

Artificial Cells, Nanomedicine, and Biotechnology

An International Journal

ISSN: (Print) (Online) Journal homepage: <https://www.tandfonline.com/loi/ianb20>

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To cite this article: Jina Ryu, Sung Jae Yang, Boram Son, Haein Lee, Jongmin Lee, Jinmyoung Joo, Hee Ho Park & Tai Hyun Park (2022) Enhanced anti-cancer effect using MMP-responsive L-asparaginase fused with cell-penetrating 30Kc19 protein, *Artificial Cells, Nanomedicine, and Biotechnology*, 50:1, 278-285, DOI: [10.1080/21691401.2022.2126851](https://doi.org/10.1080/21691401.2022.2126851)

To link to this article: <https://doi.org/10.1080/21691401.2022.2126851>



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Enhanced anti-cancer effect using MMP-responsive L-asparaginase fused with cell-penetrating 30Kc19 protein

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ABSTRACT

As the acute lymphoblastic leukaemia (ALL) develops, expression of L-asparaginase (ASNase) protein is known to decrease. Therefore, deficiency of the ASNase protein would be regarded as one of the significant indications of the ALL. For the treatment of ALL, recombinant ASNase protein derived from bacterial origin is used which causes cytotoxicity by deprivation of Asn. However, short half-life of the protein is an obstacle for medical use. In order to overcome this limit, recombinant ASNase was fused to 30Kc19 with protein-stabilizing and cell-penetrating properties. As the 30Kc19 protein may induce steric hindrance, we further added a PLGLAG linker sequence (LK) between the ASNase and 30Kc19. The treatment of ASNase-LK-30Kc19 fusion protein demonstrated enhanced stability, cell-penetrating property, and anti-cancer activity. Intracellular delivery of both the non-cleaved and cleaved forms of the protein were observed, suggesting that ASNase acted both internally and externally, performing high anti-cancer activity by effective depletion of intracellular Asn. Additionally, ASNase-LK-30Kc19 showed high selectivity towards cancer cells. In terms of the dosage, releasable ASNase from ASNase-LK-30Kc19 reached the same half-maximal inhibitory concentration at a concentration five times lower than non-releasable ASNase-30Kc19. Altogether, the findings suggest that this fusion approach has potential applications in the treatment of ALL.

ARTICLE HISTORY

Received 20 July 2022
Revised 30 August 2022
Accepted 14 September 2022

KEYWORDS





Acute lymphoblastic leukaemia (ALL); L-asparaginase (ASNase); Cleavable linker; Cell penetrating 30Kc19 protein; Matrix metalloproteinase (MMP); Fusion protein


Introduction

Acute lymphoblastic leukaemia (ALL) is a common malignant haematologic disease which is characterized by the development of large numbers of immature lymphocytes. In 2018, approximately 437,000 new cases of ALL were diagnosed, and nearly 309,000 patients died from ALL worldwide [1]. The pathological abnormality of ALL is overproduction of immature lymphoblasts in the bone marrow; it may interfere with proliferation and differentiation of normal lymphoid precursor cells. For treatment, remission induction is used for eliminating residual leukaemia cells. Remission is defined by less than 5% leukemic blasts in the bone marrow including blood, and no symptoms of leukaemia show in patients [2].

Since the finding of L-asparaginase (ASNase) as a cancer-inhibitory agent in 1950s, ASNase has been used for remission induction therapy of ALL [3,4]. Leukaemia cells which lack the asparagine synthetase (AS) usually take L-asparagine (L-Asn) directly from blood circulation. If ASNase hydrolyses

L-Asn into aspartate and ammonia, leukaemia cells are unable to use L-Asn for their metabolism, resulting in starvation and cell death [5]. However, there are some limitations for use of ASNase in practical clinics. ASNase is commonly produced in *Escherichia coli* (*E. coli*) which is the most popular host to produce recombinant proteins with high-throughput expression. Because ASNase from *E. coli* may lead to hypersensitivity, extensive purification is required to eliminate toxic impurities [6]. Moreover, repeated injection due to short half-life of ASNase can cause some side effects [7]. There have been several approaches in order to improve the applicability of ASNase. PEGylation is one possible way to minimize hypersensitivity and prolong half-life of ASNase. FDA approved to administrate Pegaspargase (Oncaspar), which is a PEGylated form of ASNase in 2006 [8,9]. However, conjugation with PEG can cause reduction in efficiency of drug delivery [10]. Also, PEGylation is not free from the immunogenicity [11]. Encapsulation of ASNase is another approach to decrease cytotoxicity and increase half-life.

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 Supplemental data for this article can be accessed online at <https://doi.org/10.1080/21691401.2022.2126851>

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Loading ASNase into nanocapsules [12] or nanoparticle [13–15] has been reported to reduce those side effects. From this point of view, erythrocytes can be used as an ASNase carrier with improvement in pharmacokinetics (PK) and pharmacodynamics (PD) [7]. However, these approaches require additional steps for loading and for optimizing the loading efficiency.

30Kc19 is a dominant protein of 30K protein family (30Kc6, 30Kc12, 30Kc19, 30Kc21, and 30Kc23) that originated from silkworm haemolymph (*Bombyx mori*) which is used for cell supplements to increase cell viability and protein production [16–25]. With molecular weight around 30 kDa, 30Kc19 protein showed cargo-delivering [26–29] as well as cell-penetrating properties by the presence of cell-penetrating peptide (CPP) domain in the protein [30,31]. In addition, we have observed enzyme-stabilizing property of 30Kc19, and thus it was used as a fusion partner [32–35]. 30Kc19 showed low cytotoxicity in various cell types including cell lines and primary cells. In addition, *in vivo* toxicity was evaluated by injecting the protein into mice, and 30Kc19 did not cause significant toxicity in long-term administration experiment [32]. Therefore, it is expected that 30Kc19 can be used as a non-toxic carrier for biological drug delivery. Previously, we conjugated 30Kc19 with ASNase to enhance the stability of ASNase and the results showed that ASNase-30Kc19 has longer stability and higher anti-cancer activity when compared with ASNase. The deactivation constant (k_d) of ASNase-30Kc19 was about 47% lower than that of ASNase.

Since the advantage of fusion with 30Kc19 was validated, here we improved the enzyme activity in fusion protein by adding a cleavable linker (LK). Although 30Kc19 can increase ASNase stability, the steric hindrance effect of 30Kc19 may reduce the ASNase activity. In order to remove the steric hindrance problem, LK was inserted between the 30Kc19 and ASNase. The linker sequenced as Pro-Leu-Gly-Leu-Ala-Gly (PLGLAG) was selected to be cleaved by matrix metalloproteinases (MMPs), which are highly abundant in leukaemia cells [36–38]. Our results indicate that by adding LK between ASNase and 30Kc19, ASNase was released from fusion proteins, and it showed significant toxicity to leukaemia cells.

Materials and methods

Cloning of pET-23a/ASNase-PLGLAG-30Kc19

pET-23a/ASNase-30Kc19 plasmid was obtained from previous studies [39]. In brief, ASNase gene was amplified using genomic DNA of *E. coli* K-12 (DH5 α , Invitrogen) through the primers as follows: 5'-GGCGGGATCCATGGAGTTTTCAAA3' for forward; and 5'-GCGGAATTCGTAAGATCTG3' for reverse. Then the product was inserted into pET23a vector between *Bam*H1 and *Eco*R1 restriction sites. 30Kc19 gene was amplified using pET23a/GFP-30Kc19. And then, the product was inserted into pET23a/ASNase, between *Eco*R1 and *Xho*I in order to construct pET23a/ASNase-30Kc19 vector. A cleavable linker (LK) sequenced as PLGLAG was inserted between ASNase and 30Kc19 to improve enzyme activity by reducing steric hindrance of 30Kc19. The forward primer 5'-GAA TTC CCA CTA GGC CTA GCC GGC GCA GAT TCC GAC GTC CCT AAC-3' including *Eco*R1 restriction enzyme site and PLGLAG

sequence as shown by the underline. The reverse primer 5'-CTC GAG GAA AGC CTT TAT ACC CCA AGC-3' with the *Xho*I restriction enzyme site was used to construct the pET-23a/ASNase-LK-30Kc19 plasmid.

Expression and purification of ASNase-30Kc19 and ASNase-LK-30Kc19 proteins

The constructed vectors were used to transform *E. coli* BL21 (Novagen, Madison, WI, USA), and cells were grown in LB-ampicillin medium at 37 °C in 200 rpm shaking incubator. After induction using isopropyl 1-thio- β -D-galactopyranoside (1 mM) with optical density of 600, cells were further incubated at 37 °C for 4 h. Harvested cells were resuspended in binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, pH 8.0), and disrupted by sonication. After centrifugation (12,000 rpm, 30 min), proteins were loaded to a HisTrap HP column (GE healthcare, Uppsala, Sweden), then non-binding proteins were washed using wash buffer (20 mM Tris-HCl, 0.5 M NaCl, 50 mM imidazole, pH 8.0). Finally, target proteins were purified by fast protein liquid chromatography (FPLC, GE healthcare) using elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 350 mM imidazole, pH 8.0). HisTrap Desalting column (GE healthcare) was used for dialysis through 20 mM Tris-HCl buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0). The purified proteins were quantitated using a Micro BCA kit (Thermo Scientific Inc., Rockford, IL, USA) with a standard bovine serum albumin solution, and then stored at –70 °C for further use.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western-blot analysis

Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using 10% polyacrylamide gels. Protein samples were mixed with reducing sample buffer, containing SDS and β -mercaptoethanol (pH 6.8), and then the samples were denatured by boiling. Each sample was separated according to size by electrophoresis. The polyacrylamide gel was immersed in Coomassie blue staining. For Western-blot analysis, the gel was transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). PageRuler™ Prestained Protein Ladder (Thermo Scientific, No. 26616) ranging from 10 to 180 kDa was used to estimate the size of the target protein. After blocking with 5% skim milk in 0.1% TBS-T, anti-30Kc19 primary antibodies were used. The band was visualized by G: BOX Chemi XL system (Syngene, Cambridge, UK).

In vitro enzyme activity assay

To optimize amount of MMP-2 for LK cleavage, recombinant ASNase-LK-30Kc19 proteins were incubated with 0.05–0.2 μ g of MMP-2 (Calbiochem) at 37 °C for 6 h. Direct nesslerization method was performed to measure the amounts of ammonia formed [40,41]. Briefly, L-Asn was added to the pre-incubated mixture of ASNase-LK-30Kc19 and 0.1 μ g MMP-2, and then incubated at 37 °C for 30 min. The reaction was stopped by adding 1.5 M Trichloroacetic acid, and then Nessler's reagent (Sigma, St. Louis, MO, USA) was added.

We prepared 1 mM ammonium sulphate $((\text{NH}_4)_2\text{SO}_4)$ and used it in the standard range of 0–100 nmol. The blank was used to filter out the artefacts ($\Delta A_{450\text{nm}}$ Standard = $\Delta A_{450\text{nm}}$ Standard – $\Delta A_{450\text{nm}}$ Blank). Coloured compound from the reaction of Nessler's reagent and ammonia was formed, and microplate reader (Thermo Scientific) was used to measure absorbance at 450 nm.

Reverse transcription–polymerase chain reaction analysis

Total RNAs were extracted from cells using a Trizol reagent, and 500 ng of each RNA was used for reverse transcription–polymerase chain reaction (RT–PCR). Gene-specific primers were shown in supplemental Figure S2. *GAPDH* was used as a reference gene. After amplification, the PCR products were analysed by 1% agarose gel electrophoresis, and bands were visualized by ethidium bromide (EtBr) staining. The predicted and observed PCR product sizes for *MMP-2*, *MMP-9* and *GAPDH* were 227, 539 and 197 bp, respectively.

In vitro cytotoxicity assay

FDC-P1 (ATCC[®] CRL-12103) and L5178Y cells (ATCC[®] CRL-9518) were seeded onto 96-well plates (Gibco, Grand Island, NY) at a density of 10,000 cells per each well in RPMI 1640 (Gibco) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (PS). Then, cells were incubated with different concentration of fusion proteins at 37 °C for 24 h. After incubation, cells were washed twice using phosphate-buffered saline (PBS) and the cellular viability was measured using CCK-8 assay kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Data were presented as mean \pm standard deviation ($n = 3$).

Immunofluorescence analysis

The purified ASNase-LK-30Kc19 protein was added to L5178Y cells. Then after 24 h incubation, protein-treated cells were washed three times with PBS, following fixation with 4% paraformaldehyde (PFA) for 15 min. Permeabilization with 0.25% Triton X-100 in PBS (PBS-T) for 10 min, and then blocking with 3% BSA in 0.1% PBS-T for 1 h were sequentially performed. The cells were incubated with anti-T7 (Abcam, Cambridge, UK) or anti-His tag (Santa Cruz Biotechnology, Dallas, TX) primary antibodies for 1 h, then Alexa Fluor 488-conjugated secondary antibody (Abcam) was used to visualize target proteins. After 10 min of incubation with DAPI (Life Technologies, Gaithersburg, MD) to stain the nuclei, cells were observed by confocal laser scanning microscopy (Olympus, Lake Success, NY).

Results

Enhanced ASNase activity after cleavage

We observed that ASNase fused with 30Kc19 had increased activity by the stabilizing property of 30Kc19 protein by

reducing the deactivation constant of ASNase-30Kc19 by 47%, compared with the native ASNase. So, we confirmed the advantageous feature of 30Kc19 protein as a fusion partner. However, the size of 30Kc19 protein (30 kDa) is considered to be too large, resulting in causing hindering effect interfering with the activity of ASNase. Therefore, in order to reduce steric hindrance, we designed a cleavage linker (LK) sequenced as PLGLAG in ASNase-30Kc19 fusion protein. ASNase-LK-30Kc19 was expressed as soluble protein in *E. coli* and purified using FPLC (Figure 1(A)). To clarify the role of LK, ASNase-30Kc19 proteins without LK were purified together. After purification, a specific band was detected in expected 67 kDa size by Western-blot analysis (Figure 1(B)). To confirm the cleavage of fusion proteins, ASNase-LK-30Kc19 was incubated with MMP-2, which can recognize the cleavage sequence, for 6 h. After optimizing the MMP concentration, 0.1 μg of MMP-2 was selected for *in vitro* activity test (supplemental Figure S1). In the presence of MMP-2, a 30Kc19 band was detected in ASNase-LK-30Kc19, while there was not in ASNase-30Kc19 (Figure 1(C)). After MMP-2 cleavage, the enzyme activity of ASNase-30Kc19 and ASNase-LK-30Kc19 were measured simultaneously by direct nesslerization method. The enzyme activity of ASNase-LK-30Kc19 was increased about 57% in unit/ml (or 22% in unit/nmole) compared with the activity of ASNase-30Kc19 (Figure 1(D)). Thus, we confirmed that MMP-2 selectively cleaved LK, and the releasable ASNase in ASNase-LK-30Kc19 had enhanced enzyme activity compared to non-releasable ASNase in ASNase-30Kc19.

Efficient anti-leukemic effects of ASNase-LK-30Kc19

We measured MMP-2/9 mRNA expressions in leukaemia cells in order to compare the sensitivity for the LK, and thus the efficacy of cleavage. Mouse normal lymphoblast (FDC-P1) and mouse lymphoma (L5178Y) cells were prepared. From RT–PCR analysis, L5178Y showed higher MMP-2/9 mRNA expression, compared with FDC-P1 cells (Figure 2(A)). MMP-2 was expressed in both cells, but the expression level was approximately 48% higher in L5178Y. Outstanding expression of MMP-2/9 in L5178Y cells indicates that the lymphoma cells are more favourable to the cleavage of ASNase-LK-30Kc19, comparing with normal lymphoblast cells. Then, in order to confirm predominant anti-cancer activity of releasable ASNase, purified ASNase-30Kc19 and ASNase-LK-30Kc19 proteins were treated on L5178Y (Figure 2(B)). The half-maximal inhibitory concentration (IC_{50}) of ASNase-30Kc19 was 10 mU/mL, whereas ASNase-LK-30Kc19 reached IC_{50} at 2 mU/mL. A similar effect was observed in cell viability. Compared to the cell viability before protein treatment (non-treated group), the cell viability reached about 30% at 100 mU/mL of ASNase-30Kc19, while it reached the similar value at 10 mU/mL of ASNase-LK-30Kc19. In terms of dosage, ASNase-LK-30Kc19 used five times lower concentration for 50% cell death, and 10 times lower concentration for 70% cell death than ASNase-30Kc19. Therefore, these results indicate that ASNase-LK-30Kc19 has higher anti-cancer effect, compared to ASNase-30Kc19.

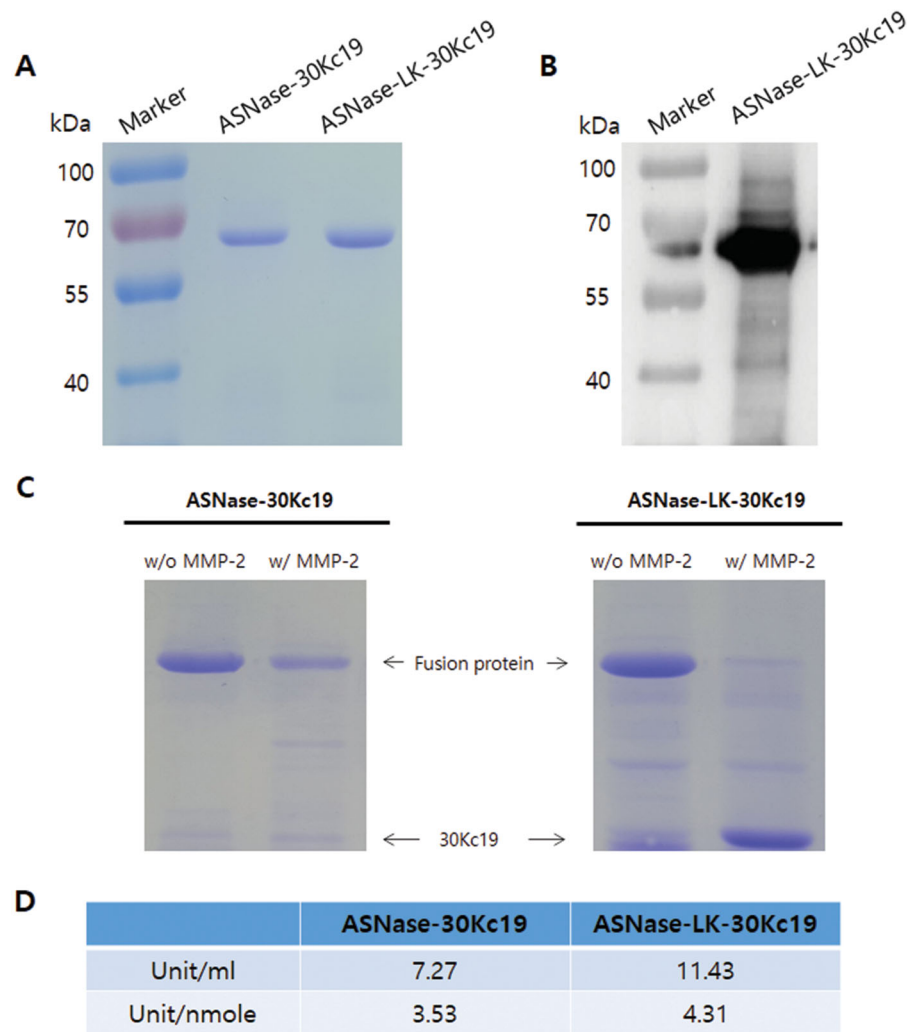


Figure 1. Protein expression and *in vitro* activity of ASNase-30Kc19 and ASNase-LK-30Kc19 fusion proteins. Purified proteins were analysed by (A) SDS-PAGE, and (B) Western blot. (C) Proteins were analysed by SDS-PAGE after incubation with or without MMP-2. (D) *In vitro* ASNase activity assay of ASNase-30Kc19 and ASNase-LK-30Kc19 was performed by direct nesslerization after incubation with 0.1 μ g MMP-2. LK: Linker sequence (PLGLAG).

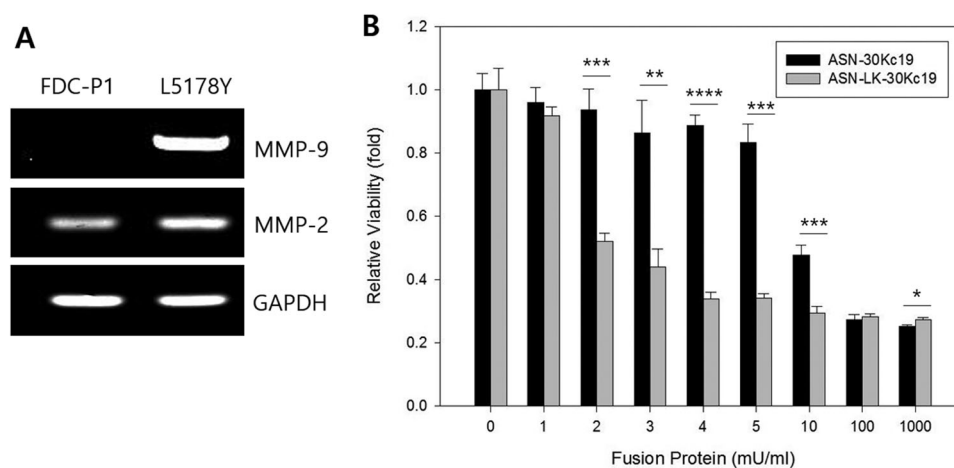


Figure 2. Effect of ASNase-30Kc19 and ASNase-LK-30Kc19 proteins on L5178Y cell viability. (A) Expressions of MMP-2 and MMP-9 in L5178Y cells. (B) The viability of L5178Y cells exposed to different fusion proteins for 24 h was determined by CCK-8 assay. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 3$.

Cellular penetration of ASNase-LK-30Kc19

The cell-penetrating and protein dragging property of 30Kc19 was reported in previous studies [28–30]. Because

MMPs exist outside and inside the tumour cell, ASNase-LK-30Kc19 has both possibilities of being cleaved before or after entering the cells (Figure 3(A)). To check cellular penetration

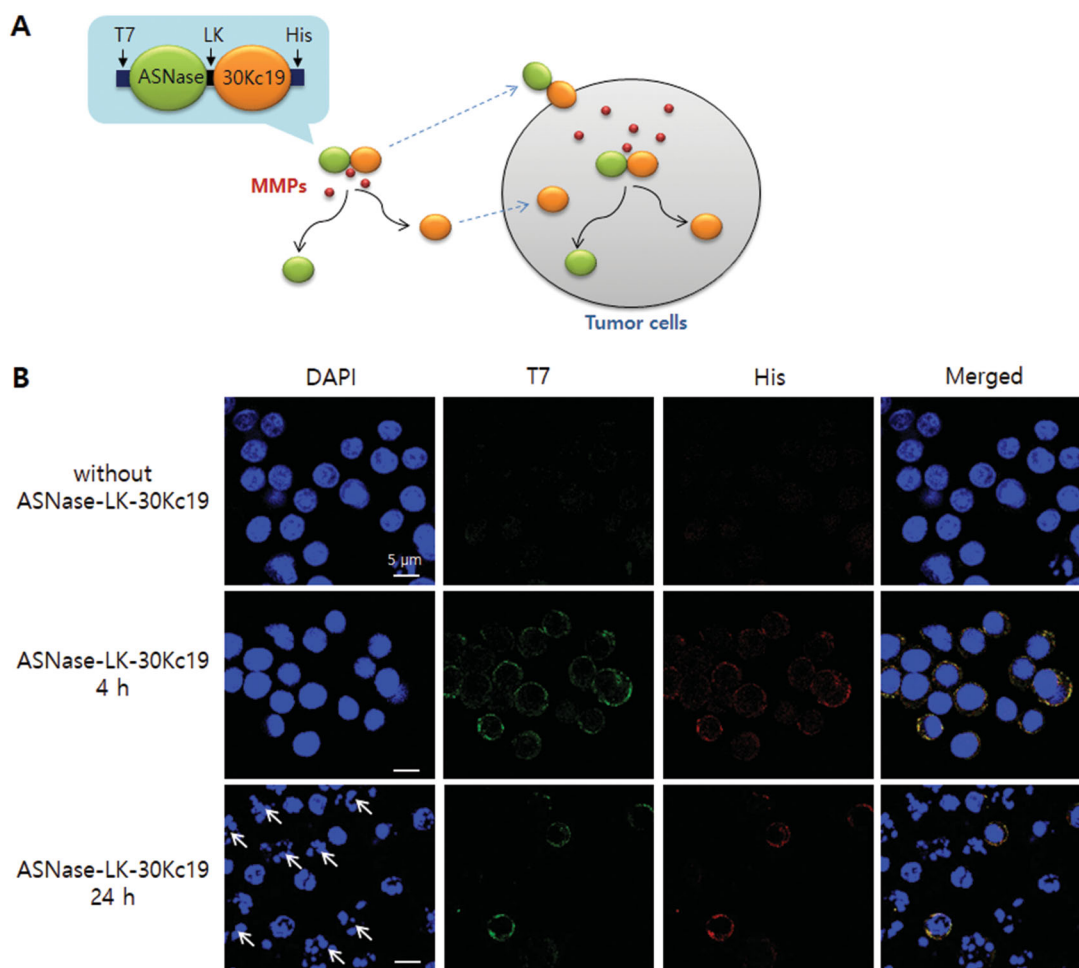


Figure 3. Cellular penetration of ASNase-LK-30Kc19. L5178Y cells were incubated with 0.1 mg/ml of ASNase-LK-30Kc19 for 4 or 24 h. (A) A schematic illustration for the cleavage and entering the cell of ASNase-LK-30Kc19 protein. (B) Confocal images of L5178Y cells after incubation without or with ASNase-LK-30Kc19 protein. T7 and His primary antibodies were used for the detection of N- and C-terminal proteins, respectively. Arrows indicate apoptotic cells. Scale bar = 5 μ m.

of proteins, L5178Y cells were incubated with 0.1 mg/mL of ASNase-LK-30Kc19 protein for 4 or 24 h. ASNase-LK-30Kc19 has two tags, T7 and His at N- and C-terminus, respectively, as shown in Figure 3(A). T7 and His antibodies were used for the detection of N- and C-terminal proteins, respectively. After 4 h incubation, we observed ASNase-LK-30Kc19 proteins by double staining of T7 and His antibodies (Figure 3(B)). Furthermore, truncated forms of the protein were observed from T7 or His tag staining at N- or C-terminal, indicating that cleavage of ASNase-LK-30Kc19 occurred. In the 24-h protein incubation, double and single stained cells were found, but most cells were induced cell death. Consequently, the cleavage of ASNase-LK-30Kc19 protein occurred both outside and inside cells.

Selective cytotoxicity of ASNase-LK-30Kc19 on L5178Y cells

Although ASNase is considered a drug for complete remission due to its cancer selectivity, side effects, such as allergenic reactions and cytotoxicity, may arise and cause problems when applying for medical treatment [5]. Thus, we evaluated the selective cytotoxicity of ASNase-LK-30Kc19 protein (Figure 4). Cytotoxicity of ASNase-LK-30Kc19 in normal cells

showed much lower value than cancer cells. With 3 mU/ml of ASNase-LK-30Kc19, half of L5178Y cells died, while the viability of FDC-P1 cells was higher than 80%. The viability of FDC-P1 cells was maintained to be higher than 70% even with high concentration of the proteins (100 mU/ml). However, in L5178Y cells, less than 20% of cells survived at the same concentration. Accordingly, ASNase-LK-30Kc19 protein demonstrated significant cancer selectivity with low normal cell cytotoxicity in comparison with ASNase alone.

Discussion

30Kc19 is a multifunctional protein with properties of increasing the yield of soluble protein expression, enzyme-stabilizing, and cell-penetrating effects [29]. In this study, we fused the 30Kc19 with ASNase to increase its activity through enhancing stability of the protein. Moreover, cleavage linker was additionally applied to this fusion protein. Cleavage linker enabled to separate ASNase from fusion protein, and thus ASNase with reduced steric hindrance had improved anti-cancer efficacy.

Structural perturbation is the major concerns for fusion protein design. The lack of structural space could interfere with protein function [42]. A cleavable linker can be applied

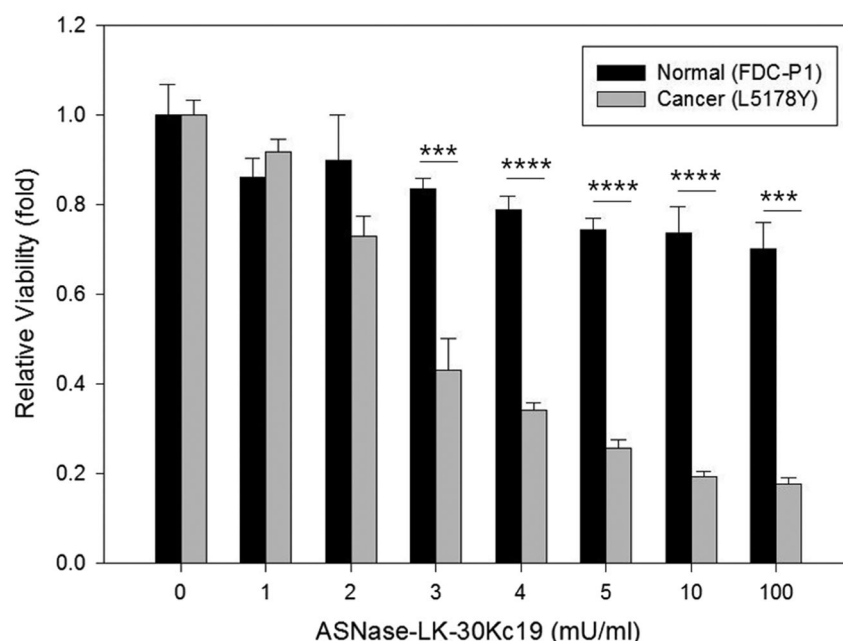


Figure 4. Effect of ASNase-LK-30Kc19 on normal lymphoblast cell line (FDC-P1) and L5178Y cells. The viability of FDC-P1 and L5178Y cells exposed to ASNase-LK-30Kc19 was determined by CCK-8 assay. *** $p < 0.001$, **** $p < 0.0001$. $n = 3$.

to a wide range of cancer types due to their ability to separate and release functional cargo proteins from the fusion protein [43]. Cleavable linkers can be categorized as chemical-cleavable or enzyme-cleavable, and exploitation of specific conditions can lead to release of the therapeutic protein at the target cell [44]. For the chemically cleavable linkers, acid cleavable linkers, reducible disulphide linkers, and cleavable by exogenous stimuli linkers are available. As for enzyme cleavable linkers, they can be used to be selectively cleaved intracellularly, and dipeptide-containing linkers, glycosidase-cleavable linkers, phosphatase-cleavable linkers are available for selective cargo release at the tumour site.

In this study, the cancer specific cleavable linker sequenced as PLGLAG was selected for separating ASNase and 30Kc19 near to cancer cells. In a previous study, dendrimeric nanoparticles coated with activatable cell penetrating peptides containing PLGLAG as linker were used because of its predominantly sensitivity to MMP-2 and MMP-9 [45,46]. We also maximized the effect of ASNase by using PLGLAG cleavable linker for targeting of MMP-abundant leukaemia cells [36–38]. When ASNase-30Kc19 and ASNase-LK-30Kc19 were treated to cancer cells, respectively, a significant difference in the cell viability was observed at 2 mU/ml concentration between the two proteins. IC_{50} of ASNase-LK-30Kc19 was 2 mU/mL, and ASNase-30Kc19 without linker was 10 mU/mL. Also, ASNase-LK-30Kc19 was able to reach IC_{50} with much less concentration than the ASNase was 240 mU/mL on IC_{50} . Therefore, the results indicated that the 30Kc19 improved the PK of the fusion protein by increasing the ASNase stability, and the cleavage linker improved the PD of the fusion protein by releasing the ASNase.

Another function of the 30Kc19 protein is the cell-penetrating property, which also delivers the fusion protein into the cell [28]. Because the fusion protein has both a cell-penetrating 30Kc19 and a cleavage linker sequence, we were

interested in whether the cleavage occurs prior or post-intracellular delivery. To check the efficiency between two properties in the fusion protein, L5178Y cells, lymphoma, were incubated with ASNase-LK-30Kc19 proteins for 2 h and analysed by confocal. Previously, it was reported that 30Kc19 can deliver a cargo protein into cells within 30 min from observation of intracellular accumulation of proteins over time [29]. As for the cleavage, it was reported that most PLGLAG sequence becomes cleaved after 30 min incubation with 50 nM of MMP-2 or MMP-9 [36]. In our study, incubation in lower concentration of MMPs required more time for the cleavage to occur (supplemental Figure S1). Interestingly, we observed ASNase-LK-30Kc19 protein in L5178Y cells by confocal microscope. The observed protein demonstrated cleavage by intracellular MMPs, and hence free ASNase was able to hydrolyse the intracellular L-Asn. In cancer cells, most MMPs are secreted, and play a degradation role for invasion of the surrounding environment. However, MMPs are also found in the cytosolic fraction and even in the nucleus [47,48]. It is thought that the intracellular ASNase delivered by 30Kc19 may have more activity than the extracellular ASNase. According to the results, ASNase-30Kc19 had more cytotoxicity than ASNase alone. We assume that this is because of enhanced stability of ASNase due to 30Kc19, but also intracellular activity of ASNase. ASNase-LK-30Kc19 proteins showed more cytotoxicity than ASNase-30Kc19 and ASNase alone (Figure 2(B)). Also, ASNase-LK-30Kc19 showed higher tumour cell selective toxicity (Figure 4). As the ASNase-LK-30Kc19 protein concentration was increased in normal or tumour cells, significant differences in the cell viability were observed starting from 3 mU/ml concentration; where the viability of normal cells was more than 80%, while the viability of tumour cell was only about half. According to related former study [49], the delivery of anti-cancer drug through the CPP-mediated internalization only at the

tumour-associated microenvironments showed enhanced anti-cancer activity. Therefore, selective release of the anti-cancer protein would achieve better toxicity towards tumour cells. Altogether these results indicate that the fusion with 30Kc19 and a cleavage linker allows ASNase to be used more efficiently as a cancer cell therapy.

Conclusion

Recombinant L-asparaginase fused and cell-penetrating 30Kc19 protein demonstrated improved stability and cell-permeability of the ASNase. Between two proteins, the linker sequence PLGLAG was inserted and showed cleavage by the MMPs, which are highly abundant in leukaemia cells. As a result, intracellular delivery of both the non-cleaved (ASNase-LK-30Kc19) and cleaved (ASNase and 30Kc19) forms of the protein were observed, suggesting that ASNase functioned both internally and externally, which led to high anti-cancer activity by effective depletion of intracellular asparagine. In terms of the dosage, releasable ASNase of the ASNase-LK-30Kc19 protein reached the same IC₅₀ at a concentration five times lower than non-releasable ASNase of ASNase-30Kc19 protein. Altogether, the findings suggest that this fusion approach has potential applications in the treatment of ALL.

Author contributions

JR and SJY carried out conceptualization, investigation, methodology, formal analysis, and visualization. BS, HL, JL and JJ assisted with the experiments and curate the data. HHP and THP supervised the whole study and acquired the funding. JR, BS and HHP write and revised the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) [No. 2020R1A4A3078645, No. 2021R1C1C1014606].

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Data availability statement

All the data generated for this study are available on request to the corresponding author.

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