L02 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MD-MB-231 cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), and incubated at 37°C in a humidified atmosphere of 5% CO₂. MD-MB-231 cells (8x10³/100 ml) were treated with *TRAIL-Wi* and *TRAIL-Mu* for 48 h in the concentrations indicated. Cell viability was assessed using a sulforhodamine B (SRB) assay (18). A549, NCI-H460 and L02 cell lines were maintained in RPMI-1640 supplemented with 10% FBS. The dose-response curves were depicted using Origin-Pro version 9.0 software (OriginLab, Northampton, MA, USA). The concentrations of *TRAIL-Wi* and *TRAIL-Mu* that induced a 50% reduction in cell viability (IC₅₀) were determined from the curves of reagent concentration vs. cell inhibition rate at 48 h of incubation for the cell line analyzed. The sensitivity of cells to the activity of the proteins was evaluated using the value of IC₅₀. IC₅₀ <10 μg/ml indicates that cells are sensitive to the biological activity of a protein, while IC₅₀ ≥10 μg/ml suggests that cells are relatively resistant to the biological activity.

Table I. Primers used for cloning of *TRAIL-Wi* and *TRAIL-Mu* constructs.

Primer name	Sequence (5'-3')	Restriction site
F1	AGCGTGGTCCGCAGCGTGTGGCTGCTCACATCACTGG	-
F2	GGT <u>CATATG</u> GTGCGTGAGCGTGGTCCGCAGCGTGTGGC	<i>NdeI</i>
F3	AGCGTGGTTCGTCAGCGTGTGGCTGCTCACATCACTGG	-
F4	GGT <u>CATATG</u> GTGCGTGAGCGTGGTTCGTCAGCGTGTGGC	<i>NdeI</i>
R	GTT <u>CTGCAGT</u> TATTAACCAACAAGGAAAGCACCGAAGAAAG	<i>PstI</i>

F1 and F2 are the forward primers for the first and second cycle of PCR for *TRAIL-Wi*, respectively. F3 and F4 are the forward primers for the first and second cycle of PCR for *TRAIL-Mu*, respectively. R is the common reverse primer for *TRAIL-Wi* and *TRAIL-Mu*. The restriction site of *NdeI* is underlined in F2 and F4. The restriction site of *PstI* is underlined in R. TRAIL, TNF ligand superfamily member 10; Wi, wild-type; Mu, mutant; F, forward; R, reverse; PCR, polymerase chain reaction.

Table II. Nucleotide sequences of *sTRAIL* (114-281aa; Gene ID, 8743), *TRAIL-Wi* and *TRAIL-Mu*.

Gene name	Sequence (5'-3')
<i>sTRAIL</i>	ATGGTTCGTGAACGTGGTCCGCAGCGT GTT
<i>TRAIL-Wi</i>	ATGGTGCGTGAGCGTGGTCCGCAGCGT GTG
<i>TRAIL-Mu</i>	ATGGTGCGTGAGCGTGGTCGTCAGCGT GTG

The base sequence of *TRAIL-Wi* was changed based on codon bias of *E. coli* in order to ensure the yield of the protein of interest; however, the amino acid sequence and the biological activity of *TRAIL-Wi* peptide was the same as *sTRAIL* (114-281 aa; Gene ID 8743; data not shown). TRAIL, TNF ligand superfamily member 10; s, soluble; Wi, wild-type; Mu, mutant.

Results

Construction of the expression vectors. The *pTWIN1* plasmid is widely used to obtain high-level expression of heterologous proteins in *E. coli*. The non-fusion *sTRAIL* fragment was subcloned using *NdeI* and *PstI* cloning sites and analyzed by PCR and restriction endonuclease digestion. The *sTRAIL* cDNA was sequenced (BGI) and confirmed to be the correct sequence (Table II).

Expression of recombinant TRAIL. A culture temperature of 20°C with IPTG induction in BL21 (DE3) was chosen to express *TRAIL-Wi* and *TRAIL-Mu* (19-21). Following centrifugation, 68 g wet bacterial weight of *pTWIN1/TRAIL-Wi* BL21 (DE3) and 65 g wet bacterial weight of *pTWIN1/TRAIL-Mu* BL21 (DE3) were collected. SDS-PAGE analysis demonstrated that 90% of the induced *TRAIL-Wi* and *TRAIL-Mu* peptides were in the soluble total protein fraction (Fig. 1). The % was evaluated by gel image gray scale quantification.

Purification of *TRAIL-Wi* and *TRAIL-Mu*. *TRAIL-Wi* and *TRAIL-Mu* proteins were purified by Ni²⁺ affinity chromatography, cation exchange chromatography and anion exchange

chromatography. The three-step purification is summarized in Table III. The obtained results were 95% pure *TRAIL-Wi* (Fig. 2) and *TRAIL-Mu* proteins (Fig. 3), as determined by SDS-PAGE analysis and gel image gray scale analysis. The purified proteins were subsequently used in further assays to determine their biological activity. Western blot analysis further confirmed that the *TRAIL-Mu* protein was successfully obtained (data not shown).

Biological activity of the novel *TRAIL-Mu* peptide. The biological activities of *TRAIL-Wi* and *TRAIL-Mu* on MD-MB-231, A549, NCI-H460, and L02 cell lines are presented in Table IV. The relative dose-response curves are depicted in Fig. 4. According to the data, *TRAIL-Mu* exerted a more pronounced anti-proliferative effect on MD-MB-231, A549 and NCI-H460 cancer cells compared with wild type *TRAIL*. However, *TRAIL-Wi* and *TRAIL-Mu* peptides did not appear to have an effect on human L02 normal liver cells.

Discussion

Sensitivity to TRAIL is exhibited by a wide range of tumor cell lines, although normal tissues are resistant to its activities, which is a notable advantage compared with other TNF family members, including TNF- α and Fas ligand (22,23). It is these properties of TRAIL which make it a potentially promising cancer therapeutic agent.

In the present study, one amino acid of the extracellular region (114-281 aa) of the wild-type TRAIL was selectively changed to form an arginine-rich sequence near the N-terminal of *sTRAIL*; this novel TRAIL mutant was termed *TRAIL-Mu*.

Following PCR amplification with designed primers, a target sequence of ~500 bp was obtained and ligated with pMD19-T and *pTWIN1* vectors successively. Following transformation into bacteria, culturing, enzyme digestion, electrophoresis and sequencing, it was confirmed that *TRAIL-Mu* was successfully synthesized. Next, positively-transformed bacteria with plasmid *pTWIN1/TRAIL-Mu* were obtained. Analysis of total protein extracts by SDS-PAGE demonstrated that the positively-transformed bacteria with plasmid *pTWIN1/TRAIL-Mu* expressed *TRAIL-Mu* protein successfully. Subsequently, Ni²⁺

Table III. TRAIL-Wi and TRAIL-Mu protein purification summary.

Purification steps	TRAIL-Wi			TRAIL-Mu		
	Volume, ml	Protein, mg/ml ^b	Overall yield, %	Volume, ml	Protein, mg/ml ^b	Overall yield, %
Crude extract ^a	40	7.16	-	40	6.9	-
Ni ²⁺ affinity chromatography	22	2.35	18.05	30	2.7	29.35
Cation exchange chromatography	9	2.33	7.32	9	4.1	13.37
Anion exchange chromatography	14	0.42	2.05	19	0.91	6.26

^aFrom 68 g wet weight of pTWIN1/TRAIL-Wi BL21(DE3) and 65 g wet weight of pTWIN1/TRAIL-Mu BL21(DE3). ^bProtein concentration determined by bicinchoninic acid assay. TRAIL, TNF ligand superfamily member 10; Wi, wild-type; Mu, mutant.

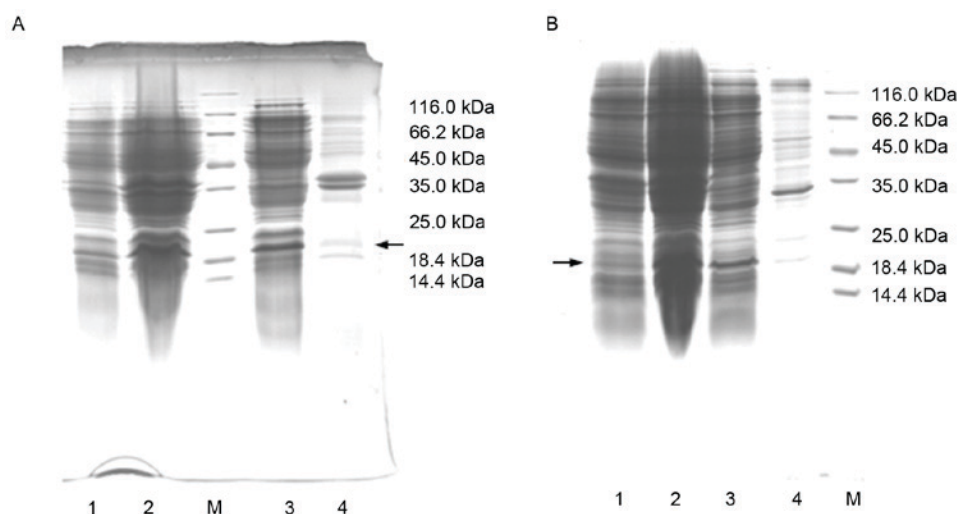


Figure 1. SDS-PAGE analysis of TRAIL-Wi and TRAIL-Mu expression. Samples were boiled for 10 min and purified by centrifugation. A total of 10 μ g of each sample lysed in SDS 2X loading buffer was loaded in each lane, separated by electrophoresis and then stained with Coomassie brilliant blue R-250. The proteins of interest are marked with arrows: (A) TRAIL-Wi and (B) TRAIL-Mu. Lanes 1 and 2, lysates of transformed bacteria prior to and following induction, respectively; lane 3, supernatant following sonication; lane 4, sediment following sonication. Comparison of lanes 3 and 4 indicated that ~90% of the induced peptide was in the soluble fraction. TRAIL, TNF ligand superfamily member 10; Wi, wild-type; Mu, mutant; M, molecular ladder.

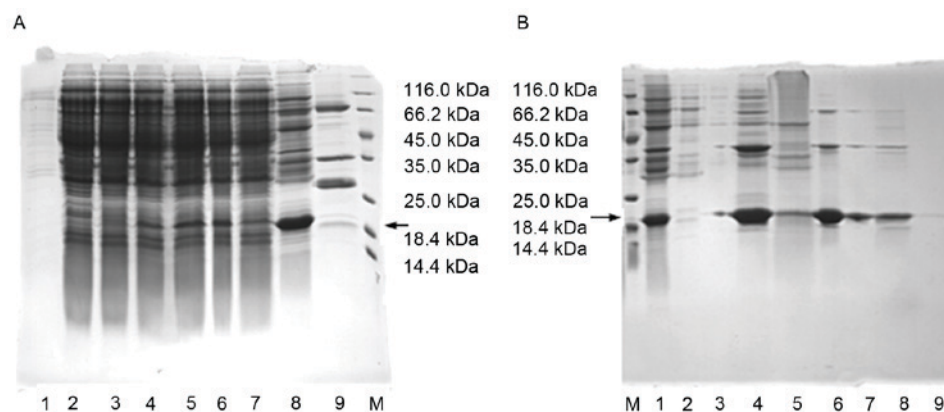


Figure 2. SDS-PAGE analysis of purified TRAIL-Wi by Ni²⁺ affinity chromatography, cation exchange chromatography and anion exchange chromatography. The primary sample (10 μ g) and other samples (20 μ g) lysed in SDS 2X loading buffer were loaded in each lane, separated by electrophoresis and stained with Coomassie brilliant blue R-250. The proteins of interest are marked with arrows. The purification process was monitored by measuring the absorbance at 280 nm. (A) Lanes 1-5, samples of penetration when the retention volume was 18, 55, 95, 135 and 180 ml, respectively; lane 6, primary sample; lane 7, final penetration sample of Ni²⁺ affinity chromatography; lanes 8 and 9, purified TRAIL-Wi following elution with 120 mM imidazole and 500 mM imidazole. (B) Lane 1, primary sample of cation exchange; lane 2, penetration sample of cation exchange; lanes 3-5, purified TRAIL-Wi following elution with 150 mM NaCl (pH 7.0), 600 mM NaCl (pH 8.0) and 0.5 M NaOH, from cation exchange; lane 6, primary sample of anion exchange; lane 7, penetration sample of anion exchange (final material); lanes 8 and 9, purified TRAIL-Wi following elution with 2 M NaCl and 0.5 M NaOH, from anion exchange. TRAIL, TNF ligand superfamily member 10; Wi, wild-type; M, molecular ladder.

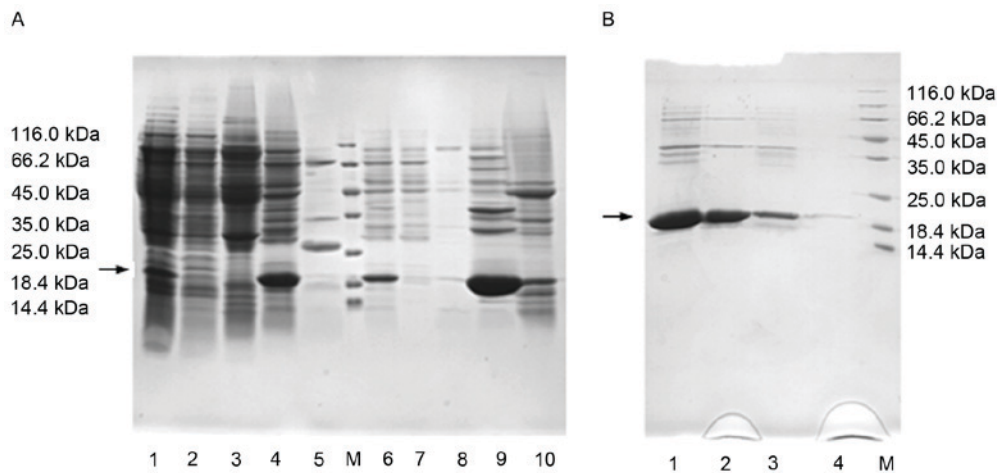


Figure 3. SDS-PAGE analysis of purified TRAIL-Mu by Ni^{2+} affinity chromatography, cation exchange chromatography and anion exchange chromatography. The primary sample (10 μg) and other samples (20 μg) lysed in SDS 2X loading buffer were loaded in each lane, separated by electrophoresis and stained with Coomassie brilliant blue R-250. The proteins of interest are marked with arrows. The purification process was monitored by measuring the absorbance at 280 nm. (A) Lane 1, primary sample; lane 2, penetration sample of Ni^{2+} affinity chromatography; lane 3, peak of 20 mM imidazole buffer elution; lanes 4 and 5, purified TRAIL-Mu following 120 and 500 mM imidazole elution; lane 6, primary sample of cation exchange; lane 7, penetration sample of cation exchange; lanes 8-10, purified TRAIL-Mu following elution with 150 mM NaCl (pH 7.0), 600 mM NaCl (pH 8.0) and 0.5 M NaOH, from cation exchange. (B) Lane 1, primary sample of anion exchange; lane 2, final penetration sample of anion exchange; lanes 3 and 4, purified TRAIL-Mu following 2 M NaCl and 0.5 M NaOH elution, from anion exchange. TRAIL, TNF ligand superfamily member 10; Mu, mutant; M, molecular ladder.

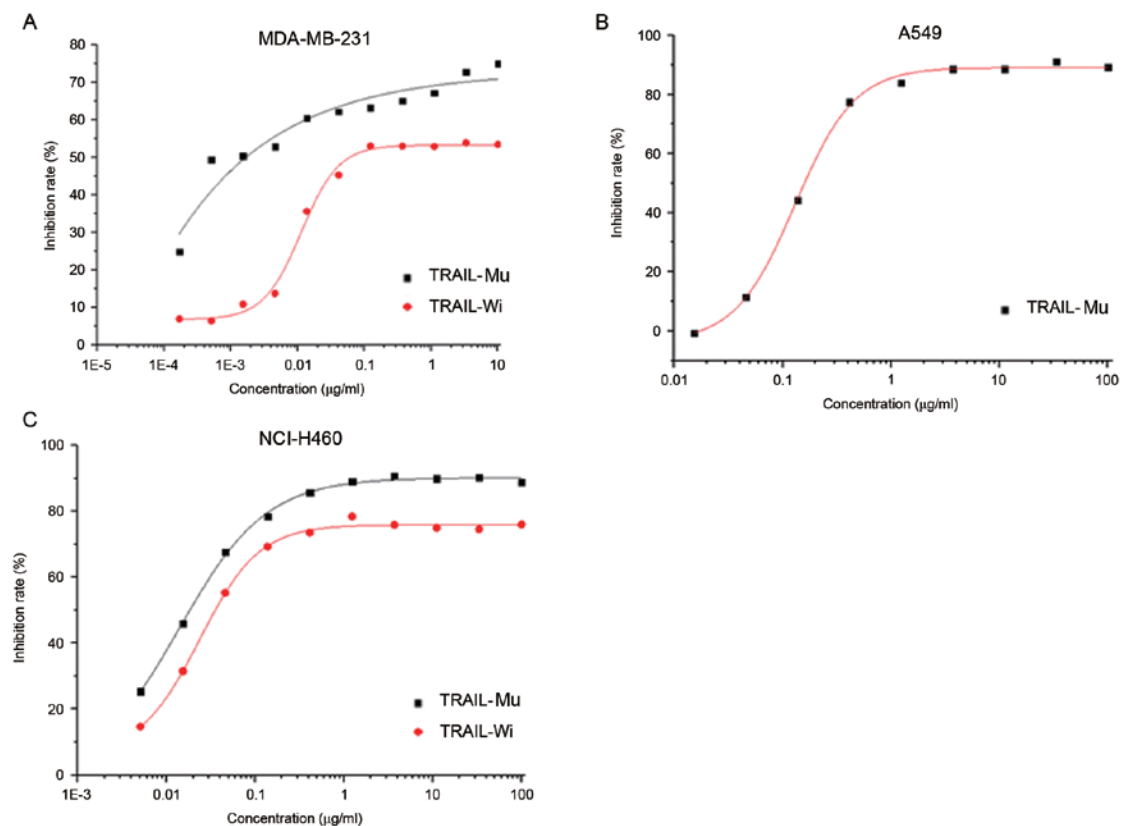


Figure 4. Cytotoxicity effects of TRAIL-Mu and TRAIL-Wi purified peptides in MD-MB-231, A549 and NCI-H460 cells. (A) Dose-response curve of the MD-MB-231 cell line to TRAIL-Mu and TRAIL-Wi. (B) Dose-response curve of the A549 cell line to TRAIL-Mu. (C) Dose-response curve of the NCI-H460 cell line to TRAIL-Mu and TRAIL-Wi. Cell viability was evaluated by sulforhodamine B assay. The concentration was converted into common logarithm. The IC_{50} of the A549 cell line to TRAIL-Wi and the IC_{50} of L02 cell line to TRAIL-Mu and TRAIL-Wi were >10 ; therefore, the dose-response curves were not depicted. TRAIL, TNF ligand superfamily member 10; Mu, mutant; Wi, wild-type.

affinity chromatography, cation exchange purification and anion exchange purification methods were used to purify the TRAIL-Mu protein. The results demonstrated that the purity

of TRAIL-Mu protein was high. Western blot analysis further confirmed that the TRAIL-Mu protein was successfully obtained (data not shown).

Table IV. Cytotoxic activities of *TRAIL-Wi* and *TRAIL-Mu* on MD-MB-231, A549, NCI-H460 and L02 cell lines.

Cell line	IC50 (μ g/ml)	
	<i>TRAIL-Wi</i>	<i>TRAIL-Mu</i>
MD-MB-231	0.0113	0.0015
A549	>100	0.1297
NCI-H460	0.0236	0.0138
L02	>10	>10

TRAIL, TNF ligand superfamily member 10; Wi, wild-type; Mu, mutant; IC50, half maximal inhibitory concentration.

Through the above experiments, a new TRAIL variant was synthesized, which successfully overcame inclusion body formation. The soluble protein expressed in *E. coli* accounted for 90% of the total cellular protein, and a purity of >95% was obtained by three-step purification.

The present study investigated the antitumor effects of TRAIL-Mu on tumor cells and normal cells. The cytotoxic effects of TRAIL-Mu and TRAIL-Wi on MD-MB-231, A549, NCI-H460 and L02 cell lines were detected using the SRB method. It was observed that TRAIL-Mu exerted significant stronger antitumor effects than TRAIL-Wi on MD-MB-231, A549 and NCI-H460 tumor cell lines. Therefore, it may be hypothesized that TRAIL-Mu may exert strong antitumor effects in TRAIL-sensitive tumor cells (MD-MB-231 and NCI-H460), and was able to, at least in part, reverse the resistance of TRAIL-resistant tumor cells (A549). In addition, TRAIL-Mu and TRAIL-Wi exhibited no significant biological effects in the normal liver cell line L02.

The three principles proposed in the present study were primarily based on codon usage of *E. coli*, which was reported in recent years. Codon bias exists in almost all synonymous codons, and certain codons which are frequently used by *E. coli* are technically associated with the abundance of their cognate tRNAs instead of the abundance of the protein (17). It is noteworthy that arginine (24-28), guanine and cytosine (29-32) and mononucleotide repeats (33,34) of heterologous genes were demonstrated to be associated with the output of target proteins in *E. coli* expression systems. In the present study, proline to arginine substitution of the 7th amino acid of the N-terminal region formed an arginine-rich sequence near the N-terminus of sTRAIL, which was confirmed to increase the soluble expression of sTRAIL; however, the mechanisms are not well-understood. It may be suggested that tRNA Arg is relatively efficient in translation in *E. coli* (35).

The present study aimed to overcome difficulties which have been raised in the progress of TRAIL production. Overcoming inclusion body formation has been an unresolved issue in *E. coli* cytoplasmic expression systems for a number of years. The present study generated a novel mutant which successfully overcame inclusion body formation and resulted in a soluble form of the TRAIL protein; in addition, TRAIL-Mu exhibited notably stronger antitumor effects compared with TRAIL-Wi on MD-MB-231, A549 and NCI-H460 cell lines *in vitro*.

However, the mechanisms underlying the antitumor effects of TRAIL-Mu remain to be fully elucidated. Future studies will complete the mechanistic investigation and examine the antitumor effects of TRAIL-Mu, as well as optimize the potential mass production of the TRAIL peptide.

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