공학석사학위논문

Enhancement of Solubility, Cell Penetration, and Stability of Arginine Deiminase Using 30Kc19α for Effective Melanoma Treatment

효과적인 흑색종 치료를 위한 30Kc19α 기반 아르기닌 디이미나아제의 수용성, 세포투과성, 안정성 증대

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Abstract

Enhancement of Solubility, Cell Penetration, and Stability

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Arginine deiminase (ADI) is an amino-acid degrading enzyme which catalyzes the conversion of L-arginine into L-citrulline. ADI originating from *Mycoplasma* has been reported to present anti-tumor activity against arginine-auxotrophic tumors, including melanoma. Melanoma cells are sensitive to arginine depletion due to reduced expression of argininosuccinate synthase (ASS), a key enzyme for arginine biosynthesis. However, clinical applications of ADI for melanoma treatment present some limitations. Since ADI is not produced by humans, it has a short circulating

half-life. The emergence of drug resistance due to the intracellular expression of once-silenced ASS also remains an unsolved problem. Moreover, ADI proteins are mainly expressed as inclusion body forms and require a time-consuming refolding process to turn back into active form. Herein, we propose conjugating 30Kc19α protein with ADI to solve these problems. 30Kc19α is a cell-penetrating protein which increases stability and soluble expression of cargo proteins. We constructed pET-23a/*ArcA-LK-30Kc19α* plasmid, overexpressed ADI-LK-30Kc19α fusion protein in *E. coli* system. Then we purified the fusion protein using fast protein liquid chromatography and verified enhanced soluble expression of ADI through fusion with 30Kc19α. Also, we conjugated cleavable linker between ADI and 30Kc19α to increase enzyme activity by reducing steric hindrance. Compared to ADI, ADI-LK-30Kc19α showed higher cytotoxicity in melanoma cells after 72 hour treatment. Fusion protein presented low cytotoxicity in normal cells regardless of the protein concentration. Recombinant ADI-LK-30Kc19α showed enhanced *in vitro* stability and penetration into melanoma cells, thus would be a promising strategy to improve treatment efficacy in melanoma.

Keyword : melanoma treatment, arginine deiminase, enzyme stability, cell penetrating protein, soluble expression, 30Kc19α

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1. Introduction

Arginine deiminase (ADI) is a prokaryotic enzyme that catalyzes the hydrolysis of L-arginine to produce L-citrulline and ammonia [1]. Arginine is nonessential for humans because it can be produced from citrulline via urea cycle through the expression of argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) in normal cells. However, arginine-auxotrophic tumors, including melanoma and hepatocellular carcinoma, cannot express ASS and these tumors depend on external supply of arginine for cell growth and survival [2, 3]. ADI orgininated from *mycoplasma hominis* is a promising anti-tumor therapeutic that converts extracellular arginine to citrulline and shows cytotoxicity in arginine-auxotrophic tumors [4, 5]. ADI treatment induces atypical or excessive autophagy that leads to cell death. This type of cell death mechanism is different from classic caspase-dependent apoptosis and combination with conventional chemotherapies can lead to synergistic tumor growth inhibition [6, 7].

However, the use of ADI enzyme as anti-tumor agent is limited by several disadvantages. Firstly, ADI is a microbial enzyme and has a short serum half-life which is less than 5 hours *in vivo* and should be administered to patients frequently. Also, ADI is strongly antigenic to mammals because of its origin [8, 9]. Since ADI mainly depletes extracellular arginine due to its inefficient internalization, subsequent ASS-mediated arginine regeneration, which is associated with intracellular ADI resistance, reduces treatment efficacy [10, 11]. Lastly,

recombinant ADI is prone to form inclusion bodies and requires extra steps to turn back into biologically active form with proper folding [12, 13].

To solve half-life and immunogenicity problems, PEGylated ADI has been used in clinical trials and studies [14, 15]. PEGylation is the chemical modification of proteins by linking polyethylene glycol (PEG) chains [16]. Conjugation of PEG allows prolonged residence in serum by increasing hydrophilic shield and protecting from proteolytic enzymes. Also, PEGylation decreases immunogenicity by masking antigenic sites [17]. However, some limitations such as the existence of anti-PEG and increased renal tubular vacuolation exist, and ADI resistance still remains as a major obstacle in clinical trials [18].

30Kc19 protein is a member of 30K family, originating from silkworm hemolymph [19]. In the previous studies, it was confirmed that 30Kc19 has cell-penetrating ability due to its intrinsic cell penetrating peptide (CPP), Pep-c19 [20, 21]. CPPs have ability to deliver various cargo proteins into cells. Unlike other CPPs, 30Kc19 also has additional functions: protein stabilization and soluble expression enhancement of cargo proteins [22, 23]. 30Kc19α is the N-terminal α-helix domain of 30Kc19 and retains functions exhibited by 30Kc19 [24]. Herein, we propose an alternative strategy to overcome short half-life, intracellular resistance and insoluble expression of ADI by fusion with the 30Kc19α protein. We investigated whether the recombinant ADI-LK-30Kc19α fusion protein could enhance protein stability, intracellular delivery, and soluble expression of ADI.

In addition, the design of linker between two functional domains of a recombinant fusion protein is indispensable. Direct fusion without a linker can cause undesirable outcomes, such as impaired bioactivity. Linkers designed by researchers can be classified into 3 categories: rigid, flexible, and cleavable [25, 26]. In this study, we

chose cleavable linker which contains a site sensitive to specific protease, MMP-2 (Matrix metalloproteinase-2). The short amino acid sequence, GGGGSPLGLAGGGGGS, was used as linker for tumor targeting effect and recovery of enzymatic activity [27, 28].

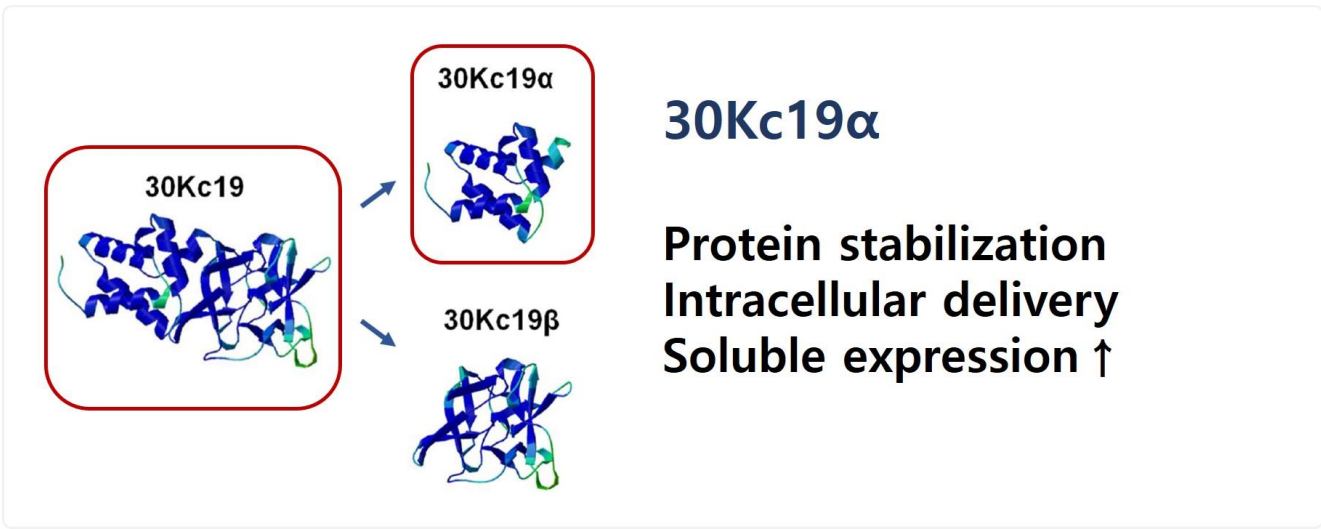


Figure 1. Structure of 30Kc19 and function of 30Kc19α subunit

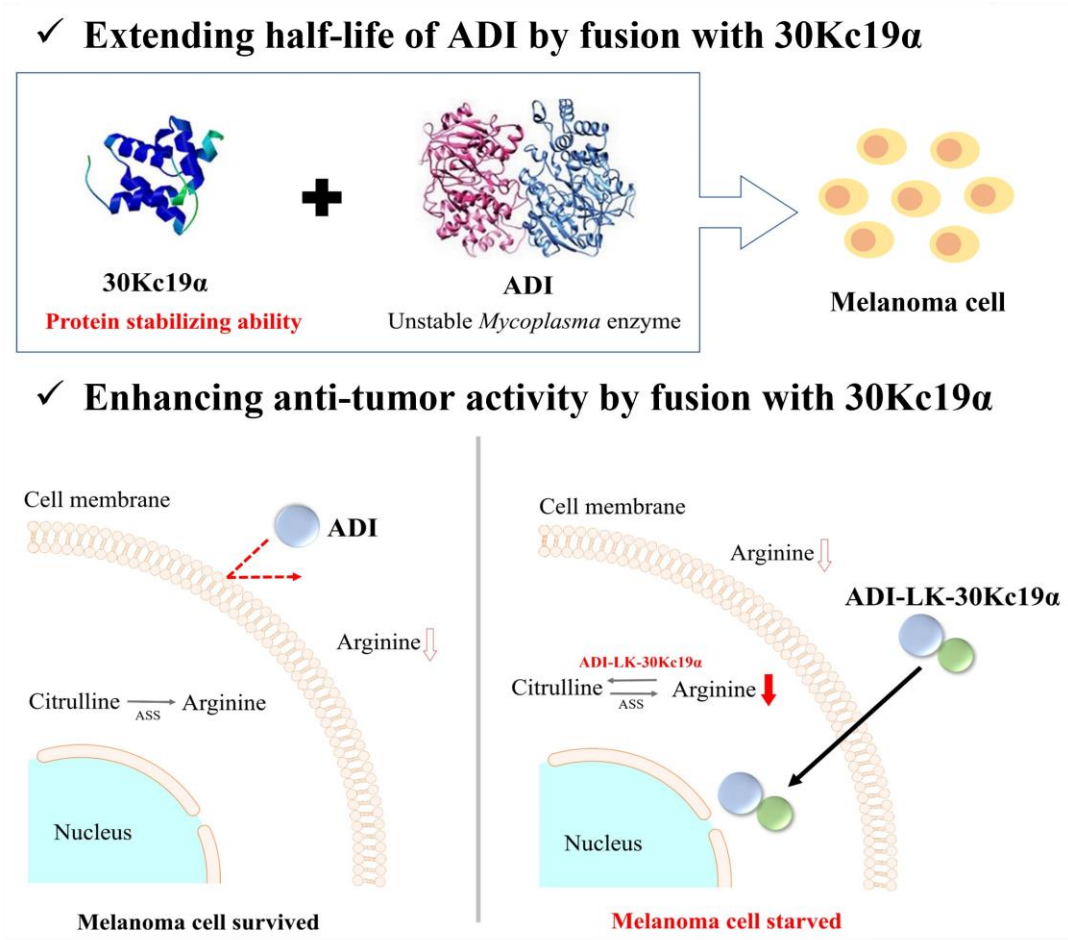


Figure 2. Research scheme

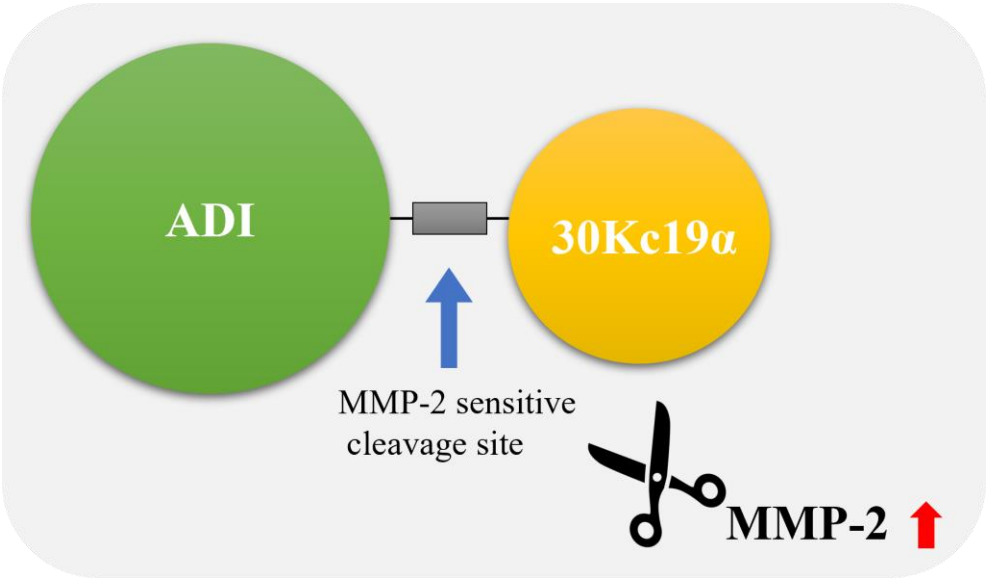
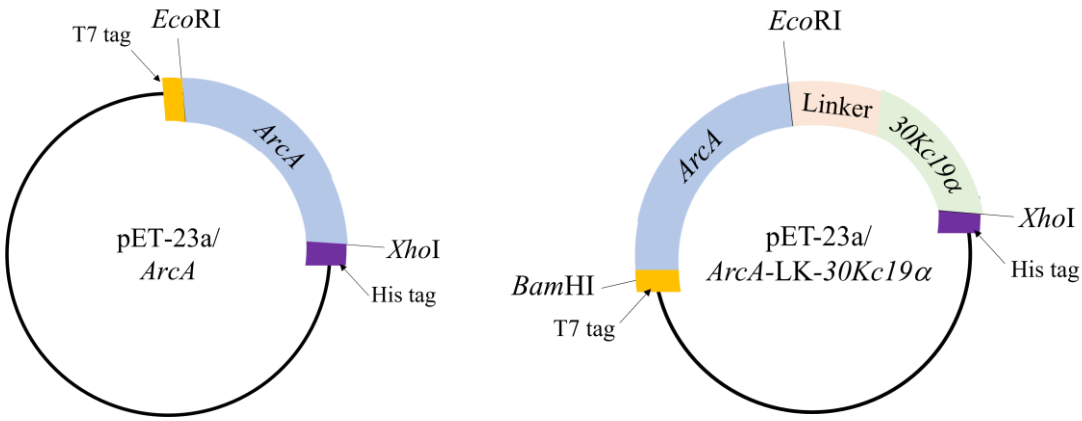


Figure 3. Cleavage of linker by MMP-2

2. Materials and methods

2.1 Plasmid construction of pET-23a/*ArcA* and pET-23a/*ArcA-LK-30Kc19α*

ADI gene was synthesized, optimized for expression in *E. coli* and cloned into pUC57 vector by Bionics Co. (Seoul, Korea). ADI gene was constructed into pET-23a expression vector (Novagen, USA) to create pET-23a/*ArcA* constructs. *Eco*RI and *Xho*I were used as restriction sites. GGGGSPLGLAGGGGGS-30Kc19α (LK-30Kc19α) gene was amplified by polymerase chain reaction (PCR) using appropriate primers containing *Bam*HI and *Eco*RI as restriction sites. The PCR products and pET-23a/ADI-PLGLAG-30Kc19α vectors obtained from previous experiments were digested with *Bam*HI and *Eco*RI restriction enzymes to create pET-23a/*ArcA-LK-30Kc19α* (LK: linker, GGGGSPLGLAGGGGGS) constructs.



ArcA: Gene name of ADI

Figure 4. Plasmid construction of pET-23a/*ArcA* and pET-23a/*ArcA-LK-30Kc19α*

2.2 Protein expression and purification of ADI and ADI-LK-30Kc19α

The constructed plasmid was transformed into *E. coli* Rosetta2 (Novagen) for recombinant protein production. The transformed *E. coli* was cultured in LB broth medium with 100μg/ml ampicillin (Sigma-Aldrich, USA) at 37℃ overnight with the agitation speed of 200 rpm. The inoculated medium was transferred to 1L LB broth medium and cultured at 37℃ with agitation speed of 180 rpm. When OD_{600} value reached 0.6, 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG; LPS solution, Korea) was added for induction and *E. coli* was further incubated at 25℃ for 6 hours. The cells were harvested by centrifuging at 7000 rpm for 10 minutes at 4℃.

After discarding the supernatant, the pellets were resuspended in His-binding buffer (20mM imidazole (Sigma-Aldrich), 20mM Tris-HCl (Sigma, USA), 500mM NaCl (Junsei, Japan), pH 8.0). Then, resuspended pellets were sonicated at 30% amplitude for 10 minutes, with 5 seconds interval between each sonication. The lysates were centrifuged at 12,000 rpm at 4℃ for 30 minutes. The supernatants were filtered with 0.45μm bottle top filter (Jetbiofil, Korea). The recombinant proteins were purified by fast protein liquid chromatography (FPLC; GE Healthcare, Uppsala, Sweden). Filtered proteins were loaded onto His-Trap HP column (GE healthcare, Sweden) filled with His-binding buffer. His-washing buffer (50mM imidazole, 20mM Tris-HCl, 500mM NaCl, pH 8.0) flowed through the column to washout unbound proteins. Remaining proteins were eluted with His-elution buffer (350mM imidazole, 20mM Tris-HCl, 500mM NaCl, pH 8.0). Finally, the buffer was changed to 20mM Tris-HCl buffer (pH 8.0) using a

desalting column (GE Healthcare, Sweden) for further use.

2.3 SDS-PAGE and western blot analysis

To identify the purified recombinant proteins, the protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. The proteins were mixed with 2× Laemmli sample buffer (Bio-Rad, USA) and loaded to 10% SDS-PAGE gel. The gel was stained with Coomassie blue R-250 (Sigma) for Coomassie blue staining. After staining, the band size was calculated using Image J software. For western blot analysis, the proteins on the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane. As T7-tag is located in the N-terminus of the recombinant protein, anti-T7 tag antibody (ab9115, Abcam, UK) was used as the primary antibody and anti-rabbit HRP-conjugated antibody (Milipore, USA) was used as secondary antibody. To visualize the purified proteins, Lumina Forte Western HRP substrate (Merck Milipore, USA) was used.

2.4 Cleavage of linker

For *in vitro* enzymatic degradation test of MMP-2 (Matrix Metalloprotease-2) cleavable linker, the purified protein samples (ADI, ADI-LK-30Kc19α) were incubated in the degradation buffer (50mM Tris-HCl, 10mM CaCl2, 150mM NaCl, 50μM ZnSo4, 0.02% NaN3, 0.05% Brij-35) for 24 h at 37℃ with 0, 1, 2, 3 or 4 μg MMP-2 (Peprotech). To confirm the cleavage of the linker, protein samples were loaded onto 10% SDS-PAGE gel and separated based on protein sizes.

2.5 Enzyme activity assay

Based on the cleavage assay results, the recombinant protein samples (ADI, ADI-LK-30Kc19α) were incubated with MMP-2 for 24 hours at 37℃. To compared the enzyme activity before and after cleavage, samples were incubated without MMP-2 under the same condition. The enzyme activity of the recombinant proteins were determined as described by Miyazaki *et al*. The reaction mixture contained 10mM L-arginine, 0.1M potassium phosphate (pH 6.5), and 0.1ml of an enzyme solution in a final volume of 1ml. The mixture was incubated at 37℃ for 3 hours, after which the reaction was terminated by adding 0.5ml of a 1:3 mixture (v/v) of H_2SO_4 and H_3PO_4. The amount of citrulline formed during the incubation was determined with diacetyl monoxime using the method of Oginsky [29]. One unit (U) of enzyme activity was defined as the amount of enzyme catalyzing 1μmol of L-arginine to L-citrulline per minute under the assay conditions used.

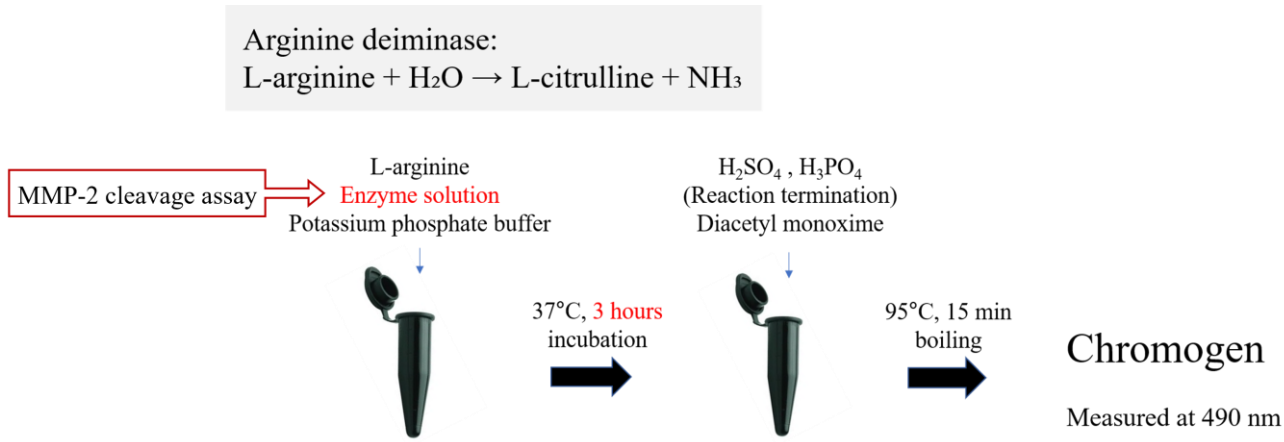


Figure 5. Enzyme activity assay

2.6 Protein stability assay

The purified enzyme solutions (ADI, ADI-LK-30Kc19α), which had been stored at -70℃, were thawed at slowly 4℃. To evaluate *in vitro* stability, 40μl of enzymes were added into 360μl of fetal bovine serum (FBS). The reaction mixture was incubated at 37℃ for 24, 48, 72, 96, and 120 hours. The enzyme activity was determined at each time point.

2.7 Detection of MMP-2 expression by western blot

Human melanoma cells (SK-MEL-2; Korean Cell Line Bank, KCLB) and human skin fibroblasts (Detroit 551; Korean Cell Line Bank, KCLB) were cultured in Dulbecco's modified Eagle's medium (DMEM; Biowest, France). Briefly, cell lysates were loaded and separated on 10% SDS-polyacrylamide gels and transferred onto a PVDF membrane. After blocking with 5% skim milk in 0.1% TBS with 0.1% tween 20 for 1 hour, rabbit anti-MMP-2 antibody (Bioworld) were used as primary antibodies. goat anti-rabbit IgG conjugated with horseradish peroxidase was used as secondary antibody. Lumina Forte Western HRP substrate (Merck Millipore, USA) was used to label the proteins.

2.8 Cell penetration assay

For cell penetration assay, SK-MEL-2 and Detroit 551 cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS, Biowest) and 1% penicillin streptomycin (PS, Sigma). The test cells (3.0×10^5 cells/well) were seeded in each well of 6-well microplates containing 2 ml of DMEM supplemented with 10% FBS and 1% PS. When confluency reached 70%, cells were treated with 1μM ADI or ADI-LK-30Kc19α. After 24-hour treatment, cells were detached and harvested by trypsin-EDTA. The harvested cells were centrifuged at 8000 rpm for 1 min at 4℃. The culture medium were removed and the cell pellets were washed with dPBS. The resuspended pellets were centrifuged again at 8000 rpm for 1 min. The remaining cell pellets were resuspended by RIPA buffer supplemented with protease inhibitor cocktail and were lysed for 20 min at 4℃. Lysates were separated by 10% SDS-PAGE and transferred onto PVDF (polyvinylidene difluoride) membrane. Rabbit anti-T7 tag IgG was used as primary antibody and goat anti-rabbit IgG conjugated with horseradish peroxidase was used as secondary antibody. Lumina Forte Western HRP substrate (Merck Millipore, USA) was used to label the proteins.

2.9 Cytotoxicity assay

The *in vitro* growth-inhibitory activities of the purified enzymes were assessed on SK-MEL-2 and Detroit 551 cell lines. The test cells (5.0×10^3 cells/well) were seeded in each well of 96-well microplates containing 0.1 ml of DMEM medium supplemented with 10% FBS and 1% PS. The cells were preincubated at 37℃ with 5% CO_2 for 24 hours. Then, the cells were treated with various concentrations of proteins and further incubated for 72 hours. 10μl of Cell Counting Kit-8(CCK-8; Dojindo, Japan) was added into each well and the cells in microplates were incubated for 2 hours. The absorbance at 450 nm was measured in a microplate reader (Tecan, Switzerland).

3. Results and discussion

3.1 Plasmid construction and soluble expression of recombinant proteins

pET-23a/*ArcA* and pET-23a/*ArcA-LK-30Kc19α* were constructed to produce recombinant ADI and ADI-LK-30Kc19α. The ligated plasmids were digested with *Eco*RI and *Xho*I for reconfirmation. The length of the restriction digests and DNA sequencing data were within their theoretical values.

According to previous studies [12, 13], most of the synthesized ADI proteins were produced in insoluble inclusion body forms. Inclusion bodies could recover their bioactivities via solubilization and refolding processes but these extra steps require much more time and effort. Therefore, for further experiments, solubility and productivity of ADI need to be increased.

High yield of soluble proteins was obtained by lowering induction temperature and using 30Kc19α as fusion partner, CPP which has the ability to express the cargo protein in soluble form. It is known that usually the expression of proteins in *E. coli* at low temperatures improves protein solubility, since low induction temperature is associated with low protein synthesis rate. Decelerating the protein synthesis can abate protein aggregation and degradation [30]. Therefore, we decreased the induction temperature from 37℃ to 25℃.

For solubility enhancement, fusion with solubility-enhancer proteins, such as

maltose-binding protein (MBP), Shistosoma japonicum glutathione S-transferase (GST) has been used as popular tool [12]. Similarly, according to the previous study [23], 30Kc19 and its subunit, 30Kc19α, increased the solubility of the fused transcription factors.

Following the expression of recombinant ADI and ADI-LK-30Kc19α in *E. coli*, the soluble fraction of proteins were purified using fast protein liquid chromatography (FPLC). The purified proteins were analyzed by SDS-PAGE and identified visually by Coomassie Blue staining. The concentration of ADI and ADI-LK-30Kc19α were 19.6 μg/ml and 222.9 μg/ml respectively, indicating the enhanced soluble expression of ADI. Western blot data showed bands on the expected sizes of ADI and ADI-LK-30Kc19α, which are 49.0 and 60.4 kDa, respectively.

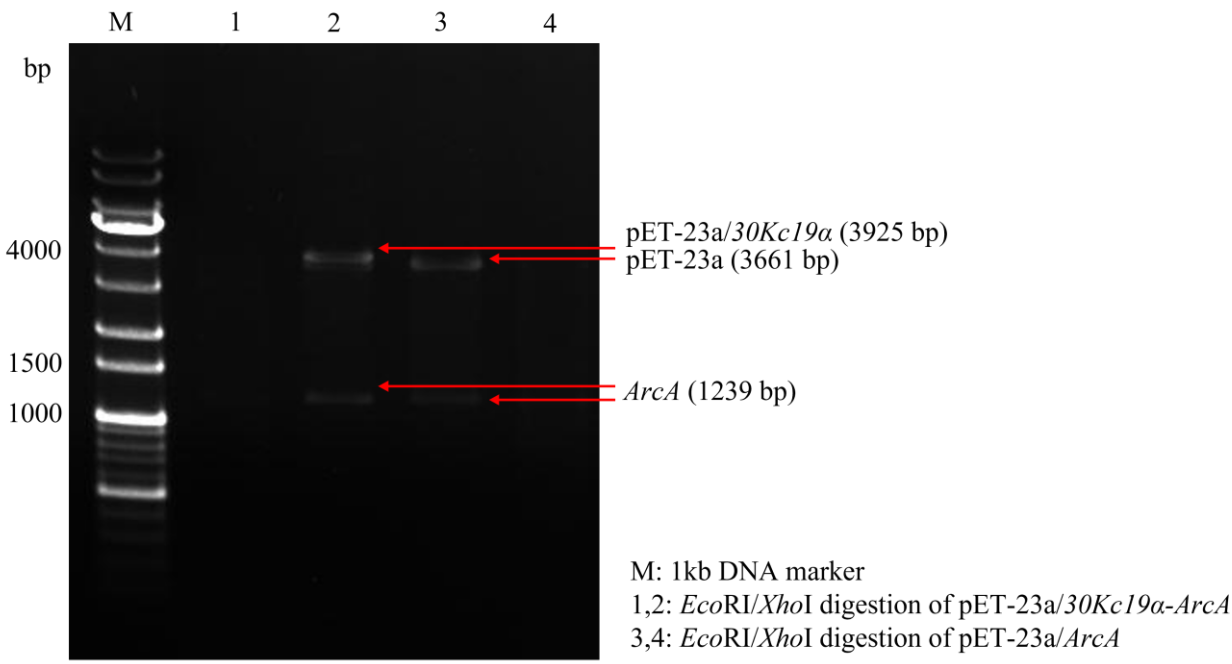
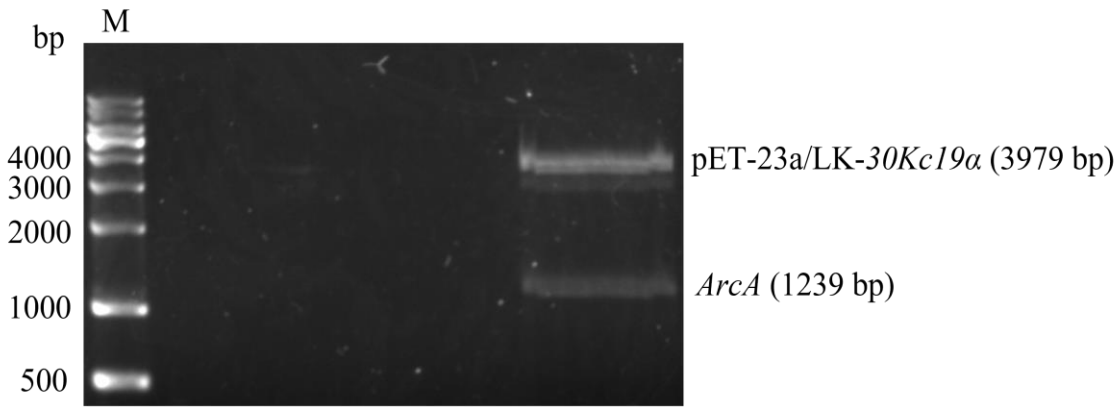


Figure 6. Restriction digestion of pET-23a/*ArcA*



M: 1kb DNA marker
*Bam*HI/*Eco*RI digestion of of pET-23a/*ArcA*-LK-*30Kc19α*

Figure 7. Restriction digestion of pET-23a/*ArcA-LK-30Kc19α*

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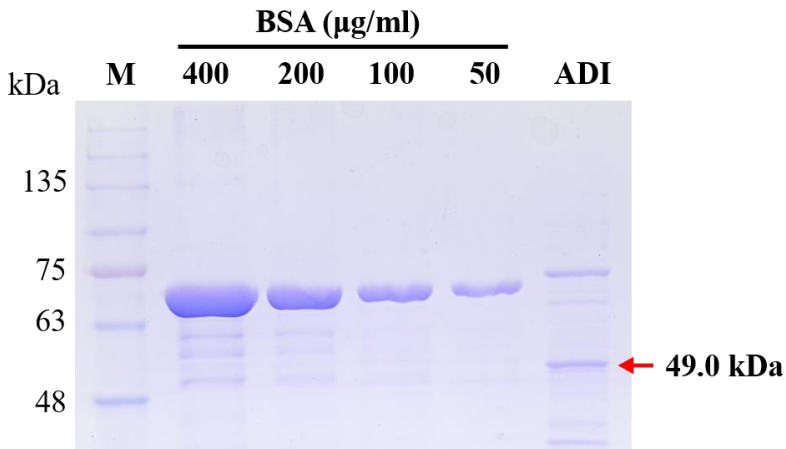
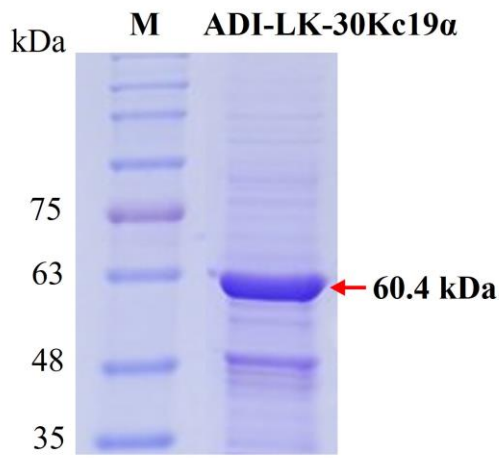
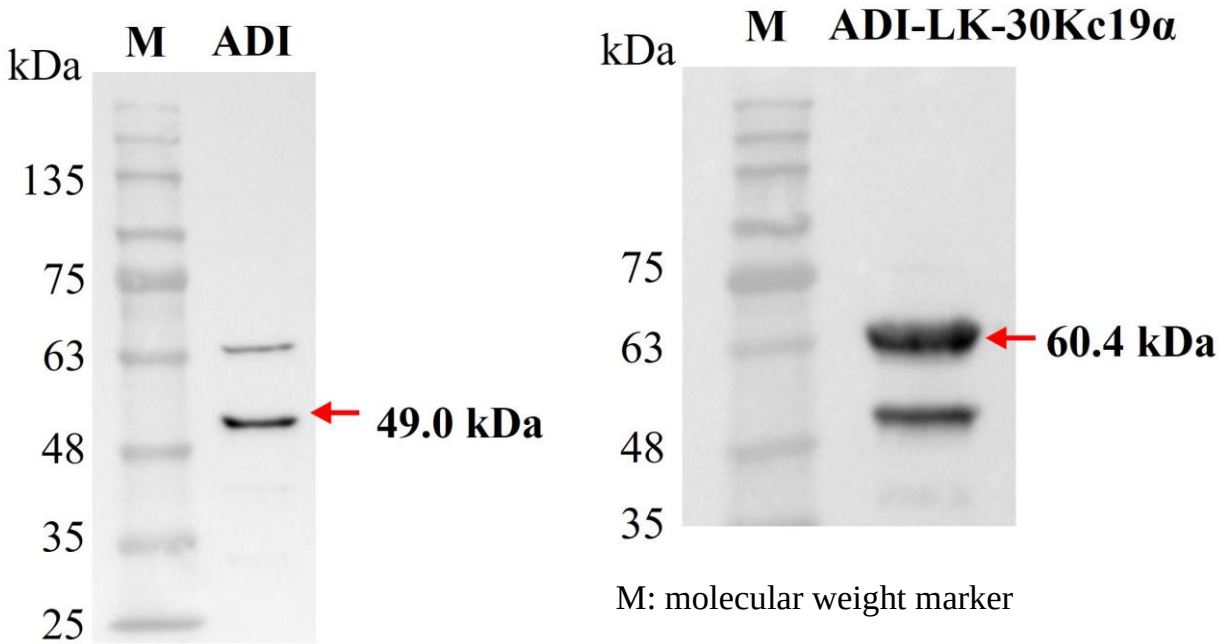




M: Molecular weight marker

Figure 8. Coomassie blue staining of ADI and ADI-LK-30Kc19α



M: molecular weight marker

Primary antibody: Rabbit anti-T7 tag IgG
Secondary antibody: Goat anti-rabbit IgG-HRP antibody

Figure 9. Western blot analysis of ADI and ADI-LK-30Kc19α

3.2 *In vitro* cleavage of linker by MMP-2

We examined if the linker inserted between ADI and 30Kc19α domains of ADI-LK-30Kc19α was indeed cleavable by MMP-2 *in vitro*. After incubation of the recombinant proteins with different concentrations of MMP-2 for 24 hours, the protein samples were separated by 10% SDS-PAGE. As the concentration of MMP-2 increased, the thickness of the band around 49.0 kDa, which is the size of ADI, increased. At the same time, the band around 60.4 kDa, which is the size of ADI-LK-30Kc19α, decreased. However, when 0 or 3 μg MMP-2 was incubated with ADI, there were no significant differences in the band thickness around 49.0 kDa. The results showed that the linker was designed sensitive to MMP-2, and ADI is robust against MMP-2 cleavage, as planned.

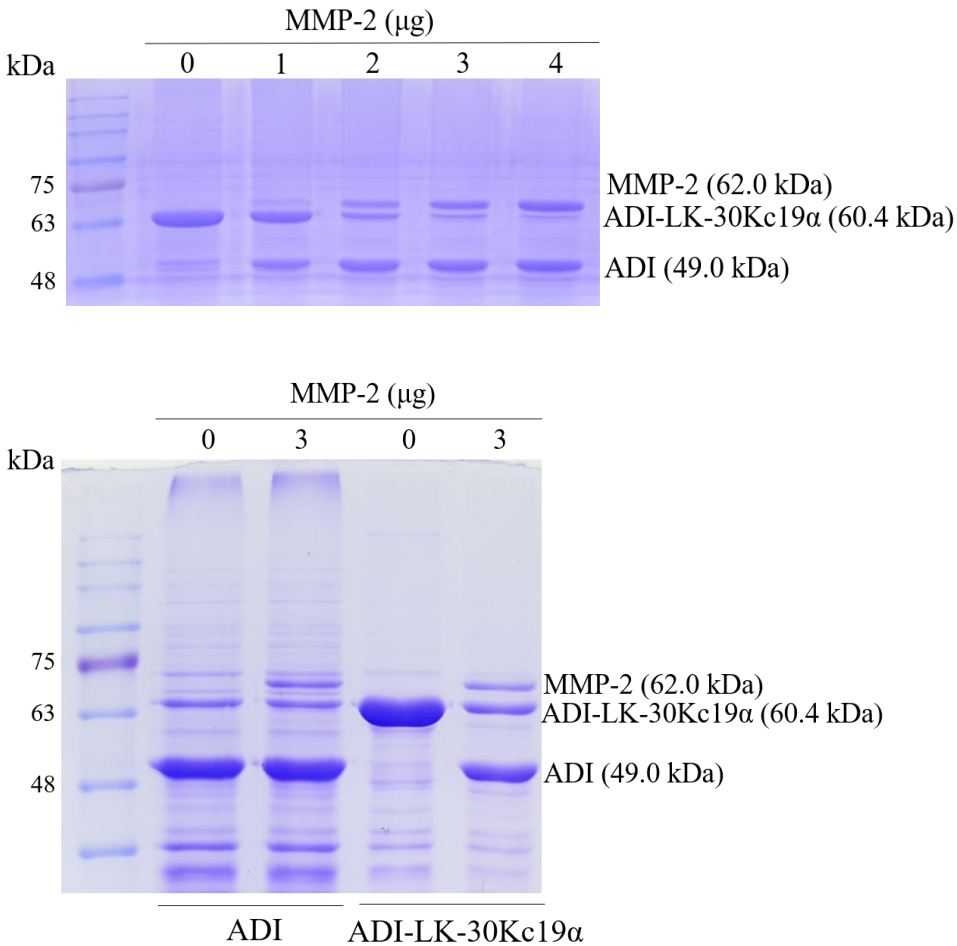


Figure 10. MMP-2 Cleavage assay of ADI and ADI-LK-30Kc19α

3.3 Enzymatic activity of the recombinant proteins

After the cleavage assay, the enzyme activity was assayed using colorimetric determination of the reaction product, L-citrulline by the method of Oginsky [29]. We observed that the molar activity of the free ADI domain of ADI-LK-30Kc19α after linker cleavage was 0.22 Unit/nmol, thereby indicating similar levels of enzyme activity to ADI. Also, we compared the enzyme activity of ADI-LK-30Kc19α before and after the cleavage of the linker. The enzyme activity of the fusion protein increased from 0.1 Unit/nmol to 0.22 Unit/nmol, which indicates that the cleavage of the linker and exposure of the ADI free domain contributed to the recovery of bioactivity.

	ADI w/o MMP-2	ADI w/ MMP-2	ADI-LK-30Kc19α w/o MMP-2	ADI-LK-30Kc19α w/ MMP-2
Molar activity (Unit/nmol)	**0.22**	**0.22**	**0.1**	**0.22**

1 Unit of enzyme activity (μmol/min): The amount of enzyme which converted 1 μmol L-arginine to L-citrulline per min under the assay conditions.

Figure 11. Enzyme activity of ADI and ADI-LK-30Kc19α

3.4 Higher expression of MMP-2 in melanoma cells

As we inserted MMP-2 cleavable sequence as linker between ADI and 30Kc19α domain of the fusion protein, we assessed MMP-2 protein expression by Western blot in SK-MEL-2 melanoma cells and Detroit 551 normal skin cells. SK-MEL-2 cells showed relatively higher expression levels of MMP-2 compared to Detroit 551 cells and indicating that MMP-2 protein expression level was upregulated in melanoma cells compared to normal cells. β-actin was used as housekeeping gene.

MMP-2, which is also known as gelatinase A, can degrade extracellular matrix (ECM) substrates and facilitates malignant behavior of tumors. It is usually known as a secreted, zinc-dependent metalloprotease. However, several studies have revealed the intracellular activation of MMP-2 and MMP-2 may cleave intracellular proteins which share the sequence cleaved by those proteolytic enzymes in ECM [31, 32, 33]. Also, figure 12 shows the intracellular expression of MMP-2 in melanoma cells. The up-regulation and higher expression of MMP-2 of tumor cleaves the MMP-2 sensitive linker. Taken together, ADI-LK-30Kc19α protein can have tumor-targeting effect.

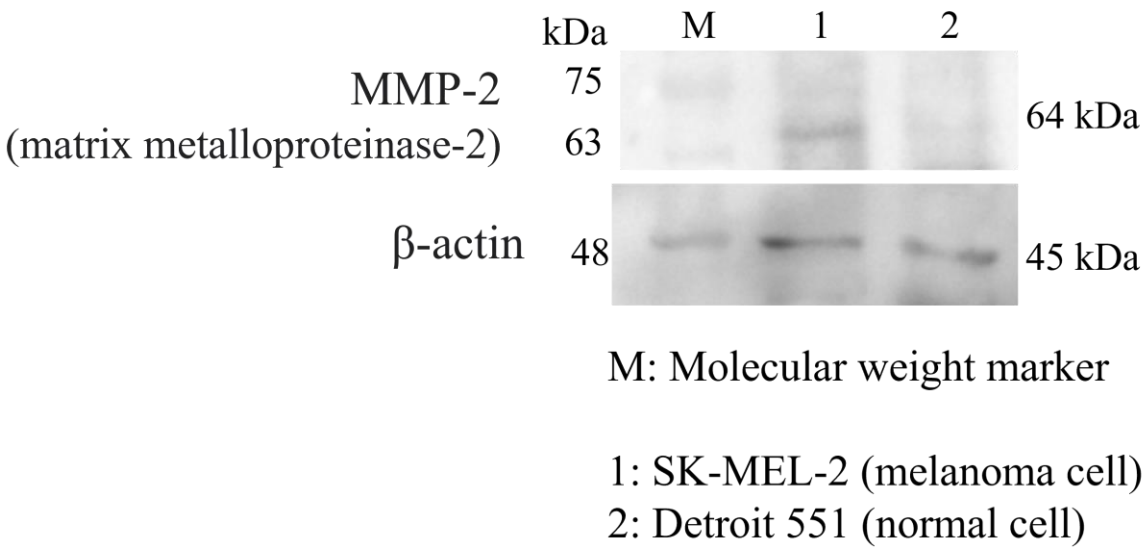


Figure 12. Expression level of MMP-2 in SK-MEL-2 and Detroit 551

3.5 *In vitro* stability of ADI and ADI-LK-30Kc19α

Stability is an important pharmacological parameter and determines the efficacy of enzymatic drugs. Hence, we investigated the effect of 30Kc19α on the stability of ADI *in vitro*. Recombinant proteins were added to Fetal Bovine Serum (FBS) and incubated at 37℃. After at regular intervals of 24 hours, enzyme activity was analyzed. Compared to ADI, ADI-LK-30Kc19α showed enhanced stability. After 4 days, the remaining activity of ADI was about 50%, while the activity of the fusion protein was more than 60%. Previous findings [22, 24] demonstrated that 30Kc19α has protein-stabilizing effect, but the underlying mechanism is not well known. Shielding effects could be derived from hydrophobic interactions, or 30Kc19α might have unknown stabilizing sequences.

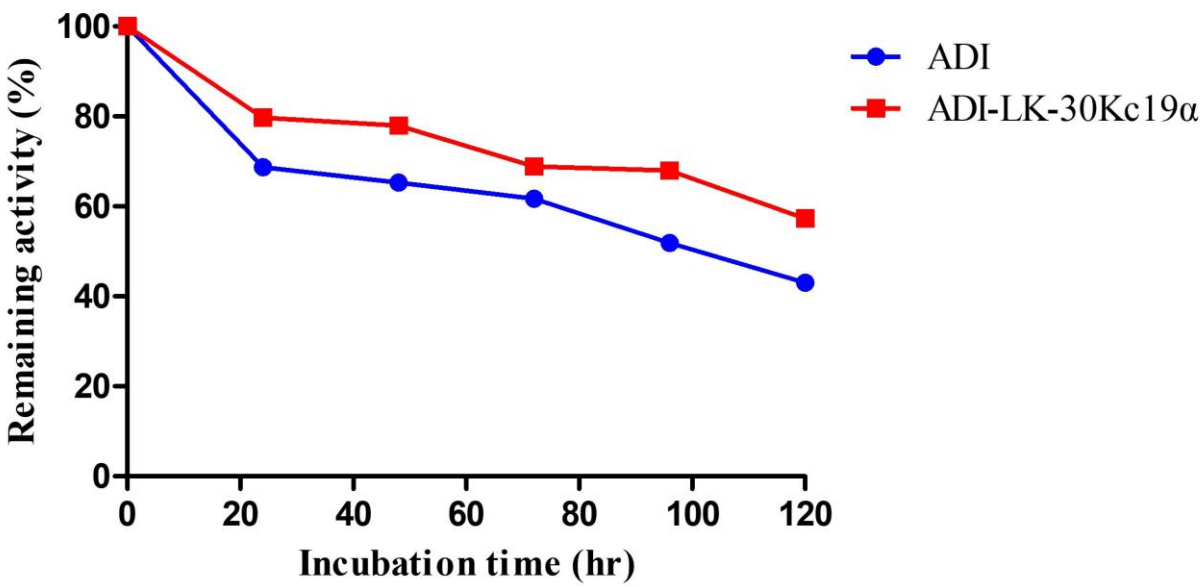
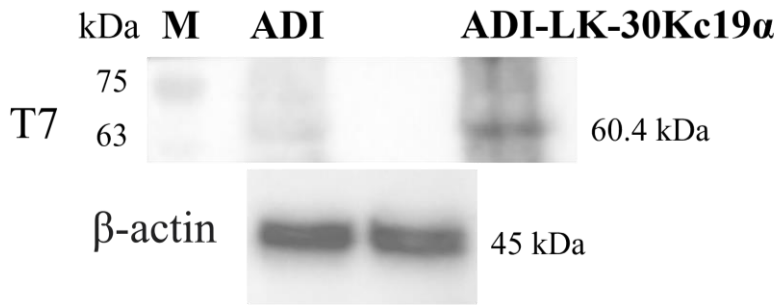


Figure 13. Deactivation profile of ADI and ADI-LK-30Kc19α

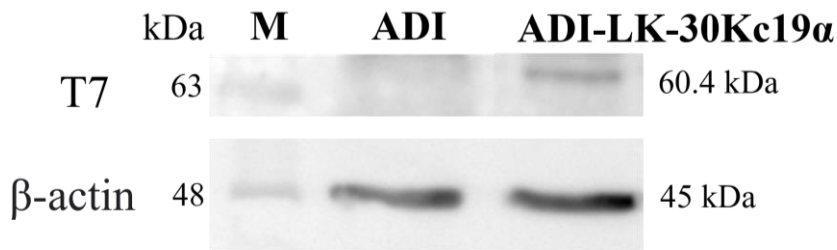
3.6 Cell-penetration ability of ADI-LK-30Kc19α

According to previous studies [34, 35, 36], ADI depletes extracellular arginine and work outside the cell because of their inefficient internalization. To evaluate the cell-penetrating ability resulting from the fusion of ADI with 30Kc19α, we added 1μM recombinant proteins to the culture media for 24 hours and analyzed by Western blotting. β-actin was used as housekeeping gene. When melanoma cells and normal skin cells were treated with ADI-LK-30Kc19α fusion protein, bands with the expected molecular weight (60.4 kDa) were detected. This indicates the fusion of 30Kc19α to ADI enhanced cellular uptake. The fusion protein can deplete not only extracellular arginine and but also the intracellularly synthesized arginine.



M: Molecular weight marker

Figure 14. Cell-penetration of ADI-LK-30Kc19α in SK-MEL-2



M: Molecular weight marker

Figure 15. Cell-penetration of ADI-LK-30Kc19α

in Detroit 551

3.7 Cytotoxicity of ADI and ADI-LK-30Kc19α in SK-MEL-2 and Detroit 551

To determine the anti-tumor effect of the purified proteins, cell viability of SK-MEL-2 melanoma cells were detected using CCK-8 assay. Both ADI and ADI-LK-30Kc19α inhibited the growth of melanoma cells after 72-hour treatment in a dose-dependent manner. Compared to native ADI, ADI-LK-30Kc19α showed greater anti-tumor activity. When 1 mU/ml of ADI was treated to SK-MEL-2 cells for 72 hours, relative cell viability was about 80%. However, less than 50% SK-MEL-2 cells survived when treated with 1 mU/ml of ADI-LK-30Kc19α for 72 hours. The sensitivity of melanoma cells to ADI treatment is related to arginine auxotrophy and native ADI only depletes extracellular arginine due to its poor internalization into cells. On the other hand, ADI-LK-30Kc19α not only depletes extracellular arginine but also intracellularly regenerated arginine from citrulline. This difference led to more effective growth inhibition.

The cell viability of Detroit 551 normal skin cells were detected in the same manner. Since normal cells can synthesize arginine intracellularly via urea cycle, more amount of enzyme had been treated to show cytotoxic effect in normal skin cells. Also, Detroit 551 cells were more sensitive to ADI than the fusion protein. When treated with 2 mU/ml of ADI for 72 hours, relative viability of Detroit 551 cells was about 60%. More than 80% of cells survived with 2 mU/ml fusion protein treatment.

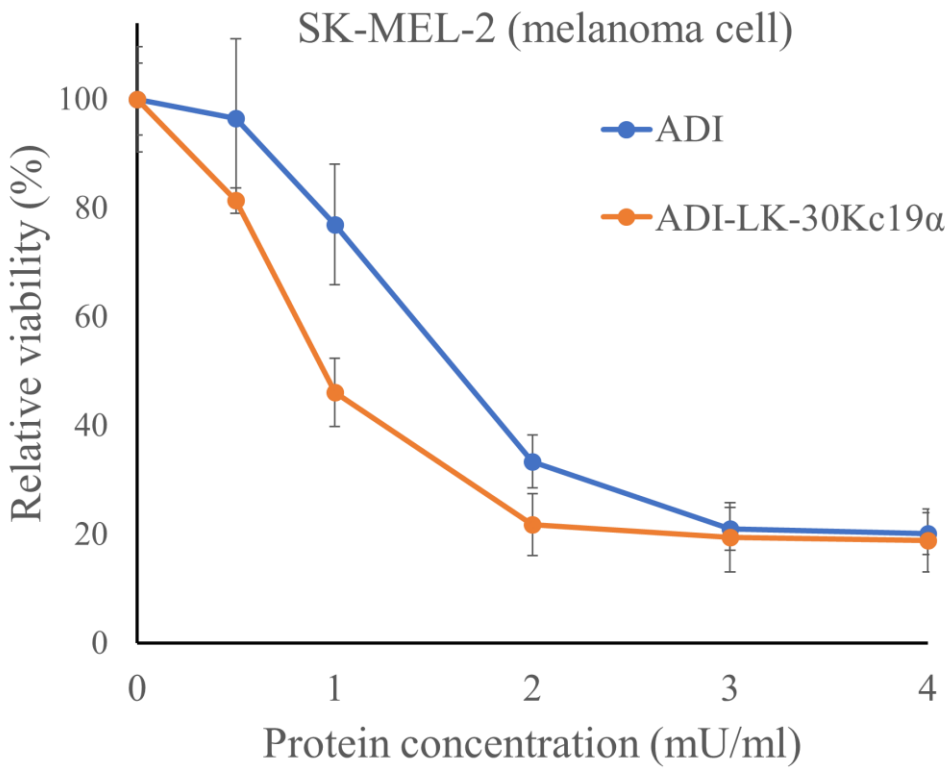


Figure 16. Cytotoxicity assay of ADI and ADI-LK-30Kc19α in SK-MEL-2

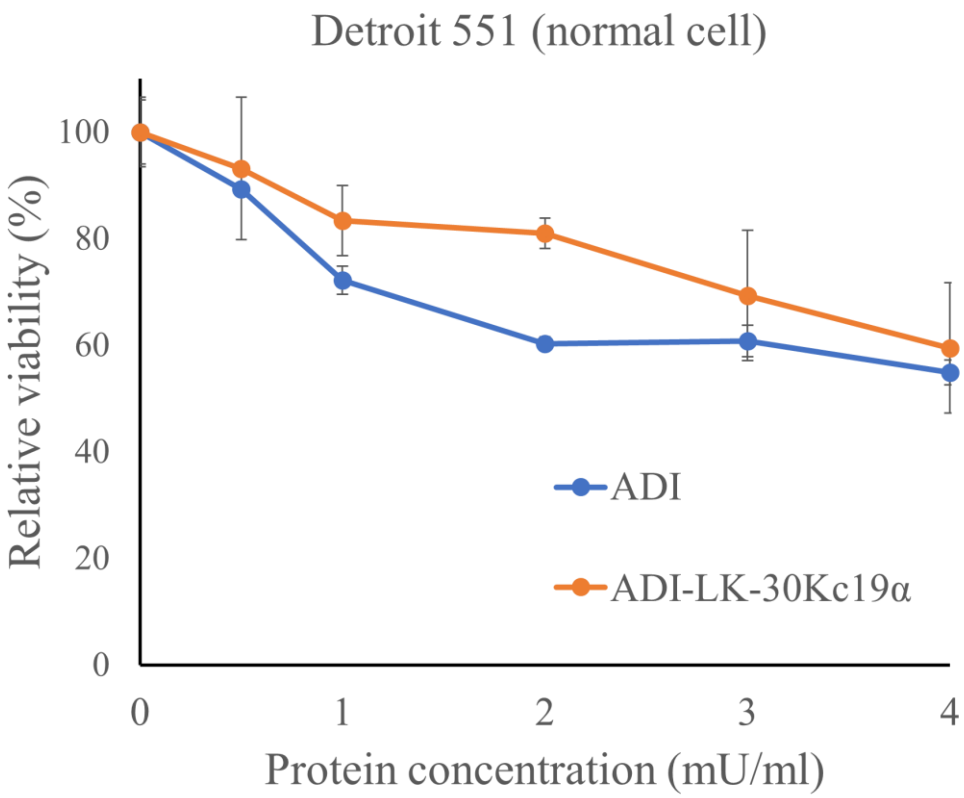


Figure 17. Cytotoxicity assay of ADI and ADI-LK-30Kc19α in Detroit 551

4. Conclusions

In this study, we fused 30Kc19α with arginine deiminase originating from *Mycoplasma hominis* to enhance solubility, stability, and cell penetration. We inserted cleavable linker between 30Kc19α and ADI for selective cleavage in melanoma cells and recovery of biological activity. The enzyme activity of recombinant fusion proteins increased after cleavage from 0.1 U/nmol to 0.22 U/nmol. Our results show that recombinant ADI-LK-30Kc19α fusion protein was more stable, efficiently delivered into melanoma cells, and could overcome ADI-induced resistance in melanoma cells. Also, the soluble expression of ADI was enhanced through fusion with 30Kc19α in an *E. coli* expression system. Overall, the intracellular delivery of ADI by multifunctional CPPs, 30Kc19α, offers a novel treatment strategy to solve ADI-resistance, short half-life, and insolubility problem and is expected to improve melanoma treatment efficacy of ADI.

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국 문 초 록

효과적인 흑색종 치료를 위한
30Kc19α 기반 아르기닌 디이미나아제의
수용성, 세포투과성, 안정성 증대

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Arginine deiminase (ADI)는 아르기닌을 시트룰린으로 전환하는 반응을 촉매하는 아미노산 분해 효소이다. *Mycoplasma*로부터 유래한 ADI는 흑색종을 비롯한 아르기닌 영양요구성 암에 대해 항암 작용을 나타낸다고 알려져 있다. 흑색종 세포는 argininosuccinate synthase (ASS)라는 아르기닌 생합성에 중요한 효소의 발현량이 적기 때문에 아르기닌이 고갈된 환경에 취약하다. 하지만 ADI를 흑색종 치료에 임상적으로 적용하기에는 몇 가지 한계점이 존재한다. ADI는 인간이 생산하는 단백질이

아니기 때문에 체내 반감기가 짧다. 또한, 기존에 세포 내에 잠재되어있던 ASS가 발현되며 나타나는 약물 저항성 문제도 여전히 해결되지 못하고 있다. 게다가, ADI 단백질은 봉입체 형태로 주로 발현되며 활성형 형태로 되돌리려면 시간이 오래 걸리는 재접힘 공정을 거쳐야 한다. 본 논문에서는 이런 문제점들을 해결하기 위해 30Kc19α 단백질을 ADI와 융합하는 것을 제안한다. 30Kc19α는 세포투과 단백질로, 카고 단백질의 안정성과 수용성 발현을 증대시킨다. 본 논문에서는 pET-23a/*ArcA-30Kc19α* 플라스미드를 구축하고, *E. coli* 시스템에서 ADI-LK-30Kc19α 융합단백질을 과발현 시켰다. 이후 융합단백질을 fast protein liquid chromatography를 이용해서 정제하였고, 수용성 발현 증대 효과를 확인하였다. 또한, ADI와 30Kc19α 단백질 사이에 cleavable linker를 삽입하여 암세포에서 선택적으로 cleavage 되게 하였고, 두 단백질 사이의 steric hindrance를 줄이고 활성을 증가시켰다. 단백질을 흑색종 세포에 72시간 처리했을 때, ADI보다 융합단백질이 더 높은 항암효과를 나타냈다. 정상세포에서 융합단백질은 상대적으로 낮은 세포독성을 나타냈다. 재조합 ADI-LK-30Kc19α 단백질은 더 높은 안정성과 세포 투과능을 나타냈고, 흑색종 치료 효율을 높일 수 있음을 확인하였다. 따라서 향후 추가적인 *in vivo* 실험과 modification을 통해 흑색종 치료제로 사용될 수 있을 것이라는 가능성을 제시하고 있다.

주요어: 흑색종 치료, 아르기닌 디이미나아제, 효소 안정성, 세포투과 단백질, 수용성 발현, 30Kc19α

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Enhancement of Solubility, Cell Penetration, and Stability of Arginine Deiminase Using 30Kc19α for Effective Melanoma Treatment

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