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**NIKITA OSKOLKOV**

# A novel strategy for peptide-mediated cellular delivery and induction of endosomal escape

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# ABSTRACT

Oligonucleotide-based gene regulation has a high potential in gene therapy, but the plasma membrane is impermeable for these large-size and hydrophilic nucleic acid polymers, consequently an efficient and non-toxic delivery vector is needed to promote their deliver into the cell. Cell-penetrating peptides are a group of delivery vectors that have been extensively exploited for intracellular delivery of nucleic acids, including plasmid DNA (pDNA) and splice-correction oligonucleotides *in vitro* and *in vivo* using covalent and non-covalent strategies for attaching the cargo.

The main aims of this thesis were to design and to synthesize novel and efficient peptide vectors for nucleic acids delivery and evaluate them for *in vitro* applications using non-covalent co-incubation strategy. We used two methodologies: gene transfection with plasmid DNA and splice-correction assay with oligonucleotides. In this thesis we present several novel CPPs with improved delivery properties, compared with parent peptides, TP10 and $(RXR)_4$, which were acquired by adding stearic acid (stearylation) and/or phosphoryl group (phosphorylation) modifications or by performing amino acid substitution in backbone of the peptide. All these modifications were done in order to facilitate better uptake of cargo into the cell and provide more efficient endosomal escape from intracellular compartments.

In papers I–II, we showed that N-terminal modification with stearic acid of chimeric and amphipatic TP10 or non-amphipatic and synthetic $(RXR)_4$ peptides resulted in efficient peptide vector for nucleic acids delivery in non-toxic fashion and in case of stearylated TP10 was preserved efficacy even in the presence of serum.

In paper III, we presented chemically modified stearyl-TP10 based delivery vectors for efficient SCO delivery, which were obtained by replacement of Ile8 by threonine in the backbone of the peptide and by modifying of tyrosine and/or threonine by their phosphorylated monomers, respectively.

In conclusion, minor modifications in peptide sequence and side chains can improve the delivery properties of CPPs in order to promote effective intracellular delivery of therapeutic molecule and we believe that our studies help to overcome the difficulty of endosomal escape providing highly potential and efficient CPPs for oligonucleotides delivery both *in vitro* and *in vivo*.

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# LIST OF PUBLICATIONS

The current thesis is based on the following original publications, which will be referred to by the corresponding Roman numerals in the text:

I. Mäe, M.; El-Andaloussi, S.; Lundin, P.; **Oskolkov, N**.; Johansson, H.J.; Guterstam, P.; Langel, Ü. (2009). A stearylated CPP for delivery of splice correcting oligonucleotides using a noncovalent co-incubation strategy. Journal of Controlled Release, 134(3), 221–227.

II. Lehto, T.; Abes, R.; **Oskolkov, N**.; Suhorutšenko, J.; Copolovici, D.-M.; Mäger, I.; Viola, J.R.; Simonsson, O.; Guterstam, P.; Eriste, E.; Smith, C.I. E.; Lebleu, B.; El-Andaloussi, S.; Langel, Ü. (2010). Delivery of nucleic acids with a stearylated $(RxR)_4$ peptide using a non-covalent co-incubation strategy. Journal of Controlled Release, 141(1), 42–51.

III. **Oskolkov, N.**; Arukuusk, P.; Copolovici, D. M.; Lindberg, S.; Margus, H.; Padari K.; Pooga, M; Langel Ü. (2011). NickFects, phosphorylated derivatives of transportan 10 for cellular delivery of oligonucleotides. International Journal of Peptide Research and Therapeutics, 17(2), 147–157.

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My personal contribution to the articles referred to in this thesis is as follows:

Ref. I – performed the synthesis of the peptides and participated in the writing of the paper

Ref. II – performed the synthesis of the peptides and participated in the writing of the paper

Ref. III – designed and synthesized the novel peptides, planned most of the experiments, performed some of them, analyzed all the data and wrote the paper as corresponding author

# ADDITIONAL PUBLICATIONS

El Andaloussi, S., Lehto, T., Mager, I., Rosenthal-Aizman, K., Oprea, II, Simonson, O. E., Sork, H., Ezzat, K., Copolovici, D. M., Kurrikoff, K., Viola, J. R., Zaghloul, E. M., Sillard, R., Johansson, H. J., Said Hassane, F., Guterstam, P., Suhorutsenko, J., Moreno, P. M., **Oskolkov, N**., Halldin, J., Tedebark, U., Metspalu, A., Lebleu, B., Lehtio, J., Smith, C. I. & Langel, Ü. (2011). Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo. Nucleic Acids Res, 39, 3972–3987.

Lehto, T., Simonson, O. E., Mäger, I., Ezzat, K., Sork, H., Copolovici, D. M., Viola, J. R., Zaghloul, E. M., Lundin, P., Moreno, P. M., Mäe, M., **Oskolkov, N**., Suhorutšenko, J., Smith, C. E. & Andaloussi, S. E. (2011). A Peptide-based Vector for Efficient Gene Transfer In Vitro and In Vivo. Molecular Therapy. In press.

Loog, M., Ek, B., **Oskolkov, N**., Närvanen, A., Järv, J. & Ek, P. (2005). Screening for the optimal specificity profile of protein kinase C using electrospray mass-spectrometry. J Biomol Screen, 10, 320–8.

Loog, M., **Oskolkov, N.,** O'Farrell, F., Ek, P. & Järv, J. (2005). Comparison of cAMP-dependent protein kinase substrate specificity in reaction with proteins and synthetic peptides. Biochim Biophys Acta, 1747, 261–6.

# ABBREVIATIONS

| | |
|---|---|
| 2’-OMe ON | phosphorothioate 2’-O-methyl RNA |
| CPPs | cell-penetrating peptides |
| Cy5 | cyanine5 |
| DCM | dichloromethane |
| DIEA | diisopropylethylamine |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMF | dimethylformamide |
| DMSO | dimethyl sulfoxide |
| FM | serum-containing medium |
| GalR | galanin receptor |
| HBTU | O-benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluoro-phosphate |
| HOBt | hydroxybenzotriazole |
| HKR | HEPES-buffered Krebs-Ringer solution |
| IMDM | Iscove’s Modified Dulbecco's Medium |
| LDH | lactate dehydrogenase |
| mRNA | messenger RNA |
| NF | NickFect |
| NMP | N-Methyl-2-pyrrolidone |
| OBzl | benzyl ester protecting group |
| OD | optical density |
| ON | oligonucleotide |
| PBS | phosphate buffered saline |
| pDNA | plasmid DNA |
| PF | PepFect |
| PMO | phosphorodiamidate morpholino oligomer |
| PNA | peptide nucleic acid |
| pre-mRNA | precursor mRNA |
| SCO | splice-correcting oligonucleotides |
| SFM | serum-free medium |
| siRNA | short interfering RNA |
| SPPS | solid-phase peptide synthesis |
| SV40 | Simian vacuolating virus 40 |
| TFA | trifluoroacetic acid |
| TIS | triisopropylsilane |
| TP10 | transportan 10 |
| TP | transportan |

# INTRODUCTION

The plasma membrane of eukaryotic cells contains a lipid bilayer into which cholesterol and various proteins are inserted. The hydrophobic interior of lipid bilayer makes it impermeable to metabolites, nutrients and other hydrophilic molecules and serves as a barrier between cytoplasm and extracellular environment. Therefore, many biologically active hydrophobic therapeutic molecules, such as nucleic acids (oligonucleotides, plasmids), proteins, etc., cannot translocate across the plasma membrane. That restricts the usage of nucleic acid molecules in gene therapy. Although few viral and non-viral strategies were designed to solve this problem in last decade, the inability to cross the plasma membrane is still the major hindrance for current drug research and development.

In recent 40 years, several oligonucleotide (ON)-based methods have been developed with the purpose of regulating gene expression. The efficiency of these methods depends, among the other factors, on the efficient uptake of ON into the cells and subsequent endosomal escape. One basic method for gene regulation involves the use of bacterial vectors to express the genes of interest. In addition to evaluation of functional aspects of different genes, this is a highly appealing strategy to utilize in clinical settings, e.g. gene therapy. Gene therapy was originally thought to serve as corrective treatment for inherited genetic diseases, e.g., by splice-correcting oligonucleotides. However, in recent 15 years, experimental gene therapy for cancer diseases has become the most frequent application and acquired diseases have also been investigated (Cross and Burmester, 2006).

Cell-penetrating peptides have great potential and promise as *in vitro* and *in vivo* delivery of therapeutic molecules. Cell-penetrating peptides (CPPs), also called protein transduction domains (PTDs) are a class of peptides that has drawn much attention in the last decades as non-toxic vehicles for delivery of wide range of cargos. These peptides are usually less than 30 amino acids in length with net positive charge and/or amphipathic nature and are able to deliver payload over cell membranes both *in vitro* and *in vivo*. Cell-penetrating peptides can be utilized as delivery vectors for peptides, proteins, nucleic acids and chemotherapeutics (Mäe and Langel, 2006, Järver and Langel, 2006).

The main aims of this thesis are to design and to synthesize novel peptide vectors for nucleic acid intracellular delivery using minor chemical modifications and evaluate them for *in vitro* and *in vivo* applications using non-covalent co-incubation strategy: gene transfection with plasmid DNA and splice-correction with oligonucleotides. In this thesis we present several novel CPPs, which were obtained by modification of known CPPs by adding stearic acid (stearylation) and/or phosphoryl group (phosphorylation) modifications or by performing amino acid substitution in backbone of the peptide.

In conclusion, minor modifications in peptide sequence and side chains can improve the delivery properties of CPPs in order to promote effective intracellular delivery of therapeutic molecule and we believe that our studies help to overcome the difficulty of endosomal escape providing highly potential and efficient CPPs for oligonucleotides delivery both *in vitro* and *in vivo*.

# 1. LITERATURE OVERVIEW

## 1.1. Cellular delivery vectors

The challenge of using different biomolecules such as siRNA, antisense oligonucleotides, plasmids, miRNA and even proteins as therapeutic agents is anchored by their low bioavailability. These molecules are quite large and often negatively charged, that make them impermeable for plasma membrane and consequently impossible to use as therapeutics.

During the last decade the topic of intracellular delivery of impermeable effector molecule in the forms of covalent or non-covalent conjugates with different delivery vectors capable to mediate membrane translocation was considerably investigated. As a result, over hundred different delivery systems were developed in order to facilitate the delivery of broad range of macrobiomolecules into the cell. An ideal gene delivery method needs to meet three major criteria: (1) it should protect the therapeutic gene (transgene) against degradation by nucleases in intercellular matrices, (2) it should bring the transgene across the plasma membrane and into the nucleus of target cells, and (3) it should have no detrimental effects (Gao et al., 2007).

All these vectors can be classified into the two major types: viral and non-viral vectors. But unfortunately most of them have some drawbacks, like high cytotoxicity, inherent immunogenicity, low transfection efficiency, etc. The applications with both disadvantages and usefulnesses of the most widely used intracellularly delivery strategies are shown in **Table 1**. Although significant progress has been made in the basic science and applications of various nonviral gene delivery systems, the majority of non-viral approaches are still much less efficient than viral vectors, especially for *in vivo* gene delivery.

**Table 1**. Comparison of different cellular delivery vectors

| Transfection reagent | Drawbacks and advantages |
|---|---|
| Viral vectors (adenoviral, recombinant lentiviral, oncoretroviral vectors ) | Endogenous recombination, oncogenic effects and immunological reactions, high cost of production, high delivery efficiency *in vitro* and *in vivo* |
| Cationic liposomes (Lipofectamine™ 2000) | High cytotoxicity, not usable *in vivo*, unable to transfect the entire cell population, high delivery efficiency *in vitro*, applicable to wide range of biomolecules |
| Peptide-based reagents (Transductin™) | Serum-sensitive, mainly used for siRNAs transfection, high siRNA concentrations are required for siRNA transfection |
| Cationic polymers (PEI) | Non-biodegradable, low solubility, high cytotoxicity |
| Physical methods(eg. electroporation, ultrasound) | Cell damage, difficulties for *in vivo* applications, low efficiency, no specificity, high cost of usage |
| Cell-penetrating peptides | Non-toxic, efficient for a wide spectrum of cargos in the entire cell population, less efficient than viral vectors |

### 1.1.1. Viral vectors

Viral vectors are an efficient tool to deliver genetic material into the cells. The main advantage of viral vectors is very efficient transfection of genetic biomolecules in cell cultures (*in vitro*) and in living organisms (*in vivo*). Viruses can efficiently transport their genome inside the cells they infect for protein expression or knockdown and even reach hard-to-transfect cells types. On the other hand the main drawback of viral vectors is safety troubles (McMenamin and Wood, 2010, Campos and Barry, 2007, Escors and Breckpot, 2010). When using viral vectors, gene is either integrated into the genome or must be continuously administrated, depending on delivery systems; as a result there is the risk of insertional mutagenesis of endogenous gene. At the same time viral vectors have shown to be prone to insert gene in active genome, causing side effects and in case of prolonged administration, there is the risk of the immune response (Lindberg S. et al., 2011). Other disadvantages of viral systems are high cost of production and limited size of cargo (up to 10 kb) (Lundstrom, 2003).

The start point to use viral vectors for gene transfection was done by Paul Berg in 1970s by modification of Simian vacuolating virus 40 (SV40) containing DNA from the bacteriophage lambda to infect monkey kidney cells maintained in culture (Goff and Berg, 1976).

There are three types of viral vectors, which have been exploited extensively for delivery: adenoviral, retroviral, and lentiviral vectors. Retroviruses are one of the keystones of gene therapy approaches. The recombinant retroviruses have the ability to integrate into the host genome in a reliable technique (Cavazzana-Calvo et al., 2000). The primary drawback to use retroviruses is the requirement for cells to be actively dividing for transduction; as a result cells become resistant to infection and transduction by retroviruses. Lentiviruses are a subclass of retroviruses. They have recently been adapted as gene delivery vectors because of their ability to integrate into the genome of non-dividing cells, as other retroviruses can infect only dividing cells. For safety reasons lentiviral vehicles never carry the genes required for their replication (Escors and Breckpot, 2010). As opposed to lentiviruses, adenoviral DNA does not integrate into the genome and is not replicated during cell division. This limits their use in basic research, although adenoviral vectors are occasionally used in *in vitro* experiments. Since humans usually get in contact with adenoviruses, they trigger a rapid immune response with potentially dangerous consequences. To overcome this problem scientists are currently investigating adenoviruses to which humans do not have immunity.

## 1.1.2. Non-viral delivery vectors

Gene delivery using non-viral approaches has been extensively studied as a basic tool for intracellular gene transfer and gene therapy. In the past, the primary focus has been on application of physical, chemical and biological principles to develop a safe and efficient method that delivers a transgene into target cells for appropriate expression. (Gao et al., 2007). Methods of non-viral gene delivery have also been explored using physical (carrier-free gene delivery) and chemical approaches (synthetic vector-based gene delivery).

### 1.1.2.1. Physical methods

Physical approaches, including needle injection (Wolff et al., 1990), electroporation (Heller et al., 2005), gene gun (Yang and Sun, 1995), ultrasound (Lawrie et al., 2000), hydrodynamic delivery (Liu et al., 1999), employ a physical force that permeates the cell membrane and facilitates gene transfer into cells *in vitro* and *in vivo*. Physical approaches induce transient injuries or defects on cell membranes, so that DNA can enter the cells by diffusion. However, the main drawbacks of physical methods are low efficiency for *in vivo* applications, high cost and usage of specific equipment (Mehier-Humbert and Guy, 2005).

Local injection of naked plasmid DNA into the muscle (Wolff et al., 1990), liver (Hickman et al., 1994), skin (Choate and Khavari, 1997), or airway instillation into the lungs (Meyer et al., 1995) is able to transfect cells and usually leads to low gene expression. Furthermore, it is attractive to many researchers because of its simplicity and lack of toxicity. Practically, airway gene delivery and intramuscular injection of naked DNA for the treatment of acute diseases and DNA-based immunization, respectively, are two areas that are likely to benefit from naked DNA-mediated gene transfer.

Electroporation, also known as electropermeabilization, a versatile method to introduce DNA into cells using an electric field has been extensively tested in many types of tissues *in vivo* (Heller et al., 2005). This technique exposes the cell membrane to high-intensity electrical pulses that can cause transient and localized destabilization of the barrier. During this perturbation, the cell membrane becomes highly permeable to exogenous molecules, such as DNA. Skin and muscles are the most extensively investigated using this method (Neumann et al., 1982), although the system should work in any tissues into which a pair of electrodes can be inserted. Gene transfer by electroporation has shown less variation in efficiency across species than direct DNA injection. Several major drawbacks exist for electroporation. First, it has a limited effective range of ~1 cm between the electrodes, which makes it difficult to transfect cells in a large area of tissues. Second, a surgical procedure is required to place the electrodes deep into the internal organs. Third, high voltage applied

to tissues can result in irreversible tissue damage as a result of thermal heating (Mehier-Humbert and Guy, 2005).

Particle bombardment through a gene gun is an ideal method for gene transfer to skin, mucosa, or surgically exposed tissues within a confined area (Yang and Sun, 1995). DNA is deposited on the surface of heavy metal particles (most commonly 1–1.5 μm), which are then accelerating a high-voltage electric spark, or a helium discharge and expelled onto cells or a tissue. The momentum allows the gold particles to penetrate a few millimeters deep into a tissue and release DNA into cells on the path. The major application of this technology is DNA-based genetic immunization, in which the skin is the target (Wang et al., 2004).

Sonoporation enhances cell permeability via the application of ultrasound mainly using sinusoidal probes at megahertz frequencies (Huber et al., 1999). Unlike electroporation, which moves DNA along the electric field, ultrasound creates membrane pores and facilitates intracellular gene transfer through passive diffusion of DNA across the membrane pores (Kim et al., 1996). The transfection efficiency of this system is determined by several factors, including the frequency, the output strength of the ultrasound applied, the duration of ultrasound treatment and the amount of plasmid DNA used. So far, the major problem for ultrasound-facilitated gene delivery is low gene delivery efficiency.

Hydrodynamic gene delivery is a simple method that introduces naked plasmid DNA into cells in highly perfused internal organs (e.g. the liver) with an impressive efficiency (Liu et al., 1999). The gene delivery efficiency is determined by the anatomic structure of the organ, the injection volume, and the speed of injection. As a result, the injection induces a flow of DNA solution in retrograde into the liver, a rapid rise of intrahepatic pressure, liver expansion and reversible disruption of the liver fenestrae (Zhang et al., 2004). The efficiency of this simple method is the highest so far and was achieved *in vivo* using non-viral approaches and it allows direct transfer of any water-soluble compounds, small colloidal particles (molecular assembly), or viral particles into cytoplasm without endocytosis.

#### 1.1.2.2. Chemical approaches

By far the most frequently studied strategy for non-viral gene delivery is the condensation of DNA into the nanoparticles by using cationic lipids or cationic polymers. The DNA-containing particles are subsequently taken up by cells via endocytosis, macropinocytosis, or phagocytosis. Small fractions of the DNA are released from intracellular vesicles into the cytoplasm and thus migrate into the nucleus, where transgene expression takes place. Chemical delivery vectors are usually safer than viral vectors, but still could have some acute immune response (Gao et al., 2007). Moreover, the cost of their production and handling is low. Practically, cationic lipids, cationic polymers, and other naturally occurring compounds have proven to be extremely effective for *in vitro* gene

delivery. However, all of the cationic-based systems mainly have failed in clinical trials because of low delivery efficiency and high toxicity, causing significant aggregation in biological matrices full of negatively charged molecules, and preventing effective release of DNA from intracellular vesicles inside the cells.

#### 1.1.2.2.1. Cationic lipids

The efficient transfection of eukaryotic cells using cationic liposomes was first described in 1987 by Felgner et al. (Felgner et al., 1987). These cationic liposomes, composed of a cationic lipid (N-trimethylammoniumchloride, DOTMA) and a natural neutral phospholipid (dioleoyl phosphatidylethanolamine, DOPE) in a ratio 1:1 (v/v), were shown to bind DNA efficiently, leading to cellular uptake of plasmid DNA and to high levels of transgene expression. Many synthetic amphiphiles have been synthesized since then that present the common features of vesicles forming in aqueous solutions, DNA binding, and more or less efficient gene transfer, reviewed in (Audouy et al., 2002). These lipids differ by the number of charges in their hydrophilic residue and by the detailed structure of their hydrophobic moiety. Although some cationic lipids alone exhibit good transfection activity, they are often formulated with a noncharged phospholipid or cholesterol as a helper lipid to form liposomes. Upon mixing with cationic liposomes, pDNA is condensed into small quasi-stable particles called lipoplexes. Lipoplexes are able to mediate cellular uptake and facilitate the release of DNA from endo-lysosomal compartments in the absence of serum. The transfection efficiency of such complexes is affected by the chemical structure of the cationic lipid, the charge ratio between the cationic lipid and the DNA, the structure and proportion of the helper lipid in the complexes, the size and structure of the liposomes, the concentration of the lipoplexes and the cell type. Unfortunately, these vectors are not suitable for *in vivo* applications, due to their sensitivity to proteins from serum (Liu et al., 2003) and cytotoxicity (Scheule et al., 1997).

#### 1.1.2.2.1. Cationic polymers

Synthetic and naturally obtained, linear or branched cationic polymers represent another category of delivery vectors. Poly-L-lysine is one of the first group used in gene transfection discovered in 1988 by George Wu (Wu and Wu, 1988). It can bind DNA in strong non-covalent and non-damaging interaction. Since that time the large range of cationic polymers have been investigated as delivery vector for gene transfer: the most studied are polyethylenimine (PEI) (Boussif et al., 1995), polyamidoamine (Haensler and Szoka, 1993) and polypropylamine dendrimers (Schatzlein et al., 2005), polyallylamine, cationic dextran (Hosseinkhani et al., 2004), chitosan (Erbacher et al., 1998), cationic proteins (polylysine, protamine, and histones) (Balicki and Beutler, 1997) and cationic peptides (Park et al., 2003). Although most cationic polymers share the function

of condensing DNA into small particles, so called polyplexes and facilitating cellular uptake via endocytosis, their transfection activity and toxicity differ significantly.

PEI is probably the most widely used polymer in gene therapy. There are two types of PEI polymers, either linear (LPEI), made by hydrolysis of poly(2-ethyl-2-oxazolium), or branched (BPEI), synthesized from aziridine ring opening reaction, which differ only in the degree and in the size of branch (Boussif et al., 1995). For transfections which employ cationic polymers, DNA:PEI ratios, the molecular weight and configuration of PEI, the concentration of polyplex particles and the ionic strength of the solvent used for polyplex preparation are important factors that determine the physical properties of the DNA/PEI complexes and their transfection activity.

The major disadvantage of PEI usage as a transfection vector is its non-biodegradable nature, and the toxicity and transfection efficacy depends on molecular weight (Fischer et al., 2003). The most active PEI from a commercial source is 25 kDa for BPEI and 22 kDa for LPEI. The polymers larger that 25 kDa is also active, but usually demonstrate high toxicity.

##### 1.1.2.2.2. Lipid-polymer hydrid systems

The reported lipid-polymer hybrid systems, which include DNA precondensed with polycations, then coated with either cationic liposomes (Gao and Huang, 1996), anionic liposomes (Lee and Huang, 1997), or amphiphilic polymers with or without helper lipids (Lee et al., 2006), can be used for gene transfection. DNA molecules are better protected in these lipid-wrapping polyplexes. The three-part system appears to be more efficient in transfection than lipid-DNA complexes *in vitro* (Gao and Huang, 1996) and is equally active *in vivo* (Lee and Huang, 1997). The cytotoxicity of such nanoparticles is reduced, making the receptor-mediated targeting possible without interference of nonspecific charge-charge interaction. The main drawback of this approach is that it typically involves tedious procedures of chemical synthesis and purification. Careful evaluations of the stability, safety and efficacy of the drug derivative are also required, which are not easy tasks in themselves (Wong et al., 2007).

#### 1.1.2.3. Cell-penetrating peptides

The research field of cell-penetrating peptides (CPPs) has increased rapidly in the last ten years. CPPs represent one of the most promising non-toxic tool for the delivery of biologically active molecules into cells and therefore play a key role in future development of therapeutics (Järver and Langel, 2004). CPPs exhibit the efficient intracellular delivery of various biomolecules, including plasmid DNA (Futaki et al., 2001a), oligonucleotides (Mäe et al., 2009), siRNA (El Andaloussi et al., 2011), PNA (El-Andaloussi et al., 2006, El-Andaloussi et al., 2007) and even proteins (Temsamani and Vidal, 2004), as well as liposome

nanoparticles (Sawant and Torchilin, 2011), into cells and tissues. Today there are more than hundred known CPPs, which can be exploited as delivery vectors for different cargos.

**Table 2**. Classification of CPPs

| CPP | Sequence | Origin | Reference |
|---|---|---|---|
| ***Protein derived*** | | | |
| **Penetratin** | RQIKIWFQNRRMKWKK[a] | *Drosophila* homeo-protein Antennapedia | (Derossi et al., 1994) |
| **Tat (48–60)** | GRKKRRQRRRPPQ | Human immuno-deficiency virus type 1 (HIV-1) Tat | (Vives et al., 1997) |
| **pVec** | LLIILRRRIRKQAHAHSK[a] | VE-cadherin | (Elmquist et al., 2001) |
| **VP22** | NAKTRRHERRRKLAIER | Herpes simplex virus | (Elliott and O'Hare, 1997) |
| ***Chimeric*** | | | |
| **MPG** | [c]GALFLGFLGAAGSTMGA WSQPKKKRKV[b] | HIV gp41 and SV40 T-antigen | (Morris et al., 1997) |
| **Pep-1** | KETWWETWWTEWSQPK KKRKV[b] | NLS from SV40 large T-antigen and reverse transcriptase of HIV | (Morris et al., 2001) |
| **Transportan /TP10** | GWTLNSAGYLLGKINLKA LAALAKKIL[a]/ AGYLLGKINLKALAALAK KIL[a] | Galanin and mastoparan | (Pooga et al., 1998a), (Soomets et al., 2000) |
| **M918** | [c]MVTVLFRRLRIRRACGPP RVRV[a] | The tumor suppressor protein p14ARF | (El-Andaloussi et al., 2007) |
| ***Synthetic*** | | | |
| **MAP** | KLALKLALKALKAALKLA[a] | Amphipatic model peptide | (Oehlke et al., 1998) |
| **Oligoarginine** | $(R)_n$[d] | Positively charged sequence | (Mitchell et al., 2000) |
| **CADY** | GLWRALWRLLRSLWRLL WRA[b] | Derived from PPTG1, W-rich peptide | (Crombez et al., 2009) |

Amide [a] , cysteamide modification [b], acetyl modification[c], n=6–12[d]

By definition, CPPs are short cationic and/or amphipatic peptides, usually 5–40 amino acids in length. They commonly have positive net charge and together with covalently or non-covalently conjugated bioactive cargo are able to translocate across the plasma membrane by means of different mechanism, mainly

including endocytosis. In contrary to other delivery vectors, CPPs are associated with low toxicity and high delivery efficacy, therefore being promising candidates for clinical trials.

In 1994 the group of Alain Prochiantz reported the peptide with cell-penetrating properties. The first presented CPP, 16-mer peptide penetratin (also named Antp) was derived from the third helix of the *Drosophila melanogaster* antennapedia transcription factor homeodomain (amino acids 43–58) (Derossi et al., 1994). During the past years several novel CPPs have been discovered and developed and some of them are presented in **Table 2**.

CPPs can be divided into several subgroups, based on their origin and sequence characteristics: protein derived, chimeric and synthetic/designed (Lindgren and Langel, 2011). The difference between chimeric and synthetic CPPs is that the core sequences in chimeric peptides partly have sequences from naturally occurring proteins. In case of synthetic peptides, the sequence of CPPs is completely designed for intracellular delivery.

Another classification system of CPPs is based on ability of CPPs to associate with lipid membranes (Ziegler, 2008). According to this definition, the CPPs can be divided into three subgroups: primary amphipatic, secondary amphipatic and non-amphipatic. Primary amphipatic peptides are able to bind both neutral and anionic lipids with high affinity, like TP10 and MPG. Secondary amphipatic peptides have poor affinity to neutral membranes (e.g. pAntp, MAP) and non-amphipatic peptides usually do not associate with membrane because they do not have hydrophobic domains (Tat, oligoarginine).

#### 1.1.2.3.1. Transportan and their deletion analogues

Transportan (TP), a 27 amino acids-long galanin-based chimeric peptide, was designed and synthesized for the first time by Ülo Langel and co-workers (Pooga et al., 1998a) as galparan analog (Langel et al., 1996). Transportan contains 12 functional amino acids from the highly conserved N-terminal part of the neuropeptide galanin and 14-mer wasp venom peptide toxin, mastoparan at *C*-terminus, which are connected via a lysine. The *N*-terminal part of transportan is GalR ligand with agonist properties (Land et al., 1991, Juréus and Langel, 1996). In *C*-terminus located mastoparan is known to increase the GTPase activity and the rate of nucleotide binding of several GTP-binding regulatory proteins (G proteins) whose function is to couple cell-surface receptors to intracellular mediators (Higashijima et al., 1988). Negatively charged phosphatidylglycerol remarkably enhance the mastoparan-lipid interactions and as a result, mastoparan penetrates the cell membrane by creating short-living pores in the lipid bilayer and translocating into the inner leaflet (Matsuzaki et al., 1996). Transportan penetrates into the cells in rapid and efficient way. It localizes mostly in intracellular endolysosomal compartments and conveys into the nuclei, where it concentrates in the nucleoli and at moderate concentrations. TP is not toxic to the cells. TP has been shown to deliver antisense PNAs into the cell (Pooga et al., 1998b). TP shows an inhibitory effect on basal GTPase

activity in Bowes melanoma cell membranes, which is probably caused by the mastoparan part of the peptide. Although the inhibitory effect of TP is detectable at higher concentrations of peptide vector than commonly used for delivery experiments, this feature could be a drawback for carrier peptide. Therefore studies on the structural requirements for the cell membrane penetrating activity of TP have been performed (Soomets et al., 2000). As a result, shorter TP analogue, TP10 has been indentified. In this deletion analogue six amino acid residues from TP's *N*-terminal part are omitted missing while mastoparan part remains intact. TP10 retains the efficient internalization properties of the parent peptide and also does not modulate the basal GTPase activity at high concentrations nor is it recognized by GalR features which recommend TP10 as a promising delivery vector.

## 1.2. CPP mediated cargo delivery

In general, delivery systems based on CPPs can be classified into two major categories: non-covalent complex formation, in which negatively charged DNA or RNA molecules are simply co-incubated with cationic CPP, or covalent conjugation in which bioactive molecule coupled with CPP via covalent bound, for example, a disulfide bridge.

The non-covalent strategy was firstly introduced by group of Heitz and Givita in 1997 (Simeoni et al., 2003) and it is effective only for negatively charged cargos, because nanocomplexes are formed with cationic CPP molecules via electrostatic or hydrophobic interactions. The main advantage of this method is its simplicity, considering that only mixing of CPPs and cargo molecules is required for condensation of CPP:cargo nanoparticles, which is followed by addition to the cell culture.

Since the CPPs are usually exploited in a charge or molar ratios compared to negatively charged cargo in order to neutralize them, different ratios yielded complexes of various sizes. This could be the disadvantage in therapeutic applications the requirement of defined size of nanocomplexes (Oehlke et al., 2002, Morris et al., 1999).

In case of covalent conjugation, one molecule of CPP is conjugated to one molecule of bioactive cargo in order to get pre-defined compound. The advantage of this strategy is that it is suitable for therapeutic applications, the final conjugate can be confirmed by mass-spectrometry analysis and low peptide concentrations are needed for conjugation, which usually is associated with low cytotoxicity. The drawbacks of this strategy are that the preparation of covalent conjugates is difficult, more expensive and laborious and it is compatible only with uncharged nucleic acids, like PNA or PMO. The methods used for conjugation of CPP molecule with cargo depends on nature of cargo. Direct chemical synthesis can be exploited for the preparation of CPP and other peptide-based bioactive molecule conjugates, like apoptotic peptides (Jones and Howl, 2011).

Another widely utilized technique for covalent conjugation is disulfide bond linkage, which demonstrates the applicability of this system to a wide variety of cargoes, for example, for PNA conjugation (Pooga et al., 1998b). This involves the introduction of a free thiol group into both the cargo and vector molecule and subsequent oxidation of them in order to form a covalent disulfide bridge.

### 1.2.1. Splice-correcting oligonucleotides

Oligonucleotides, negatively charged as well as uncharged ON-analogs, have been exhibited promising therapeutic potential in regulation of gene expression. All these molecules share the common feature of being impermeable over plasma membrane and therefore require efficient delivery vehicle to be delivered into the cell. Therefore, CPPs have been extensively used for delivery of SCOs and their analogs, as PNAs, PMOs and 2'-OMe RNAs (El-Andaloussi et al., 2006, Abes et al., 2007a, Moulton et al., 2007).

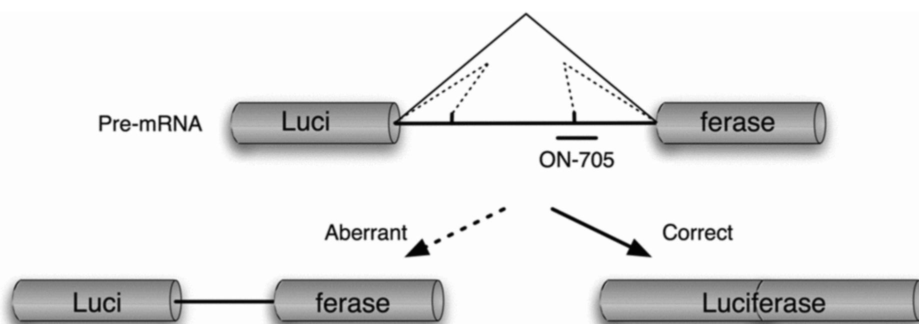


**Figure 1** . HeLa pLuc 705 reporter system. Luciferase pre-mRNA was modified with insertion of β-globin intron 2 carrying a point mutation at nucleotide 705. Blockage of this site with antisense ON redirects splicing towards the functional mRNA (Guterstam et al., 2008).

In 1998 Kole and his co-workers have been developed the functional splice-correction assay for characterization of cellular delivery efficiency of oligonucleotides by transport vectors. This assay is based on Hela pLuc 705 cell line, which is HeLa cell line stably transfected with the recombinant plasmid (pLuc/705) (Kang et al., 1998). This plasmid carries the luciferase gene, interrupted by a mutated human beta-globin intron 2. This intron mutation cause

aberrant splicing of the luciferase pre-mRNA, thus resulting in a non-functional mRNA not able to translate into the luciferase enzyme. However, upon treatment of the cells with an ON targeted to the mutation site this aberrant splicing can be corrected, leading to accurate pre-mRNA splicing and a functional luciferase enzyme **(Fig. 1)**. These cells, referred to as HeLa pLuc 705 cells, provide a positive read-out assay in order to quantify ON delivery into the nucleolus by various delivery vectors.

Using this assay the delivery efficiency of different CPPs was analyzed during the last decade and the CPP-mediated ON delivery is overviewed in **Table 3**.

**Table 3**. Examples of CPP-mediated SCO delivery

| CPP | Cargo | Targeted RNA | References |
|---|---|---|---|
| Tat, Pen | 2'-OMe RNA | Luciferase | (Astriab-Fisher et al., 2002) |
| Tat | PMO | Luciferase | (Moulton et al., 2003) |
| Tat, TP, Pen | PNA | Luciferase | (El-Andaloussi et al., 2006) |
| M918 | PNA | Luciferase | (El-Andaloussi et al., 2007) |
| MAP | PNA | Luciferase | (Wolf et al., 2006) |
| $(RXR)_4$ | PMO | Dystrophin | (Moulton et al., 2007), (McClorey et al., 2006) |
| Pip2 | PNA | Luciferase, Dystrophin | (Ivanova et al., 2008) |
| R6-penetratin | PNA | Luciferase | (Abes et al., 2007b) |
| Stearyl-TP10 | 2'-OMe RNA | Luciferase | (Mäe et al., 2009), Paper I |
| Stearyl-(RXR)4 | 2'-OMe RNA | Luciferase | (Lehto et al., 2010), Paper II |
| PF14 | 2'-OMe RNA | Luciferase | (Ezzat et al., 2011) |

As the starting point, several classic CPPs like Tat or Penetratin have been chosen for ON delivery (Astriab-Fisher et al., 2002). Mainly covalent conjugation strategy has been utilized to bind CPP with ON, while later non-covalent co-incubation strategy has been found to be more potent for this application (Lehto et al., 2010, Mäe et al., 2009). The non-covalent co-incubation strategy to form stable CPP:ON nanocomplexes is a promising approach for future application compared with covalent conjugations of the moieties due to the simplicity, low cost and small quantities that has to be used.

### 1.2.2. Plasmids

Cell-penetrating peptides have been actively exploited as delivery vectors for pDNA transfection, but in most cases the delivery efficacy was quite low compared to viral vectors (Rittner et al., 2002, Kilk et al., 2005, Ignatovich et al., 2003, Liu et al., 2005), probably due to endosomal entrapment or inadequate CPP:pDNA complex formation. To overcome these problems in order to achieve the efficient transfection, so some CPP modifications or novel synthetic CPPs are necessary. Fatty acid modification with stearic acid (stearylation) of well-known CPPs like TP10, Arg9 and $(RXR)_4$ has shown to improve significantly endosomal escape and stable CPP:pDNA nanocomplexes formation and as a result the pDNA transfection efficiency was enhanced (Futaki et al., 2001a, Lehto et al., 2010, Paper II).

Short overview of CPP-mediated pDNA cellular delivery is presented in **Table 4**.

**Table 4**. Examples of CPP-mediated pDNA transfection

| CPP | Biological effect | References |
|---|---|---|
| **KALA** | Luciferase expression | (Wyman et al., 1997) |
| **MPG** | Luciferase expression and cell arrest | (Morris et al., 1999) |
| **Stearyl-Arg9** | Luciferase expression | (Futaki et al., 2001a) |
| **ppTG1, ppTG20** | Luciferase expression | Rittner, 2002 |
| **Poly-Tat** | Luciferase expression | (Liu et al., 2005) |
| **Stearyl-$(RXR)_4$** | Luciferase and EGFP expression | (Lehto et al., 2010), Paper II |
| **Stearyl-TP10** | Luciferase and EGFP expression | (Lehto et al., 2011) |
| **Stearyl-NLS-Arg8** | Luciferase expression | (Wang et al.) |
| **Tat** | Luciferase and EGFP expression | (Saleh et al., 2010) |

In conclusion, CPPs can condensate plasmids into stable nanoparticles and deliver pDNA into the cell for gene expression *in vitro* and *in vivo* applications. But the delivery efficiency of CPPs is less efficient compared with the delivery provided by viral vectors. In order to enhance the transfection efficiency of plasmids mediated by CPPs, the properties of CPP vectors should be significantly improved.

## 1.3. Endosomal escape

Successful uptake of CPP-cargo complexes does not assure that the cargo reached the target inside the cell, nucleus or cytosol. The main problem of cellular internalization, performed by endocytic pathways is endosomal entrapment in intracellular compartments (Lundin et al., 2008). Therefore, endosomal entrapment is the main limitation for intracellular delivery by CPPs. To overcome this obstacle, different strategies can be exploited to disturb the endosomal membrane, for example, to design delivery vectors that contain molecules with endosomotropic properties like viral fusogenic peptide, polymers, fusogenic lipids or membrane-disruptive peptides (Wadia et al., 2004, El-Sayed et al., 2009, Nakase et al., 2009, Varkouhi et al., 2011, Endoh and Ohtsuki, 2009).

Viral vectors, taken up by endocytosis, have developed specific peptides, also named fusion peptides, which promote escaping from endosomes. The same strategy also has been used in the field of CPPs. The 20 amino acids sequence from the N-terminal part of the influenza protein hemagglutinin (HA2) is known to promote endosomal escape upon acidification. At pH 5, the peptide is able to change conformation, with a subsequent insertion into the endosomal membrane, which results in disruption of membrane and escape the CPP particles into cytosol (Wadia et al., 2004). Histidine-rich motifs have been also utilized for endosomolysis (Lundberg et al., 2007) by being protonated at low pH.

Chloroquine is a weak base that prohibits maturation of endosomes, thereby giving CPP-cargo opportunity to escape to the cytosol. Improved cellular response to cargo activities upon chloroquine treatment has been reported frequently (Wolf et al., 2006, Shiraishi et al., 2005). Unfortunately chloroquine is toxic at higher concentrations and is not suitable for *in vivo* applications.

Stearyl acid modification of CPPs has also shown to improve endosomal escape of different CPP:cargo systems: pDNA (Futaki et al., 2001b, Khalil et al., 2004, Lehto et al., 2010), SCOs ((Mäe et al., 2009, Lehto et al., 2010, Ezzat et al., 2011, Oskolkov et al., 2011) and siRNAs (El Andaloussi et al., 2011, Nakamura et al., 2007, Tonges et al., 2006).

## 1.4. Uptake mechanisms of CPPs

Although CPPs have been extensively used as highly efficient delivery vectors, the internalization mechanism is still under debates. The new studies in this field of research have been done by many research groups in order to understand how CPPs enter into the cell interior. It is known that there are two pathways that cell-penetrating peptides can exploit for internalization of cell membrane: endocytic pathways (active) or direct penetration (passive). Passive transport does not require the metabolic energy or the help of transport protein, but depends on the permeability of the cell membrane. The active pathways require the cellular energy to translocate bioactive materials over the plasma

membrane against their concentration gradient. It is important to know the uptake mechanism in order to rationally design novel more efficient delivery vectors or to improve well-known CPP for delivering the cargoes into specific intracellular compartments. The primary internalization studies were done only by using fluorescent-labeled peptide for evaluation of CPP uptake into the cell without considering biological activity or delivery efficiency of CPP vector. As a result, it was shown that uptake process was not inhibited at +4°C or by using any endocytosis inhibitors (Vives et al., 1997) which means that CPP internalization mechanism is fully energy-independent.

Most of the large and polar macromolecules can enter cells via energy-dependent and receptor-dependent or receptor-independent process known as endocytosis. There are two different endocytic mechanisms: phagocytosis and pinocytosis. Phagocytosis is highly regulated actin-mediated process, which is specialized in ingestion of large molecules (0.1–10 μm) like bacteria, cell debris or solid particles. Pinocytosis mediates the uptake of fluids, smaller particles (50–1000 nm) and membrane components and is carried out practically by all cells (Conner and Schmid, 2003). At least four different types of pinocytosis can be distinguished: clathrin-and caveolae-mediated endocytosis, macropinocytosis and clathrin-and caveolae-independent endocytosis, which are extensively described by Conner (Conner and Schmid, 2003). However, direct penetration cannot be excluded. The choice of uptake mechanism depends on structure and nature of CPP or cargo, concentration, cargo conjugation strategy, cell lines, etc.

# 2. AIMS OF THE STUDY

The thesis is focused on intracellular delivery of nucleic acids (RNA, pDNA) mediated by cell-penetrating peptides.

**Paper I–II**
Assessment of well-known CPPs with N-terminal stearylation in cellular delivery of different nucleic acids by using non-covalent co-incubation strategy and comparison with commercially available transfection reagent Lipofectamine$^{TM}$2000.

**Paper III**
Rational design of novel CPPs vectors based on stearylated TP10 for intracellular delivery of modified splice-correcting oligonucleotides using non-covalent co-incubation strategy.

# 3. METHODOLOGICAL CONSIDERATIONS

The methods and materials used in this thesis are described in each paper in details, therefore only some theoretical and practical aspects will be discussed here

## 3.1. Cell-penetrating peptides: design and choice

Several peptides were utilized in this thesis, some of them are well-known cell-penetrating peptides, like oligoarginine or transportan10 and others are newly designed peptides **(Table 5)**.

In paper I–II, several well-established CPPs were utilized as delivery vector for splice-correcting oligonucleotides: oligoarginine, penetratin, $(RXR)_4$, MPG and transportan10. The main problem for CPPs taken up by endocytic pathways is the escape from endolysosomal compartments. In order to promote more efficient endosomal escape we introduced two modifications to the peptides – N-terminal stearylation and C-terminal cysteamidation. Newly designed peptides as well as unmodified peptides were exploited as delivery vectors for intracellular delivery of SCO, PMO and plasmids.

In previous studies was shown, that addition of stearyl group to N-terminal position of TP10 (PF3) significantly increased the endosomal escape of peptide:ON complexes from intracellular compartments. In paper III we designed new peptide vectors, named NickFects, in order to enhance the interaction between peptide and plasma membrane, to improve physicochemical properties of stearylated TP10 peptide (average hydrophilicity, iso-electric point, ON:peptide non-covalent complex formation) and to render peptide vector pH-sensitive. By the replacement of Ile8 by a more hydrophilic amino acid Thr, an amino acid that is similar to isoleucine, in the middle of peptide sequence we still preserved the stearylated-TP10 idea, while increasing the hydrophilicity of peptide. The introduction of phosphoryl group to Tyr3 or Thr8 in stearylated TP10 (PF3) sequence further increased hydrophilicity, reduced the overall charge and led to a pH-dependent peptide vector.

**Table 5**. Sequences of cell-penetrating peptides and 2'-OMe RNA used in this thesis

| **Names** | **Sequence** | **References** |
|---|---|---|
| Penetratin, Pen | [a]RQIKIWFQNRRMKWKK-$NH_2$ | (Derossi et al., 1994) |
| Transportan 10, TP10 | AGYLLGKINLKALAALAKKIL-$NH_2$ | (Pooga et al., 1998a, Soomets et al., 2000) |
| PepFect3, stearyl-TP10,PF3 | Stearyl-AGYLLGKINLKALAALAKKIL-$NH_2$ | (Mäe et al., 2009)I |
| Oligoarginine, Arg9 | RRRRRRRRR-$NH_2$ | (Mitchell et al., 2000) |
| MPG | [c]GALFLGFLGAAGSTMGAWSQP KKKRKV[b] | (Morris et al., 1997) |
| $(RXR)_4$ | RXRRXRRXRRXR-$NH_2$ | (Rothbard et al., 2002) |
| Stearyl-$(RXR)_4$ | Stearyl-RXRRXRRXRRXR-$NH_2$ | (Lehto et al., 2010) |
| Stearyl-Arg9 | Stearyl-RRRRRRRRR-$NH_2$ | (Futaki et al., 2001a) |
| phospho-PepFect3, p-PF3 | Stearyl-AGY$(PO_3)$LLGKINLKALAALAK KIL-$NH_2$ | (Oskolkov et al., 2011) |
| NickFect1, NF1 | Stearyl-AGY$(PO_3)$LLGKTNLKALAALAK KIL-$NH_2$ | (Oskolkov et al., 2011) |
| NickFect2, NF2 | Stearyl-AGYLLGKT$(PO_3)$NLKALAALAK KIL-$NH_2$ | (Oskolkov et al., 2011) |
| NickFect3, NF3 | Stearyl-AGY$(PO_3)$LLGKT$(PO_3)$NLKALA ALAKKIL-$NH_2$ | (Oskolkov et al., 2011) |
| NickFect11, NF11 | Stearyl-AGYLLGKTNLKALAALAKKIL-$NH_2$ | (Oskolkov et al., 2011, Abes et al., 2008) |
| $PMO_{705}$ | XB-CCT CTT ACC TCA GTT ACA-3′ | (Abes et al., 2008) |
| Phosphorothioate 2′-OMe RNA, 2′-OMe ON | [d]5′-CCU CUU ACC UCA GUU ACA | (Guterstam et al., 2008) |

.[a] N–terminal stearyl-modification
[c] N–terminal acetyl-modification
B – β-alanine
X – aminohexanoic acid (Ahx)
. [b] C–terminal cysteamide modification
[d] Cy5 labeled

$(PO_3)$ – phosphoryl group $(PO_3^{2-})$ on either Thr or Tyr

## 3.2. Peptide and ON synthesis

### 3.2.1. Solid-phase peptide synthesis (SPPS)

All peptides used in this thesis were synthesized using solid-phase peptide synthesis technique, which was introduced in 1963 by Bruce Merrifield (Merrifield, 1963). SPPS is based on the repeated cycles of amino acid couplings and deprotection of protected amino acids, carried out on a solid polymer. Amino acid side chains are protected, thus ensuring that the activated carboxylic acid is only reacting with the α-amino group. However, if the α-amino group was free the amino acids not yet coupled to the peptide could react with each other. Therefore, the α-amino group is protected with either tert-Butyloxycarbonyl (t-Boc) or 9-fluorenylmethyl-oxycarbonyl (Fmoc), depending on which type of chemistry is applied. This protective group is removed before every new amino acid is coupled, while the side chain protective groups are present until the final cleavage of the peptide from the resin is performed.

In these studies all peptides were synthesized in stepwise manner at 0.1 mmol scale on an automated peptide synthesizer using Fmoc (fluorenylmethyloxycarbonyl) solid-phase peptide synthesis strategy (Fields and Noble, 1990) with Rink-amide MBHA (methylbenzylhydrylamine) resin or cysteamine-2-chlorotrityl resin as solid phase to obtain C-terminally amidated peptides and cysteamide modified peptides, respectively. The stearic acid was coupled manually to the N-terminus of the peptide by treatment of peptidyl-resins with 5 eq. stearic acid, 3 eq. HOBt and 3 eq. HBTU, 6 eq. DIEA in dimethylformamide/dichloromethane (1:1) overnight at room temperature. For synthesis of phosphorylated peptides phosphothreonine Fmoc-Thr(PO(OBzl)OH)-OH (Fluka, Germany) and phosphotyrosine Fmoc-Tyr(PO(OBzl)OH)-OH (Merck, Germany) monomers were used and the coupling was carried out manually by treatment of peptidyl-resin with 3 eq. of a phosphomonomer, 3 eq. HOBt and 3eq. HBTU, 6 eq. DIEA for 3 h at room temperature in DCM/DMF/NMP/DMSO (3:3:3:1; v:v:v:v) mixture to increase the yield. C-terminally cysteamide modified TP10 was assembled by Fmoc chemistry using cysteamine-2 chlorotrityl resin (CBL Patras, Greece). The final cleavage was performed using standard protocol (95% TFA/2.5% TIS/2.5% $H_2O$). The crude products were precipitated using diethyl ether and then freeze dried.

### 3.2.2. Purification and identification of synthesized peptides

Peptides were purified by using preparative reversed phase high pressure liquid chromatography (RP-HPLC) with C18 or C4 column and a 5–100% acetonitrile/water gradient containing 0.1% TFA. The fraction containing the correct product was identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using α-cyano-hydroxycinnamic acid as matrix.

### 3.2.3. Oligonucleotide synthesis

Phosphorothioate 2′-O-methyl RNA oligonucleotides **(Fig. 2)** were synthesized on an ÄKTA™ oligopilot™ plus 10 synthesizer with Oligosynt™ 15 (GE Healthcare, Sweden) using disposable pre-packed synthesis columns with polystyrene-based solid support, functionalized for synthesis of ON sequences with 2'-O-Methyl RNA monomers at the 3' –end. 5'-labelling was carried out using Cy5 amidite (Guterstam et al., 2008). For 5′-labeling, a molar excess of 10 equivalents Cy5 amidite (Amersham Biosciences, USA) at 0.1M was used and Cy5 amidite recycling went on for 10 min. Crude oligonucleotide was purified by anion exchange chromatography (AEC), desalted and freeze dried. The molarity of ONs was determined by OD-measurements.

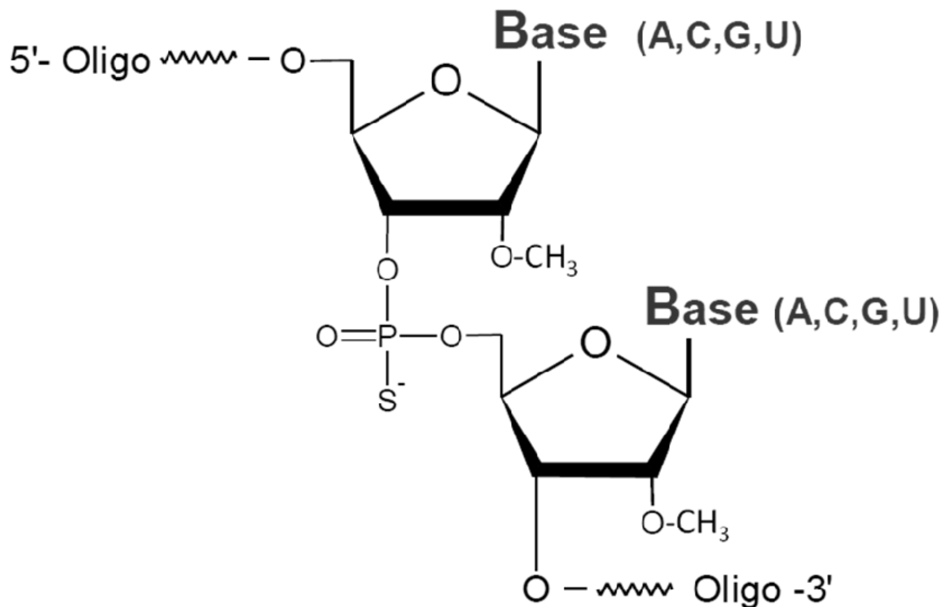


**Figure 2**. Phosphorothioate linkage

## 3.3. Complex formation between CPPs and nucleic acid

### 3.3.1. Non-covalent and covalent strategies for cargo attachment

There are two major strategies for conjugation of CPPs to different cargos: covalent coupling, where cargo and CPP are conjugated via covalent bond, or non-covalent complexation, where the cargo is simply co-incubated with the CPP.

In case of covalent conjugation the nucleic acid polymer (PNA, PMO) is usually coupled to the cell-penetrating peptide using a reducible disulfide bridge. The rationale behind this technique is that the intracellular glutathione cleaves the disulfide bridge and as a result release the cargo inside the cell. The main advantage of this method is that quite stable CPP-nucleic acid conjugate is obtained; however the strategy is rather laborious, has low synthesis yield and requires extensive purification after each step of synthesis. Synthesis of covalent conjugate was done as described above by S. Abes et al. 2006 (Abes et al., 2006).

The non-covalent co-incubation strategy basically involves a simple mixing of CPP and nucleic acid cargo (plasmid or oligonucleotide) at a specified ratios, charge and molar ratios for plasmids and oligonucleotides, respectively, resulting in the formation of nanoparticle complexes. The principle for the complex formation is based on electrostatic interactions between the polycationic CPPs and the polyanionic nucleic acid polymer. The co-incubation strategy generally needs smaller amounts of material for efficient transfection than covalent conjugation and often nanocomplexes show high level of serum stability. The advantage of this strategy is the simplicity of making nanocomplexes, but peptide:nucleic acid complexes usually are not so stable compared with covalent bound peptide:nucleic acid complexes.

In order to obtain peptide:ON nanocomplexes, 2′-OMe oligonucleotides were mixed with CPPs at different molar ratios (1:3 – 1:10) in $H_2O$ in 10% of the final treatment volume (i.e. 50 µl). Complexes were formed for 1 h at room temperature and meanwhile the cell medium was replaced in 24-well plates to fresh serum free DMEM (450 µl). Thereafter complexes were added to each well. For peptide:plasmid complexes formation, 0.5 µg of luciferase expressing plasmid (pGL3) or enhanced green fluorescent protein expressing plasmid (pEGFP-C1) were mixed with CPPs at different charge ratios (CRs) (1:1–1:5) in $H_2O$ in 10% of the final treatment volume (i.e. 50 µl). CRs were calculated theoretically, taking into account the positive charges of the peptide and negative charges of the plasmid. For instance, final concentration of stearyl-$(RXR)_4$ was 0.38 µM at the 1:1 CR. Complexes were formed for 1 h at room temperature. Meanwhile, cell medium was replaced in 24-well plates for fresh serum-free media (450 µl). For all experiments Lipofectamine™ 2000 was used as positive control. When using Lipofectamine™ 2000 (Invitrogen, USA), the complexes were prepared according to manufacturer's protocol in Opti-MEM medium (Invitrogen, USA).

### 3.3.2. Characterization of nucleic acid condensation

Ethidium bromide (EtBr) is a large molecule which has low fluorescence until it binds to DNA. When exposed to ultraviolet light, it will fluoresce with an orange colour, intensifying almost 20-fold after binding to DNA. In this work, EtBr exclusion assay and agarose electrophoresis stained with EtBr were used

for examination of nanoparticles formation. Briefly, non-covalent CPP:nucleic acid complexes were formed as described above. After 1 h incubation, 135 μl MQ water was added to each sample and transferred into a black 96-well plate (NUNC, Sweden). Thereafter, 15 μl of EtBr solution was added to give a final EtBr concentration of 400nM. After 10 min, fluorescence was measured on a Spectra Max Gemini XS fluorometer (Molecular Devices, Palo Alto, CA, USA) at $\lambda_{ex}$=518 nm and $\lambda_{em}$=605 nm. Results are given as relative fluorescence and a value of 100% is attributed to the fluorescence of naked DNA with EtBr. Stability of plasmid:stearyl-$(RXR)_4$ complexes were evaluated in the presence of serum. Briefly, complexes were formed as described above. Thereafter, serum was added to the complexes at different concentrations (5, 10 and 50%) and incubated over different periods of time. At 0, 1, 4 and 24 h samples were loaded on an agarose gel (2%) and imaged by staining the gel with EtBr (0.5 μg/ml).

The formation of peptide:ON complexes was analyzed using fluorescence measurement of Cy5 labeled ON, previously run in 2% agarose gel in 0.5X TAE buffer for 1 h at 100 V, by Typhoon Variable Mode Imager (Amersham, Sweden) or by electrophoresis on a 6% agarose gel in TBE buffer, containing ethidium bromide (Sigma, Sweden), for 1h at 100V.

## 3.4. Cell culture

Different cell lines have several characteristics that distinguish them, e.g. in their rate of protein expression, proliferation metabolism, etc. Therefore, the choice of cell line is of outmost importance when performing *in vitro* studies to measure peptide uptake and transfection efficacy. In addition, cells were chosen in order to make comparisons of newly designed peptide vectors with previously published data.

### 3.4.1. CHO cells

Chinese hamster ovary (CHO)-K1 cell line is the most commonly used cell line in studies of genetics, toxicity screening, and gene expression. It is a subclone of the parental CHO cell line, which was derived from the ovary of an adult Chinese hamster (Tjio and Puck, 1958); it contains a slightly lower amount of DNA than the original CHO. These cells have been widely used in CPP field.

### 3.4.2. HeLa pLuc 705 cells

HeLa cells are an immortalized cell line derived from a cervical cancer obtained from Henrietta Lacks in 1951. From this time, HeLa cells have been widely used within various research areas, especially in cancer research. The main

desirable features of this cell line are that it grows rapidly and is very robust. HeLa pLuc 705 cell line, which is HeLa cell line stably transfected with the recombinant plasmid (pLuc/705) was mentioned above (Kang et al., 1998).

### 3.4.3. HEK293 cells

Human Embryonic Kidney 293 (HEK293) cell line is a specific cell line originally derived from human embryonic kidney cells grown in tissue culture in early 1970s by transformation of cultures of normal human embryonic kidney cells with sheared adenovirus 5 DNA in Alex Van der Eb's laboratory in Leiden, The Netherlands (Graham and Van Der Eb, 1973). The human embryonic kidney cells were obtained from a healthy aborted fetus and originally cultured by Van der Eb himself, and the transformation by adenovirus was performed by Frank Graham who published his findings in the late 1970s after he left Leiden for McMaster University in Canada (Graham et al., 1977). They are called HEK for human embryonic kidney, while the number 293 comes from Graham's habit of numbering his experiments; the original HEK 293 cell clone was simply the product of his 293rd experiment. HEK 293 cells are very easy to grow and transfect very readily and have been widely-used in cell biology research for many years. They are also used by the biotechnology industry to produce therapeutic proteins and viruses for gene therapy.

### 3.4.4. BHK21 cells

Baby Hamster Kidney fibroblast (BHK21) is an adhesive cell line used in molecular biology. The cells were derived in 1961 by I. A. Macpherson and M. G. P. Stoker (Stoker and Macpherson, 1964). Nowadays, subclone 13 is used, which originally was derived by single-cell isolation from the kidneys of five unsexed, 1-day-old hamsters. The BHK-21 cells are mainly useful for transformations and for stable and temporary transfections.

## 3.5. Characterization of CPP mediated uptake and cargo delivery

Several methods have been introduced during the last decades to characterize the uptake and delivery efficacy of CPPs. The most widely used of them were utilized in this thesis in order to analyze the properties of newly designed CPPs. The most common and applied method to study and quantify the uptake of CPPs is by labeling of one of the component from peptide:cargo complex with fluoresceinyl moiety. Different labeling opportunities can be used, e.g. addition of the dye either in peptide backbone or in nucleic acid polymer. For evaluation

of delivery efficacy of peptide:nucleic acid nanoparticles non-covalent coincubation strategy has been used, due to its simplicity.

### 3.5.1. Quantitative uptake

The cellular uptake quantification method was described in detail by Holm, et al. 2006 (Holm et al., 2006). In short, freshly prepared Cy5-labeled CPP:oligonucleotide complexes at different molar ratios (5:1,:10:1, 20:1) were incubated with the cells. After incubation for 1 h, cells were washed twice with HKR buffer and peptides bound to the cell membrane were removed by trypsination. The cells were lysed in sodium hydroxide solution and centrifugated, in order to eliminate the risk of measuring peptides on the cell membrane. Fluorescence was measured at 635/670 nm on FlexStation II fluorescence reader (Molecular Devices, USA) and the amount of internalized cargo was normalized against the total protein content in the cell lysate using DC protein determination assay (Bio-Rad, USA) for protein concentration measurement. This normalization helps to correct small variances in the amount of cells from different experiment.

Even though the method is straightforward and quantifies the amount of internalized cargo, one should always consider the possible false interpretations of the results. For instance, it is impossible to differ between cytosolic, nuclear, or endosomal localization of the peptide.

### 3.5.2. Confocal microscopy

Fluorescence microscopy is an useful method for localization studies of fluorescein-labeled peptide:cargo complexes inside the cell or in tissue sections. The microscopy studies of CPP:plasmid nanocomplexes were performed on fixed cells, using 4% formaldehyde solution for fixation. However, the fixation step appeared to cause artifacts on the CPP uptake pattern and a reevaluation of the uptake mechanism led the field to use unfixed, live cells. Images were captured using 60-fold objective on Nikon Eclipse TE2000-U inverted microscope and a Digital Camera DXM1200C and processed with EZ-C1 software V.2.30 (Nikon, Japan). Confocal microscopy was used where only light from a specific focal plane is detected. By scanning a focal plane crossing the nucleus of a cell, there will be reduced risk of detecting fluoresceinyl-labeled peptide bound to the outside of the cell membrane.

### 3.5.3. Flow cytometry

Fluorescence-activated cell sorting (FACS) is a technique for counting and examining microscopic particles, such as cells and chromosomes, by sus-

pending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. In this work, HeLa pLuc 705 cells were seeded prior to experiment. Thereafter, cells were washed twice with PBS and detached from the plate by trypsinization. Cells were suspended in ice-cold PBS and centrifuged. Supernatant was removed and the resulting cell pellet was resuspended in ice-cold PBS and 0.05 mg/ml propidium iodide (PI) (Molecular Probes, Eugene, OR, USA) for staining cells with non-intact plasma membrane. Flow cytometry analysis was carried out with a BD FacsCanto flow cytometer using FacsDiva software (BD Biosciences, San Jose, CA, USA).

Population of viable cells was determined from a scatter plot (forward scattered light (FSC) vs. side scattered light (SSC) plot). PI staining was used as a control to verify that plasma membranes of cells in the viable population were intact. A minimum of 10,000 events from the viable cell population per sample were analyzed. The percentage of transfected cells was determined by counting the cells displaying Cy5 fluorescent signal above a threshold level. The uptake was characterized by calculating mean Cy5 fluorescence value for transfected cells and comparing it with the same parameter of untreated cells.

### 3.5.4. Splice correction assay

The splice-correction assay has been widely used to estimate the delivery efficiency of CPPs, providing additional information about intracellular localization of non-covalent conjugates. The splice-correction method developed by Kole and co-workers in 1998 was previously described in this thesis (p. 22). In addition, besides giving information about the delivery efficiency of peptide vector, it also shows the sub-cellular localization of conjugates. In order to perform this assay, the cells were treated with peptide:ON complexes at six different molar ratios (1:1–15:1) at 200 nM oligonucleotide concentration for 4 h in serum-free or serum-containing media, followed by the addition of serum-containing medium and incubated for additional 20 h. Thereafter, the cells were washed with PBS buffer and lysed using 0,1% Triton X-100 in HEPES–Krebs–Ringer (HKR) buffer. Luciferase activity was measured using Promega's luciferase assay system on GLOMAX™ 96 microplate luminometer (Promega, Sweden) according to suggestion by manufacturer and normalized to protein content by using DC protein determination assay (Bio-Rad, USA) for protein concentration measurement. Lipofectamine™ 2000 was used as positive control for measuring transfection efficiency and naked oligonucleotides were used as negative control.

In experiments with sodium 1-naphthyl phosphate monohydrate – N7000, inhibitor was added to the medium 30 min prior to treatment of cells with peptide:ON complexes. 2 h later, medium was removed and replaced with fresh medium in order to avoid toxicity effects of inhibitor. In experiments with chlo-

roquine, chloroquine was added to cells along with peptide:ON complexes in order to promote endosomal escape. 4 h later, medium was replaced with fresh medium in order to avoid toxic effects of chloroquine.

### 3.5.5. Plasmid delivery assay

Transfection of eukaryotic cells utilizing cell-penetrating peptides complexed to plasmid DNA has become an increasingly important model for study and development of intracellular gene delivery. The mechanisms by which CPPs mediate intracellular delivery of plasmid DNA are currently not well understood. However limitations of gene delivery systems like plasmid:peptide complexes have led to the development of potentially more versatile encapsulated DNA particles that exploit the ability of biological membranes to act as protective barriers to exterior nucleases and DNases found in the serum.

In this thesis different cell lines (CHO, BHK 21 and HEK293) were used for transfection with plasmid:peptide nanocomplexes. Briefly, the cells were seeded 24 h before the experiment and then treated with plasmid:CPP complexes at different charge ratios (1:1, 1:2, 1:3, 1:5) for 4 h in serum-free media followed by addition of full growth media and incubated for another 20 h. Thereafter, the cells were washed twice with HKR and lysed using 0.2% Triton X-100 in HKR buffer. Luciferase activity was measured using Promega's luciferase assay system on GLOMAX™ 96 microplate luminometer (Promega, Sweden) and normalized to protein content (Lowry, BioRad, USA). Lipofectamine™ 2000 (Invitrogen, Sweden) was used according to the manufacturer's protocol.

### 3.5.6. Internalization and intracellular localization by transmission electron microscopy

Electron microscopy has a number of benefits over the fluorescence microscopy. First, the resolution of light microscopy is not significant to differ small objects in a detail manner (shape and size of the nanoparticle, membranes of internal structures of the cells. Secondly, the signal of fluorescent-labeled markers and CPP-nucleic acid complexes must be optimized in order to avoid signal cross-talk and background. Therefore, electron microscopy approach provides a higher resolution and magnification than a light microscopy.

In order to characterize the interaction sites and localization of the cargo molecule delivered into the cultured cells by peptide transport vector, the transmission electron microscopy (TEM) has been used. For this purpose 705 pLuc splicing switching oligonucleotide carrying a thiol group at the 5' end was tagged with nanogold (NG) cluster (Monomaleimido Nanogold, Nanoprobes, NY, d 1.4 nm) by forming a covalent bond between the thiol group on oligonucleotide and the maleimide group of label (Padari et al., 2010). After conjugation with nanogold, the mixture was concentrated and the ON-NG conjugate

was purified using Superdex 75 (HR10/30, GE, UK) equilibrated in MilliQ water. For treatment with nanogold-labeled oligonucleotides (NG-ON) and peptide complexes HeLa pLuc 705 cells were seeded onto glass coverslips in 24-well plates, grown to 90–100% confluency, and incubated with the complexes of nanogold-labeled ON (NG-ON) and peptide in serum-free culture medium at 37 °C for 4 h. The complexes were preformed as described above and resulting solution was diluted with serum-free IMDM to the final peptide concentration of 1.4 or 2.8 µM and SCO concentration of 0.2 or 0.4 µM respectively and applied to the cells. After treatment with complexes, the cells were washed 2 times with serum-free IMDM, fixed with 2.5% glutaraldehyde in cacodylate buffer (pH 7.4) at room temperature (RT) for 1 h, and washed three times for five minutes with cacodylate buffer. The nanogold label on ON was revealed as described earlier for peptides (Padari et al., 2010). Finally, the coverslips were dipped into acetone and embedded in epoxy resin (TAAB Laboratories Equipment Ltd., UK). Ultrathin sections were cut in parallel with the coverslip and contrasted with 2% uranyl acetate in 50% ethanol for 2 min and in standard lead citrate staining solution for 2 min. The sections were examined with JEM-100S (JEOL, Tokyo, Japan) transmission electron microscope at 80 kV. The scanned electron microphotos were analyzed and processed with Adobe Photoshop CS4 software.

## 3.6. Toxicity studies

CPPs constitute a new class of delivery vectors with high pharmaceutical potential. However, the abilities of these peptides to translocate through cell membranes can be accompanied by toxic effects resulting from membrane perturbation at higher peptide concentrations (Saar et al., 2005). The cell viability, proliferation and cytotoxicity studies induced by CPPs or by CPP:cargo complexes can be evaluated by using different cell-based assays.. Several methods have been developed to investigate the short term membrane disturbance (eg. LDH leakage assay) or long term effects on cellular proliferation (eg. MTS assay , WST-1 assay).

### 3.6.1. LDH leakage assay

The LDH Leakage assay determines the membrane integrity/extent of LDH leakage from cells with a damaged membrane under the influence of peptide: cargo complexes (Decker and Lohmann-Matthes, 1988, Legrand et al., 1992).

The assay is a fluorometric measure of the release of lactate dehydrogenase (LDH) from cells with a damaged membrane by conversion of resazurin into resorufin that is measured by change in absorbance at 490 nm **(Fig. 3)**. Briefly, cells were seeded 1 day before treatment into 96-well plate. Cells were treated with cargo:CPP complexes at different charge ratios in serum-free media for 30

min. Thereafter, LDH leakage assay was used according to the manufacturer's protocol. Untreated cells were defined as no leakage and 100% leakage was defined as total LDH release by lysing cells in 0.2% Triton X-100 in HKR buffer.

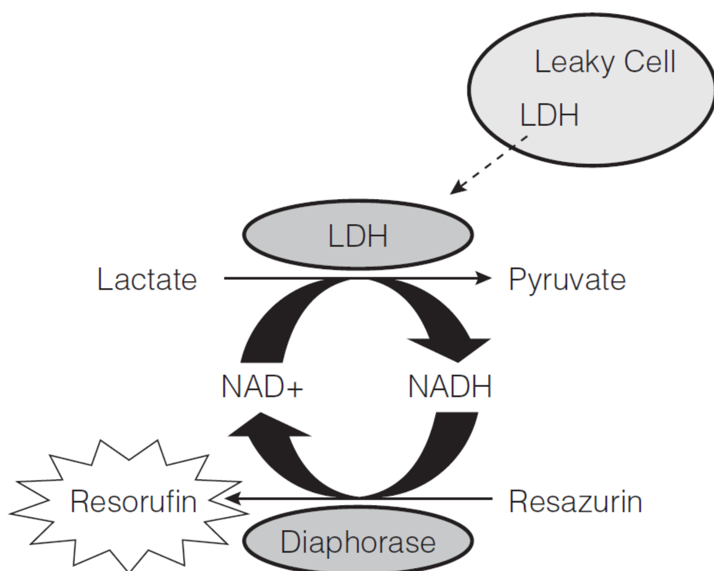


**Figure 3**. Release of LDH from damaged cells is measured by supplying lactate, $NAD^+$, and resazurin as substrates in the presence of diaphorase. Generation of the fluorescent resorufin product is proportional to the amount of LDH.

### 3.6.2. Cell proliferation assay

For investigation of long-term toxicity effects on cells treated with CPP:cargo complexes tetrazolium salt based colorimetric WST-1 or MTS proliferation assay were used. The idea of these assays is to measure the activity of mitochondrial dehydrogenases, which convert tetrazolium salts to formazan in viable cells **(Fig. 4)**.

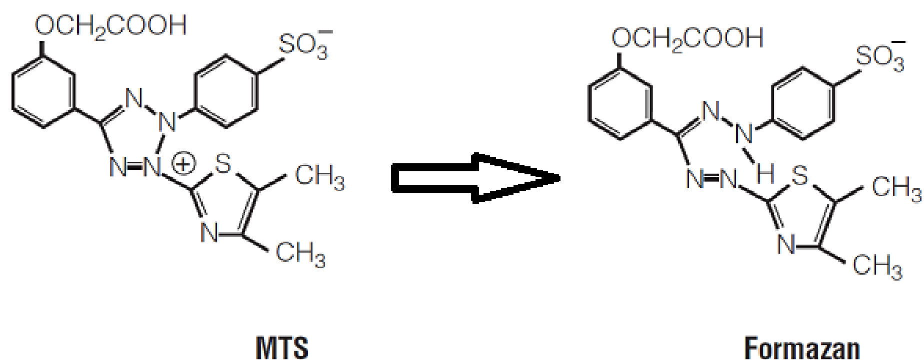


**Figure 4**. Structures of MTS tetrazolium salt and its formazan product

Briefly, cells were seeded 1 day prior to the experiment in complete medium. The cells were treated with peptide:cargo complexes for 4 h in serum-free medium followed by the addition of serum containing medium and incubated for another 20 h. Wst-1 or MTS assays were added according to the manufacturer's protocol. Absorbance was measured on Digiscan absorbance reader at 490 nm (Labvision via AH Diagnostics AB, Sweden). The percentage of viable cells was determined using the GraphPad Prism software 4.0 (GraphPad Software, CA).

## 3.7. DLS and Z potential measurements

Dynamic light scattering, also known as photon correlation spectroscopy or quasi-elastic light scattering is a technique, which can be used to determine the size distribution profile of small particles in suspension or polymers in solution (Pecora, 1964, Urban and Schurtenberger, 1998).

Hydrodynamic mean diameter of the particles resulting from the condensation of the nucleic acid polymer with the different peptides was determined by DLS studies using a Zetasizer Nano ZS apparatus (Malvern Instruments, United Kingdom). Particle formulations of peptide:cargo nanocomplexes, freshly prepared in solution, were obtained using the same protocol as for *in vitro* transfections and diluted in Opti-MEM® to a final volume of 500 µl. All data were converted to "relative by intensity" plots from which the mean hydrodynamic diameter was derived. Measurements were performed with the apparatus set to automode and a number of 5 runs were recorded.

# 4. RESULTS AND DISCUSSION

The articles included in this work demonstrate how the introduction of minor modifications in peptide sequence can improve the delivery properties of well-known cell-penetrating peptides for *in vitro* applications without exhibiting any cytotoxicity. For this purpose non-covalent co-incubation strategy of CPPs:cargo (nucleic acid) has been used. In paper I, well-known CPP TP10 has been modified by introduction of fatty acid residue N-terminally in order to increase intracellular uptake of splice-correcting oligonucleotides *in vitro*. In paper II, several CPPs have been N-terminally modified by stearylation to promote better intracellular delivery of nucleic acid polymers in *in vitro* applications. In paper III, introduction of phosphoryl group and substitution of Ile8 by Thr in stearylated TP10 peptide has been done in order to improve the delivery of splice-correcting phosphorothioate 2'-OMethyl RNA into the cells *in vitro*. In all papers commercially available transfection agent Lipofectamine$^{TM}$2000 has been used as a positive control.

## 4.1.1. Stearylated CPPs for phosphorothioate 2'-OMethyl RNA delivery using non-covalent co-incubation approach (Paper I)

The utilization of CPPs to promote intracellular uptake of various types of ONs holds great promise for future therapeutic applications. During the last decade, mainly covalent binding has been used for conjugation of CPPs and ONs that made CPP:ON conjugate preparation process laborious and time-consuming. Non-covalent co-incubation methods for preparation of CPP:ON complexes, where cationic peptides and the negatively charged ONs are mixed together at certain molar ratios in order to form stable nanoparticle complexes have been initiated (Meade and Dowdy, 2008). Recently, two chemical modifications, stearylation and C-terminal cysteamidation, have been used in order to improve the non-covalent cellular delivery of ONs (Futaki et al., 2001a).

In paper I, we modified three different CPPs – TP10, penetratin and oligoarginine by adding stearyl moiety at N-terminal position and compared their transfection efficiency with unmodified peptides. The commercially available transfection agent Lipofectamine™ 2000 and previously published MPG peptide were used as positive control. First, the transfection efficiency of Cy5-labelled phosphorothioate 2'-OMe RNA with unmodified CPPs TP10, penetratin, and oligoarginine were assessed using splice-correction assay. Although all peptide:ON complexes were taken up by the cells, no significant splice correction was detected, probably because the complexes were entrapped inside endolysosomal compartments. This assumption was confirmed by performing the same splice-correction assay in the presence of lysosomotropic agent, chloroquine, in order to promote endosomal escape. As a result, the splice correction

was significantly increased. N-terminal stearylation of TP10, Pen, and Arg9 did not show any difference between the quantitative uptake of peptides. No significant increase in splice correction was observed for stearyl-Pen and -Arg9 in complex with 2'-OMe ON, but the use of stearyl-TP10 on the other hand, resulted in the discovery of an exceptionally potent transfection agent. Thus, apparently the stearylation does not potentiate all CPPs. Furthermore, it is worth mentioning that correctly spliced luciferase intensity reached levels comparable with results achieved with Lipofectamine™ 2000. Since the cationic lipids are associated with quite severe cellular toxicity we evaluated the effect of stearylated TP10 on cell proliferation. Stearyl-TP10 displayed no long-term toxicity even at the highest CPP:ON ratios, whereas Lipofectamine™ 2000 reduced live-cell population already after 24 h.

Conclusively, our studies showed that stearylation of TP10 results in a non-toxic and highly efficient peptide delivery vector for ON delivery *in vitro* and which is comparable with commercially available transfection agent Lipofectamine™2000, despite not displaying any of the toxicity associated with cationic lipid-based delivery. Interestingly, stearylation did not improve splice correction mediated by Pen and Arg9, leaving their transfection efficiencies on modest levels. The potency in splice-correction observed in the presence of serum with the non-covalent CPP (stearyl-TP10):ON complexes holds great promise for future *in vivo* applications.

## 4.2. Intracellular delivery of splice-correcting oligonucleotides and plasmids using stearyl-$(RXR)_4$ peptide vector (Paper II)

The $(RXR)_4$ peptide has been successfully used in recent years for the delivery of covalently linked SCOs (Abes et al., 2006, Abes et al., 2008), was conjugated to PMO molecule, and has been already exploited in pre-clinical trials (Moulton, 2007). To our knowledge, there are no reports showing that the $(RXR)_4$ peptide would be able to facilitate delivery of plasmids. The aim of this work was to modify $(RXR)_4$ peptide with introduction of a stearyl moiety and to utilize newly designed $(RXR)_4$ analogs for SCOs and plasmids delivery using non-covalent strategy, methodology which was successfully used in the previous research.

Nanoparticle size of plasmid:stearyl-$(RXR)_4$ complexes was around 370–450 nm depending on charge ratio 1:5 and 1:3, respectively. Furthermore such nanoparticles were capable to mediate efficient intracellular delivery of luciferase expressing plasmid DNA. The plasmid delivery experiments were carried out in three different cell lines: CHO, BHK21 and HEK293 cells. The obtained data were compared with the data observed by using stearylated oligoarginine, which was also capable to transport plasmids into the cell, as it was shown before (Futaki et al., 2001a). Although stearylated oligoarginine showed some

capacity for pDNA delivery into the cell, it was less significant compared to stearyl-$(RXR)_4$.

Lipofectamine™ 2000, the most widely used transfection agent, was used as a positive control to compare the delivery efficacy with CPPs and it was superior to stearyl-$(RXR)_4$ in terms of transfection efficiency, but at the same time the difference between toxicity profiles of stearyl-$(RXR)_4$ and Lipofectamine™ 2000 were observed. Viability of the cells was reduced more than 30% as compared to untreated cells by Lipofectamine™ 2000 in case of long-term toxicity studies (MTS assay), that is common for cationic lipid-based reagents (Nguyen et al., 2007), $(RXR)_4$ analogs did not show any toxicity. To address the fraction of cells transfected with plasmid:stearyl-$(RXR)_4$ complexes, we transfected CHO cells with EGFP expressing plasmid and analyzed them by confocal microscopy. Plasmid:stearyl-$(RXR)_4$ nanocomplexes transfected uniformly most of the cell population, in line with the result obtained with SCOs. However, the Lipofectamine 2000 was not able to transfect the entire cell population.

As mentioned before, the $(RXR)_4$ peptide has been successfully exploited when conjugated to SCOs, both *in vitro* and *in vivo* (Lebleu et al., 2008, Fletcher et al., 2007, Ivanova et al., 2008). However, when utilizing a non-covalent co-incubation protocol, the peptide was unable to mediate any splice correction in HeLa pLuc 705 cell (Kang et al., 1998). After confirming that stearyl-$(RXR)_4$ can be efficiently exploited for the delivery of plasmid DNA, we characterized its ability to convey antisense SCOs into cells using non-covalent approach. As a result stearyl-$(RXR)_4$ promoted efficient splice correction in the same co-incubation setting. Remarkably, concentrations needed to obtain efficient splice correction with stearyl- $(RXR)_4$:SCO complexes are up to 20-fold lower compared to the concentrations needed when utilizing the covalent strategy of $(RXR)_4$-PMO, which are rather in µM range (Abes et al., 2008), 200nM and 4 µM respectively. We have previously reported that stearyl-TP10 is an efficient vector for the delivery of SCOs [Paper I]. Stearylated TP10 is slightly more potent for the delivery of 2′-OMe SCOs than stearyl-$(RXR)_4$, the difference in efficiency was at least 2 times. Nevertheless, these $(RXR)_4$ peptides did not reach the transfection efficiency levels of Lipofectamine™ 2000. Interestingly, stearylation of Arg9 did not have any effect on the delivery of 2′-OMe SCO, indicating that the successful implementation of stearylation is dependent on the properties of the used CPP. Moreover delivery efficiency is highly dependent on the cargo, as stearyl-Arg9 was relatively effective for plasmid delivery but failed to deliver SCOs. Generally it seems that hydrophobicity plays a key role in the general success of CPPs to deliver plasmids and SCOs. Stearylation of transportan 10 had an even greater effect on SCO delivery, than stearyl-$(RXR)_4$ used in this study, which can be explained by the amphipathic/hydrophobic nature of transportan. Also, as it was seen that $(RXR)_4$ and stearyl-$(RXR)_4$ complexes with ON were taken up in comparable quantities, it is reasonable to assume that minor differences in uptake could not

account for the big differences in biological activity, indicating the central role of the stearic acid moiety. Furthermore, if these complexes are expectedly taken up by endosomal pathway, it is another indication that stearic acid enables endosomal escape, at least in case of $(RXR)_4$ peptide.

In conclusion, stearyl-$(RXR)_4$ promotes efficient delivery of plasmid DNA and SCOs using non-covalent co-incubation strategy in non-toxic manner. Moreover, dose-dependent splice correction was in parity with $(RXR)_4$-PMO covalent conjugates, but at least 10-times lower concentration of peptide was used in the non-covalent strategy. These features make stearic acid modified analog of $(RXR)_4$ an promising vector for future *in vivo* experiments.

## 4.3. NickFects mediated delivery of splice-correcting oligonucleotides (Paper III)

Oligonucleotide-based gene regulation has a high potential in gene therapy, but the plasma membrane is impermeable for nucleic acid polymers and, consequently, an efficient and non-toxic transfection agent is needed for their delivery into the cell. Recently, stearylation has been used to improve the delivery efficacy of oligonucleotides using non-covalent strategy (Futaki et al., 2002, Mäe et al., 2009, Lehto et al., 2010). In this study we present a novel series, NickFects, of chemically modified TP10 peptide-based delivery vectors used for the cellular delivery of single-stranded oligonucleotides. These carriers were obtained by replacement of Ile8 by threonine in stearyl-TP10 and by modifying of tyrosine and/or threonine, respectively, with phosphorylation. We hypothesized that the modification of PF3 sequence by addition of a phosphoryl group, one of the functional units in the membrane constituents, could induce a more efficient endosomal escape due to pH-responsiveness and can also change the properties of the complex with oligonucleotides (Fattal et al., 2004, Niidome et al., 1999). For that we used phosphorylated Tyr3 and in the middle of the peptide we replaced Ile8 with a threonine, whereas retaining all the rest of PF3 sequence. The resulting delivery vehicles, NF1 and NF2, were significantly efficient in targeting of splice-correcting oligonucleotides into the cells compared to PF3 peptide in serum-free medium. However, in the serum-containing medium NF1 was more efficient than NF2, which could probably be caused by the different stability of complexes either in extracellular medium or in endosomal vesicles.

Remarkably, these peptides were at least 4 times more efficient in the splice correction assay compared to widely used transfection reagent Lipofectamine™ 2000 and also displayed low long-term cytotoxicity at efficient molar ratios. However, the efficient transfection by Lipofectamine™ 2000 is often accompanied by a high level of cytotoxicity. Furthermore, when HeLa pLuc 705 cells were treated with peptide:ON complexes in the presence of chloroquine, we observed only 20–30% splice-correction increase in the case of NF1 and NF2.

These results demonstrate that modification of Tyr3 or Thr8 with phosphoryl moieties in PF3 peptide sequence significantly increases both the uptake and endosomal escape of NF:ON complexes.

NickFects efficiently formed stable nanoparticles with oligonucleotides with a size of 300–500 nm and negative surface charge, as was measured by dynamic light scattering. Particles with different surface charges are expected to have distinct interactions with the plasma membrane, which should affect the uptake mechanism and efficacy of the delivery peptide vector. We expected that the amphipathic peptide NF1, which carries four positive charges, will form net positive surface charge with ON nanocomplexes. In contrary to our expectations the zeta potential analyses revealed that NF1:SCO complexes possessed a net negative surface charge. The previous studies in this field demonstrated that negatively charged nanocomplexes can exhibit good transfection efficacy *in vitro* and *in vivo* as well (Son et al., 2000b, Son et al., 2000a). The value of Z-potential value showed that formed nanocomplexes are inherently instable and therefore prone to release oligonucleotide molecule from the peptide:ON complex inside the cell.

Characterization of the peptide:ON complexes structure, their internalization process and nuclear localization transmission electron microscopy were performed, in which ONs were tagged with nanogold particle. NF1 and NF2 packed oligonucleotides in dense and regular spherical particles, which avidly associated with plasma membrane and then were efficiently engulfed by cells. Internalized complexes were present in endolysosomal compartment, but a substantial amount of these had escaped from vesicles and localized free in cytosol followed by translocation to nucleus. The abundance of NF1/2–ON complexes in cytoplasm and translocation to nucleus is in a very good concordance with their high efficiency in splice correction. Although PF3–ON complexes stably associated with cells, these were endocytosed less actively and cannot efficiently destabilize endosomes and, consequently, were mostly entrapped in endosomal vesicles, yielding low concentration of oligonucleotides in cytoplasm and modest splice-correction. In contrary, particles of NF1/2–ON break endosomes more actively and dissociate in cytosol to rather small clusters, most probably due to the less stable interaction caused by negative charge introduced into peptide by phosphoryl group.

In summary, we presented two very promising delivery vectors (NF1 and NF2) for *in vitro* applications. Non-covalent NickFect-SCO nanocomplexes induced splice-correction at rates higher than lipid-based transfection vector Lipofectamine™ 2000 in non-toxic manner and remained active in the presence of serum. NF1 is probably more suitable for *in vivo* applications than NF2 in future due to its higher efficacy in serum-containing medium.

# 5. CONCLUSIONS

The main findings of this work are listed and described below:

**Paper I**
Introduction of stearic moiety in *N*-terminal position of amphipatic TP10 drastically increased intracellular delivery of splice-correcting oligonucleotides using non-covalent co-incubation strategy. In addition, the delivery efficiency is exhibited also in serum-containing medium and the peptide:oligonucleotide nanocomplexes did not show any significant cytotoxicity to the cells, that recommends it as a promising delivery peptide vector for future *in vivo* applications.

**Paper II**
In this study, we evaluated the effect of *N*-terminal stearylation of the $(RXR)_4$ and oligoarginine (Arg9) peptides on the delivery of modified splice-correcting oligonucleotides and pDNA using non-covalent co-incubation strategy. Our results show that stearylation significantly increase plasmid and oligonucleotide delivery with no cytotoxicity. Stearyl-$(RXR)_4$ was clearly superior to both unmodified $(RXR)_4$ and stearyl-Arg9 in intracellular delivery, but was not as efficient as commercially available transfection reagent Lipofectamine$^{TM}$2000. We also demonstrated, that the splice-correction effects of stearyl-$(RXR)_4$, utilizing the same co-incubation approach was in parity with that of the pre-clinically used $(RXR)_4$-PMO conjugate. The versatility of the stearyl-$(RXR)_4$ in combination with its non-toxic properties makes it an interesting vector for further *in vivo* applications.

**Paper III**
In this paper, we presented two very promising delivery vectors (NickFect1 and NickFect2) which were successfully used in *in vitro* applications. These were obtained by modifying the chimeric stearyl-TP10 (PepFect3) sequence. The biological efficiency was obtained by introduction of one phosphoryl group in backbone of the peptide, attached either to Tyr3 or Thr8 and replacement of Ile8 by Thr. These peptides formed stable complexes with oligonucleotides, which were efficiently taken up intracellularly in non-toxic manner, resulting in efficient splice correction in serum-free and serum-containing media.

In conclusion, in this thesis we confirmed that minor modifications in peptide sequence and side chains can improve the delivery properties of CPPs without any toxic side effect for effective intracellular delivery of therapeutic molecules such as splice-correcting oligonucleotides or plasmid DNA. We believe that our studies help to overcome the difficulty of endosomal escape providing highly potential and efficient CPPs for nucleic acids delivery both *in vitro* and *in vivo*.

# SUMMARY IN ESTONIAN

## Peptiidide rakkudesse sisenemise ja rakusisestest endotsütootilistest vesiikulitest vabanemise uudne strateegia

Oligonukleotiididel põhinev geeni regulatsioon omab suurt potentsiaali geeni teraapias. Kuid kuna suured ja hüdrofiilsed nukleiinhapete polümeerid ei suuda läbida plasmamembraani, on vaja efektiivset ja mitte toksilist transportvektorit, et soodustada nende transporti rakku. Rakku sisenevad peptiidid (RSP-d) on grupp transportvektoreid, mida on laialdaselt kasutatud nukleiinhapete rakku viimiseks, k.a. plasmiidne DNA (pDNA) ja splaissingut korrigeerivad oligonukleotiidid (SKO), nii *in vitro* kui *in vivo* tingimustes kasutades kovalentset või mitte-kovalentset peptiid:oligonukleotiid kompleksi moodustamise strateegiat.

Käesoleva töö peamiseks eesmärgiks oli disainida ja sünteesida uued ja efektiivsed peptiidsed vektorid nukleiinhapete transpordiks rakku ning hinnata nende rakendusvõimalusi *in vitro* kui ka *in vivo* tingimustes, kasutades mitte kovalentset koinkubatsiooni strateegiat. Me kasutasime kahte metoodikat: geeni transfektsiooni plasmiidse DNAga ja splaissingu korrigeerimist oligonukleotiididega. Antud töös me esitleme mitmeid uusi ja võrreldes algse TP10ga täiustatud transpordi võimega RSPsid, mis saadi stearüülhappe (stearüleerimine) ja/või fosforüülrühma (fosforüleerimine) lisamisel või peptiidi peaahelas aminohappe asenduse tulemusena. Kõik nimetatud modifikatsioonid tehti eesmärgiga, et parandada transporditavate molekulide rakku sisenemist ja et soodustada nende vabanemist rakusisestest endotsütootilistest vesiikulitest.

Esimeses ja teises töös me näitasime, et kimäärse ja amfipaatse TP10 ning mitteamfipaatse ja sünteetilise $(RXR)_4$ peptiidi stearüülhappega N-terminaalse modifitseerimise tulemuseks on peptiid, mis on võimeline efektiivselt ja mitte toksiliselt nukleiinhappeid sihtkohta transportima. Stearüleeritud TP10 säilitas oma efektiivsuse isegi seerumi juuresolekul.

Kolmandas töös me esitleme keemiliselt modifitseeritud stearüül-TP10-l põhinevaid transpordivektoreid efektiivseks SKO rakku viimiseks, mis saadi asendades peptiidi peaahelas 8 positsioonil olev isoleutsiin treoniiniga ja modifitseerides vastavalt türosiini ja /või treoniini nende fosforüleeritud monomeeridega.

Kokkuvõttes, väike modifikatsioon peptiidi peaahelas võib oluliselt parandada RSP, kui transpotvektori omadusi ja soodustada ravimite efektiivset rakku viimist. Peptiid:oligonukleotiid komplekside vabanemine endosoomidest on siiani olnud peamine takistus molekulide sihtmärgini jõudmisel. Käeosolevas töös disainitud ja modifitseeritud RSP-d transportisid oligonukleotiide efektiivselt rakku nii *in vitro* kui ka *in vivo* tingimustes soodustades transporditavate molekulide vabanemist rakusisestest endotsütootilistest vesiikulitest. Käesoleva töö tulemusena saadi mitmed perspektiivikad RSP-d, mida tulevikus on võimalik kasutada geeni teraapias.

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# PUBLICATIONS

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## List of publications


1. **Oskolkov, N**.; Arukuusk, P.; Copolovici, D. M.; Lindberg, S.; Margus, H.; Padari K.; Pooga, M; Langel Ü. (2011). NickFects, phosphorylated derivatives of transportan 10 for cellular delivery of oligonucleotides. International Journal of Peptide Research and Therapeutics, 17(2), 147–157.
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