A SYNTHETIC CELL-PENETRATING PEPTIDE (CPP) WITH PROTAMINE CONJUGATE UTILIZED FOR GENE DELIVERY

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ABSTRACT

Background

There are two main gene transfer techniques, a non-biological method such as liposome/polycations (lipofection), electroporation and gene gun. A biological method like transduction, synthetic biomolecules and virus mediated transfer.

Objective

The objective of this study is to utilize Cell penetrating peptides (CPPs) as a novel tool for transferring and delivery of therapeutically active macromolecules and drugs *in vitro* and *in vivo*.

Materials and Methods

In this study, we utilized two synthetic CPPs, based on HIV-TAT protein, a minimal sequence of 11 amino acids with transducing domain activity. TAT is a subclass of CPPs, which often been used for intracellular targeting of proteins, but has not explored their application for DNA expression vectors.

Results and Conclusion

Here, we conjugated TAT to a DNA/RNA condensation moiety of Protamine, so called TAT-Protamine (TAT-P) and a branched dual TAT-Protamine (dTAT-P). Our results show that dTAT-P has both transduction capacity and DNA-condensation ability to deliver oligonucleotides and plasmid DNA into living cells. Experiments on living cells shows that there are many advantages in using dTAT-P for genetic delivery, expression plasmids of human proteins actin and Btk fusion with Green Fluorescent Protein (GFP, in addition to FITC-labelled synthetic oligonucleotides.

***Keywords*:** *HIV-TAT protein; Protamine ;Ttransfection; Gene delivery; Cell penetrating peptides (CPPs)*

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INTRODUCTION

Gene therapy is a strategy to replace defective genes with normal or "wild type" form genes responsible for specific diseases, which caused from inherited gene [(1)]. Many genetic disorders are discussed as candidates for gene therapy like Parkinson's and Alzheimer's diseases, arthritis, and heart disease but the most success in application of gene therapy has taken place in monogenic diseases [(2)]. Theoretically there are two types of gene therapy: somatic cell therapy, which makes it possible by modifying defective gene to a correct form in somatic cells, and germ line cell therapy, where the germ line cells modified to correct a genetic defect. In combination to these cell therapy results, researchers may use at least one of several approaches in order to correct the defective genes such as: (a) Insertion normal gene into a non-specific location within the genome to replace a non-functional gene (most common). (b) Exchange an abnormal gene to a normal gene through homologous recombination. (c) Repair the abnormal gene through selective reverse mutation, which returns the gene to its normal function. (d) Change the regulation or expression (the degree to which a gene is turned on or off) of a particular gene [(3)].

Carrier molecule in form of DNA vectors must be fused with the interested gene designed especially for patient target tissues or cells. The humanized viral genome is the most common gene delivery vectors, after it has been genetically modified to become harmless and to carry normal human DNA. By using the capability of viruses to deliver their genes to human cells in a pathogenic manner the scientists have used these advantages and altered the virus genome to replace the abnormal genes with the normal one. The principle for viral gene therapy is that the target cells such as patients liver cells will infect with the viral vectors. After infection, the genetic material containing the therapeutic human gene unloads into the target cells. As a result of a functional protein produced from the therapeutic gene, the target cell corrected to a normal state [(3)]. Negatively charged liposome-encapsulated drugs were introduced three decades ago and even have been used as vehicles for gene delivery into the cells. But the problem with the efficiency of nucleic acid encapsulation caused the development of positively charged liposome (cationic liposome), which was able to bind to the negatively charged DNA leading to the cluster of aggregated vesicles along the nucleic acid. The advantage of the cationic liposome was to bind to the negatively charged cell membrane more effective than the classical liposome. The main factors involved in transfection efficiency are structure of cationic lipid and the ratio of the cationic lipid to DNA, but still the method suffers from low transfection efficiency compared with viruses and its cytotoxicity in vitro according to clinical research. Besides more studies on liposome/DNA complex in mice have been showed that the high concentration of this complex can cause histopathological changes and suggesting its risks in clinical research[4].

The naked plasmid-based DNA is the simplest gene transfer system. The simplicity of this approach made it possible to use it in many of clinical protocols especially in cancer gene therapy where the DNA can be injected directly into the tumour cells (even useful system to treat genetic diseases like tissues available for direct injection such as skin) or into the muscle cells to express tumour antigens which can work as a cancer vaccine. In spite of advantages for this system there are some disadvantages like low level of gene expression and limited tissue specificity and accessibility [(4)].

Cell penetrating peptides (CPPs) are a new tool, which even if it describes as a water-soluble natural and synthetic peptide, has ability to translocation through various cell membranes. The discovery of CPPs and their efficient transduction into the cells opened new opportunities for medical treatment and delivery of therapeutic agents across cells *in vivo*. There are some specific qualities for this system such as, high efficiency, biologically inert, harmless, cheap and degradable inside cells [(5,6)]. The mechanism of CPP translocation is still mostly unknown, but we know more about the structural entities facilitates the peptide-membrane interaction and its role for translocation process [(5)]. Larger CPP molecules are able to translocate when they bind covalently with a cargo like enzyme or even larger molecules such as polypeptides, oligonucleotides, lyosomes or nano-particles, which are many times heavier than their own molecular mass but still keep their translocating ability. There are many known types of peptides such as TAT peptide, penetratin, transportan; anopidia peptides possess functional CPP activity [(5,6)].

The aim of this research is to use cell-penetrating peptides (CPPs) to carry macromolecules and transport impermeable drugs across cell membranes as a new strategy for gene transfer therapy. In this study we used two different TAT-P sequences and tried to find out if these peptides are more efficient for gene delivery into different cell types by fluorescent microscopy. In addition, the transfection efficiency was tested to determine capacity of TAT-Protamine carrier to introduce oligo-nucleotides into cells.

MATERIAL AND METHODS

Cell Culture

African green monkey cell line COS-7, rat mast cell line RBL-2H3 and human uterine cervical cell line HeLa were cultured in Dulbecco's modified Eagles medium (DMEM) (Invitrogen) supplemented with 10% FBS and 1% PEST (Penicillin and Streptomycin). The cell culture incubated in 5% CO_2 enriched air at 37 ^oC.

CPP-Transfections

The COS-7, RBL-2H3, Hela and A20 cell lines were transfected with FITC-labelled oligonucleotides using CPPs (dTAT-P) in serum free DMEM (Invitrogen). The DMEM medium were mixed with CPPs solution peptide (0.6 mg/ml in Tris-Sulphate pH=7.2). The CPPs solution added separately to 1 µg FITC-labeled oligonucleotides, incubated for 15-30 min in RT. Oligonucleotides of 21- and 45-bases were synthesized and chemically conjugated with FITC (IDT-Denmark). The sequence is customized to be unique with 50% CG ratio and FITC-labelling were essential to track the oligonucleotide intake inside cells. The pre-incubated sample were added to 6-well plate drop wise and incubated in 5% CO_2 enriched air at 37^oC for 4h. After 4h incubation, DMEM medium + 12% FBS were added to each dish, incubated in 5% CO_2 enriched air at 37^oC for 36-48h.

Fluorescent Microscopy

For uptake of plasmid DNA and oligonucleotides, cells were grown on the cover slips. After PBS washing, cells were fixed by 3% formaldehyde solution in PBS for 10 min in RT. To permeabilize cells, 0.1% Triton-X is added and diluted in PBS, incubated for 15 min in RT and followed by series of PBS washing. For blocking, the cells were incubated in 0.1% BSA-C/PBS, 5% serum for 1-2 h in RT. Then the cover slips were washed with 0.1% TWEEN diluted in PBS. For staining of nucleus DAPI is used with 1:1000 dilution and incubated for 20-30 min in dark at RT. The cells were washed extensively with 0.1% TWEEN diluted with PBS and finally mounted on glass slides. The cells were analyzed by fluorescent microscopy and image analysis performed using SlideBook™ (Intelligent Imaging Innovations, USA) and Photoshop™ Software (Adobe Systems, USA).

RESULTS

Protein Transduction Domain (PTD) of TAT

There are many members of CPPs acting as transporter into cells, a short list summarized here (Table 1). TAT peptide is a portion of protein transduction domain (PTD) and is a subclass of CPPs family, which derived from the TAT protein of the HIV virus. The TAT-PTD peptide is a motif located between amino acids 46 and 64 of the 86 residues of complete TAT protein. TAT-PTD is able to facilitate intracellular delivery of proteins and small colloidal particles. The cell entry of whole TAT protein follows binding to the cell surface heparin sulphate proteoglycans and required for efficient transcription of the viral genome. Additionally it has been shown that the shortest PTD sequence of TAT (48-57) is sufficient for membrane translocation and carry proteins and fluorescent molecules into cells *in vivo* or in culture. The identification of TAT-PTD peptide mediated translocation may clarify the key steps of virus penetration into cells and makes it easier in building an effective antiviral defence. Besides, as passing through cellular membranes which represents a major barrier for efficient delivery of macromolecules into cells, the TAT-PTD peptide facilitates transport various drugs into mammalian cells *in vitro* and *in viv*o. TAT peptide can induce growth and differentiation of blood vessels. It can also activate the VEGF receptors (which express on endothelial cells)**.** Therefore, it might be used as an ideal vehicle for a pharmacological intervention aiming at influencing angiogenesis *in vivo* as well as anti-angiogenic therapies.

Table 1. A small catalogue of various CPPs sequences used for gene therapy applications. These CPP moieties have been applied to mediate proteins and DNA constructs into mammalian cells. The tryptophan (*W in italic*) and charged residues (underlined) are highlighted [5,7]

Peptide	Sequence
Pentratin	RQIKI*W*FQNRRMK*W*KK
Pentratin-2W2F	RQIKIFFQNRRMKFKK
pIsl	RVIRV*W*FQNKRCKDKK
Transportan	*W*TLNSAGYLLGKINLKALAALAKKIL
TAT-peptide	YGRKKRRQRRR
pVEC	LLIILRRRIRKQAHAHSK

Design of a novel CPP Carriers: TAT conjugated to Protamine

The TAT-Protamine (TAT-P) sequence (Table 2A) consists of 46 amino acids with three tandem functional motifs. At the N-terminal, contains 11 amino acids of TAT is responsible for cell penetrating capability and at C-terminal Protamine consists of 34 amino acids which has DNA condensation capacity. The third part contains triplet of glycine residues acts as linker between TAT and Protamine. The dTAT-P sequence (Table 2B) is similar to TAT-P, but the only difference dTAT-P contains a dual TAT sequence at the N-terminal. The TAT sequences links to the triplet glycine residues via a poly-lysine backbone. In this study, we only studied the efficacy of dTAT-P as carrier to deliver oligonucleotides and plasmid DNA into various types of cells.

Table 2. Primary sequence of the synthesized TAT-protamine conjugates, (A) TAT-Protamine (TAT-P) and (B) dTAT-Protamine (dTAT-P). The synthesis of dTAT-P was done as a branched derivative of TAT-P. Lysine residues were used in the backbone for the branched molecule accurately. These fusion peptides has both trans-membrane permeability (TAT) as well as DNA condensation (protamine) moiety. In this study, only dTAT-P was studied extensively.

(A) TAT-P structure

TAT-sequence	Poly-glycine-linker	Protamine
YGRKKRRQRRR	-GGG-	-PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR

(B) dTAT-P structure

TAT-sequence	Poly-glycine-linker	Protamine
YGRKKRRQRRR-K\ K- YGRKKRRQRRR-K/	-GGG-	-PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR

Oligonucleotide Transfections by dTAT-P

To investigate the transporter effect of dTAT-P peptides on synthetic oligonucleotides of 21 bases and 45 bases delivery to the double trypsinized adherent cells. Transfection is done using equal amount of synthesized oligonucleotide-FITC (1µg) but different peptide dTAT-P concentrations (0.1-10 µg) to RBL-2H3 and Cos 7 cells. The results of this screening indicated that cell colonies of RBL-2H3 cells transfection were optimal at 1:1 ratio (Figure 1A), similar results were found in Cos 7 cells (Figure 1B). At excess concentrations of 5:1 ratio of dTAT-P to DNA oligonucleotides were cytotoxic and induce apoptotic cells. However, we kept the transfection reagents to DNA ratio in the optimal range 1:1 ratio (Figure 1). On-the-other hand, the cytotoxicity level of TAT-P is generally higher compared to dTAT-P peptide at higher ratios 4:1 and 5:1 (data not shown).

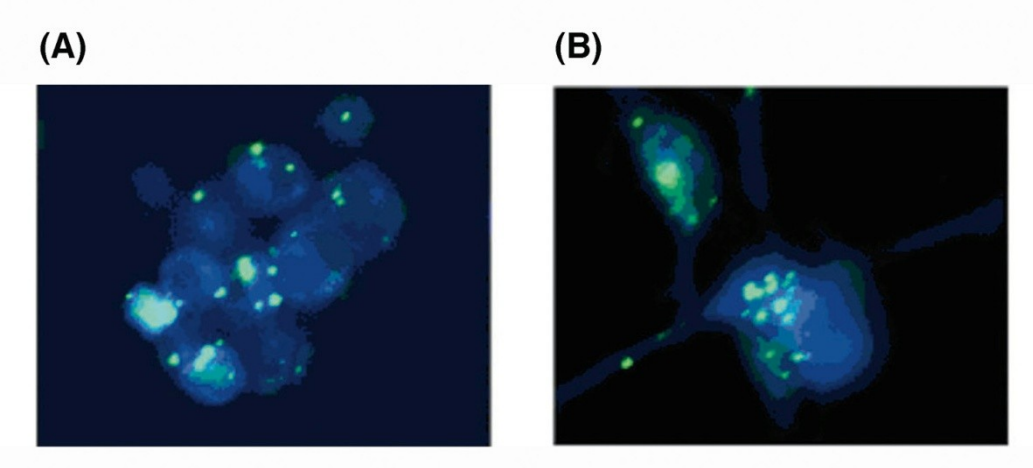

Figure 1. Uptake of FITC-labeled synthetic oligonucleotide into (A) RBL-2H3 and (B) COS-7 cell lines by dTAT-P (1 µg) at 1:1 ratio. The transfection efficiency in RBL-2H3 and Cos-7 cells was 50% and 80%, respectively. These images were representative data of 4-independent experiments.

Transfection Efficiency by dTAT-P

Cos-7, RBL-2H3 and HeLa cells were transfected with different plasmid DNA to monitor expression of mammalian proteins. To this end, cell lines were separately transfected with protein expression vector for GFP-Actin (1µg) and GFP-Btk (1µg). The post-transfected cells were analyzed 48 hours later by Fluorescent microscopy. Fluorescent microscopy images show clearly expression of actin and Btk genes in Cos-7 (Figure 2). However, elevated expression was observed after 72 h post-transfection in Cos-7 (Figure 2A and 2B), HeLa cell lines (Figure 3A and 3B). The data shows that dTAT-P peptide has ability to deliver both plasmid DNA of GFP-Actin and GFP-Btk to the adherent cells.

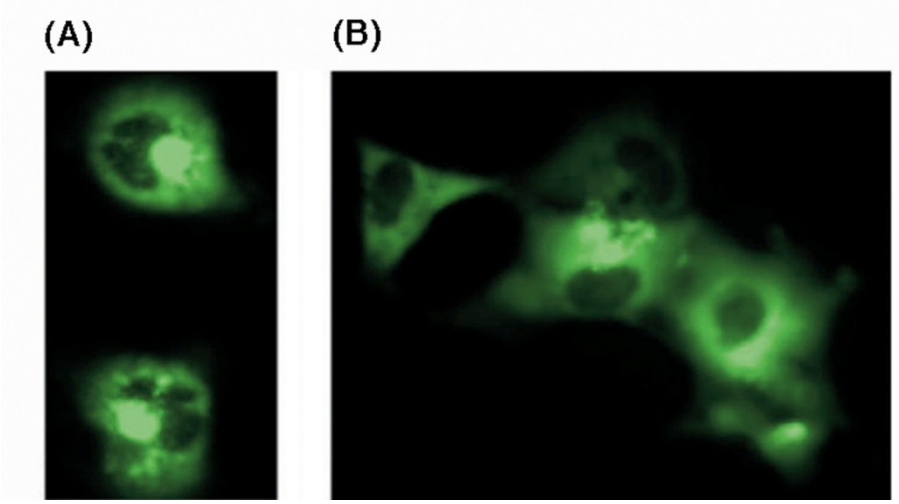

Figure 2. The dTAT-P peptide efficiency delivers (A) GFP-Actin and (B) GFP-Btk plasmids in Cos-7 cells. Expression of Actin and Bruton's tyrosine kinase (Btk) in cells was monitored by green fluorescent protein (GFP).

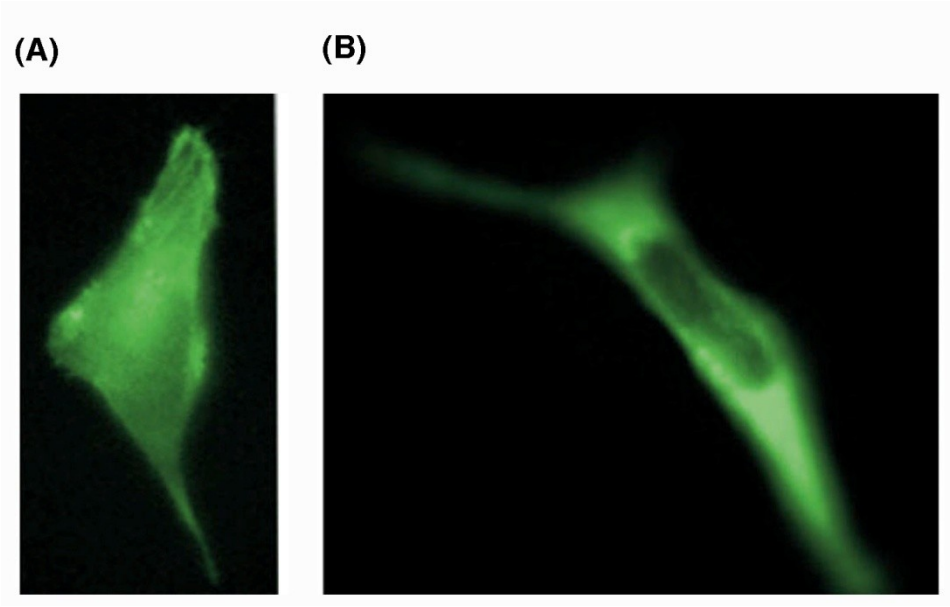

Figure 3. The dTAT-P peptide efficiency delivers (A) GFP-Actin and (B) GFP-Btk plasmids in Hela cells. Expression of Actin and Bruton's tyrosine kinase (Btk) in cells was monitored by green fluorescent protein (GFP).

DISCUSSION

Improving the performance of non-viral gene-delivery vehicles that consist of synthetic compounds and nucleic acids is a key to successful gene therapy. Our data for oligonucleotide transfection by using synthetic oligonucleotide-FITC shows higher transfection efficiency in dTAT-P, but it is not clear how close the oligonucleotide particles to the cytoplasm or nucleus are. In this case it needs more research to clarify the exact localization using various probes. In this study we could also show dTAT-P transfection efficiency by expressing fusion GFP with actin and Btk into the cells and follow the expression in living cells expressing GFP. We propose that dTAT-P bind to the cell surface and

are taken up into the cells, by ionic interactions between the positively charged peptides and negatively charged cell membrane.

Our results indicate that these basic peptides can penetrate effectively and deliver DNA, siRNA, drugs and active compounds inside cells, but still we lack specificity in targeting, since these complexes penetrate many types of cells. However, this research is relevant to measure efficacy of biologically active vehicles (TAT-P and dTAT-P) to deliver various macromolecules.

The non-viral gene delivery tools are still in its infancy and further improvement and optimization of the delivery system is required. In spite of many studies on the CPPs, they are still unknown concerning cell surface interactions and intracellular translocation mechanisms. But any progress in this approach depends on a better understanding of the cellular and *in vivo* barriers of the gene transfer. To achieve more optimization, further investigation has to be performed. For quantitative analysis and accurate comparison, other methods like FACS and immune gold labelling for electron microscopy analysis could be applied.

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