ORIGINAL ARTICLE

Bacterial Expression of TMTP1-Fused L-Asparaginase for Targeting Leukemia and Metastatic Tumor Cells

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Abstract- L-asparaginase is recognized as a first-line anticancer drug for acute lymphoblastic leukemia (ALL); however, low-substrate specificity and exhibiting glutaminase activity cause various off-target toxicities on normal cells. In the following study, we functionalized wild-type asparaginase with the TMTP1 targeting peptide which specifically targets a variety of hematological and metastatic cancer cells. The peptide sequence was genetically added to the N-terminal end of the asparaginase using the restriction endonuclease-free cloning method. Wild-type and engineered asparaginases were expressed in *E. coli* and purified by Nickel affinity chromatography column. The *in vitro* activity of both types of enzymes was evaluated by Nessler's method. The sequencing results showed that the TMTP1 sequence was added in the correct frame to the asparaginase. Wild-type and TMTP1-fused asparaginases were produced in a soluble state with the specific activity of 172 U/mg and 153 U/mg, respectively. The evidence from this study suggests that TMTP1-fused asparaginase could preserve its solubility and activity compared to the wild-type species and can be proposed for future research in anticancer therapies.

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Introduction

Cancer is the first or second death threat in most countries, according to the world health organization (WHO). About 19.3 million new cancer patients and 10 million deaths globally were reported in 2020 (1). While cancer treatment is on the way to development, chemotherapy, and radiotherapy still stand on the first line of cancer therapy; however, the administration of these conventional drugs has been hampered due to their off-target toxicities on the various organs of the patient's body. These complexities comprise cardio-toxicity, neuro-toxicity, and hepato-toxicity caused by chemotherapeutics and vascular and neuronal damages from radiation therapy which can significantly affect the life quality and expectancy of cancer cases (2,3).

To answer the limitation of conventional drug delivery, targeted drug delivery systems functionalize the pharmaceutical agents with targeting moieties to direct them only to the desired part of the body. They act as ligands that target specific or overexpressed receptors on the surface or inside the diseased cells and induce drug accumulation and uptake. Different ligands have been used till now for active targeting, including aptamers, peptides, proteins, and monoclonal antibodies (mAbs) (4). mAbs are the most applied targeting moiety in pharmaceuticals. They were exploited as naked antibodies or as antibody-drug conjugates (ADC).

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Although several mAbs (e.g., Trastuzumab) and ADCs (e.g., Trastuzumab emtansine) have been approved by the FDA; developing such complex therapeutics is laborious and requires extensive investment. Moreover, in some cases, they may cause severe immune responses (4,5).

Peptides are a newer class of targeting ligands with a low number of amino acids (less than 40 amino acids as defined by the FDA) (6) that resemble the structure of antibodies for targeting receptors. Their small size makes them cost-effective and easy to engineer. Furthermore, they are generally known not to promote immune responses. Tumor-homing peptides (THPs) can specifically bind to tumor cells; additionally, by conjugating them to the cytotoxic payload, peptide-drug conjugates (PDCs) can be created. Despite ADCs, due to their smaller dimensions, PDCs can penetrate deep into the tumor (7). ^{177}Lu-DOTATATE is the first FDA-approved PDC for the treatment of gastroenteropancreatic neuroendocrine tumors; consisting of somatostatin-targeting peptide and lutetium-177 (^{177}Lu) as a radiotherapeutic agent (8). There are more PDCs in different clinical phases or under investigation to be used in a variety of cancers.

L-asparaginase is a potential anticancer therapeutic which catalyzes the conversion of L-asparagine to aspartic acid and ammonia. Lymphoblastic leukemia cells are deficient in the production of L-asparagine amino acid due to the lack of the asparagine synthesis enzyme; therefore, unlike healthy cells, L-asparagine is an essential amino acid for them. Asparaginase is not only used in the first-line therapy of acute lymphoblastic leukemia (ALL) but has also shown promising outcomes in the treatment of ovarian and breast cancers as well as autoimmune diseases. However, a body of research concluded that asparagine is not the only substrate of asparaginase, and the enzyme shows glutaminase activity as well, resulting in a decreased rate of protein synthesis in normal cells and causing several side effects such as pancreatitis and leucopenia (9-11).

As an attempt to functionalize the L-asparaginase enzyme with a targeting moiety, here we applied the TMTP1 homing peptide. The TMTP1 peptide consists of 5 amino acids (NVVRQ) that specifically binds to a series of hematological cancer cells, including HL60 and K562 cell lines. TMTP1 could also target highly metastatic cancerous cells (12,13).

In this regard, the sequence of TMTP1 peptide was genetically fused to the N-terminus of the L-asparaginase by using the site-directed mutagenesis method. Wild-type and targeted asparaginase were then expressed in the *Escherichia coli* host. The biological activity of both enzymes was also evaluated.

Materials and Methods

Epitope tagging

To create engineered TMTP1-fused asparaginase (henceforth named ASPase) for targeting hematological and metastatic cancerous cells, we aimed to add TMTP1 sequence (NVVRQ) to the N-terminal end of asparaginase by site-directed mutagenesis method (Figure 1). The wild-type ASPase in pET26b vector was used as a template. The first PCR was performed to amplify the chimeric asparaginase gene consisting of the pET26b plasmid backbone and two-copy of TMTP1 sequences with a primer set; the forward primer included 5' end complementary to the pET26b backbone, TMTP1 sequences, and 3' end complementary to the ASPase gene. The reverse primer was complementary to the pET26b and 3' end of the ASPase. The synthesized amplicon was cleaned up and employed in Quick-Change PCR as a megaprimer to complete the rest of the plasmid using a high-fidelity CloneAMP premix (Takara Bio, Japan). The resulting product was treated with DpnI restriction enzyme (Thermo Fisher Scientific, USA) to remove the template vector (i.e., pET26b-ASPase) and subsequently transformed into the DH5α bacteria. The construct (pET26b-TMTP1-ASPase) was verified by PCR and Sanger sequencing.

Expression and purification

Wild-type ASPase and TMTP1-ASPase expression constructs were introduced into the *E. Coli* BL21 (DE3) for recombinant protein expression. Following transformation, some of the resulting colonies were cultured in Luria Bertani (LB) broth media, and when culture absorbance at 600 nm reached 0.4-0.6, the bacteria cells were induced by 0.1 mM IPTG. The protein expression before and after induction was analyzed with 10% SDS-PAGE gel.

A single colony with the highest expression was selected and used for the inoculation of 200 ml LB broth. The culture medium was incubated at 37° C and shaken at 150 rpm. After reaching the OD600 nm of 0.4-0.6, the culture was induced by 0.1 mM IPTG and incubated overnight. The BL21 pellet was collected by centrifugation and resuspended in lysis buffer (500mM NaH_2PO_4 and 300 mM NaCl; pH:8) and sonicated on ice (10 s pulses for 20 times at 40% amplitude with 20 s intervals). The supernatant was then harvested by centrifugation at 12,000 rpm for 20 min and loaded onto the Nickel Sepharose (Ni-NTA) column (Qiagen Inc.).

Following washing the column with 15 ml washing buffer (lysis buffer with 20 mM Imidazole), the protein fractions were eluted using elution buffer (lysis buffer with 300 mM Imidazole). The purity of each enzyme was evaluated by 10% SDS-PAGE, and the protein concentration was calculated by the Bradford assay.

Immunoblotting

The purified sample of TMTP1-ASPase enzyme was loaded on 10% SDS-PAGE gel and bands were transferred onto a nitrocellulose filter membrane by the semi-dry procedure. The membrane was blocked using 3% w/v skim milk in phosphate buffer saline (PBS) at 4˚ C overnight. After thrice washing the membrane with TBST buffer (Tris-buffered saline with 0.1% Tween 20), it was subjected to HRP-conjugated anti-polyhistidine antibody (Sigma-Aldrich, USA). Washing was done four times again, and a His-tagged enzyme band was developed by the ECL detection kit (Amersham).

Enzymes activity

The biological activity of the purified enzymes was evaluated by Nessler's method described previously by Wriston (14). Reactions containing 50 mM Tris-HCL and 10 mM L-asparagine were incubated to reach the temperature of 37˚ C. 10 µl of wild-type or TMTP1-fused ASPase was added to each reaction and incubated at 37˚ C for 15 min. The reactions were stopped by adding 1.5 M trichloroacetic acid (TCA). Nessler's reagent measures the ammonia released by the hydrolyzing of L-asparagine. The absorbance of each reaction was read at 436 nm, and the released ammonia was calculated by plotting the standard curve of ammonium sulfate. The experiment was performed in triplicate. The unit (IU) of ASPase is described as the amount of the enzyme required to release 1 mM of ammonia at 37˚ C.

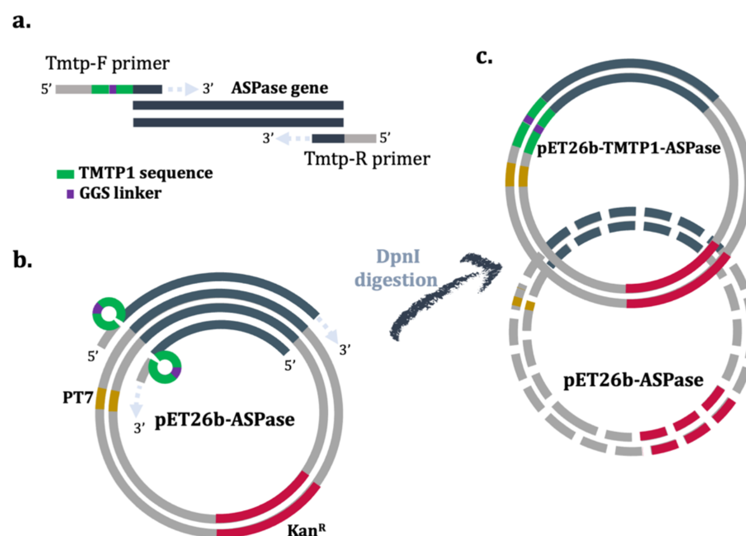

Figure 1. Schematic representation of site-directed mutagenesis to create TMTP1-fused asparaginase. (a) The first PCR was performed to create a chimeric ASPase gene by using Tmtp-F and Tmtp-R primers. (b) The product of the first PCR was used in Quick-change PCR to synthesize the pET26b-TMTP1-ASPase construct. (c) Following treatment with the DpnI restriction enzyme, the template vectors (pET2b-ASPase) were digested due to having methylated sites

Results

Molecular cloning

Two copies of the TMTP1 peptide were successfully added to the N-terminus of the ASPase gene by polymerase chain reaction using extended primers. Figure 2a shows the desired band size of 1.1 kb on the 1% agarose gel. Two copies of the peptide were designed to better target cancer cells. Peptides were fused to each other and to the ASPase enzyme by GGS and GS linkers, respectively. The TMTP1-ASPase amplicon was then subcloned to the pET26b vector using the restriction endonuclease-free cloning method (Figure 2b). The precise insertion of sequences was confirmed by Sanger sequencing (Figure 2c).

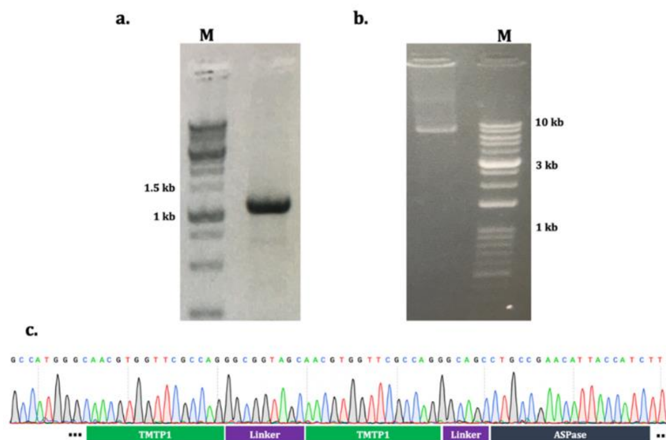

Figure 2. Precise insertion of (TMTP1)-ASPase into the pET26b vector. (a) Mega primer product: a simple PCR was performed to synthesize the mega primer (near 1.1 kb) by adding the TMTP1 peptide sequence to the N-terminus of ASPase using a set of primers (M, 1 kb DNA marker). (b) Quick-change PCR product; the product of the first PCR was used to synthesize the whole plasmid (i.e., pET26b-TMTP1-ASPase). (c) The generated construct was sent for Sanger sequencing using a universal T7 promoter primer. The results did not show any undesired mutations within the construct

Expression and purification

The *E. Coli* BL21 host cells expressed wild-type and TMTP1-fused asparaginases. Figures 3a and 3b depict the desired bands following induction with 0.1 mM IPTG. Each recombinant enzyme was then purified by Ni-NTA affinity column in soluble form (Figures 3a and 3b). The concentration of each enzyme was measured to be 298 µg/ml and 209 µg/ml for ASPase and TMTP1-ASPase, respectively. The identity of the TMTP1-ASPase enzyme was also verified by immunoblotting analysis (Figure 3c).

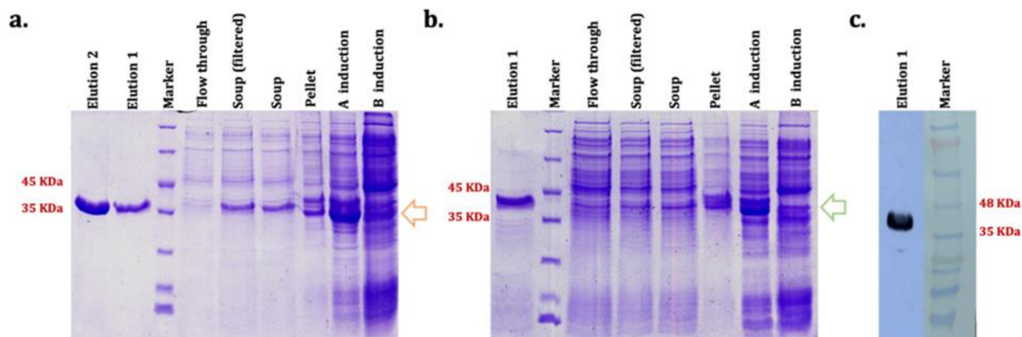

Figure 3. Expression and purification of wild-type ASPase and TMTP1-ASPase. (a) And (b) Depicts the 10% SDS-PAGE gel results of expression and purification results of wild-type and TMTP1-fused asparaginases, respectively. (B induction; before induction with 0.1 mM IPTG and, A induction; after IPTG induction). (c) The result of immunoblotting analysis of TMTP1-asparaginase

Activity assay

The *in vitro* enzyme activity of each enzyme was assessed by Nessler’s method. The specific activity of wild-type ASPase was 172 U/mg, and TMTP1-ASPase was 153 U/mg, relatively. The lower activity of the engineered enzyme could be due to N-terminal end epitope tagging.

Discussion

The lack of targeting in conventional drug delivery approaches has led to the use of high doses of drugs to deliver a sufficient amount of active pharmaceutical agents to diseased cells. This can cause adverse toxicities on healthy cells that affect the patient's life quality (15).

Deficiency or insufficiency of asparagine synthetase, as the only enzyme to synthesize asparagine amino acid in Leukemia cells, makes asparagine an essential amino acid for the growth and division of hematological malignant cells. L-asparaginase, by depleting serum asparagine, plays a vital role as an anticancer drug in acute lymphoblastic leukemia (ALL) (11). Currently, several asparaginases present in marketing are derived from *E. coli* (e.g., Oncaspar) or *E. chrysanthemi* (e.g., Crisantaspase) (16). In this study, the wild-type asparaginase was recombinantly expressed in *E. coli* BL21 host and purified in the soluble state with a concentration of 298 µg/ml.

Recent studies have shown that L-asparaginase can bind to glutamine with lower affinity and exhibit glutaminase activity (17,18). Consequently, at high doses, the enzyme can reduce protein synthesis in healthy cells and cause several side effects. Here, we tried to make the L-asparaginase specific for leukemia cells by using a peptide as a targeting moiety. Targeting or homing peptides can bind to the specific or overexpressed receptors on the diseased cells and deliver the conjugated drug to them (7). The TMTP1 peptide was exploited in the present research to functionalize the asparaginase enzyme. Previous studies showed that this five-amino acid peptide has a promising ability to target hematological malignant cells (12). It also specifically binds to metastatic tumors (13). Two copies of the peptide were added to the N-terminus of asparaginase by restriction endonuclease-free cloning method (19). This method allowed us to insert the peptide sequences into the vector in the right frame without additional nucleotides to prevent any adverse effects on protein structure or biological activity. The TMTP1-ASPase was also purified, and the concentration was measured to be 209 µg/ml. The biological activity of both types of asparaginase was also calculated by Nessler's method. The engineered asparaginase exhibited lower activity compared to the wild-type enzyme (153 U/mg vs. 172 U/mg). This could be a result of N-terminal epitope tagging of asparaginase, which is consistent with a previous report that the addition of scFv antibody to the N-terminus of asparaginase reduced its biological activity by about 20% (20).

Taken together, TMTP1 targeting peptide could be exploited as a targeting moiety for asparaginase enzyme to reduce its side effects on healthy tissues. By imposing minimal effect on the specific activity and having a small size compared to the ADCs, this fusion can be proposed for further research in cancer therapy.

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