Specificity of antisense oligonucleotide derivatives and cellular delivery by cell-penetrating peptides

Peter Guterstam

Stockholms universitet

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A good time to keep your mouth shut is when you are out in deep water.

-Sidney Goff

List of publications

The thesis is based on the publications below, referred as paper I – V:

I. EL Andaloussi, S., Guterstam, P., and Langel, Ü.
Assessing the delivery efficacy and internalization route of cell-penetrating peptides.
Nature Prot., (2007) 2, 2043 – 2047.

II. Lundin,P., Johansson,H., Guterstam,P., Holm,T., Hansen,M., Langel,Ü., and EL Andaloussi,S.
Distinct uptake routes of cell-penetrating peptide conjugates.
Bioconj.Chem., (2008) *19(12), 2535-2542*

III. Guterstam, P., Lindgren, M., Johansson, H., Tedebark, U., Wengel, J., EL Andaloussi, S., and Langel, Ü.
Splice switching efficiency and specificity for oligonucleotides with locked nucleic acid monomers.
Biochem. J., (2008) 412 (2), 307 - 313.

IV. Mäe, M., EL Andaloussi, S., Lundin, P., Oskolkov, N., Johansson, H.J., Guterstam, P., and Langel, Ü
A stearylated CPP for delivery of splice correcting oligonucleotides using a non-covalent co-incubation strategy.
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Elucidating cell-penetrating peptide mechanisms of action for membrane interaction, cellular uptake, and translocation utilizing the hydrophobic counter-anion pyrenebutyrate.
BBA Biomembranes, (2009) *in press*

Additional publications

- Lehto T, Abes R, Oskolkov N, Suhorutšenko J, Copolovici DM, Mäger I, Viola JR, Simonsson O, Guterstam P, Eriste E, Smith CI, Lebleu B, Samir El Andaloussi, Langel Ü
  Delivery of nucleic acids with a stearylated (RXR)(4) peptide using a non-covalent co-incubation strategy.
  J Contr. Release. (2009) *in press*

- Guterstam,P., EL Andaloussi,S, and Langel,Ü.
  Evaluation of CPP uptake mechanisms using the splice correction assay.
  Cell-Penetrating Peptides. Methods and Protocols. Methods in Molecular Biology, Humana Press. (2010) *in press.*

Abstract

Atypical gene expression has a major influence on the disease profile of several severe human disorders. Oligonucleotide (ON) based therapeutics has opened an avenue for compensating deviant protein expression by acting on biologically important nucleic acids, mainly RNAs. Antisense ONs (asONs) can be designed to target complementary specific RNA sequences and thereby to influence the corresponding protein synthesis. However, cellular uptake of ONs is poor and is, together with the target specificity of the asONs, the major limiting factor for the development of ON based therapeutics.

In this thesis, the mechanisms of well-characterized cell-penetrating peptides (CPPs) are evaluated and CPPs are adapted for cellular ON-delivery. The functionality of ON derivatives in cells is investigated and by optimization of asONs, targeting pre-messenger RNA, high efficiency and specificity is achieved. The optimization of the asONs is based on sequence design and through the choice of nucleic acid analogue composition. It is concluded that asONs, partly composed of locked nucleic acids are attractive for splice-switching applications but these mixmers must be designed with limited number of locked nucleic acid monomers to avoid risk for off-target activity. A protocol allowing for convenient characterization of internalization routes for CPPs is established and utilized. A mechanistic study on cellular CPP uptake and translocation of associated ON cargo reveals the importance of the optimal combination of for example charge and hydrophobicity of CPPs for efficient cellular uptake. Formation of non-covalent CPP:ON complexes and successful cellular delivery is achieved with a stearylated version of the well-recognized CPP, transportan 10.

The results illustrate that CPPs and ON derivatives have the potential to become winning allies in the competition to develop therapeutics regulating specific protein expression patterns involved in the disease profile of severe human disorders.

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Abbreviations

2OMe	2'-*O*-Methyl
2MOE	2'-*O*-Methoxyethyl
AEC	Anion exchange chromatography
asON	Antisense oligonucleotide
BMD	Becker muscular dystrophy
CQ	Chloroquine
CF	Carboxyfluorescein
CFTR	Cystic fibrosis transmembrane conductance regulator
CME	Clathrin-mediated endocytosis
CPP	Cell-penetrating peptide
DCC	Dicyclohexylcarbodiimide
DIC	Diisopropylcarbodiimide
DIEA	Diisopropylethylamine
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
FACS	Fluorescence-assisted cell sorting
FITC	Fluorescein isothiocyanate
Fmoc	*9*-Fluorenylmethyloxycarbonyl
GABA	γ-Aminobutyric acid
HATU	*2*-(*7*-Aza-1H-benzotriazole-*1*-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate
HKR	HEPES-Krebs Ringer buffer
HOBt	Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HS	Heparane sulfate
LDH	Lactate dehydrogenase
LNA	Locked nucleic acid
LUV	Large unilamellar vesicle
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MAP	Model amphipathic peptide

MBHA	*4*-Methylbenzhydrylamine
MEND	Multifunctional envelope-type nano-device
miR	Micro RNA
Npys	*3*-Nitro-*2*-pyridinesulfenyl
ON	Oligonucleotide
PB	Pyrenebutyrate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethylenimine
PenArg	Penetratin - arginine
PG	Proteoglycan
PMO	Phosphorodiamidate morpholino oligonucleotide
PNA	Peptide nucleic acid
PO	Phosphodiester
POPC	Palmitoyl-2-oleoyl-phosphatidylcholine
POPG	Palmitoyl-2-oleoyl-phosphatidylglycerol
PS	Phosphorothioate
pTat48-60	Trans-activator of transcription protein peptide 48-60
pVEC	Vascular endothelial cadherin protein peptide
R9	Nona-arginine
R12	Dodeca-arginine
RFU	Relative fluorescence units
RISC	RNA-induced silencing complex
RLU	Relative luminescence units
RNA	Ribonucleic acid
RNAi	RNA interference
RP	Reversed phase
RT-PCR	Reverse transcriptase polymerase chain reaction
siRNA	Short interfering RNA
SPPS	Solid phase peptide synthesis
SSO	Splice switching oligonucleotide
t-Boc	*tert*-Butyloxycarbonyl
TBTU	*O*-(Benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium tetrafluoroborate
TFA	Trifluoroacetic acid
TP10	Transportan 10

Introduction

Advances in molecular biology have improved our understanding about biological qualities governing regulatory processes in humans and other organisms. This understanding has not only given us indications about the biological organization, but it has also led to the identification of specific genes and proteins that are involved in the progression of many diseases. Identification of an alteration in gene- or protein-function associated with a particular disease provides potential sites for novel therapeutic compounds. The conventional mechanism of action for most current pharmaceuticals is to alter the function of specific proteins utilizing small molecules, or lately, also antibodies. In this way, a certain disease-related physiological process can be inhibited. There are many examples of successful therapeutic compounds developed on the basis of this approach. The disadvantage with the current approach for drug development is that it relies on complicated screening and optimization strategies that can take years or even decades, and may still be limited by accessibility, target specificity and efficacy (**Leaman**, **2008**). Rational screening for functional compounds is not the only origin for drug discoveries. Serendipity has played an important role. Even if serendipitous discoveries usually strike a prepared mind it is not desirable to be dependent on them. The most well-known example of fortunate leaps in drug discovery is probably Fleming's finding of penicillin (**Fleming**, **1947**)**.** Serendipity still plays an ineligible role in drug development. This is exemplified by the discovery of sildenafil citrate, more known under its commercial name Viagra, which was discovered in a project screening for potential compounds for treatment of hypertension (**Raja and Nayak**, **2004**). The ability to bypass the screening strategies and directly target the aberrant proteins at gene or ribonucleic acid (RNA) level, based on the sequence of the human genome, has been considered a more convenient alternative. Drug targeting at gene or RNA level is a flexible and easily manipulated approach that would shorten lead times and potentially enhance target specificity. Thus, regulation of gene expres-

sion is an attractive method on which to base novel therapeutics, or to use as a tool for target validation of other therapeutic compounds.

Oligonucleotide derivatives and protein expression

Cellular protein synthesis can be manipulated to achieve desired protein expression patterns. By utilizing antisense oligonucleotides (asONs), which bind complementary to its target oligonucleotide (ON), most often RNA, the protein expression from a specific gene can be modulated (**Green, et al**, **1986**). A great number of diseases arise from atypical protein expression and specific regulation of gene expression is therefore of great interest from a therapeutic point of view. Several synthetic nucleic acid analogues with improved properties for targeting of biologically important RNAs have been developed (**Kurreck**, **2003**). Functional, non-toxic and dynamic vectors for cellular ON delivery are scarce and this is an issue limiting the initiated development of ON based therapeutics.

The understanding of the gene expression mechanisms from chromosomal deoxyribonucleic acid (DNA) level to functional proteins has resulted in deeper insights into the background of several diseases. The basis for these insights is the association of genetic research with research about protein function and malfunction. Extensive cell biological research explaining the proteomic background to diseases and the full sequencing of the human genome (**Venter, et al**, **2001**) have given opportunity to develop novel therapeutic compounds. ON mediated regulation of gene expression to inhibit disease related proteins is about to transform the development of novel drugs. Active regulatory asONs can bind to target RNA and influence cellular processes, such as messenger RNA (mRNA) degradation, translation in the ribosomes and pre-mRNA splicing. Another opportunity to manipulate gene expression is inhibition of transcription at the chromosomal level. This implies asONs targeting chromosomal DNA by acting as sequence-specific triplex forming ONs (**Besch, et al**, **2004**).

Standard asONs are used to block translation by targeting mature mRNA and hindering the protein synthesis at ribosomal level. These asONs are either active as steric block asONs or RNAse H recruiting asONs. The steric block asONs target mature mRNA and thereby inhibit the single stranded mRNA from being processed in the ribosome. This results in down-regulation of the specific protein (**Stephenson and Zamecnik**, **1978**). The RNAse H recruiting asONs also

target mature mRNA, but in this case the mRNA is degraded by the ribonuclease RNAse H. This ribonuclease acts on DNA:RNA duplexes in a non sequence specific manner. Cellular treatment with an RNAse H recruiting asON targeting the sequence of a specific mRNA results in down-regulation of the specific protein by degradation of its mRNA (**Walder and Walder**, **1988**). The advantage with RNAse H recruiting asONs is that the asON itself is not degraded and therefore act catalytically. The drawback with this type of asONs is that its chemical structure must not differ too much from the structure of the DNA, otherwise RNAse H will not recognize the asON:mRNA duplex as a substrate (**Inoue, et al**, **1987**). To avoid the need for RNAse H recruitment, asONs attached to ON-based artificial nucleases have been evaluated. The artificial nucleases are zinc ion dependent and have built in activity to degrade complementary RNA sequences. Hence, catalytic activity can be built into an asON by attachment of a synthetic catalytic group (**Åström and Strömberg**, **2004**).

Another mechanism that modulate gene expression is RNA interference (RNAi) (**Fire, et al**, **1998**,**Elbashir, et al**, **2001a**,**Elbashir, et al**, **2001b**). The RNAi-pathway is active in most eukaryotic cells controlling the activity of specific genes. The trigger molecules for initiating the RNAi-pathway are short (21 – 28 nucleotides) double stranded RNA sequences. Long double stranded RNA can be derived from various sources such as the simultaneous sense and antisense transcription of a specific gene, gene transcripts forming hair-pin loops or from viral replication etc. (**Meister and Tuschl**, **2004**). The maturation of small RNAs is a stepwise process catalyzed by double stranded RNA-specific endonucleases creating short interfering RNA (siRNA). The siRNA is then bound to RNA-binding proteins forming the cytosolic RNA-induced silencing complex (RISC) (**Peters and Meister**, **2007**,**Hammond, et al**, **2000**). One of the siRNA strands is released and the other integrates to RISC as a template for further binding to other, longer, single stranded RNAs, for example mRNA, in an antisense manner. RISC generates cleavage of the target RNA sequence and, consequently, allows specific protein down-regulation. The RISC-mediated cleavage of complementary RNA is not limited to one single substrate sequence but acts catalytically, mediating cleavage of several substrate sequences, for example mRNA sequences. By introducing exogenous siRNA duplexes into cells it is possible to mediate specific protein down-regulation.

One explicit type of endogenous ONs that partly employs the RNAi-pathway is micro-RNAs (miRs). The miR genes are

typically evolutionary conserved and the miRs have a similar mechanism of action as siRNA. The gene products of miR genes are processed in several steps generating single-stranded RNA sequences of approximately 22 nucleotides length. The miR has the potential to follow the RNAi-pathway by being incorporated into RISC mediating cleavage of complementary RNA-sequences present in the cytoplasm. However, this has mainly been reported for plants and the main mechanism of action for miRs in animals is inhibition of protein synthesis by targeting partially complementary sequences located within the 3'-untranslated region of mRNAs (**Meister and Tuschl**, **2004**,**Ambros**, **2004**). The miR activity within eukaryotic cells is likely to hold enormous potential for complex genetic regulatory interactions involving hundreds of miRs and their numerous targets. By introducing specific sequence complementary exogenous asONs, anti-miRs, into cells it is possible to target miRs to inhibit the miR activity and thereby influence gene expression. The anti-miRs are ON derivatives that bind complementary to miRs. The anti-miRs have generated extraordinary attention as means to delineate the mechanisms of miRs and also for potential therapeutic applications (**Stenvang and Kauppinen**, **2008**). The advantage with anti-miRs is that such ONs can be modified with nucleic acid analogues to fine-tune serum-stability target specificity while there are limitations for the use of nucleic acid analogues in siRNAs.

Another cellular process where asONs can be utilized for regulation of gene expression is to influence splicing of pre-mRNA. In the splicing process, introns are separated from exons and the exons are fused into mRNA. Splicing of pre-mRNA is the target cellular process for the asONs utilized in this thesis, see details below. The asONs influencing pre-mRNA splicing are called splice-switching oligonucleotides (SSOs) and they have capacity to, e.g., mask mutations giving aberrant pre-mRNA splicing patterns and thereby alleviate the effect of such mutations (**Busslinger, et al**, **1981**).

Constitutive and alternative splicing

Post transcriptional modifications, including pre-mRNA splicing (i.e. removal of introns and fusion of exons), are fundamental for generating mRNAs that can be translated into proteins. In contrary to constitutive splicing, where the immature pre-mRNA transcript always is processed in the same manner, generating only one type of mRNA, alternative splicing produces various mRNAs with different se-

quences, and concomitantly, different protein isoforms with potentially different functions. Considering that an average gene encodes pre-mRNAs with eight different exons and that approximately 70% of all genes undergo alternative splicing, alternative splicing is the most likely major source of protein diversity present in human cells (**Lander, et al**, **2001**). Pre-mRNA splicing is an essential, precisely regulated, process that occurs in the nucleolus of cells. As the pre-mRNA is assembled by the RNA polymerase, it immediately becomes packaged with various RNA binding factors. A specific subunit of the RNA polymerase recruits, to the emerging transcript, available RNA processing factors that are crucial for the subsequent splicing events. The RNA processing factors are involved in a multi-component protein complex, known as the spliceosomal machinery, capable of discriminating between exons and introns.(**Moore and Sharp**, **1993**). The rate of pre-mRNA synthesis by the polymerase can influence splicing patterns by accelerated or delayed synthesis speed. The speed of polymerase elongation affects the alternative splicing activity since the access for various RNA binding factors to sequence dependent recognition sites, splice sites or splice regulatory elements, depends on the pre-mRNA elongation speed. Consequently, the essential outcome of pre-mRNA splicing is already influenced at the level of pre-mRNA synthesis by the RNA polymerase (**Matlin, et al**, **2005**). It is in this dynamic setting that alternative splicing occurs.

In constitutive splicing the spliceosomal activity is limited to recognizing exon-intron boundaries, splice sites, followed by accurate cleavage and rejoining of exons (**Figure 1a**). The splice-sites are determined by invariant GU and AG intronic nucleotides at the 5´ and 3´ intron-exon junctions respectively (**Figure 2)**.

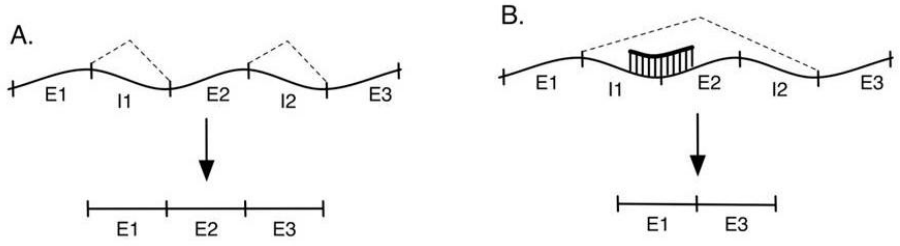

Figure 1: Example of spliceosomal activity at pre-mRNA level showing exon skipping induced by a splice-switching oligonucleotide (SSO) **A**. Normal splicing of the pre-mRNA. **B**. An SSO interferes with the splicing machinery by blocking the 5'-splice site. This gives rise to another mRNA, lacking one exon, and thereby it will give rise to another protein isoform.

Introduction of an SSO to the cell can direct splicing to the desired protein isoform (**Figure 1b**). The SSO can target the consensus splice site sequences, i.e. intron-exon boundaries or more variable auxiliary elements, like splicing enhancers or silencers that are involved in defining both constitutive and alternative exons. The SSO may also target the branch-point, which is an intronic site where the 5'-end of the intron binds when removing an intron from the exonic parts of a pre-mRNA strand (**Ruskin, et al**, **1985**) (**Figure 2**). Hence, there are numerous potential sites to target for SSOs and there are several considerations, specific for each case, which have to be taken into account when searching for a suitable target sequence. In this thesis a well-established read-out assay for cellular delivery of SSOs was utilized (**Kang, et al**, **1998**). This assay is based on a plasmid with a reporter gene interrupted by the mutated intron 2 from a thalassemic human β-globin gene. This intron carries a mutation which creates an aberrant splice site generating a pseudo-exon and thereby inhibits the normal processing of the pre-mRNA. The hybridization of an SSO masks the cryptic splice site and redirects the splicing machinery towards the complete removal of intron 2, thereby allowing correct pre-mRNA processing and expression of the reporter protein (**Kang, et al**, **1998**).

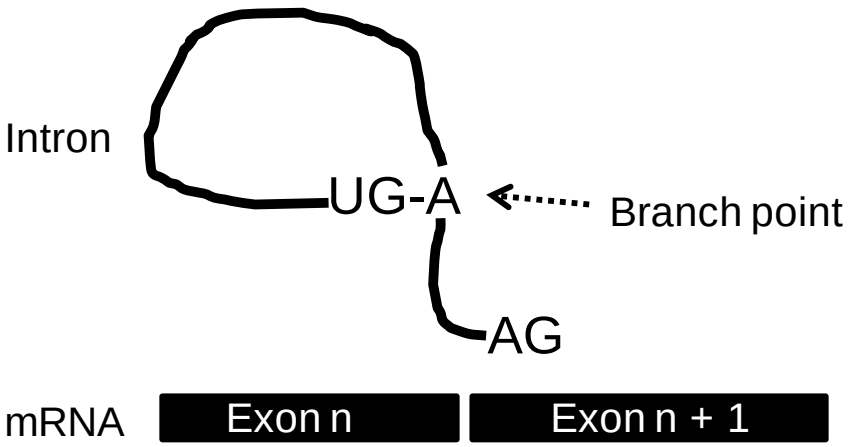

Figure 2: Removal of intron from exons. The 5'-end of the intron forms 2'-5' phosphodiester bond (RNA branch) to a single adenosine residue (branch point) catalyzed by the spliceosome complex.

Splicing and diseases

Several diseases, including β-thalassemia, cystic fibrosis, and muscular dystrophies, are associated with alterations in alternative splicing, caused by mutations affecting the splicing process (**Black**, **2003**,**Faustino and Cooper**, **2003**,**Pajares, et al**, **2007**). It is estimated that 20-30% of all disease causing mutations affects pre-mRNA splicing. Mutations solely affecting the pre-mRNA to be spliced are called *cis*-acting mutations and they either disrupt existing splice sites, generating intron inclusions or exon exclusions, or produce novel splice sites (**Faustino and Cooper**, **2003**). The *trans*-acting splicing mutations can lead to joining of different, independently transcribed, pre-mRNAs, affecting the function of the basal splicing machinery, or affecting the factors that regulate alternative splicing, resulting in changed preferences of choice of splice sites (**Mercatante, et al**, **2001**). The *cis*-acting mutations are often the targets for SSOs since the asONs can mask the mutations at the pre-mRNA level.

One of the first described mutations affecting pre-mRNA splicing was found in β-thalassemia patients. It was found that a mutation in intron 2 of β-globin pre-mRNA created an aberrant 5´splice site, concomitantly activating a cryptic 3´splice site which leads to an intron inclusion (pseudo-exon), and therefore, expression of β-globin proteins with hampered functionality (**Busslinger, et al**, **1981**). The same types of mutations have been identified in the cystic fibrosis trans-membrane conductance regulator (CFTR) gene, resulting in aberrant splicing and development of cystic fibrosis (**Rowntree and Harris**, **2003**). Duchenne muscular dystrophy (DMD), characterized by progressive degenerative myopathy, and its milder allelic disorder, Becker muscular dystrophy (BMD), are both caused by mutations in the dystrophin gene. The mutations giving DMD shift the open reading frame leading to premature termination of translation giving a non-functional dystrophin protein. Mutations giving BMD maintain the open reading frame giving truncated but semi-functional dystrophin protein (**Koenig, et al**, **1989**). Some of the DMD mutations can be converted to less severe forms by treatment with SSOs that induce exon skipping, restores the open reading frame. This gives rise to a truncated but partly functional dystrophin, similar to dystrophin proteins found in the milder BMD disorder (**Koenig, et al**, **1989**,**van Deutekom, et al**, **2007**,**Kinali, et al**, **2009**,**Jearawiriyapaisarn, et al**, **2009**). SSOs have also been used to modulate splicing of a tumor necrosis factor receptor pre-mRNA. The SSO induces skipping of exon 7, which codes for the trans-membrane domain of the receptor, and

switches endogenous expression from the membrane-bound form to a soluble, secreted form of the receptor. This soluble decoy receptor accumulates in the circulation, *in vivo*, and antagonizes tumor necrosis factor α, and alters thereby disease patterns of arthritis (**Graziewicz, et al**, **2008**).These are mere examples of diseases caused by alterations in alternative splicing, for reviews see (**Pajares, et al**, **2007**,**Garcia-Blanco, et al**, **2004**,**Cooper, et al**, **2009**).

Oligonucleotide derivatives

ON derivatives based on synthetic nucleic acid analogues for regulation of gene expression should preferably be stable in serum, hybridize effectively to target RNA, and be non-toxic. The asONs used for inhibition of protein expression at the mRNA-level should also, preferably, recruit the RNA cleaving enzyme RNAse H whilst the asONs for splice switching should not recruit RNAse H (**Kurreck**, **2003**). The first generation of ON derivatives, represented by phosphorothioate (PS) DNA (**Figure 3**), has a modification on the phosphodiester (PO) linkage. It recruits RNAse H and has high serum stability (**Campbell, et al**, **1990**,**Spitzer and Eckstein**, **1988**). Spiegelmers or *L*-DNA is the *L*-ribose modified form of the natural *D*-DNA, thus the enantiomeric form of natural DNA. This nucleic acid analogue has high serum-stability due to its enantiomeric structure, but it has dubious base-pairing properties for complementary RNA strands and does not recruit RNAse H (**Klussmann, et al**, **1996**,**Wlotzka, et al**, **2002**).

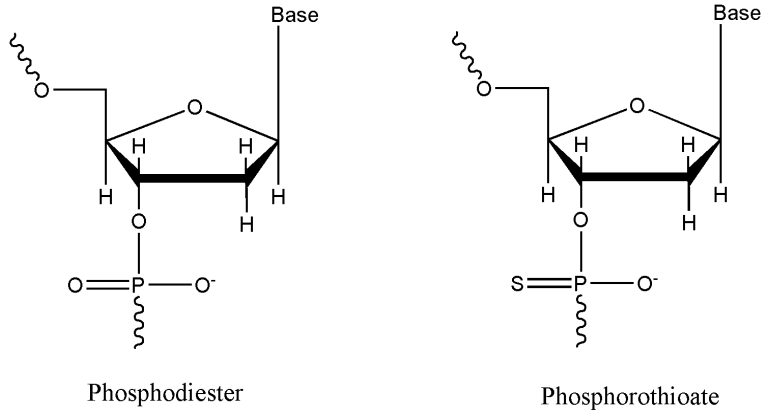

Figure 3: Phosphate backbone modification of DNA. Natural DNA has phosphodiester (PO) backbone. One of the oxygen atoms in the phosphate is replaced by sulfur in the first generation ON derivatives, phosphorothioate (PS) DNA.

The second generation of ON derivatives have 2'-*O*-modifications, such as 2'-*O*-methyl (2OMe) RNA (**Figure 4**) or 2'-*O*-methoxyethyl RNA (2MOE RNA), resulting in increased melting temperatures when hybridized to RNA. They also have favorable serum stability. Modification of the ribose sugar ring most often implies that such ON derivatives does not recruit RNAse H. (**Monia, et al**, **1993**). The second generation of ON derivatives is therefore suitable as SSOs since degradation of the target RNA sequence is undesirable for splice-switching applications.

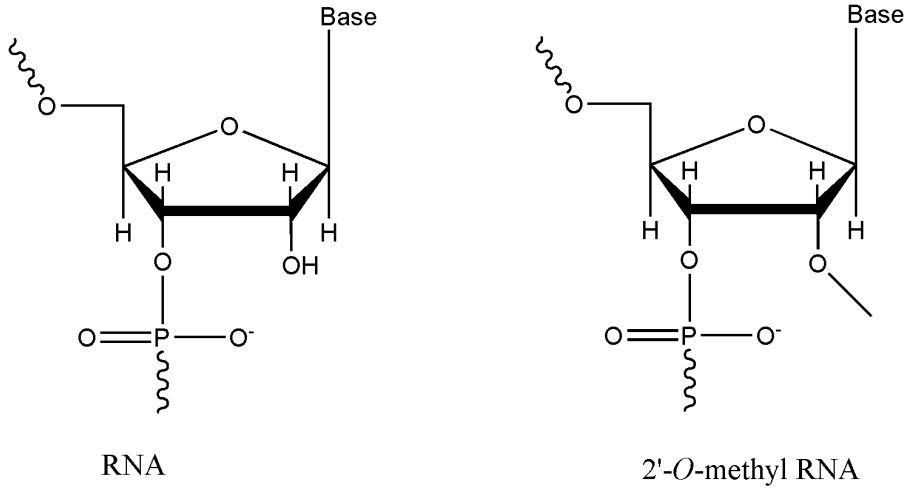

Figure 4: Ribose modifications characterize the second generation ON derivatives, here exemplified by 2'-*O*-methyl RNA. The 2'-*O* modifications increase binding affinity and abolish RNAse H recruitment.

Third generation asONs are characterized by further modifications of the ribose moiety and/or other components of the backbone. Nucleic acid analogues with constrained ribose ring forming bicyclic nucleic acids, such as locked nucleic acid (LNA), have been developed (**Figure 5**). LNA has considerably higher affinity for target RNA than asONs from the second generation, confirmed by an increased melting temperature for LNA strands hybridized to RNA (**Kumar, et al**, **1998**,**Bondensgaard, et al**, **2000**). Another type of third generation ON derivatives is peptide nucleic acid (PNA), which is achiral and has an uncharged backbone. PNA comprises most often N-(2-aminoethyl) glycine units, where nucleobases are attached to central amines, with a methylene carbonyl chain (**Nielsen, et al**, **1991**) (**Figure 5**). Oligomers of PNA have high sequence specificity, induce low extent of non-antisense activities, such as protein binding, and,

have been shown useful for several applications. Examples of such applications are visualization of specific mRNAs by magnetic resonance imaging utilizing antisense PNA coupled to magnetic resonance contrast agents (**Mishra, et al**, **2009**) and inhibition of ribosome function by antisense binding to ribosomal RNA crucial for the ribosomal accuracy of mRNA decoding (**Hatamoto, et al**, **2009**,**Ogle, et al**, **2001**). The latter application may be suitable as a novel antibiotic since specific bacterial ribosomal RNA sequences can be targeted (**Hatamoto, et al**, **2009**). Oligomers of PNA are less water soluble than most other ON derivatives but the solubility can be improved by the addition of positively charged lysine residues to the sequence (**EL Andaloussi, et al**, **2006**,**Fabani and Gait**, **2008**). Phosphorodiamidate morpholino oligonucleotides (PMOs) are also uncharged, displaying properties similar to PNA. The PMOs have a backbone where the deoxyribose sugar of DNA is replaced by a six membered ring, and the phosphodiester linkage is replaced by a phosphorodiamidate linkage.

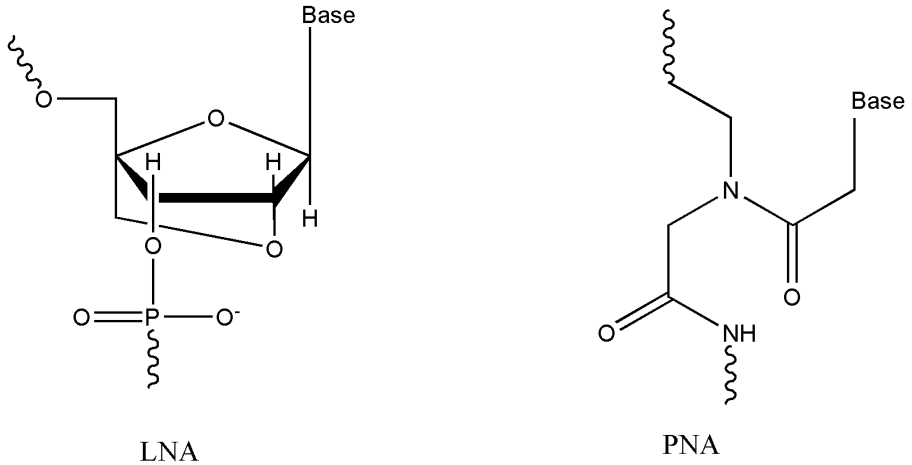

Figure 5: Third generation ON derivatives have significant ribose modifications as for the bicyclic LNA or complete replacement of the ribose phosphate backbone as in the uncharged PNA.

Since second and third generations ON derivatives do not recruit RNAse H, their utility as asONs targeting mRNA is limited. To avoid this drawback, asONs with an internal stretch of 7-10 PS DNA monomers flanked by for example LNA monomers are designed to achieve RNAse H mediated cleavage of target mRNA (**Jepsen and Wengel**, **2004**). This type of asONs is called gapmers due to

the ‘gap’ in the middle of the sequence holding DNA nucleotides whereas the ON is flanked by nucleic acid analogues. The gapmers have a slightly lower affinity to target RNA compared to the corresponding mixmers, which have an even sequence distribution of different nucleic acid analogues (**Petersen and Wengel**, **2003**). Hence, mixmers containing, for example PS DNA and LNA monomers, do not recruit RNAse H, and have advantageous affinity for target RNA. These are desirable characteristics for SSOs.

Due to the intensive research in the field of novel ON derivatives, and as a result of the introduction of third generation asONs, issues with serum stability and target affinity are, to a great extent, already solved, whilst target specificity and delivery issues remain.

Specificity to complementary strands in cells

The advantage with ON-based sequence specific regulation of gene expression is the ability to fine-tune the expression levels of specific proteins. Successful choice of set-up for ON-based regulation of gene expression in clinical settings will be useful for a wide range of disorders. By modifying the ON sequence, a target of choice can be exposed to treatment. Given that the toxicity for ONs with novel nucleic acid analogues is low (**Kaur, et al**, **2007**), a fundamental, and still indistinctly clarified, prerequisite for universal application of ON-based therapeutics is high target specificity. Low target specificity is exemplified by non-specific binding to innate proteins and by binding to off-target semi-complementary ON strands within the cell. The binding to non-target biologically active RNAs can create alterations in the gene expression for additional genes, other than the target gene. Such interactions may be harmless but they may also create adverse side-effects, e.g. if they happen to influence the activity of a transcription factor.

One way to delineate the ability of asONs to mediate off-target effects based on binding to non- or semi-complementary strands is to analyze the biological effect of a corresponding control ON-sequence carrying mismatches to target sequence. If the control sequence induces similar biological effects in the applied read-out system as the correct sequences it indicates possibilities for binding also to un-related RNA sequences. To avoid unspecific binding, the asON can be optimized by fine-tuning the melting temperature to the complementary RNA strand. Such improvements can be done by

changing the length of the asON or by altering its chemical composition. Different ON derivatives display different binding affinities to complementary nucleotides. For example, inclusion of one LNA nucleotide in the DNA strand of a 9-mer DNA/RNA duplex increases the melting point by 9.6 ^{o}C as compared to the unmodified duplex (**Bondensgaard, et al**, **2000**). Inclusion of other nucleic acid analogues also mediates higher affinity to the complementary RNA strand than the corresponding unmodified DNA strand. Examples of such nucleic acids are 2OMe RNA, 2MOE RNA, PNA or PMO (**Braasch and Corey**, **2001**). By introducing L-DNA, unlocked nucleic acid (**Jensen, et al**, **2008**) or abasic nucleic acids (**Kvaerno, et al**, **2000**) into an asON, the melting temperature to complementary RNA can be lowered. The unlocked nucleic acids are acyclic with no bond between the 2'- and 3'- carbon in the ribose and abasic nucleic acids have no purine or pyrimidine nucleobases attached to the 1'-carbon of the riboses.

Cellular delivery of oligonucleotide derivatives

The main obstacle associated with the use of asONs regulating gene expression patterns is the low bioavailability of asONs due to their charged anionic nature. Most delivery vectors available to date are far from optimal and they have mainly been formulated and optimized for the delivery of gene expressing plasmids. For example, several viral vectors have been developed and utilized in gene therapy. Despite being very efficient, the viral vectors potentially suffer from several detrimental effects such as acute immune responses, immunogenicity, and viral recombination. Therefore, methods of non-viral gene delivery have been explored using various physical and chemical approaches. Physical approaches include needle injection, electroporation, gene gun, and ultrasound, with the common denominator being the employment of physical force to permeate the cell membranes. These approaches have been utilized both *in vitro* and *in vivo* with varying degrees of success, as reviewed by Gao (**Gao, et al**, **2007**). The main impediments to these methods are practical issues *in vivo*, cytotoxicity associated with plasma membrane perturbation, and that they are optimized for plasmid delivery. By far the most frequently utilized strategy in non-viral gene delivery is formulation of DNA into condensed particles using cationic lipids or cationic polymers. These particles are subsequently taken up by cells via endocytosis into ve-

sicles, from which a small fraction of DNA is released into the cytoplasm (**Gao, et al**, **2007**,**Xu and Szoka**, **1996**).

The viral vectors are limited to delivery of plasmids whereas the non-viral vectors are suitable for cellular delivery of gene regulatory ONs. A relatively non-toxic technique for non-viral ON delivery is employment of cell-penetrating peptides (CPPs). The CPPs have gained increasing attention since their initial discovery in 1994 (**Derossi, et al**, **1994**) due to their remarkable ability to penetrate cells and convey cargo, such as asONs. Understanding the mechanisms for CPP-mediated delivery and ON-induced regulation of protein expression may prove to be a prerequisite for therapeutic applications of asONs. Direct translocation across cellular plasma membranes was first assigned as the mechanism of action of CPPs. This view is slowly being altered in favor for the endocytotic uptake mechanisms. Based on the understanding of specific mechanisms, CPPs that employ the endocytotic uptake pathway have been optimized to avoid being trapped in endocytotic vesicles. The endosomal escape for CPPs is a crucial topic within the field of CPP-development. In the consecutive text, cellular uptake is defined as the accumulation of the CPP and potentially associated cargo within the cell, irrespectively of its intracellular localization and translocation is defined as direct access to non-vesicular compartments, e.g. cytosol and/or nucleus (**Joliot and Prochiantz**, **2008**).

Non-viral transfection

Cationic lipids have been used frequently since Felgner and coworkers' initial discovery, in 1987. They found that a double-chain monovalent quaternary ammonium lipid, referred to as lipofectin, could efficiently bind to and convey DNA into cultured cells (**Felgner, et al**, **1987**). Mixing of cationic lipids and ONs creates small nuclease protected particles, liposomes, that allows cellular uptake and facilitate the release from endosomal structures (**Xu and Szoka**, **1996**). Although cationic lipids have been successfully exploited *in vivo*, most of these vectors are not well suited for *in vivo* use, as a result of their sensitivity for serum proteins. A dramatic change in surface charge and size occur when cationic lipids in complex with ONs are exposed to overwhelming amounts of negatively charged proteins that are abundant in the blood and elsewhere extracellularly (**Gao, et al**, **2007**). Artificial phospholipid vesicles, liposomes, can be loaded with a variety of cargo molecules. Liposomes used *in vivo* are often

coated with polyethylene glycol (PEG). By PEGylating the liposomes they stay in the blood long enough to accumulate in various pathological areas with impaired or leaky vasculature, such as tumors (**Torchilin**, **2008**).

Apart from liposomes, cationic polymers represent the other large group of carriers that have been applied widely for ON-delivery. These linear or branched conformation polymers range from DNA condensing polylysine to the more extensively used polyethylenimine (PEI) (**Boussif, et al**, **1995**) One major drawback using PEI as a transfection reagent is its non-biodegradable nature, raising toxicity concerns (**Kunath, et al**, **2003**). Cellular uptake through receptor-mediated endocytosis in absence of any ON condensing agent is another approach. Instead of any ON-condensing agent, cholesterol or non-toxic polyethers are linked to the ON enhancing the ON-stability and time in circulation prior to renal clearance (**Soutschek, et al**, **2004**).

Tissue-specific homing peptides or ON-based aptamers have been introduced (**McNamara, et al**, **2006**,**Dassie, et al**, **2009**,**Myrberg, et al**, **2008**,**Ruoslahti and Rajotte**, **2000**). Specific delivery to target cells is a plausible way to avoid side effects stemming from unwanted delivery to non-targeted cells and decreasing doses required to attain desirable biological response *in vivo*. Aptamers are one group of targeting ligands with high specificity that have been used for delivery of siRNAs to prostate cancer cells both *in vitro* (**Chu, et al**, **2006**) and *in vivo* (**McNamara, et al**, **2006**,**Dassie, et al**, **2009**). Aptamers are ON sequences that have been engineered through repeated rounds of selection to have affinity for a specific molecular target (**Ellington and Szostak**, **1990**). Since aptamers are ONs themselves it is possible to covalently fuse asON and aptamer through continuous synthesis. Liposomes can be functionalized with targeting ligands, such as aptamers or homing peptides (**de Fougerolles, et al**, **2007**).

Even though the mentioned vectors are only a limited selection of non-viral delivery vehicles, it is essential to find more efficient and non-toxic vectors for the transport of ONs. Delivery efficacy, tissue targeting, and toxicity are the main concerns for ON delivery vectors and these concerns are central for the development of ON-based therapeutics.

Cell-penetrating peptides

Proteins with the ability to penetrate cells were first identified while investigating proteins involved in infection of mammalian cells by HIV-virus. The trans-activator of transcription protein (Tat) was found to penetrate adjacent cells. (**Frankel and Pabo**, **1988**,**Green and Loewenstein**, **1988**). The first CPP, pAntp or penetratin, was discovered when truncating the antennapedia homeoprotein in *Drosophila* (**Derossi, et al**, **1994**). Screening of the cell-penetrating properties of the Tat protein revealed that a positively charged peptide, pTat 48-60, is capable to penetrate into cells (**Vivés, et al**, **1997**). Furthermore, promising chimerical peptides like transportan, have been developed (**Pooga, et al**, **1998a**), together with its deletion analogue, transportan 10 (TP10) (**Soomets, et al**, **2000**). More simple and non-natural designs like polyarginines have also been found potent CPPs (**Rothbard, et al**, **2000**). The α-helical model amphipathic peptide (MAP) is designed with respect to hydrophobicity, hydrophobic moment and charge to possess cell-permeable properties (**Oehlke, et al**, **1998**). Other examples of CPPs are M918 derived from the tumor suppressor protein p14ARF (**EL Andaloussi, et al**, **2007b**), and pVEC, derived from the cell adhesion molecule vascular endothelial cadherin protein (**Elmquist, et al**, **2001**). The CPPs utilized or discussed in this thesis are presented below (**Table 1**).

Table 1: Name and sequences of CPPs utilized or discussed in this thesis.

Peptide	Sequence	Reference
R9	H-RRRRRRRRR-NH_2	**Rothbard, et al, 2000**
pTat 48-60	H-GRKKRRQRRRPPQ-NH_2	**Vivés, et al, 1997**
Penetratin	H-RQIKIWFQNRRMKWKK-NH_2	**Derossi, et al, 1994**
PenArg	H-RQIRIWFQNRRMRWRR-NH_2	**Thorén, et al, 2003**
pVEC	H-LLIILRRRIRKQAHAHSK-NH_2	**Elmquist, et al, 2001**
M918	H-MVTVLFRRLRIRRASGPPRVRV-NH_2	**EL Andaloussi, et al, 2007**
Transportan	H-GWTLNSAGYLLGKINLKALAALAKKIL-NH_2	**Pooga, et al, 1998**
TP10	H-AGYLLGKINLKALAALAKKIL-NH_2	**Soomets, et al, 2000**
MPG	H-GALFLGFLGAAGSTMGAWSQPKKKRKV-NH_2	**Morris, et al, 1997**
MAP	H-KLALKLALKALKAALKLA-NH_2	**Oehlke, et al, 1998**
$(RXR)_4$[1]	H-RXRRXRRXRRXR-OH	**Abes, et al, 2006**

[1]X – 6-aminohexanoic acid

As the name indicates, CPPs were originally thought to directly penetrate the plasma membrane and gain access to the cytoplasm. It was well-recognized that CPPs were taken up at 4 ^{o}C, thus energy independently. This conclusion was later found to partly be based on an experimental artifact and the uptake pathway for most CPPs was revised to endocytotic pathways (**Lundberg, et al**, **2003**,**Richard, et al**, **2003**). Nevertheless, this topic is still under debate and a few CPPs are considered to be capable of entering cells via non-endocytotic pathways (**Simeoni, et al**, **2003**). CPPs are alternatives to cationic polymers, liposomal or viral delivery vectors promoting cellular uptake of asONs.

There are two strategies for CPP-mediated ON delivery, namely covalent attachment of cargo to CPP, and a strategy based on non-covalent interactions, mainly electrostatic interactions, between anionic ON and cationic peptide.

Non-covalent peptide and oligonucleotide complexes

Non-covalent peptide and ON complexes are formed by mixing the two entities, CPP and ON. Usually an excess of peptide is used. The two components are allowed to form complexes based upon electrostatic interactions between anionic ONs and cationic peptides. The complexes formed are then used for cellular transfection. Stearylation of CPPs is one way to facilitate endosomal release and potentially also influence the non-covalent CPP:ON complex formation. N-terminal stearylation has proven applicable for polyarginines (**Futaki, et al**, **2001**) and in this thesis the use of stearylation has been further exploited.

Liposomes can be functionalized with PEG, as described above, but also in other ways, e.g. with CPPs to improve cellular uptake. However, liposomes modified with both PEG and CPP result in low transfection of cells because of steric hindrances for the liposome-to-cell interaction due to the PEG coat, which shields the surface-attached CPPs. To avoid this phenomena one can introduce PEG to liposomes coupled via a low pH-detachable linker. This construct enables removal of PEG under the action of the decreased intratumoral pH leading to the exposure of the liposome-attached CPPs (**Kale and Torchilin**, **2007**).

Multifunctional envelope-type nano device (MEND) is a special liposome-based approach for cellular delivery of nucleic acids encapsulated in a lipid envelope that can be functionalized. The

MEND encapsulates nucleic acids that are condensed by a polycation, e.g. poly-lysine. The condensed ONs are coated with a lipid bilayer, analogous to envelope-type viruses. Functional devices introduced into the outer lipid layer include e.g. specific ligands, to permit its entry into cells, and fusogenic lipids to fuse with the endosomal membrane (**Kogure, et al**, **2004**). A promising approach for functionalizing MENDs is to PEGylate them with a linker that has a peptide sequence that is cleaved in the presence of matrix metalloproteinase, a protease that is specifically secreted from tumor cells. Using this type of PEGylation in combination with a pH-sensitive fusogenic peptide has proven successful for siRNA delivery (**Hatakeyama, et al**, **2009**). A pH-sensitive fusogenic peptide has the capability to, upon reduction of the pH, switch conformation from random coil to α-helix and insert into lipid bilayers, forming pores of a defined size (**Nir and Nieva**, **2000**). This is an attractive capability to enhance release from intracellular endocytotic vesicles that interiorly are acidic.

Covalently formed peptide and oligonucleotide conjugates

For uncharged ONs like PNAs or PMOs, the opportunities to utilize classical non-viral vectors, such as cationic lipids, or non-covalent CPP-ON complexes are limited. Instead a number of covalent coupling strategies to assemble the asON and the peptide have been developed. Continuous peptide synthesis can be employed. More general strategies are fragment coupling, creating thiol-maleimide, thioether, ester or disulfide linkages (**Lebleu, et al**, **2008**,**Pooga, et al**, **1998b**). By using covalent coupling, there is a defined entity, which is advantageous for potential therapeutic applications from a regulatory point of view. The drawback with covalent conjugates is the rather cumbersome protocols for their synthesis. One CPP used for clinical purposes is the $(RXR)_4$ peptide, composed of arginines spaced by 6-aminohexanoic acids, covalently conjugated to PMO (**Abes, et al**, **2006**,**Rothbard, et al**, **2002**). The application of this conjugate is splice-switching activity, primarily for treatment of DMD (**Amantana, et al**, **2007**,**Youngblood, et al**, **2007**). The $(RXR)_4$ CPP has recently been tailored for non-covalent cellular delivery of SSOs by N-terminal stearylation (**Lehto, et al**, **2009**).

CPP-mediated cellular delivery of synthetic asON analogues, for example PNA, is often performed as disulfide conjugates since this linker is cleaved when exposed to a reductive intracellular environment thus releasing the ON from CPP (**Pooga, et al**,

1998b,**Hällbrink, et al**, **2001**). However, a recent report highlights that free reactive thiols can be detected at the cell surface enabling thiol/disulfide exchange reactions. These exchange reactions can lead to the reduction of disulfide conjugates before cellular entry, impairing the translocation efficiency (**Aubry, et al**, **2009**).

The advantages with non-covalent complexes, as compared to covalent conjugates, are that most often non-cumbersome procedures for mixing the two entities are employed, relatively low concentration of the cargo molecule is usually needed and that the non-covalent strategy is useful for anionic cargoes such as ON derivatives or plasmids. The disadvantage associated with non-covalent particles is that the structure of the active compound is not as well-defined as covalent CPP-ON conjugates. This is a potential limitation from a regulatory point of view.

Cellular uptake and translocation of CPPs

The use of CPPs as delivery vectors is nowadays considered to be a functional and effective method for cellular delivery of ONs, while the underlying mechanism for cellular uptake is a controversial matter (**Fonseca, et al**, **2009**). The hydrophobic interior of the lipid bilayer constituting the plasma membrane represents a highly impermeable barrier to most polar molecules (**Miller**, **2003**). In order to translocate molecules and ions into or out of cells, the plasma membrane contains gated ion channels and pumps. Cellular influx and efflux are, for some molecules, regulated by selective membrane-bound transporter proteins. Receptor-dependent or -independent endocytosis is generally involved in cellular internalization for large molecules (**Tréhin and Merkle**, **2004**). Despite these mechanisms, CPPs and potentially associated cargo were initially thought to directly penetrate cell membranes by an energy-independent route (**Derossi, et al**, **1998**). For some CPPs, e.g. MPG (**Morris, et al**, **2001**,**Morris, et al**, **1997**), it has been shown that the major cellular translocation mechanism is independent on the endosomal pathway and involves transient membrane disorganization associated with folding of the carrier into either an α-helical or β-sheet structure within the phospholipid membrane (**Deshayes, et al**, **2004**,**Morris, et al**, **2008**). For most other CPPs it is now generally concluded that endocytosis is involved in the cellular uptake at low, e.g. non-toxic, treatment concentrations (**Duchardt, et al**, **2007**). Different CPPs, concentrations, incubation times and vo-

lumes, cell type, cargo type, and cargo coupling methodology are factors that affect the cellular uptake (**Saar, et al**, **2005**,**Jones, et al**, **2005**,**EL Andaloussi, et al**, **2007a**). Insignificant cytotoxicity is reported for most CPPs at appropriate concentrations but the origin for observed toxicity is seldom delineated. It may origin from general membrane disturbance but also by influencing specific cellular mechanisms, for example inhibition of kinase activity (**Ward, et al**, **2009**).

Direct translocation

Several mechanisms for direct cellular translocation of CPPs have been proposed. Direct penetration of CPPs most likely destabilizes the plasma membrane. This does not, however, necessarily mean that it affects the long-term viability since membrane disturbances may be transient and also most mammalian cells can induce a membrane resealing response (**McNeil and Steinhardt**, **2003**). Most of the proposed mechanisms for direct translocation are based on initial CPP assembly on the outer cell membrane, e.g. interaction with extracellular proteoglycans (PGs) or with phosphates of the phospholipids (**Abes, et al**, **2006**,**Rothbard, et al**, **2004**), followed by direct penetration partly dependent on cell-membrane potential (**Rothbard, et al**, **2002**,**Terrone, et al**, **2003**,**Herce, et al**, **2009**). The PGs having heparane sulfate (HS) moieties have proven especially important for the initial extracellular CPP interaction (**Wadia, et al**, **2004**). A well-received hypothesis for direct translocation that was proposed for the penetratin CPP is the inverted micelle model. This model depends on the formation of a transient, inverted micelle phase consisting of bilayer lipids and CPP. An initial charge–charge interaction between the peptide and the bilayer followed by hydrophobic interactions between the bilayer and tryptophan residues would cause bilayer disruption, resulting in the move of the peptide into the bilayer while being entrapped in an inverted micelle. This micelle is then thought to pass to the opposite side of the bilayer and release its contents directly into the cytosol (**Derossi, et al**, **1998**). The inverted micelle model was confirmed to be independent of membrane proteins, extracellular structures or membrane sub-structures such as caveolae or cholesterol rafts in vesicle uptake studies (**Thorén, et al**, **2000**). Later studies revealed that translocation of CPPs across lipid bilayers depends on the model system utilized, thus there is no simple correlation between the results for peptide translocation in model systems and cellular uptake.

Peptide-lipid interactions alone can therefore not explain the mechanism of cellular uptake of CPPs (**Thorén, et al**, **2004**).

The presence of extracellular structures is not the only factor influencing whether direct cellular translocation occurs. The concentration of CPP applied to the cells has also shown to influence translocation. Cellular uptake studies with fluorescently labeled dodeca-arginine (R12) revealed that R12 peptides cluster into endocytotic structures at relatively low concentrations. When raising the peptide concentration to exceed a certain threshold, no such clustering occurs and the peptide diffuses instead freely into the cytosol indicating direct penetration of the peptide through the plasma membranes (**Duchardt, et al**, **2007**,**Kosuge, et al**, **2008**). Therefore, at low CPP-concentrations, endocytosis is suggested to be the dominant pathways for the cellular uptake of CPPs, at least arginine-rich CPPs, whereas a pathway involving direct translocation through plasma membrane could dominate at higher concentrations (**Nakase, et al**, **2009**).

Translocation via endosomal uptake

Endocytosis is a complex and ambiguous process involving several pathways (**Jones**, **2007**). The main endocytotic pathways can be generally summarized in macropinocytosis, endocytosis dependent on the coat proteins clathrin or caveolin, and pathways independent of clathrin and/or caveolin (**Mayor and Pagano**, **2007**). Dynamin is a protein involved when membrane invaginations are budding off from the plasma membrane to form independent vesicles which later, to various extents, end up in early endosomes (**Mayor and Pagano**, **2007**) (**Figure 6**).

In clathrin-mediated endocytosis (CME), the cytoplasmic domains of plasma membrane proteins are specifically recognized by adaptor proteins and packaged into clathrin-coated vesicles that are brought into the cell (**Grant and Donaldson**, **2009**,**Roth**, **2006**). The uptake is mediated by trimers of clathrin, triskelions, which assemble and coat the intracellular part of the membrane, induce invagination and generate a vesicle. Dynamin is needed for vesicle scission in some types of CME and the vesicles formed are a few hundred nanometers in diameter (**Mayor and Pagano**, **2007**). Endocytosis can also be dependent on caveolin for invagination and the vesicles formed by caveolin mediated endocytosis are 50-80 nm (**Mayor and Pagano**, **2007**), limiting this pathway for large CPP-ON complexes. In resemblance with macropinocytosis, there are also endocytotic pathways, generat-

ing small vesicles, which are independent of clathrin and caveolin. In general, all the cellular endocytotic uptake pathways described result in early endosomes that are mildly acidic (**Mayor and Pagano**, **2007**,**Grant and Donaldson**, **2009**).

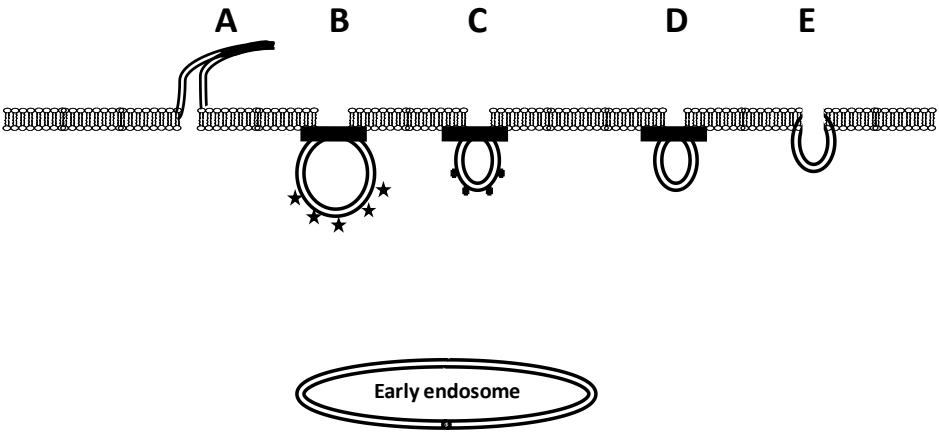

Figure 6: Scheme over pathways for macropinocytosis and endocytosis from cell exterior (upper part) to cell interior (lower part). Macropinocytosis is dependent on actin filaments in the plasma membrane (A). Clathrin-mediated endocytosis (B), and caveolin-dependent endocytosis (C) are dependent on dynamin for vesicle scission. Clathrin- and caveolin-independent pathways can either be dependent (D) or independent (E) of dynamin. Clathrin is represented by stars, caveolin by dots, and dynamin by solid line.

To assess CPP uptake mechanisms, the experiments are performed under conditions where one or several pathways are inhibited. The most widely used strategy has been to treat cells at 4 ^{o}C where all energy dependent processes, i.e. endocytosis, are slowed down so that potentially energy independent pathways can be detected (**Langel**, **2006**). Initial interaction between CPP and the cell is often mediated by PGs with anionic HSs on the cell surface (**Abes, et al**, **2006**,**Wadia, et al**, **2004**). By pre-treating cells with the enzyme heparinase, these structures are cleaved from the cell surface, and the importance of CPP interactions with HSs for cellular uptake can be elucidated. Uptake pathways can also be specifically manipulated by treating cells with endocytosis inhibitors (**Table 2**) prior to peptide exposure (**Sieczkarski and Whittaker**, **2002**). This approach has been greatly exploited in attempts to demarcate endocytotic pathways for different CPPs. The main setback with endocytosis inhibitors is

specificity of the agents since no inhibitor can completely shut down a specific pathway (**Sieczkarski and Whittaker**, **2002**). To confirm endocytosis as the uptake mechanism, chloroquine (CQ) can be added to the cells simultaneously with the cellular CPP treatment. CQ buffers intracellular vesicles delaying the endosomal pathway into lysosomes, and thereby facilitates potential endosomal release (**Bevan, et al**, **1997**). Another approach to assess CPP uptake mechanisms makes use of tracer molecules to determine the endocytotic routes that CPPs are utilizing. The cellular uptake of labeled pathway specific markers in conjunction with CPP-treatment indicates whether the CPP employs the specific pathway.

Table 2: Examples of treatments used to elucidate uptake mechanisms for CCPs. Cellular uptake is affected when pre-treating cells with inhibitors. Summarizing results from various treatments and uptake measurements gives indication of endocytotic pathway utilized by specific CPPs.

Cell treatment	Mechanism of action	Suggested pathway if cellular uptake is:		
		Increased	Unchanged	Decreased
+ 4 °C	Inhibits endocytosis	Not endocytosis		Endocytosis
Wortmannin	Inhibits macropinocytosis and CME	CME and macropinocytosis independent uptake		Macropinocytosis or CME
Cytochalasin D	Inhibits macropinocytosis	Not macropinocytosis		Macropinocytosis
Chlorpromazine	Inhibits CME	CME-independent uptake		CME
Chloroquine	Promotes endosomal release	Endocytosis	Not endocytosis	
Heparinase III	Cleaves extracellular heparan sulfates	Uptake independent of heparan sulfates		Heparane sulfate dependent

CME – clathrin mediated endocytosis

Pyrenebutyrate and peptide hydrophobicity

The crucial molecular properties of CPPs are still not defined. Guidelines for CPP design have been proposed, but universally applicable rules are lacking. Arginine content is one important factor for the functionality of some CPPs. Cationic charge in general, and the hydrophobicity, or more specifically the amphipathic properties, of CPPs are other important factors. The guanidinium head group of arginine residues enables bidentate hydrogen bonding to counter-anions, forming, for example, guanidinium -phosphate, -sulfate or -carboxylic complexes (**Sakai and Matile**, **2003**). The ability for bidentate hydrogen bonding is most likely the reason why oligoarginines, in the presence of hydrophobic counter-anions, have a higher octanol/water partition coefficient than corresponding ornithine oligomers in the presence of hydrophobic counter-anions (**Rothbard, et al**, **2004**,**Sakai, et al**, **2005**). It has been shown that the ability of the guanidium groups of arginine residues to form bidentate hydrogen bonding is also important for cellular uptake (**Rothbard, et al**, **2004**). Oligoarginine peptides with methyl modified guanidium groups showed significantly lower cellular uptake than the corresponding oligoarginine peptides without guanidinium modifications (**Figure 7) (Rothbard, et al, 2004)**. Cellular uptake of arginine-rich CPPs seems consequently, to be partly due to the ability of the guanidinium groups to interact with the phosphate groups of phospholipids in the cellular membrane or, if present, with the hydrophobic counter-anions. Such anions have the capability to partition into the hydrophobic interior of the lipid bilayer in the plasma membranes and influence CPP-uptake.

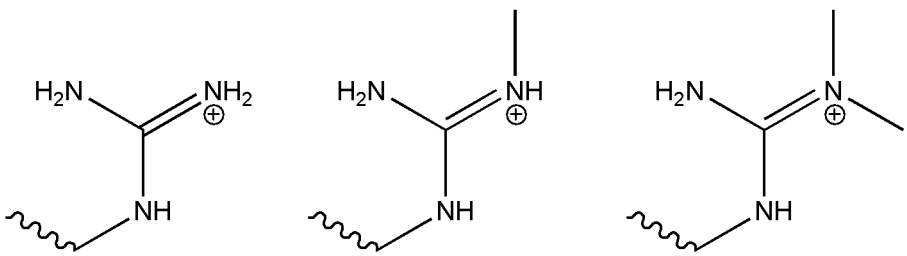

Figure 7: Methyl modifications of guanidinium groups. Oligoarginine peptides without methyl modification (left) are taken up by cells to a significantly higher extent than the corresponding methyl modified peptides (middle and right) revealing the impact of ability for bidentate hydrogen bonding (**Rothbard, et al**, **2004**).

Cellular uptake of arginine-rich CPPs can be improved through the addition of a hydrophobic counter-anion (**Takeuchi, et al**, **2006**). Pyrenebutyrate (PB) has been suggested as a suitable counter-anion to increase such solubility of oligoarginine. Addition of PB to oligoarginine phase-transfer experiments entails significant transfer of the peptides from the aqueous phase to the hydrophobic phase (**Sakai and Matile**, **2003**). Complex formation of PB and oligoarginines results in the addition of hydrophobicity to the hydrophilic oligoarginine peptide (**Figure 8**) (**Sakai and Matile**, **2003**). Altered amphipathic characteristics probably increase the potential of oligoarginine peptides to interact with the lipid bilayers, and, thereby, promote cellular uptake.

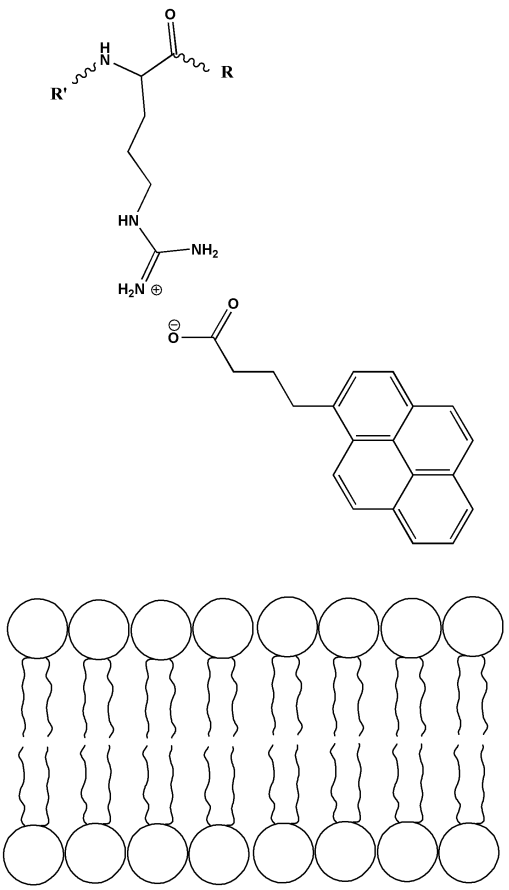

Figure 8: Suggested interaction between guanidinium groups in oligoarginine CPPs and the hydrophobic counter-anion pyrenebutyrate. The positively charged guanidinium group of arginine residues can form bidentate hydrogen bonding to the carboxyl of PB.

Aims of the study

The objectives in this thesis have been to investigate the most critical problems associated with ON-mediated regulation of gene expression. These issues are, firstly, the development of cellular delivery vectors applicable for ONs and, secondly, to assess the ON-induced biological activity in terms of antisense binding to complementary RNA. The specific aims of each paper are presented below:

I. Describe a protocol that allows for convenient assessment of CPP-mediated cellular ON-translocation mechanisms.

II. Determining whether the CPP-mediated cellular ON-translocation mechanisms correlates with the chemical nature of well-characterized CPPs.

III. Screen ON derivatives with the purpose of finding SSOs that display high activity at low concentrations and to investigate the impact of mismatches to target pre-mRNA

IV. Investigate the cellular ON-delivery properties for a chemically modified CPP, stearylated TP10.

V. Assess the impact of charge, hydrophobicity and buffering capacity of CPPs for cellular uptake efficiency and influence on uptake pathway.

Methodological considerations

The experimental details used in this thesis are described in each paper. This chapter will, therefore, discuss the methods with some theoretical aspects. The rationales for the methodological design are more thoroughly described in this chapter than in the respective papers.

Cell Culture

Different cell lines have different properties and respond differently to treatments. For initial screening of CPP uptake mechanisms and evaluation of properties for antisense ONs it is adequate to keep as many parameters as possible fixed and therefore the common accessible and well-characterized HeLa cell line has been utilized. For further rigorous establishment of cellular translocation of CPP and associated cargo it is highly relevant to examine numerous cell-lines and complement with *in vivo* experiments.

HeLa cells

HeLa cells are immortalized and human cervical cancer derived. Initially this cell line was taken from a patient who died from her cancer as far back as in 1951 (**Jones, et al**, **1971**). The HeLa name originates from the patient who was named Henrietta Lacks. HeLa cells are widely used as they are robust and proliferate rapidly. Ordinary HeLa cells or HeLa cells stably transfected with p705, thoroughly described below, have been used in this thesis (**Figure 10**) (**Kang, et al**, **1998**).

Synthesis

The methods for chemical synthesis, purification and analysis of utilized peptides, oligonucleotides and conjugates thereof are here described. The methods apply for all synthesis with exception for *L*-

DNA ONs which were provided by Noxxon Pharma AG and LNA containing ONs which were provided by RiboTask ApS.

Oligonucleotide synthesis

The solid-phase ON synthesis and subsequent purification are extensively described in the supplementary data of paper III. Synthesis of PS DNA and 2OMe RNA were performed using phosphoroamidite chemistry on an ÄKTA^{TM} oligopilot^{TM} plus 10 synthesizer. The synthesis scale was 15 µmol utilizing disposable Oligosynt^{TM} 15 columns. The columns were manually packed with polystyrene based solid support, Primer Support ^{TM} 200. The solid support was functionalized with the respective 3'-end monomer at relatively low loading (~40µmol/g) to achieve high purity of the crude material. Coupling of DNA amidites were performed with 3 equivalents molar amidite excess for 2.5 min while coupling of the more bulky 2OMe RNA amidites had to be performed with 5 equivalents molar excess for 6 minutes. For 5'-labeling of ONs with Cy5, 10 equivalents excess was used and the coupling had to proceed for 10 min to achieve acceptable (>99%) coupling efficiency.

Purification of PS DNA and 2OMe RNA was performed utilizing a previously described anion exchange chromatography (AEC) protocol (**Shanagar**, **2005**), modified to suit the employed synthesis scale. The buffer system was based upon a NaCl gradient, implying that purified ON had to be desalted before analysis and experimental use.

Peptide and peptide nucleic acid synthesis

All peptides and PNA oligomers were synthesized by solid phase peptide synthesis (SPPS), a strategy introduced by Bruce Merrifield in 1963 (**Merrifield**, **1963**). Peptides and PNAs were assembled by *t*-boc chemistry using *4*-methylbenzhydrylamine (MBHA) polystyrene resin to generate amidated C-terminus. C-terminally cysteamide modified TP10 was assembled using 9-fluorenylmethyloxycarbonyl (Fmoc) SPPS with cysteamine-2 chlorotrityl resin. The loadings of resins used for peptide synthesis was approximately 1 mmol/g, whilst the loading was lowered tenfold for PNA synthesis due to the bulky nature of PNA monomers.

Amino acids were coupled as hydroxybenzotriazole (HOBt) esters using a mixture of HOBt and *O*-(benzotriazol-1-yl)-

N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) activators. Alternatively, amino acids were coupled with HOBt and dicyclohexylcarbodiimide (DCC) using an Applied Biosystems 433A peptide synthesizer.

All PNA monomers were coupled on the Applied Biosystems 433A peptide synthesizer, but in contrast to the peptide synthesis another activator was used for PNA synthesis, *2*-(*7*-aza-1H-benzotriazole-*1*-yl)- *N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU), to achieve high coupling efficiency. PNA used for uptake studies was labeled with carboxyfluorescein at the **ε**-amino group of the C-terminal lysine using 5 equivalents of 5,6-carboxyfluorescein, 5 equivalents of diisopropylcarbodiimide (DIC), 5 equivalents of HOBt, and 20 equivalents of diisopropylethylamine (DIEA) for 24 hours.

Fluorescein isothiocyanate (FITC) labeled peptides, used for FACS and confocal microscopy in paper V, were chemically synthesized by Fmoc SPPS. The solid support used here was an Fmoc-rink-amide MBHA resin generating peptides with amidated C-terminus. The N-terminals were modified with a γ-aminobutyric acid (GABA) residue for further attachment of FITC to the peptides as previously reported (**Nakase, et al**, **2004**).

Following cleavage of peptide and PNA oligomers from the solid support by either anhydrous hydrogen fluoride or hydrous trifluoroacetic acid (TFA), peptides or PNA were filtrated to remove the resin and then lyophilized by freeze drying. Crude products were finally purified using preparative reverse-phase high performance liquid chromatography (HPLC) and analyzed using matrix-assisted laser desorption/ionization-time of flight (MALDI–TOF) mass spectrometer. The peptides and PNA were stored at -20 ^{o}C and before initiating a new set of experiments the integrity of the molecules were confirmed by MALDI-TOF analysis.

Conjugation of peptide and peptide nucleic acid oligomers

Conjugation of PNA to CPP was made by generating a disulfide bridge between the two sulfhydryl moieties of cysteines, one positioned N-terminally on the peptide and the other on the PNA. Peptides used for conjugation were synthesized with a 3-nitro-2-pyridinesulfenyl (Npys) derivative of cysteine, which rapidly reacts with thiols to form disulfides (**Figure 9**). Cysteine with Npys was coupled N-terminally to all peptides, except transportan and TP10

where it was coupled to the ε-amino group of lysine$_{13}$ and lysine$_7$, respectively. This results in branched conformations of the TP-PNA and TP10-PNA conjugates. The peptide with Npys was mixed with PNA, holding a cysteine residue, in 20% acetonitrile/water containing 0.1% TFA, incubated over night, purified by HPLC, and thereafter characterized by MALDI-TOF.

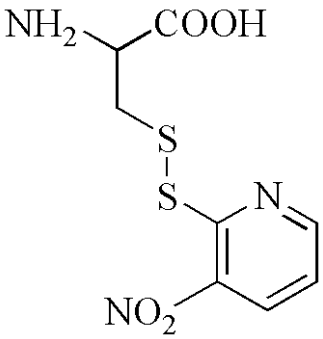

Figure 9: Cysteine activated with 3-nitro-2-pyridinesulfenyl (Npys) used for formation of a disulfide bridges in CPP-PNA conjugates.

Non-covalent complex formation

For cellular delivery of anionic ONs with CPPs, non-covalent complexes were prepared by mixing the peptide and ON in water, serum free cell culture media or buffer e.g. phosphate buffered saline (PBS) or HEPES-Krebs Ringer buffer (HKR) at a concentration 10 times greater than the intended cellular treatment concentration. Complexes were allowed to form by incubation for 30-60 min at room temperature and thereafter the mixture was added to the cells. Optionally, the endosome buffering reagent CQ was also added to the cells at this stage.

Studies of cellular CPP uptake and translocation

One of the major tasks within this thesis is the study of cellular uptake and translocation of CPP and potential cargo ONs. To do this reliable techniques and protocols are essential. Each method has its benefits and flaws and therefore a combination of different protocols are needed to generate trustworthy interpretations of the cellular uptake mechanisms.

Quantitative cellular uptake by fluorometry

The most commonly used method to study cellular uptake of CPPs is to utilize a fluorescent marker cargo covalently attached to the peptide vector. The advantage with this method is that it is quick, easy, and relatively economical. In addition, since the complete cell is lysed before measurement is performed, no artifacts due to different fluorescence quenching properties in different organelles will occur. One major drawback with this method is the lack of discrimination between labeled peptides and associated cargo trapped within intracellular vesicles, e.g. endosomes, and translocated molecules having access to the cell interior, e.g. cellular bio-molecules in the cytoplasm or nucleus. Another disadvantage with fluorometric measurements is the difficulty to fully remove extracellular CPPs, tightly bound to outer part of the plasma membrane. Trypsin treatment has been used within this thesis and it has been suggested that trypsin may not fully remove all extracellular peptides (**Mueller, et al**, **2008**). However, the variation of amount extracellular bound peptides is small for a wide range of different CPPs and similar artifactual contribution of extracellularly bound CPPs can be assumed (**Mueller, et al**, **2008**).

Confocal Microscopy

To provide spatial information of the intracellular localization of CPPs taken up by cells confocal microscopy has been utilized. This method provides opportunity to discriminate between surface or extracellularly bound peptides and internalized peptides. One limitation with confocal microscopy is the detection limit exemplified by the uptake experiments performed in paper V with Cy5-labeled ON and the R9 peptide. The confocal microscopy experiments indicated that the Cy5-ON was trapped within intracellular vesicles and very limited amounts of ON could be visualized in the nucleus. However, ON-induced splice-switching, which takes place in the nucleoli of the nucleus, could be observed in complementary experiments. This indicate that low amounts of ON in the nucleus is sufficient to induce splice-switching activity.

Fluorescence assisted cell sorting

Flow cytometry is a tool for counting and sorting suspended cells. Fluorescence assisted cell sorting (FACS) is a flow cytometry technique used to sort cells based on their fluorescence and this method

has been used for quantification of cellular uptake of FITC-labeled CPPs. In resemblance with quantitative cellular uptake determination by fluorometry, analysis by FACS cannot discriminate between peptides trapped in intracellular vesicles and peptides that have translocated into the cytosol. The potential variations of fluorescence quenching in different organelles cannot be taken into consideration when using FACS. In contrast, FACS-analysis gives an indication of the proportion of the cell population that has taken up CPP.

Characterization of uptake pathways

The most commonly used method to characterize CPP-uptake mechanisms has been employment of cellular treatment at 4 ^{o}C (**Langel**, **2006**). The rational for such methodology is to determine whether the cellular CPP uptake relies on energy-dependent mechanism or not. The impact of extracellular PGs, such as HS can be assessed by pretreating cells with heparin or heparinase. The contribution of endosomal uptake can be evaluated by adding CQ to the treatment regime. CQ buffers intracellular vesicles delaying the lysomal pathway for endosomes, and thereby facilitates potential endosomal release (**Bevan, et al**, **1997**).

To characterize the endocytotic pathways a number of endocytosis inhibitors can be employed. There is a wide arsenal of such inhibitors available and most of them originate from extensive virology research where they have been used as means to assess the cellular entry mechanisms for viruses (**Forest, et al**, **2005**,**Kim, et al**, **2007**). The main setback with the use of such inhibitors is that specificity has been challenged (**Sieczkarski and Whittaker**, **2002**) and that it is difficult to determine the optimal treatment concentration, simultaneously avoiding low inhibiting activity and low specificity. To avoid the flaws associated endocytosis inhibitors a combination of several endocytosis inhibitors should be utilized to give premises of the cellular uptake pathway for a specific CPP and potentially associated cargo. Other possibilities to circumvent the flaws with potentially unspecific inhibitors is to use cells with tetracycline controlled transcription of e.g. clathrin or caveolin (**Ter-Avetisyan, et al**, **2009**) and siRNA to control gene expression of proteins crucial for certain uptake pathways (**Säälik, et al**, **2009**).

Luciferase splicing reporter assay

To facilitate evaluation of SSOs, Kole and co-workers have constructed a splicing reporter system (**Kang, et al**, **1998**,**Dominski and Kole**, **1993**) with a plasmid carrying a luciferase-coding sequence that is interrupted by an insertion of intron 2 from β-globin pre-mRNA, carrying an aberrant splice site that activates a cryptic splice site. Unless this aberrant splice site is masked by an SSO, the pre-mRNA of luciferase will give rise to expression of non-functional luciferase (**Figure 10**). By using HeLa pLuc 705 cells which are stably transfected with this plasmid, it is possible to evaluate various SSOs by measuring induced luciferase activity and thereby generate a positive read-out (**EL Andaloussi, et al**, **2006**). Luciferase activity in lysate from HeLa pLuc 705 cells has been an indicator for successful cellular delivery of SSOs (paper I, II, IV and V) and as a tool to verify splice switching efficiency and specificity for various ON derivatives (paper III).

The HeLa pLuc 705 splice-switching reporter assay has by no means any clinical relevance but it is a suitable assay for delineating the properties of antisense ONs developed for the purpose of targeting disease related protein isoforms. The splice-switching reporter assay is also a suitable tool for evaluation of CPP-mediated translocation of bioactive ONs.

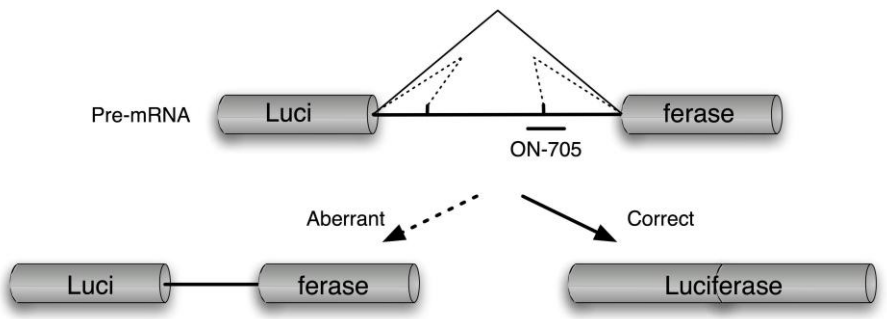

Figure 10: Reporter system for splice switching based on a plasmid, pLuc 705, carrying a luciferase-coding sequence with insertion of intron 2 from β-globin pre-mRNA containing an aberrant splice site that activates a cryptic splice site. Unless the aberrant splice site is masked by a splice switching oligonucleotide, ON-705, non-functional luciferase is expressed (**Kang, et al**, **1998**).

Reverse-transcriptase polymerase chain reaction

Quantification of splice-switching activity by analysis of functional luciferase, as carried out here, is a sensitive and convenient assay. It allows for easy comparison of different sorts of SSOs in terms of efficiency or specificity. It allows also for evaluation of various cellular translocation vehicles, such as the CPPs investigated. However, the data observed from analysis of functional luciferase are expressed in relative light units (RLU) and do not allow direct determination of the extent to which aberrant splicing has been corrected (**Abes, et al**, **2007**). To get an indication of the ratio of correct and aberrant mRNA, reverse-transcriptase polymerase chain reaction (RT-PCR) can be employed (**Mahbubani, et al**, **1991**). For absolute determination of the pool of aberrant and correctly spliced mRNA it is accurate to use a better quantitative method like real-time PCR.

In RT-PCR, the mRNA from treated cells is extracted and transcribed to DNA using a polyT primer. Subsequently, the DNA strands of interest are amplified by PCR using specific primers. The RT-PCR products from the aberrantly and correctly spliced luciferase pre-mRNA can easily be separated by gel electrophoresis, thus allowing evaluation of the extent of splice switching. RT-PCR measurements in combination with luciferase luminescence measurements is an excellent procedure to ascertain splice switching activity. The details for RT-PCR analysis is described in paper II and III.

Toxicity measurements

Assessing cell membrane integrity is one of the most common ways to measure cytotoxic effects. Several methods have been developed to evaluate integrity of cells after treatment with different compounds. Some are addressing the effects on membrane integrity while others are measuring long-term toxic effects in terms of metabolic activity. The cytotoxicity, as evaluated by cell integrity analysis, of CPPs included in this thesis does not induce significant leakage at the concentration applied (**EL Andaloussi, et al**, **2007b**,**EL Andaloussi, et al**, **2007a**,**Takeuchi, et al**, **2006**,**Thorén, et al**, **2003**).

Wst-1 assay

Long-term cell viability measurements are included to address the toxic profile of the cellular treatments applied (Paper II-V). Several

assays to evaluate cell viability have been developed based on tetrazolium salts that are, in viable cells, converted to different formazan products by mitochondrial dehydrogenases. One such assay is the Wst-1 assay. In this assay, Wst-1 is cleaved by the mitochondrial succinate-tetrazolium-reductase system to generate formazan (**Figure 11**). Quantification of the formazan dye produced by metabolically active cells was conducted on a spectrophotometer by measuring absorbance (420 nm) 1 - 4 h after addition of Wst-1 to the cells. The absorbance is directly correlated to mitochondrial dehydrogenase activity and, hence, to the number of viable cells (**Scudiero, et al**, **1988**).

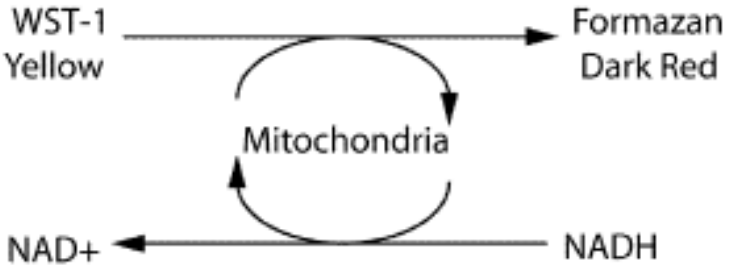

Figure 11: Proliferation assay. The wst-1 salt is cleaved by mitochondrial succinate-tetrazolium reductase forming the soluble formazan dye, which is detected by absorbance measurements as an indicator of viable cells.

Lactate dehydrogenase leakage assay

The cellular leakage of the enzyme lactate dehydrogenase (LDH) was used to measure integrity of the plasma membrane after treatment with CPPs. In cells, LDH is responsible for the conversion of lactate and pyruvate with concomitant exchange of NAD^+ and NADH. Release of LDH from damaged cell membranes is measured in the surrounding media using an enzymatic assay, CytoTox-ONE^{TM}, where the substrate reazurin is converted to resorufin, which can be quantified by fluorescence measurements at λ_{ex}= 560 nm and λ_{em} = 590 nm (**Figure 12**). A potential short-coming with the LDH leakage assay is that the protein detected is relatively large protein and, hence, small pores created by CPPs could be difficult to detect.

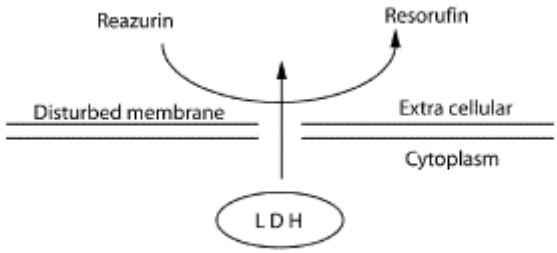

Figure 12: LDH leakage assay. LDH released from the cell is measured in the surrounding media. The substrate reazurin is converted by LDH to resorufin, which is fluorescent and can therefore easily be quantified.

Calcein-release from large unilamellar vesicles

An helpful method to assess the effect of peptides on vesicle integrity is to employ a calcein release assay. The calcein release assay provides important information on the membrane perturbation effects of the peptide. Calcein release is commonly used as a means of quantifying the interaction and perturbation caused by CPPs or antimicrobial peptides on lipid bilayers (**Magzoub**, **2004**). The advantages of using calcein include its high solubility, the fact that calcein has a negative net charge at neutral pH, which minimizes its interaction with membranes especially when anionic lipids are used, and the fact that calcein is self-quenched at high concentrations (**Chen and Knutson**, **1988**). Any changes to the vesicle integrity resulting in release of entrapped calcein and its dilution in the surrounding buffer can be observed as an increase in the fluorescence intensity, i.e. de-quenching of the calcein dye.

Model membranes were used for the calcein release since cellular membranes are beyond the scope of most spectroscopic techniques employed in biophysical studies. Simplified model membranes were used although they lack the proteins, glycolipids, cholesterol and coating carbohydrates that are found in cell membranes. Large unilamellar vesicles (LUVs) are perhaps the most biologically relevant vesicle system available, in that they size-wise are more similar to natural cells than micelles or small unilamellar vesicles and they are more stable membranes than giant unilamellar vesicles (**Magzoub**, **2004**).

Results and discussion

The reports included in this thesis aim for delineating critical issues associated with ON-mediated regulation of gene expression and, to some extent, finding solutions to these issues. The objectives are different between the papers. In paper I, II, IV and V, the opportunities to use CPPs as vectors for bioactive ONs are investigated. In paper III, the ON derivatives *per se* are investigated with focus on their properties in terms of biological activity and specificity.

Paper I

This paper describes in detail a protocol to assess CPP activity based upon the splice switching assay in combination with regular quantitative uptake measurements by fluorometry. The HeLa pLuc 705 cells were previously exploited with the aim of discovering efficient and non-toxic ON-delivery vectors. In different reports, the experimental settings are divergent in terms of the treatment and incubation times, making comparisons between studies difficult. This particular protocol presented here is optimized to delineate the uptake mechanisms of specific CPPs. We describe not only the use of covalent conjugates of CPP and PNA, but also the possibility to employ the non-covalent strategy between CPPs and the negatively charged 2OMe RNA, targeting the same aberrant splice site.

When comparing the cellular delivery of different CPP-PNA conjugates promoting splice-switching, M918 was found to be the most potent peptide followed by the amphipathic peptides transportan and TP10. pTat(48-60)-PNA display very low cellular uptake and induces hardly any splice-switching. Since most studies using CPPs have employed penetratin or some version of pTat (**Park, et al**, **2002**), the role of endocytosis in the uptake of penetratin-PNA was further investigated. The pTat(48-60)-PNA conjugate was omitted in this study since the levels of splice switching activity was very low. In accordance with M918-PNA, penetratin conferred internalization and splicing increased dose-dependently with increasing concentrations of

the conjugate. Furthermore, inhibitors of macropinocytosis significantly decreased the splicing efficiency, which is in line with the results concerning M918. However, unlike M918-PNA, penetratin-PNA is dependent on initial interaction with HS for subsequent internalization, in accordance with previous reports (**EL Andaloussi, et al**, **2007b**).

Finally, paper I is a convenient protocol for the evaluation of CPP delivery efficacy and this protocol presents a robust means of evaluating the efficacy of CPPs and to investigate the influence of various endocytotic pathways.

Paper II

This study assesses efficacy and evaluates mechanistic properties of previously well-characterized CPPs using the protocol (Paper I) for CPP-PNA conjugates. The CPP selection includes representation of CPPs with high degree of hydrophobicity and a group of CPPs with cationic and more hydrophilic nature. The involvement of endocytosis was initially confirmed through the use of the endosomal buffering agent, CQ, in combination with cellular CPP-PNA treatments at 4 ^{o}C. In order to elucidate the endocytotic mechanisms, heparinase III in combination with commonly utilized endocytosis inhibitors and pathway specific tracer molecules were exploited. However, not all peptides examined were able to successfully mediate translocation of the PNA SSOs. This highlights the necessity of using a biological readout assay when evaluating CPPs.

Cellular pre-treatment with the chlorpromazine inhibitor, which limits CME, significantly decreased ON-translocation mediated by the relatively hydrophobic CPPs, transportan and TP10. The ON-translocation mediated by CPPs displaying less hydrophobicity and more cationic chemical properties was reduced after pre-treatment of the macropinocytosis inhibitors, wortmannin and cytochalasin D. The tendency that arginine-rich CPPs employ macropinocytosis and more hydrophobic CPPs, transportan and TP10, employ CME for cellular uptake was confirmed in experiments using the pathway specific tracer molecules. The influence of HSs, an anionic extracellular PG, on CPP-mediated translocation was investigated by pre-treating cells with heparinase III. Removal of extracellular HSs lowered the CPP-mediated ON-translocation for all CPPs examined except for the cationic M918. This indicates that cellular uptake of M918 may involve

another cell surface component that becomes accessible when removing HSs. Minor dependence of extracellular PGs for cellular uptake of M918, compared to other CPPs like penetratin or TP10, is confirmed by a report where glycosaminoglycan-deficient chinese hamster ovarian cells have been used (**EL Andaloussi, et al**, **2007b**).

The results indicate differences in cellular ON-uptake mediated by different CPPs but the results show no evident correlation between the chemical nature of the CPPs and ON-translocation, as observed in the splice-switching assay.

Paper III

The use of LNA for antisense applications is emerging since its discovery, a decade ago (**Kumar, et al**, **1998**). The potential therapeutic antisense applications discussed are mainly for targeting micro-RNA (**Stenvang and Kauppinen**, **2008**) and mRNA (**Jepsen, et al**, **2004**), but its potential as SSO is also a relevant therapeutic application (**Roberts, et al**, **2006**). In this paper, the splice-switching activity of SSOs holding nucleic acid analogues was investigated using a commercial transfection reagent. It became evident that chimerical SSOs holding high percentage of LNA monomers unambiguously displayed the most prominent splice-switching activity. The splice-switching activity induced by a chimerical LNA/2OMe RNA SSO, i.e. mixmer, holding 50% LNA monomers was more than double that compared to the corresponding ON-sequence, with only 2OMe RNA monomers. However it was established that the mismatch control sequence for chimerical LNA/2OMe RNA SSO induced similar splice-switching activity, revealing low specificity for the LNA/2OMe RNA mixmer. Even when reducing LNA content to 33%, the corresponding mismatch control sequence induced high splice-switching activity. This observation is obviously important and must be carefully considered when developing ON derivatives holding LNA monomers for regulation of gene expression.

The other nucleic acid analogues examined, PS DNA and L-DNA, induced lower splice-switching activity than the LNA-mixmers, but the ratio to the effect induced by the mismatch control sequences were higher, indicating better specificity to target pre-mRNA. For PNA, the target specificity was striking but the induced splice-switching activity could not be compared to the anionic ONs examined since the PNA SSO had to be transfected as a CPP-

conjugate due to its uncharged nature. The M918 CPP was used as a cellular vector for the PNA sequences.

Subsequent to the publication of paper III, induced splice-switching activity for a number of truncated versions of the LNA/2OMe RNA mixmer holding 33% LNA monomers were investigated. It became evident that that shortening of the sequence length from 18 to 12 monomers provided target specificity for the LNA sequences (**Table 3**) without severely compromising efficiency. Shortening the SSO sequences will enable higher doses, in molar, when performing *in vivo* experiments since such experiments are limited by the amount of active substance per dose in weight units. The truncated 12-mer version of the LNA/2OMe mixmer was used in paper V for splice-switching experiments.

Table 3: Sequences of truncated SSOs and corresponding luciferase activity (RLU). Treatments were performed with 100 nM SSO and Lipofectamine 2000™. Splice switching activities for sequences with mismatches are related to respective correct sequence to give an estimation of specificity. None of the 9-mers induced any splice switching activity as compared to untreated cells. Experiments are performed in triplicate (n=2).

Name	Sequence	Mis-matches	Length	LNA mono-mers	Activity* (%)
2OMe	5'- CCU CUU ACC UCA GUU ACA	0	18	0	**28**
2OMeinv	5'- CCU CUU ACA CUC GUU ACA	4			
LNA1	5'- cCU cUU aCC UcA GUt ACa	0	18	6	**99**
LNAinv1	5'- cCU cUU aCA CtC GUt ACa	4			
LNAtr1	5'- cCU cUU aCC UcA GUt	0	15	5	**74**
LNAtrinv1	5'- cCU cUU aCA CtC GUt	4			
LNAtr2	5'- cUU aCC UcA GUt	0	12	4	**17**
LNAtrinv2	5'- cUU aCA CtC GUt	4			
LNAtr3	5'- cUU aCC UcA	0	9	3	**No activity detected**
LNAtrinv3	5'- cUU aCA CtC	4			

*Percentage of induced luciferase activity for control sequences with mismatches compared to activity for correct sequences.

Paper IV

In order to achieve successful cellular ON transfection, efficient delivery vectors are necessary. In this study we present one CPP-based vector, the chemically modified TP10, displaying prominent properties for *in vitro* delivery of anionic splice-switching ONs. Due to the non-toxic nature of this peptide-based vector it has the potential for *in vivo* applications as well.

Covalent attachment of ONs or other cargo molecules to CPPs has been considered the way to go for taking CPP-mediated ON-delivery techniques from the lab bench to clinical settings. Therefore, much effort has been put into developing convenient conjugation strategies, to attach one or rather several CPPs to each ON (**Turner, et al**, **2007**,**Takayama, et al**, **2009**). Non-covalent strategies generating nano-particles of cationic CPPs and anionic ONs have been suggested (**Morris, et al**, **2008**). The non-covalent strategy generally employs a simple co-incubation approach, where cationic peptides and the negatively charged ONs are mixed at certain molar ratios in order to create nanoparticle complexes that are added to the cells (**Morris, et al**, **1997**,**Kumar, et al**, **2007**). The two chemical modifications, N-terminal stearylation and C-terminal cysteamidation of CPPs, have been shown to be critical for improving the non-covalent cellular delivery of ONs (**Futaki, et al**, **2001**,**Gros, et al**, **2006**). Therefore, we modified three peptides- TP10, penetratin and R9 N-terminally with a stearyl moiety and compared their transfection efficiency with the commercially available transfection agent Lipofectamine™ 2000 and previously published MPG peptide.

First, the transfection efficiency of Cy5-labeled PS 2OMe RNA with unmodified CPPs TP10, penetratin, and R9 was evaluated. In spite of considerable quantitative uptake of ONs no significant splice switching activity was detected. This contradiction can be explained by entrapment of the bioactive ON inside endosomes, which was confirmed in the case of TP10 and penetratin. The splice switching activity increased significantly after ON-transfection mediated by TP10 and penetratin if the endosomal buffering agent, CQ was added to the cells simultaneously with the non-covalent CPP ON complexes. The ON-translocation efficiency mediated by the unmodified and cysteamidated CPPs was low. After stearylation of the CPPs, no significant difference between in terms of quantitative ON uptake could be detected. However, stearylated TP10 proved to be exceptionally effec-

tive for mediating cellular translocation of the SSOs. The levels of correctly spliced functional luciferase reached levels comparable with results achieved for ON transfection with the commercial reagent Lipofectamine™ 2000.

Since the cationic lipids are associated with quite severe cellular toxicity, we evaluated the effect of stearylated TP10 on cell proliferation. Stearylated TP10 displayed no long-term toxicity even at the highest employed concentrations. Conversely, Lipofectamine™ 2000 induced significant impairment of metabolic activity at the concentration suggested by the manufacturer.

Stearylation of the cationic TP10 results in altered composition of hydrophobic and hydrophilic surfaces of the CPP and thereby its capacity to form stable complexes with the various surfaces of the ON cargo may be enhanced (**Futaki, et al**, **2001**,**Berman**, **1986**). The capacity to execute endosomal escape is probably influenced positively since the bioactivity mediated by the ON cargo increases with the stearyl modification of TP10, whilst the increase in quantitative uptake is less pronounced by the stearylation. TP10 differs from the other two CPPs included in this study, penetratin and R9, since it is relatively amphipathic and it does not have any arginine residues in the sequence. Additionally, TP10 has been assigned to be taken up by CME and this is not the case for any other well-characterized CPP, apart from transportan (Paper II). The non-covalent CPP-ON complexes are probably size-wise a border-line case for being able to employ CME as cellular uptake pathway. Provided that stearylated TP10 in a non-covalent complex with ONs employs CME as the cellular uptake route, this may explain why stearylation has different impact for TP10 than for penetratin and R9, which utilizes other pathways. The positive effects of stearylation, generates, obviously, beneficial characteristics that have a greater impact for TP10 than for penetratin and R9.

In conclusion, we have shown that stearylation of TP10 results in a non-toxic and highly efficient ON delivery vector reaching transfection efficiencies comparable with the commercial transfection agent Lipofectamine™ 2000. Moreover, the ON-induced splice-switching activity mediated by stearylated TP10 was high even when transfections were performed in the presence of serum.

Paper V

The aim with this paper is to investigate the impact of PB on cellular CPP-uptake in different set-ups to bring new light onto the complicated and debated issue of the mechanisms for CPP-uptake. There is no evident commercial interest in using PB for cellular transfection since this hydrophobic counter anion easily mediates toxic effects and CQ is usually needed for successful cargo translocation. By investigating CPP uptake in the presence of PB, an indirect addition of hydrophobicity to the CPPs is achieved and the impact of this additional hydrophobicity can be evaluated. The investigated CPPs have been assigned a theoretical and relative hydrophobic ranking according to the von Heijne scale for biological hydrophobicity to accurately estimate the hydrophobicity of the CPPs *per se* (**Hessa, et al**, **2005**). This scale was introduced as a tool to predict transmembrane helices in amino acid sequences of membrane proteins and to estimate the membrane insertion competence of peptide segments.

The CPPs included in this study have different arginine content and hydrophobic properties. The CPPs are divided into three subgroups based upon their hydrophobic characteristics. The subgroups are hydrophilic (R9 and pTat48-60), intermediately hydrophobic (penetratin, PenArg, pVEC, and M918), and hydrophobic (TP10). The CPPs have been studied for two biological assay endpoints, the cellular uptake of FITC-labeled CPPs and the splice-switching assay, and one chemical assay endpoint, calcein release from LUVs, and the effect of PB on these three endpoints.

When pre-treating cells with PB followed by treatment with arginine-rich CPPs, the PB counter-anion may first inserts into the cell membrane followed by hydrogen bonding to the guanidinium groups of surrounding CPPs (**Takeuchi, et al**, **2006**). Subsequent to CPP interaction with membranes or with PB, inserted into the membranes, the cellular membrane potential may have an influence on the uptake, inducing direct membrane penetration (**Rothbard, et al**, **2004**). On the other hand, guanidinium groups have been suggested to promote endocytosis by interacting with extracellular structures (**Rothbard, et al**, **2002**,**Duchardt, et al**, **2007**,**Nakase, et al**, **2007**).

The results in paper V suggest distinct patterns of cellular uptake and translocation mechanisms, varying for the peptides examined. Overall, two major cell entry pathways have been observed: the endosomal pathway composed of two steps, namely endocytotic entry followed by endosomal escape and direct cell membrane pene-

tration. PB has a positive effect on cellular uptake for arginine-rich CPPs, both in the cellular uptake experiments with FITC-labeled CPPs and in the splice-switching assay where asONs were delivered. The interpretation of this observation is not straightforward since different cellular treatment regimes had to be used in the two experimental set-ups. The PB concentration is considerably higher in cellular uptake experiments with FITC-labeled CPPs than in the splice-switching assay. Additionally, in the splice-switching assay CQ had to be added to achieve translocation of the asONs, and this is not case for the uptake experiments where diffuse intracellular distribution of FITC-labeled CPPs were observed without CQ. Based on these differences, the suggested interpretation for the hydrophilic and arginine-rich CPPs, in particular R9, is that direct penetration is the major pathway for FITC-labeled CPPs in the presence of high PB concentration. Conversely, an endocytotic pathway, presumably arginine-induced macropinocytosis, dominates in the splice-switching assay where significantly lower concentration of PB was applied in the cellular treatments. The shift to an endocytotic pathway may be due to lower PB concentration used in the splice-switching assay or due to the treatment with the non-covalent complex of ON and CPPs instead of treatment with free peptides or a combination thereof. The need for CQ to achieve successful ON-translocation indicates that potential CPPs with a buffering capacity *per se*, counteracting acidification in the endosomal pathway, may delay the formation of late endosomes, lysosomes, and associated degradation. The opportunity for endosomal release will then be improved and thereby increase the possibility for successful translocation of CPP and associated cargo.

The hydrophobic CPPs examined are not influenced by PB in any of the two biological endpoints. However PB, has positive influence on the hydrophilic CPPs both in the biological endpoints and calcein release from palmitoyl-2-oleoyl-phosphatidylcholine (POPC) LUVs is promoted. The LUV results, thus, supports the positive effect of PB in the biological endpoints, i.e. cellular uptake of FITC-labeled CPPs and of asONs in the splice-switching assay for the hydrophilic and arginine-rich CPPs.

The effect of PB on calcein release from POPC LUVs is less extensive for the hydrophilic CPPs than for the intermediately hydrophobic CPP. The rate limiting step for cellular uptake of the intermediately hydrophobic CPPs should therefore not be linked to direct membrane perturbations, neither on the plasma membrane nor on intracellular endosomal vesicles. The rate-limiting step for ON-

translocation mediated by these CPPs is rather the endocytotic entry process or the transfer from early endosomes to late endosomes, influencing the opportunities for endosomal release.

In summary, the cellular delivery of hydrophilic arginine-rich CPPs and cellular translocation of associated ON become more effective when PB is present. These findings will influence rational development of new covalently modified guanidinium-rich CPPs with favorable properties in terms of hydrophobicity. Such CPPs will more easily be released from endosomes and be suitable for *in vivo* conditions where the opportunity to use hydrophobic counter-anions is restricted. For the more hydrophobic CPPs, there is room for improved cellular translocation efficiency by modifications that enhance the endocytotic entry, the endosomal release and modifications that delays the intracellular pathway to late endosomes and associated degradation.

Conclusions

The scope of this thesis is to address two major issues associated with ON-mediated regulation of gene expression; the target specificity of asONs and their cellular delivery. The conclusion of this thesis is based on the essential findings in each of the five papers included, as described below.

Paper I

We establish a universally applicable protocol for determination of cellular CPP-uptake pathways and translocation of associated cargo. By treatment with endocytosis inhibitors, we conclude that the uptake mechanism of penetratin can be ascribed macropinocytosis. Finally, we demonstrate the importance of using functional biological assays to address CPP-mediated ON translocation.

Paper II

The chemical nature of CPPs does not necessarily influence the efficacy of CPP-mediated translocation of splice-switching PNAs. However, chemical characteristics of the CPPs influence uptake pathway; hydrophilic and cationic CPPs tend to mediate cellular uptake via macropinocytosis while more hydrophobic CPPs tend to exploit CME.

Paper III

Strikingly enhanced splice-switching activity but lowered specificity is observed when introducing LNA monomers to SSOs. The results highlight the importance of rational sequence design to allow for high efficiency with simultaneous high mismatch discrimination for SSOs and suggest that splice switching activity is tunable by utilizing LNA monomers.

Paper IV

Introduction of an N-terminal stearyl moiety to the amphipathic CPP TP10 significantly improves cellular delivery of splice-correcting oligonucleotides in non-covalent CPP:ON complexes. Furthermore, the efficacy is preserved in serum and the complex do not display any significant cytotoxicity − characteristics being of great importance for future *in vivo* applications.

Paper V

The hydrophobic counter-anion PB facilitates cellular uptake and translocation of ON mediated by the R9 CPP. More hydrophobic CPPs are significantly less influenced by presence of PB. By combining the different experimental results, we conclude that for R9, direct translocation occurs in the presence of high PB concentration while endocytosis dominates at lower PB concentrations. We suggest that PB has different sites of action for the two pathways, direct translocation and endocytosis. These findings will have implication on the development of new covalently modified CPPs, in line with the new CPP presented in paper IV. Similar peptides or peptide derivatives may well have applications for drug delivery, in particular for ON-based regulation of gene expression.

Populärvetenskaplig sammanfattning på svenska

I alla kroppens celler finns kromosomer som består av DNA. Kromosomernas DNA är vår arvsmassa och innehåller all information som krävs för att kroppen och dess celler ska fungera. Informationen i arvsmassan är grundritningar till de proteiner som varje cell tillverkar. Med hjälp av RNA förmedlas dessa ritningar från cellkärnan till cellens cytoplasma där tillverkningen av protein sker. Felaktig tillverkning av proteiner påverkar sjukdomsbilden i ett flertal vanliga och allvarliga sjukdomar. Läkemedel som består av syntetiskt tillverkade derivat av oligonukleotider (ON) gör det möjligt att åtgärda felaktig syntes av proteiner på ett tidigt stadium i den cellulära tillverkningsprocessen. De syntetiska ON-derivaten interagerar med cellens RNA på ett specifikt sätt och beroende på formgivningen av ON kan tillverkningen av ett specifikt protein påverkas. På detta sätt kan ON-baserade läkemedel ändra sjukdomsbilden på ett specifikt sätt.

För att ON-derivat ska kunna reglera genomets funktion, eller mer specifikt, påverka tillverkningen av ett visst protein, krävs att den kommer in i cellen. Antingen till cellens kärna eller till dess cytoplasma, beroende på vilken mekanism man har för avsikt att påverka. Ett stort problem är att det cellulära upptaget är mycket begränsat. Därför krävs metoder för att förbättra det cellulära upptaget av ON-derivat. I denna avhandling har, för detta ändamål, ett antal cell-penetrerande peptider (CPP) undersökts och utvecklats. En annan begränsning för användningen av ON är deras specificitet. Det krävs att ON-derivat enbart påverkar tillverkningen av det protein som avses för att undvika biverkningar.

Funktionaliteten och specificiteten för olika syntetiska ON undersökts. Baserat på dessa resutat presenteras riktlinjer för hur genreglerande ON bör formgivas. Dessutom har mekanismer för peptid-baserad cellulär leverans undersökts och det har klarlagts att hydrofobicitet är viktigt för effektiv ON-leverans med CPPer. En stearyl-modifierad version av CPPn TP10 har utvecklats och den har mycket goda egenskaper att transportera ON in i celler utan att påverka deras allmäntillstånd.

Resultaten visar att CPP och ON-derivat har möjlighet att tillsammans bli ett vinnande koncept för att utveckla nya läkemedel. Dessa läkemedel kan formges för att påverka funktionen av vår arvsmassa och på så sätt kan ett stort antal allvarliga sjukdomar behandlas.

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