# Optimization and Application Postprint of a Novel Human-Derived Cell-Penetrating Peptide for Tumor Drug Therapy

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**Date:** 2018-05-16T00:00:00+00:00

## Abstract

Cell-penetrating peptides (CPPs) have been extensively studied and applied as carriers for the transmembrane delivery of macromolecular drugs in vivo. Midkine (MK) is a human growth factor that possesses a heparin-binding domain (HBD). This study reports that a gene sequence within the HBD of MK that is rich in basic amino acids (designated MK-S0), when fused with enhanced green fluorescent protein (EGFP) and expressed, can effectively deliver EGFP into cells, with a transmembrane transport efficiency higher than that of the classical cell-penetrating peptide Tat. MK-Δ4, obtained through further mutational optimization of the MK-S0 sequence, exhibits a cell-penetrating efficiency more than 16-fold higher than that of the naturally derived MK-S0, and the cell-penetrating transport activity of MK-Δ4 is applicable to various tumor cells. Mechanistic analysis reveals that MK-Δ4 can bind to cell surface heparan sulfate and is subsequently internalized into cells via macropinocytosis. Cell growth inhibition assays detected by the MTT method demonstrate that MAP30, a ribosome-inactivating protein derived from bitter melon and conjugated with MK-Δ4, exhibits a 5.8-fold lower IC50 value against HeLa tumor cells compared to MAP30 alone, substantially enhancing the tumor cell killing efficacy of this therapeutic protein. These results indicate that this mutationally engineered MK-Δ4, derived from MK, can serve as a novel and highly efficient cell-penetrating peptide for the effective intracellular delivery of therapeutic proteins to exert antitumor effects.

## Full Text

# The Optimization of a Novel Human-Derived Cell-Penetrating Peptide for Anti-Cancer Treatment and Its Application

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## Abstract

Cell-penetrating peptides (CPPs) have been extensively studied and applied as carriers for transmembrane delivery of macromolecular drugs *in vivo*. Midkine (MK) is a heparin-binding growth factor that contains a heparin-binding domain (HBD). This study reports that a gene sequence from the HBD of MK, rich in basic amino acids and designated as MK-S0, when fused with enhanced green fluorescence protein (EGFP), can effectively deliver EGFP into cells with higher translocation efficiency than the classical CPP Tat. Further mutational optimization of the MK-S0 sequence yielded MK-Δ4, which exhibited over 16-fold higher cell-penetrating efficiency compared to the native MK-S0 sequence. Moreover, MK-Δ4 demonstrated broad applicability across multiple tumor cell lines. Mechanistic analysis revealed that MK-Δ4 binds to cell surface heparan sulfate and subsequently enters cells via macropinocytosis. Cell growth inhibition assays using the MTT method showed that MAP30, a ribosome-inactivating protein from bitter melon, when conjugated with MK-Δ4, exhibited a 5.8-fold lower IC value against HeLa tumor cells compared to MAP30 alone, substantially enhancing the tumoricidal efficacy of this therapeutic protein. These findings demonstrate that the mutationally optimized MK-Δ4, derived from MK, can serve as a novel and highly efficient human-derived cell-penetrating peptide for effective intracellular delivery of therapeutic proteins to exert anti-tumor effects.

**Keywords:** Midkine; heparin-binding domain; cell-penetrating peptide; drug delivery; anti-tumor drug

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## Introduction

Many anti-tumor drugs, such as ribosome-inactivating proteins (RIPs), cannot autonomously cross the cell membrane barrier to exert their pharmacological effects intracellularly. Cell-penetrating peptides, however, can transport these proteins or macromolecular drugs into cells to fulfill their therapeutic functions. CPPs typically consist of no more than 30 amino acid residues and possess

the ability to penetrate cell membranes autonomously while carrying macromolecules into cells without compromising the biological activity of the cargo. Consequently, CPPs represent a promising novel drug delivery vector with broad application prospects in anti-inflammatory therapy, nucleic acid delivery, and protein delivery.

Human cell surfaces universally express a negatively charged layer of heparan sulfate proteoglycans (HSPGs), which are involved in numerous biological functions including mediating cell-extracellular matrix interactions, intercellular communication, and regulation of extracellular matrix structure and function. HSPGs play important roles in cancer, wound healing, infectious diseases, and inflammatory responses. Many human proteins, such as proteases, growth factors, chemokines, and pathogen proteins, contain a special structural domain known as the heparin-binding domain (HBD) that can interact with cell surface HSPGs and participate in various physiological, biochemical, and pathological processes. Since these HBDs are largely composed of multiple basic amino acid residues, their structure resembles that of classical CPPs. Our research group previously developed a novel human-derived cell-penetrating peptide from the carboxy-terminal heparin-binding domain of human extracellular superoxide dismutase (EC-SOD). Other studies have also reported the development of CPPs from the HBDs of insulin-like growth factor binding protein and human heparin-binding epidermal growth factor-like growth factor.

Human midkine (MK) is similarly a heparin-binding growth factor/cytokine that promotes cell survival, migration, differentiation, and gene expression, and is associated with cell regeneration and repair. MK is abnormally expressed in certain tumor tissues, such as pancreatic tumors, and is considered to play a potential role in tumorigenesis and carcinogenesis, serving as a cancer biomarker. The HBD of MK contains two spaced heparin-binding sites rich in basic amino acid residues. We selected the C-terminal amino acid sequence from positions 101 to 121 of MK (TKPCTPKTKAKAKAKKGKGKD, designated MK-S0) for investigation of its cell-penetrating activity. Since the native MK-S0 is located at the C-terminus of MK and previous successful CPPs derived from various heparin-binding domains were also placed at the C-terminus of EGFP, we mimicked the natural structure of MK by constructing MK-S0 at the C-terminus of enhanced green fluorescence protein (EGFP) to evaluate its ability to transport EGFP into cells. Subsequently, we further optimized the structure of MK-S0 to obtain a CPP with higher penetrating efficiency. This study further analyzes the cell spectrum and internalization pathway of this novel CPP. Additionally, we fused this CPP with MAP30, a ribosome-inactivating protein from bitter melon, to investigate whether the introduction of this novel CPP can significantly enhance the anti-tumor efficacy of the MAP30 fusion protein. This research provides a useful reference for developing novel human-derived cell-penetrating peptides from HBD structures.

## Materials and Methods

### 1.1 Materials and Reagents

The MK-S0 gene sequence, derived from the HBD of human MK, was synthesized by Sangon Biotech and constructed in the pET28a expression vector. *E. coli* BL21 (DE3) host strain, EGFP-HBD-pET28a, EGFP-Tat-pET28a, EGFP-R9-pET28a plasmids, human cervical cancer cells (HeLa), non-small cell lung cancer cells (95D), human gastric cancer cells (MGC8-3), human lung cancer cells (A549), human colorectal cancer cells (LoVo), and mouse melanoma cells (B16) were all maintained in our laboratory. Primers for vector construction were synthesized by Sangon Biotech. Kanamycin, isopropyl -D-1-thiogalactopyranoside (IPTG), and thiazolyl blue tetrazolium bromide (MTT) were obtained from Beyotime Biotechnology. DNA sequencing was performed by BGI.

#### 1.2.1 Construction of Recombinant Plasmids

Using EGFP-MK-Δ4 as a template, the target fragment containing BamHI-MK-Δ4-XhoI sites was amplified by PCR. Both the target fragment and the MAP30-HBD-pET28b vector plasmid were digested with BamHI and XhoI restriction endonucleases. The recombinant plasmid MAP30-MK-Δ4-pET28b was obtained through ligation and transformed into *E. coli* DH5 competent cells. After colony PCR verification, positive clones were sent for sequencing to confirm the correct recombinant plasmid sequence.

#### 1.2.2 Expression and Purification of Recombinant Proteins

The successfully constructed recombinant plasmids were transformed into *E. coli* BL21 (DE3) competent cells. Positive clones were inoculated into 30 mL LB medium (containing 50 g/mL kanamycin) and cultured at 37°C for 12 h. Then, 2% of the culture was transferred to 200 mL fresh medium. When the OD reached 0.4–0.6, protein expression was induced with 1 mmol/L IPTG at various temperatures (16°C for 16 h, 25°C for 12 h, 37°C for 8 h) to determine the optimal expression temperature. All EGFP-containing recombinant proteins were induced at 25°C, while MAP30-containing recombinant proteins were induced at 16°C. After induction, cells were harvested by centrifugation at 3000 r/min for 20 min, resuspended in lysis buffer (20 mmol/L Tris-HCl, 10% glycerol, 0.5 mol/L NaCl, pH 8.5), and disrupted by sonication. The supernatant was collected after centrifugation at 12000 r/min and purified using Ni-NTA affinity chromatography. Target proteins were collected through washing and elution with buffers containing different imidazole concentrations. After dialysis against buffer (20 mmol/L Tris-HCl, 10% glycerol, 0.5 mol/L NaCl, pH 7.2) to remove imidazole, samples were analyzed by SDS-PAGE. Purified proteins were sterile-filtered and stored at -80°C.

### 1.2.3 In Vitro Cell Culture

HeLa, A549, MGC8-3, and SMMC cells were cultured in RPMI-1640 medium (containing 10% fetal bovine serum and 1% penicillin-streptomycin), while 95D cells were cultured in DMEM medium (containing 10% fetal bovine serum and 1% penicillin-streptomycin). All cells were maintained at 37°C in a 5% CO incubator.

### 1.2.4 Fluorescence Microscopy Assay

Various cell lines were seeded in 24-well plates at a density of 1×10 cells per well and cultured for 12 h. Cells were then incubated with 5 M of each EGFP-fused protein for 12 h. After washing with PBS, cells were fixed with 4% paraformaldehyde at room temperature for 20 min, washed again with PBS, and stained with DAPI for 1–2 h. Intracellular fluorescence intensity was observed under a fluorescence microscope.

### 1.2.5 Flow Cytometry Analysis

HeLa cells were cultured in 6-well plates for 12 h, then incubated with fusion proteins for 12 h. After washing three times with PBS, cells were trypsinized and collected. Intracellular fluorescence intensity was analyzed by flow cytometry (excitation wavelength 488 nm, emission wavelength 530 nm).

### 1.2.6 Investigation of Cell-Penetrating Pathways

HeLa cells were seeded in 6-well plates at a density of 1×10 cells per well and cultured for 12 h. Various endocytic pathway inhibitors were used to pretreat HeLa cells for 30 min, including: 1 mg/mL heparin sodium (competitive inhibitor of cell surface heparan sulfate proteoglycans), 10 mM sodium azide (ATP metabolism inhibitor), 10 g/mL chlorpromazine hydrochloride (clathrin inhibitor), 10 g/mL -cyclodextrin (lipid raft inhibitor), and 10 g/mL cytochalasin D (F-actin inhibitor). Fusion proteins EGFP-MK-Δ4 and EGFP-MK-S0 were then added to each well. After 12 h incubation, cells were processed and collected, and intracellular fluorescence was detected by flow cytometry.

### 1.2.7 Analysis of Tumor Cell Inhibition by Therapeutic Proteins

The inhibitory effects of the therapeutic protein MAP30-MK-Δ4 on various tumor cells were analyzed by MTT assay. Cells were seeded in 96-well plates at a density of 1×10 cells per well and cultured for 12 h. After treatment with different concentrations of the therapeutic protein (0, 0.1, 0.5, 1, 2, 3 M) for 24 h, the original medium was removed and cells were incubated with 5 mg/mL MTT solution at 37°C for 3–4 h. The MTT solution was then discarded and 150 L dimethyl sulfoxide (DMSO) was added to each well. After the formazan crystals were dissolved, absorbance at 490 nm was measured using a Thermo microplate reader to calculate cell viability.

## Experimental Results

### 2.1 Expression and Purification of Recombinant Protein EGFP-MK-S0

The recombinant plasmid EGFP-MK-S0-pET28a synthesized by the company was transformed into *E. coli* BL21 (DE3) competent cells. The EGFP-MK-S0 recombinant protein was successfully expressed and purified according to the method described in section 1.2.2. As shown in [Figure 1: see original paper], the recombinant protein EGFP-MK-S0 was expressed in soluble form at 25°C and purified by Ni-NTA affinity chromatography to obtain the target protein EGFP-MK-S0 (32.02 kDa). Gel scanning software analysis indicated that the purity of the purified protein exceeded 90%. After sterile filtration, the protein was used for subsequent cell experiments.

### 2.2 Cell-Penetrating Efficiency of MK-S0 Fused with EGFP

MK-S0 is a sequence from the heparin-binding growth factor MK that is rich in basic amino acids and contains heparin-binding sites. To investigate whether MK-S0 possesses protein transduction capability, we first used enhanced green fluorescence protein (EGFP) as a tracer to evaluate the efficiency of MK-S0 in delivering EGFP into HeLa cells. HeLa cells were incubated with 5  M EGFP-MK-S0 recombinant protein for different durations (1, 4, 12, 24 h). Fluorescence microscopy revealed no green fluorescence signal from EGFP alone in HeLa cells, whereas EGFP fused with MK-S0 exhibited green fluorescence, and the uptake efficiency of EGFP-MK-S0 by HeLa cells showed a positive correlation with incubation time ([Figure 2a: see original paper]). Similarly, when HeLa cells were incubated with different concentrations (1, 2, 5, 10  M) of EGFP-MK-S0 fusion protein for 12 h, intracellular fluorescence intensity increased in a concentration-dependent manner ([Figure 2b: see original paper]).

Furthermore, quantitative flow cytometry analysis comparing the protein transduction activity with Tat, R9, and the human-derived HBD previously reported by our group showed that compared to EGFP alone, the cell-penetrating efficiencies of EGFP-HBD, EGFP-Tat, and EGFP-R9 were enhanced by 4.36-fold, 2.58-fold, and 4.66-fold, respectively, while EGFP-MK-S0 showed the most significant enhancement at 7.52-fold ([FIGURE:2c, 2d]). These results demonstrate that MK-S0, derived from a growth factor HBD, can carry biological macromolecules into HeLa cells with higher efficiency than classical CPPs such as Tat, and exhibits typical time- and concentration-dependent characteristics of cell-penetrating peptides.

### 2.3 Sequence Optimization Design of MK-S0 Cell-Penetrating Peptide

Basic amino acids in CPPs are generally considered to make important contributions to membrane penetration. The guanidinium groups of basic amino acids form hydrogen bonds with anionic groups such as sulfate and phosphate groups

on HSPGs at the cell surface, while positively charged amino acids interact with negatively charged groups in the lipid bilayer, followed by internalization via one or more endocytic pathways. The MK-S0 heparin-binding domain is rich in basic amino acids. MK-Δ1, a mutant in which some non-basic amino acids in MK-S0 were replaced, showed no change in cell-penetrating activity compared to MK-S0, indicating that non-basic amino acids contribute little to the membrane penetration of MK-S0 ([Figure 3a: see original paper]).

To obtain a novel CPP with higher penetrating efficiency, we generated several mutants of MK-S0 with increased proportions of basic amino acids. Purified mutant proteins were incubated with HeLa cells for 12 h, and changes in penetrating activity were examined by fluorescence microscopy observation of intracellular fluorescence intensity ().

The results showed that KA repeat sequences are a structural feature inherent to the MK-S0 penetrating peptide. Additionally, structural modeling of the MK-S0 penetrating sequence revealed that KA repeats are located within an -helix of MK-S0 (the sequence PKTKAKAKAKK between amino acids 6 and 16 forms an -helical structure) ([Figure 3b: see original paper]). The mutant MK-Δ2, designed to increase the proportion of basic amino acids by adding KA repeat sequences to MK-S0, showed approximately 1.5-fold higher penetrating efficiency than MK-S0. The mutant MK-Δ3, in which lysine residues were replaced with the more basic arginine residues, exhibited 1.39-fold higher penetrating efficiency than MK-S0. Combining these two mutational strategies, we further designed the MK-Δ4 mutant. The results showed significantly enhanced fluorescence in HeLa cells, with MK-Δ4 achieving nearly 16-fold higher penetrating efficiency than the original MK-S0 sequence, demonstrating that increasing the proportion of basic amino acid residues and substituting them with more basic residues in MK-Δ4 substantially enhances penetrating efficiency in HeLa cells while maintaining the time- and dose-dependent characteristics of CPPs ([Figure 3: see original paper]).

### 2.4 Cell-Penetrating Efficiency of the High-Efficiency Peptide MK-Δ4 in Different Tumor Cells

Since MK-Δ4 demonstrated significantly higher penetrating efficiency than the original MK-S0 sequence, we subsequently examined the cell-penetrating efficiency of this optimized peptide in various tumor cell lines (HeLa, SMMC, MGC803, A549, 95D). The purified EGFP-MK-Δ4 fusion protein showed purity exceeding 90% by gel scanning software analysis and was sterile-filtered for subsequent cell experiments ([Figure 4: see original paper]).

After incubating EGFP-MK-Δ4 (5 M) with various cell lines for 12 h, fluorescence microscopy revealed that EGFP-MK-Δ4 could transduce into all five tumor cell types, demonstrating broad-spectrum cell-penetrating activity, with the strongest penetrating activity observed in HeLa cells ([Figure 5: see original paper]). The varying penetrating efficiencies of MK-Δ4 in different tumor cells

may be attributed to differences in the content and properties of heparan sulfate on the surface of different tumor cells.

### 2.5 Internalization Mechanism Analysis of High-Efficiency Peptide MK-Δ4

CPPs primarily deliver macromolecular drugs into cells through endocytosis. To elucidate the internalization mechanism of MK-Δ4, we investigated its uptake using various endocytic pathway inhibitors, including heparin sodium (competitive inhibitor of HSPGs), sodium azide (ATP metabolism inhibitor), chlorpromazine hydrochloride (clathrin inhibitor),  -cyclodextrin (lipid raft inhibitor), and cytochalasin D (F-actin inhibitor). HeLa cells were seeded in 6-well plates at a density of 1×10  cells per well, treated with various inhibitors for 30 min, then co-incubated with EGFP-MK-Δ4 (5  M) for 12 h. Intracellular fluorescence intensity was analyzed by flow cytometry.

As shown in [Figure 6: see original paper], compared to the EGFP-MK-Δ4 positive control, treatment with heparin sodium and cytochalasin D inhibited intracellular fluorescence intensity, whereas fluorescence was not reduced by  -cyclodextrin, chlorpromazine hydrochloride, or sodium azide. These results indicate that MK-Δ4 transduces into cells by binding to cell surface heparan sulfate and subsequently enters via an endocytic pathway, with macropinocytosis being the primary internalization mechanism for MK-Δ4.

### 2.6 Application of MK-Δ4 in Anti-Tumor Drug Delivery

MAP30 is a type I ribosome-inactivating protein derived from bitter melon, but its low cell-penetrating efficiency limits its application in tumor therapy. MK-Δ4 is a high-efficiency CPP optimized from the heparin-binding domain of the growth factor MK. To investigate the delivery capacity of MK-Δ4 for the anti-tumor protein MAP30, we first examined whether the fusion protein EGFP-MK-Δ4 had any growth-promoting effects on tumor cells, using EGFP alone as a control. HeLa cells were incubated with EGFP and EGFP-MK-Δ4 (0–5  M) for 24 h. As shown in [Figure 7: see original paper], HeLa cell growth was not inhibited by EGFP protein alone, and cell viability remained unchanged after treatment with EGFP-MK-Δ4 fusion protein, indicating that MK-Δ4 itself does not possess tumoricidal activity.

Subsequently, the MK-Δ4 sequence was fused with MAP30. The fusion protein MAP30-MK-Δ4 was successfully expressed and purified using the method described in section 1.2.2 ([Figure 8: see original paper]). The growth inhibitory effects of the recombinant protein MAP30-MK-Δ4 on different tumor cells (HeLa, SMMC, MGC80-3) were then evaluated. As shown in [Figure 9: see original paper], compared to MAP30 without the CPP, MAP30-MK-Δ4 inhibited the growth of different tumor cells to varying degrees. For HeLa cells, at the same protein concentration (1  M), cell viability was approximately 73% after incubation with MAP30 alone, but decreased to approximately 38% after treatment

with MAP30-MK-Δ4. The IC  of MAP30 against HeLa cells was 2.576  M, whereas the IC  of MAP30-MK-Δ4 was 0.444  M ([Figure 9a: see original paper]), representing a 5.8-fold decrease. As shown in , under the same conditions, the IC  values of MAP30-MK-Δ4 against SMMC and MGC80-3 tumor cells decreased by 5.3-fold and 4.1-fold, respectively, compared to MAP30 alone ([FIGURE:9b, 9c]). MAP30-MK-Δ4 exhibited the strongest inhibitory effect against HeLa cells, with a 5.8-fold enhancement in efficacy. These MTT results were generally consistent with the cell-penetrating efficiency of MK-Δ4 in different tumor cells ([Figure 5: see original paper]).

## Discussion

Cell-penetrating peptides have emerged as a research hotspot as novel drug delivery vectors in anti-tumor therapy. Numerous studies have demonstrated that proteins with heparin-binding domains can bind to cell surface heparin/HS, and some can be internalized into cells. Our group previously obtained a cell-penetrating peptide from the heparin-binding domain of EC-SOD and identified a heparin-binding peptide (HBP) with CPP properties, both of which were successfully applied in anti-tumor therapy. MK, as a heparin-binding growth factor, can promote cell growth, survival, and migration. The C-terminal region of MK contains two heparin-binding sites, with the second site being a region rich in basic amino acids. Since most CPPs rely on positively charged amino acids (arginine and lysine) for cell entry, such as Tat, we first investigated whether the C-terminal heparin-binding site sequence MK-S0 from MK possessed protein transduction capability. The results demonstrated that MK-S0 derived from the heparin-binding growth factor MK is a novel CPP with higher efficiency than classical CPPs like Tat, and exhibits time- and dose-dependent characteristics.

Literature reports have indicated that arginine-rich basic amino acids facilitate CPP entry into cells, and the charge effect from increasing the proportion of basic amino acids in peptides plays an important role in CPP internalization. To further improve the efficiency of MK-S0 in carrying macromolecules into cells, we optimized the MK-S0 sequence through mutagenesis. By increasing the proportion of basic amino acids or substituting them with more basic residues, we designed several mutants. The results showed that the optimized MK-Δ4 exhibited significantly increased penetrating efficiency, approximately 16-fold higher than the original MK-S0 sequence.

MK-Δ4, a novel CPP derived from the basic amino acid-rich heparin-binding domain, was found to have heparan sulfate playing an important role in its internalization, as evidenced by the significant reduction in EGFP-MK-Δ4 uptake after treatment with heparin sodium. We hypothesize that the first step of MK-Δ4 penetration involves clustered basic amino acids binding to cell surface HS through electrostatic interactions, followed by internalization via endocytosis. Studies on MK-Δ4 penetrating efficiency in different tumors revealed that MK-Δ4 can enter various cell types with different efficiencies, possibly due to differences in heparan sulfate properties on different tumor cell surfaces, though

the mechanism requires further investigation.

Our research on the application of MK-Δ4 as a novel high-efficiency CPP in anti-tumor therapy demonstrated that MK-Δ4 can enhance the inhibitory effects of MAP30 on different tumor cells. Compared to MAP30 without the CPP, MAP30-MK-Δ4 showed significantly improved efficacy against HeLa, SMMC, and MGC803 tumor cells, indicating that the introduction of CPP MK-Δ4 effectively promoted MAP30 entry into cells and greatly enhanced its tumoricidal activity.

In this study, we investigated the basic amino acid-rich heparin-binding domain sequence of human midkine growth factor. Through mutational modification of the original MK-S0 sequence, we obtained a novel high-efficiency MK-Δ4 CPP and investigated its penetrating pathway and properties. Tumor cell growth inhibition experiments demonstrated that MAP30 fused with the high-efficiency CPP MK-Δ4 exhibited significantly higher tumoricidal activity against multiple tumor cell lines than MAP30 alone.

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