From DEPARTMENT OF LABORATORY MEDICINE
Karolinska Institutet, Stockholm, Sweden

GENE TARGETING AND DELIVERY OF THERAPEUTIC OLIGONUCLEOTIDES

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Stockholm 2017

Published by Karolinska Institutet.
Printed by Eprint AB 2016

ISBN 978-91-7676-555-5

Gene targeting and delivery of therapeutic oligonucleotides
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my wife and family

ABSTRACT

Research in gene therapeutic strategies involving oligonucleotides (ONs) constitutes a growing field with several clinical products already approved and many more under intense investigation. The recent advances have mainly involved the Antisense ON platform, with the aim of modulating gene expression on the RNA level. In contrast, targeting DNA using anti-gene ONs is a much less explored therapeutic option, but has been shown to be able to provide a potent alternative for the modulation of gene expression. Many genetic diseases originate in mutations in the genome, and by directly targeting these using anti-gene ONs could potentially have advantages over RNA based options, including lower dosage and reduced toxicity.

In this thesis, several anti-gene therapeutic approaches involving ONs are presented, optimized and evaluated both *in vitro* and in cells. In Paper I, a novel approach for the generation of Zorro-LNA using click chemistry is developed, in order to join the two anti-gene ONs involved in a 3′-5′- 5′-3′orientation. This strategy replaces the use of reverse LNA phosphoroamidites and provides a screening platform suitable for optimizing new Zorro-LNA constructs. In paper II, single stranded ONs targeting the CAG-repeat region in the *Huntingtin* gene is used to down-regulate the mutant protein responsible for Huntington´s disease. The ONs are active in patient-derived fibroblasts and can efficiently reduce both mRNA and protein levels up to seven days following transfection and naked uptake delivery strategies. We also demonstrate, in different assays, that the mode of action is through ON binding to DNA, and not through RNA interactions.

In paper III, we optimize different bisLNA anti-gene ON constructs for efficient DNA strand invasion using novel chemistries and intercalators. We demonstrate the selective binding through both Hoogsteen and Watson-Crick hydrogen bonding to supercoiled DNA in a physiological environment. In addition, the DNA binding is assessed in bacterial cells and detected using rolling circle amplification. Paper IV evaluates the specific delivery to cancer cells using aptamer-mediated uptake of LNA-containing ONs. The effect on the aptamer plasticity is investigated using chemical modifications, both in the cargo ON and in the aptamer itself, as well as the influence of the construct properties on cellular uptake in two different cell lines. Taken together, the results presented in this thesis aims at advancing the anti-gene based ON therapeutic strategies in terms of efficacy and specific delivery.

LIST OF SCIENTIFIC PAPERS

The following papers are included in the thesis:

I. O. Gissberg, M. Jezowska, E. M. Zaghloul, N. I. Bungsu, R. Stromberg, C. I. Smith, K. E. Lundin, and M. Honcharenko, **Fast and Efficient Synthesis of Zorro-Lna Type 3'-5'-5'-3' Oligonucleotide Conjugates Via Parallel in Situ Stepwise Conjugation**, Org Biomol Chem, 14 (2016), 3584-90
II. Zaghloul E, Gissberg O, Moreno P, Hällbrink M, Zain R, Jorgensen A, Wengel J, K.E. Lundin, C.I. E. Smith. **GTC repeat-targeting oligonucleotides for down-regulating Huntingtin expression**. (Manuscript 2016)
III. S. Geny, P. M. Moreno, T. Krzywkowski, O. Gissberg, N. K. Andersen, A. J. Isse, A. M. El-Madani, C. Lou, Y. V. Pabon, B. A. Anderson, E. M. Zaghloul, R. Zain, P. J. Hrdlicka, P. T. Jorgensen, M. Nilsson, K. E. Lundin, E. B. Pedersen, J. Wengel, and C. I. Smith, **Next-Generation Bis-Locked Nucleic Acids with Stacking Linker and 2'-Glycylamino-Lna Show Enhanced DNA Invasion into Supercoiled Duplexes**, Nucleic Acids Res, 44 (2016), 2007-19.
IV. O. Gissberg, E. M. Zaghloul, K. E. Lundin, C. H. Nguyen, C. Landras-Guetta, J. Wengel, R. Zain, and C. I. Smith, **Delivery, Effect on Cell Viability, and Plasticity of Modified Aptamer Constructs**, Nucleic Acid Ther, 26 (2016), 183-9.

Other publications by the author not included in the thesis:

I. A. Berglof, J. J. Turunen, O. Gissberg, B. Bestas, K. E. Blomberg, and C. I. Smith, **Agammaglobulinemia: Causative Mutations and Their Implications for Novel Therapies**, Expert Rev Clin Immunol, 9 (2013), 1205-21.
II. K. E. Lundin, O. Gissberg, and C. I. Smith, **Oligonucleotide Therapies: The Past and the Present**, Hum Gene Ther, 26 (2015), 475-85..

CONTENTS

LIST OF ABBREVIATIONS

2′-*O*-Me	2′-*O*-Methyl
A	Adenine
Ac	Acetylation
AGO	Anti-gene oligonucleotide
bp	Base pair
C	Cytosine
Cas	CRISPR-associated protein
cDNA	Complementary DNA
CMV	Cytomegalovirus
CPG	Controlled pore glass
CRISPR	Clustered regularly interspaced short palindromic repeats
Da	Dalton
ddNTPs	Dideoxy-nucleotides
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DSI	Double strand invasion
G	Guanine
G4	G-quadruplex
glyLNA	2′-glycylamino-LNA
H	Histone
HD	Huntington’s disease
HEG	Hexaethylene glycol
HG	Hoogsteen
HPLC	High pressure liquid chromatography
HTS	High throughput screening
HTT	Huntingtin
K	Lysine
LNA	Locked nucleic acid
Me3	Trimethylation
miR	Micro RNA

mRNA	Messenger RNA
MS	Mass spectrometry
nc	Non coding
NCL	Nucleolin
nt	Nucleotide
ON	Oligonucleotide
PEI	Polyethyleneimine
PLP	Padlock probe
PMO	Phosphorodiamidate morpholino oligomer
PNA	Peptide nucleic acid
PO	Phosphodiester
PS	Phosphorothioate
RCA	Rolling circle amplification
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
S	Serine
SELEX	Systematic evolution of ligands by exponential enrichment
siRNA	Small interfering RNA
SNPs	Single-nucleotide polymorphisms
SSO	Splice-switching oligonucleotide
T	Thymine
TALEN	Transcription activator-like effector nuclease
TFO	Triplex forming oligonucleotide
TINA	Twisted intercalating nucleic acid
*T*m	Melting temperature
UNA	Unlocked nucleic acid
WC	Watson-Crick
ZFN	Zinc finger nuclease

1 INTRODUCTION

1.1 GENE THERAPY

The use of gene therapy to restore dysfunctional genes in disease has come a long way since the first steps were taken some 50 years ago (1-4). 25 years ago the first clinical trials involving gene therapy were conducted (5), and even though they were unsuccessful they marked the beginning of a rapid development and expansion of the field in the last decades. Looking at the combined US and EU market today, five therapeutic products have been approved using genetic material to treat diseases (6, 7): **Fomivirsen** for cytamegalovirus retinitis in 1998 (US), **Pegaptanib** against age-related macular degeneration in 2004 (US), **Apolipogene tipavovec** for lipoprotein lipase deficiency in 2012 (EU), **Mipomersen** for familiar hypercholesterolemia in 2012 (US), **Strimvelis** for severe combined immunodeficiency due to adenosine deaminase deficiency in 2016 (EU) and recently **Eteplirsen** for Duchenne muscular dystrophy in 2016 (US). Given the technical progress seen in the last 10 years together with the detailed mapping of the human genome, no doubt the future holds great promise when it comes to treating genetic diseases using many of the possible gene therapeutic strategies.

Mutations in protein coding or regulatory genes can give rise to proteins that either lack correct function, gained a new pathological function or exist in suboptimal quantities. These changes in normal protein physiology are in many cases associated with disease progression. Dominant diseases carry such mutations in the hererozygous state, i.e. when the mutation is present on one of the gene copies (allele). In families, dominant diseases affect the dominant parent carrier and the offspring inheriting the mutant allele. Recessive diseases need both alleles to be mutated for symptoms to appear. Affected offspring must have parents with one mutant allele each, which will be asymptomatic since they are only carriers for the trait. Depending on the molecular origin and pathophysiological mechanism underlying a certain genetic disease, multiple targeting options exist along the pathway of gene transcription and protein expression. In general, many gene therapeutic approaches aim to replace, add or edit the mutated gene, either given systemically or as *ex vivo* cell therapy. Even if only the coding part of a gene sequence is used therapeutically, the size in many cases involves several kilo base pairs (kb), which normally require the use of viral vectors for efficient gene transfer (8). In contrast, non-viral gene delivery strategies lack certain risks associated with viral systems such as insertional mutagenesis, instead taking advantage of various transfection methods, nanoparticle formulations or complexes with targeting moieties to reach the desired tissue or cell *in vivo* (2).

During the past ten years and most recently, the field of gene editing using targeted endonucleases has made some impressive progresses. The concept relies on the precise targeting of sequences in the genome for the direct editing of DNA. Following strand breaks and the subsequent repair, these approaches either introduce point mutations or aim at transgenic integration of a larger sequence of the desired DNA (9). The most promising

techniques used are Zinc finger nucleases (ZFN) (10-16), Transcription activator-like effector nucleases (TALENs) (17-21), and Clustered, regularly interspaced, short palindromic repeats (CRISPR) together with a CRISPR-associated (Cas) protein (22-24). These techniques have made it possible for the gene-editing concept to advance into a clinical setting for various disorders such as infection, inflammation and cancer (25, 26).

The DNA targeting approach also includes modulating the transcription of the gene, by using oligonucleotides (ONs) or other molecules capable of inducing, stalling or blocking of the polymerase activity. Targeting RNA for correction or replacement has also been heavily pursued both in academia and in industry (27-29). Finally, the protein itself can be targeted using ONs that block signaling or other undesired interactions from a mutated or dysfunctional protein (30, 31). This thesis examines the effect of DNA binding therapeutic ONs and the use of non-viral delivery applications for specific cell targeting.

1.2 THERAPEUTIC OLIGONUCLEOTIDES

ONs are linear polymers of nucleic acids and their analogues, usually between 8-50 bases in length. Using ONs for therapeutic purposes goes hand in hand with the development and understanding of gene regulation and nucleic acid chemistry in general, and dates back to the 1960s (6), beginning with the discoveries of the 2′-fluoro (2′-F) and 2′-*O*-methyl (2′-*O*-Me) sugar modifications providing increased DNA and RNA binding affinity and resistance to degradation by nucleases, and the phosphorothioate (PS) backbone substitution for prolonged halflife and bioavailability (32-35). Later during the 1970s and 1980s, technical land-winnings and the possibility for automated synthesis of longer ONs (36-38) together with the introduction of the antisense concept (39-41), the advantages of using ONs in therapeutic strategies became more and more evident. At present, an expanded use of ONs in different settings exists, taking advantage of various cellular control mechanisms for modulating gene expression (42, 43). Together with protein binding ONs, figure 1 summarizes the most advanced techniques used at the RNA level (Antisense approaches).

1.2.1 Antisense oligonucleotides

In Antisense ON (AON) applications, gene expression can be modulated after AON binding primarily to a messenger RNA (mRNA) target, even though other RNA targets are possible including non-coding and regulatory transcripts. Depending on the mode of action, modulation of expression can occur through inducing enzymatic cleavage of the target mRNA or by the steric blocking of translational events (29, 44). Enzymatic cleavage of the mRNA can be mediated by activation of RNase H, capable of recognizing DNA:RNA duplexes. For optimal RNase H-recruited cleavage, typically the AON “gapmer” design is applied where the AON has a gap of six to eight DNA nucleotides (nts) surrounded by chemically modified nts for enhanced target binding and stability (45, 46). The RNase H-inducing strategy is one of the most mature AON technologies, and constitutes a major part of AONs in clinical development at present.

Another method used for inducing cleavage and subsequent degradation of the target mRNA is by taking advantage of the RNA interference (RNAi) pathway in which small interfering double stranded (ds) RNA (siRNA), consisting of a guide and passenger strand, is loaded into the protein complex known as RNA-induced silencing complex (RISC), after which the siRNA looses its passenger strand (47). One component of RISC is Argonaute 2 which is the enzyme responsible for the cleavage of the target mRNA after its association to the RISC complex and the complementary siRNA guide strand. siRNAs tend to tolerate modified ribonucleotide incorporations to a lesser extent. For example, when placed in the passenger strand modified nts can impair its degradation (48). Nonetheless, siRNA has been made more stable and efficient using modified bases at optimized positions, for instance in a design called "internally segmented siRNA" (49, 50). In general, changes to the phosphate backbone seems to be more tolerated, and alternating modified bases with unmodified RNA has been a quite successful approach (51).

ASOs can also be designed to inhibit RNA processing by sterically blocking regulatory or translational events. This ASO category include splice switching ONs (SSOs) (52, 53), translational arrest ONs (54), anti-microRNA (anti-miR) ONs (antagomirs) and miR mimics (55). Recently, some success at inducing translation by ASOs targeting regulatory sequences in the 5′ UTR region of certain mRNAs has also been made (56). All these approaches have in common that they bind to RNA and hinder regulatory factors (or miRs) from recognizing and binding to their target sequence.

1.2.2 Anti-gene oligonucleotides

It is generally believed that anti-gene ONs (AGO) could be used to modulate gene expression at the DNA level by either interfering with transcription factor binding or by stalling the RNA polymerase during transcription (50, 57-59). In addition, AGOs have been used to induce or increase transcription from the displaced strand in DNA, which forms a so-called "D-loop" after AGO binding to the opposite strand (60). In an alternative approach, the AGO was shown to act in a similar way as a regulatory non-coding (nc) RNA, capable of binding to antisense transcripts that overlap the promoter region of the targeted gene. This can lead to an effect on gene transcription as the ON can act via steric blocking or a mechanism similar to RNAi (61, 62). At present, there is strong evidential support for the theory that AGOs can directly invade or bind chromosomal DNA and block transcription (63, 64). This thesis will focus on DNA binding AGOs.

Some of the earliest attempts to target the genome involved the use of Peptide Nucleic Acid (PNA) analogues (65, 66), which were further modified and developed to increase their ability to bind DNA and affect gene transcription (67-69). Since then, different anti-gene strategies involving ONs have evolved using novel chemistries, where most bind to their target sequence by either i) forming a triplex via Hoogsteen (HG) interactions or by ii) strand invasion of the DNA double helix via WC base-pairing, or iii) through a combination of HG and WC binding by the use of a clamp construct.

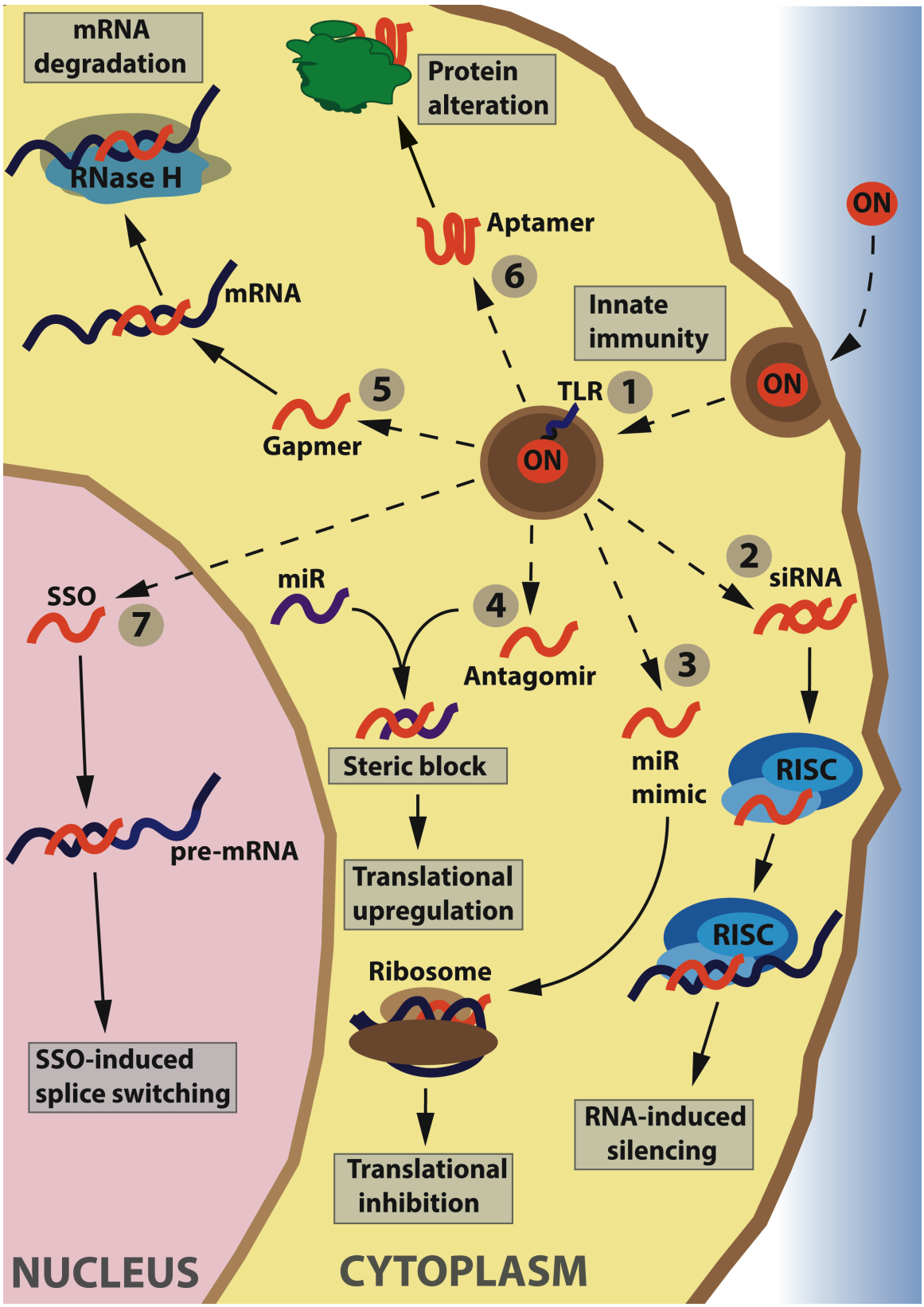


Figure 1. **Different antisense and protein binding strategies for ONs.** (**1**) Endosomal binding of toll-like receptors (TLRs). (**2**) Small interfering RNAs (siRNAs). (**3**) Micro-RNA(miR) mimics. (**4**) Antagomirs, for endogenous blocking of miRs sterically. (**5**) Gapmer Antisense ONs (AONs) for inducing RNase H degradation (steric block ONs also exist). (**6**) Aptamers, for binding and affecting proteins. (**7**) Splice switching ONs (SSOs). Reprinted from ref (6) with permission from Mary Ann Liebert Inc. publishers.

Several chemical modifications used in ASOs can also be employed in the design of AGOs to achieve higher target affinity and DNA complex stability. These aspects of AGO binding are addressed in more detail later in the thesis.

Given the increased complexity compared to RNA when targeting DNA in its genomic environment, the requirements for successful transcriptional manipulation place a heavier demand on the AGO used. First, like SSO approaches, the AGO needs to be delivered to the nucleus. In addition, once binding has occurred, the AGO also needs to be capable of i) staying bound, competing with regulatory factors and proteins, and ii) if binding through WC interactions, competing with reformation of the dsDNA, and iii) withstand the polymerase during transcription. Thus, the AGOs must have enough affinity to increase the probability of binding to the target, and at the same time have minimal off-target effects in the genome and complementary RNA. This balance will greatly affect success, and AGO strategies will also ultimately face the same challenges as ASOs when it comes to cellular targeting, delivery and bioavailability.

Successful ON binding of chromosomal DNA is a highly dynamic process depending on several factors. When active gene transcription occurs, the DNA in this region forms a less densely packed structure within the chromatin, which in combination with the dynamic breathing effect induced by supercoiling and stand separation by RNA polymerases, creates an environment favorable of ON binding (70, 71). WC-binding AGOs are typically single stranded and invade DNA at these transiently accessible regions formed mainly during transcription.

Triplex forming ONs (TFOs) can bind to the major groove of the DNA double helix, which has been explored in different applications, such as transcriptional activation (72), blocking of transcription (73-76), recruitment of transcription factors (77, 78), fluorescent *in situ* hybridization of repetitive chromosomal regions (79) and TFO-padlock mediated tagging of DNA fragments (80). TFOs can even bind to DNA at transcriptionally silent *loci,* however, binding was dramatically increased upon targeting actively transcribed genes (81, 82).

Another class of AGO is the Zorro-LNA, which invades supercoiled DNA and simultaneously binds to opposite strands forming Z-shaped structures by WC binding. The Zorro-LNA has been shown to invade and bind two adjacent target sites in supercoiled DNA, and further shown capable of blocking transcription (83). Since then, the Zorro-LNA has been extensively developed using different linkers and chemistries (84). The first generation of Zorro-LNA had the two arms connected through a sequence complementary linker to which both arms could hybridize. Later, the efficacy was improved by using chemical linkers such as the double HEG-linker, which showed higher strand invasion capacity (see figure 4G and ref (84)). This single stranded Zorro-LNA needs to be synthesized using reverse phosphoroamidites, addressed in more detail later in connection to paper I. Examples of AGO binding double-stranded (ds)DNA are shown in figure 2.

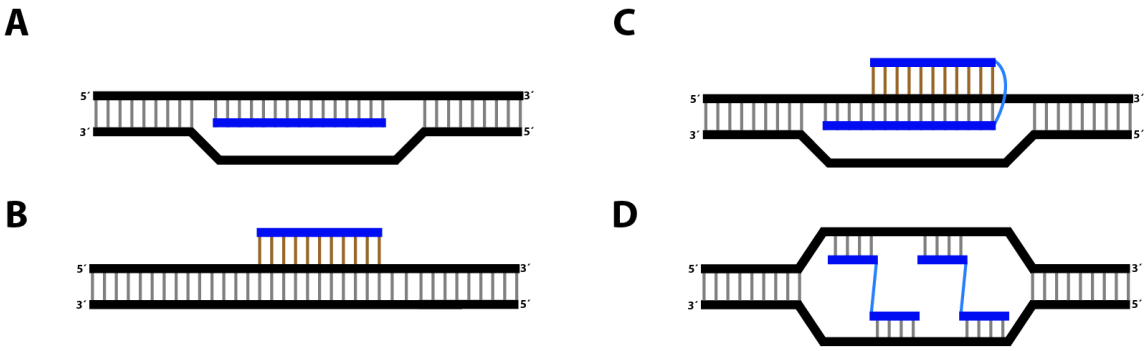


Figure 2. Different principles of dsDNA binding using AGOs. **A** shows the invasion using single stranded AGO, **B** shows TFO binding, **C** shows the binding and invasion using the combined TFO and strand invading AGO and **D** shows the Zorro-LNA binding concept.

1.2.3 Aptamers

Aptamers are ONs that interact with molecules through their ability to form secondary structures, allowing the aptamer to selectively bind to its target. The first aptamers were developed in the early 1990s (85, 86), and since then a substantial amount of both RNA and DNA aptamers have been developed, some of which have reached clinical trials (6). Aptamers commonly serve as ligands to proteins and receptors and are developed using the systematic evolution of ligands by exponential enrichment (SELEX) process in which the affinity of a large pool, or library, of RNA sequences are exposed to a target protein during repeated rounds of binding selections intermixed with PCR amplification, until only the most potent aptamer remains. The SELEX technology has been developed greatly in the last decades, introducing methods such as cell internalization-SELEX (87), where the aptamer library is enriched together with whole cells, and cell-SELEX combined with high throughput screening (HTS) (88, 89), and *in vivo*-SELEX trying to assess potent aptamer sequences in living organisms (90). These approaches typically employ post-SELEX modifications to enhance their stability and nuclease resistance (91, 92).

Even though highly successful in obtaining aptamers with great affinity for various proteins, the early SELEX based approaches selecting for binding in solution sometimes did not directly correlate with effects *in vivo* due to conformational differences in protein binding. This was to some extent overcome by using the cell-based SELEX approaches in which the aptamers were enriched in an environment closer to physiological conditions, thus providing binding kinetic parameters during natural protein foldings (92).

Despite some promising and successful aptamers being generated through cell-based SELEX, obtaining good clinical effects still remains a major hurdle in many cases. Adding to the complexity is the fact that higher number of rounds during SELEX can introduce selection bias. Some sequences will gain evolutionary advantages (93), either by sequence composition, structural conformations or stability (92). These can be more potent than components enriched in previous rounds. However, recently, attempts have been made to discover potent aptamers in early rounds in complex pools using post-SELEX based HTS

characterization combined with deep sequencing (94). Using aptamers for the specific uptake in cells is discussed further below.

1.3 OLIGONUCLEOTIDE CHEMISTRY

1.3.1 Oligonucleotide synthesis

With the introduction of automated synthesis of ONs in the 1980s, the ability for therapeutic applications and related mechanistic studies grew enormously (37, 38). The synthesis is based on the stepwise introduction of special nucleosides carrying an *N*′,*N*′-diisopropyl phosphoroamidite group attached to the 3´-hydroxyl of the sugar, with all funcional groups protected before synthesis. Through a series of deprotection, coupling and capping, each new phosphoroamidite nucleoside is added to the previous at the 5´-hydroxyl group, the first unit being attached to solid support made of Controlled Pore Glass (CPG) at its 3´ end. In the final step the nt attached to CPG is released and deprotected, yielding a single stranded ON that can be purified using HPLC techniques and qualitatively assessed by mass spectrometry (MS).

When producing ONs containing different modified nts, the majority can be commercially obtained and introduced as phosporoamidites during synthesis. More complex ON synthesis can in some cases require some custom separate modifications for the introduction of the desired phosphoroamidite. A special case relevant to this thesis is the requirement of reverse phosphoroamidites when connecting two ONs of different polarity, such as 3´-5´- 5´-3. This has been successfully applied when synthesizing single stranded Zorro-LNA containing different synthetic linkers such as an extended hexaethylene glycol (HEG) linker connecting the two ONs (see figure 4G) (84). However, only normal reverse phosphoroamidites of non-modified nucleotides are commercially available. Thus, this requires rather complex and expensive in house synthesis of such building blocks, which is why other strategies are highly desirable for reversing strand polarization.

1.3.2 Click chemistry

One alternative to using reverse phosphoroamidites to connect two Zorro arms with different polarities is to link the arms post synthesis using 1,3-dipolar cycloaddition or “click” chemistry (see figure 4H) (95). This approach can efficiently join a terminal alkyne group to an azide group under aqueous conditions using Cu(I) as a catalyst, and has been widely used in different settings since it was reported in 2002 (96-98). Five years later, a copper-free click chemistry procedure was published using a substituted cyclooctyne with similar high coupling efficiency (99). This group however, is much more expensive compared to the alkyne group used in Cu(I)-mediated click chemistry, but has more interesting applications *in vivo* since Cu(I) is not needed for the click reaction to occur.

1.3.3 Base and sugar modifications

Improvements in ON stability and target affinity were early identified as major factors for the successful use of ONs as modulators of gene expression. Unless stability and protection can be achieved through ON structure, chemically modified nts are needed to prevent enzymatic cleavage and also for increasing target affinity. Indeed, the cells’ own modification of natural

ribonucleotides provides a crucial fundamental mechanism to prevent degradation, such as the epigenetically important methylation of cytosine in DNA (100), or the many post-transcriptional RNA modifications that act as stabilizing or structural components important for folding of the transcript (101, 102). The therapeutic ON platform takes advantage of some of these or related base modifications, such as the pseudoisocytidine (103). The other large group of modifications involves the sugar at the 2′-position (see figure 3). This affects the ribose conformation to a north or C3′-*endo* like state, which gives greater binding affinity mimicking the A-conformation in duplex RNA.

1.3.4 2′-Fluoro- and 2′-*O*-Methyl modifications

The 2′-deoxy 2′-fluoro (2′-F) was the first sugar modification introduced in 1964, which later was shown to provide higher duplex stability and increased nuclease resistance compared to unmodified nucleotides (104, 105). The 2′-*O*-Methyl (2′-*O*-Me) modification adds increased binding affinity in a similar way, and both have been extensively used since then to enhance ON design, even though they were ultimately found not to have optimal drug like metabolic stability, unless combined with the phosphorothioate (PS) backbone described below (106).

1.3.5 Locked Nucleic Acids

Another important sugar modification is the Locked Nucleic Acid (LNA) in which a methylene bridge connects the 2′ oxygen to the 4′ carbon, which creates a conformational constrain, forcing the ribose into a near C3′-*endo* state which gives it an RNA-like conformation (107, 108). LNA nucleotide phosphoroamidites were developed independently in 1997/1998 by Wengel and Imanishi, and could be incorporated during ON automated synthesis (109, 110). LNA can increase the thermal stability of a duplex with 2-8°C per nt depending on the number of incorporated LNAs and which base is involved in forming the WC base pair (111). Much of the increase in affinity stems from an energetically favorable conformation that also involves base stacking (112, 113). TFOs containing LNA were shown to be more stable than their DNA counterparts due to lower dissociation rates, however, TFOs containing only LNA are not able to form a triplex at all (114-116).

In addition to binding affinity, LNA-containing ONs can also increase resistance to degradation by enzymes present both in serum and in the cell. Studies show that introducing at least two LNA nts in the 3′ end of the ON increases protection from 3′ exonuclease activity (117). As with other 2′-modifications, stability can be much enhanced if LNA is combined with PS backbone modification (118, 119). The combination with PS backbone also greatly improves the pharmacological and cell internalization properties of the ON (120). Thus, LNA-containing ONs have successfully been used in different therapeutic applications including antisense (121), siRNA (122, 123), SSO (124-126), anti-miR (127), and aptamers (128).

Several analogues have been derived from LNA. The 2′-amino-LNA provides a convenient chemical handle to which other groups can be conjugated, while at the same time maintaining the conformational properties associated with bridged nts (129). Examples of functional

groups attached to LNA ONs include moieties introducing a positive charge for enhanced duplex and triplex stability (130, 131), and cholesterol for increased efficiency of a miR knock down probe (132). Data suggest that 2´-amino-LNA seems to destabilize triplexes. In contrast, the use of 2´-glycylamino-LNA can stabilize triple helices by 1.7-3.5 °C per substituted nt (133).

Ethylene-bridged Nucleic Acids (ENA) nts are locked in the north conformation just as LNA, but instead the methylene bridge is exchanged for ethylene (134). ENA does not stabilize duplexes as good as LNA, however, triplexes can be readily stabilized using ENA containing TFOs (135).

1.3.6 Unlocked Nucleic Acids

A special case of novel modifications introduced in the last years is the Unlocked Nucleic Acid (UNA) (136), which in contrast to LNA lacks both constraint and the bond between C2´and C3´ in the ribose ring (see figure 3). Due to increased flexibility of the phosphodiester (PO) backbone, UNA reduces the thermal stability when hybridized to both DNA and RNA (137, 138). Depending on the nt position, the decrease in stability is between 1-10°C, with greater effect in the center of a duplex and less effect towards the ends (139). Applications where UNA has been found useful includes its incorporation into siRNA to reduce off-target effects by destabilizing involved regions (140), and change polarity of the RISC-loaded siRNA strand (141), and in aptamers where it can reduce conformational restrain in loop regions which increases aptamer folding kinetics and efficacy (142).

1.3.7 Phosphorothioate and backbone modifications

Also of great importance are the backbone modifications used to enhance ON performance (see figure 3). The most mature of these modifications is the inclusion of sulfur instead of oxygen in the PS backbone, changing the interactions and stability of PS-containing ONs. The PS group provides a chiral center, thus two stereoisomers exist called Rp and Sp in a 1:1 ratio giving the ON its characteristic properties (143). The simultaneous ability to resist nuclease degradation and at the same time allow other enzymatic activity (such as RNase H), is perhaps the most striking enhancement the PS modifications give. This can be traced back to the stereoisomeric properties of such PS ONs, the Rp configuration gives higher binding affinity to RNA and induce RNase H, compared to the Sp form that does not, but instead promotes higher nuclease stability (144). However, it has been shown that the combination of Rp and Sp form is desirable for antisense applications (145, 146). Because of these properties, PS ONs have been used with success in many different settings. Most importantly, they have good pharmacological properties since the bioavailability and serum stability is high due to plasma protein binding, and cellular uptake is enhanced compared to PO-containing ONs (144).

1.3.8 Peptide Nucleic Acids

PNA represents a potent nucleic acid analogue where the sugar phosphate backbone has been replaced by *N*-(2-ethylamine)glycine polyamides (65). The achiral PNA oligomers are highly flexible and have a neutral backbone, which give them good hybridization properties and high *in vivo* stability (147). Under low salt conditions, PNA have had excellent properties binding to DNA, however, at physiological salt concentration this effect is much reduced (148). Single stranded PNA oligomers experiences some issues regarding solubility under aqueous conditions where it readily forms aggregates (149), especially when GC content is over 60% and if the oligomer is longer than 12 nts (150). However, low solubility can be counteracted by introducing positively charged peptides, such as lysines. Additionally, due to the lack of charges, the PNA oligomers are not well taken up by cells; they are often combined with various methods to assist the oligomer internalization, such as electroporation, liposomal transfection agents or by direct conjugation to Cell Penetrating Peptides (CPPs) (151). Allthough, over the years many different PNA analogues have been synthesized that can overcome some of the limitations owning to poor solubility and uptake (150).

PNA has been used to target RNA in cells with some success, including inhibition of translation (152), splice correction (153), and as antimiRs (154). The antimiR approach was recently also successfully combined with CPPs for the delivery to breast cancer cells and a lymphoma animal model directed towards mir-221 and mir-155 respectively (155, 156). These latest studies provide a good example to the fact that PNA could still be a promising chemistry in conjugation to various active biomolecules.

In anti-gene approaches, PNA can bind to DNA both by strand invasion and triplex formation via strand displacement (157). Due to less electrostatic repulsion of the dsDNA to the uncharged PNA, binding is strong compared to negatively charged ONs, but neutral pH is not favoring triplex formation in polypyrimidine TFOs containing many Cs, unless pseudoisocytidine is used introducing a hydrogen bond without the need for the C protonated state (158). Also of interest is the bisPNA construct, in which two polypyrimidine PNA ONs are connected via a linker region to form a clamp. These constructs effectively bind DNA through WC and HG interactions under low salt conditions, and by extending this construct with a tail sequence it could circumvent the need for long polypurine stretches (159, 160).

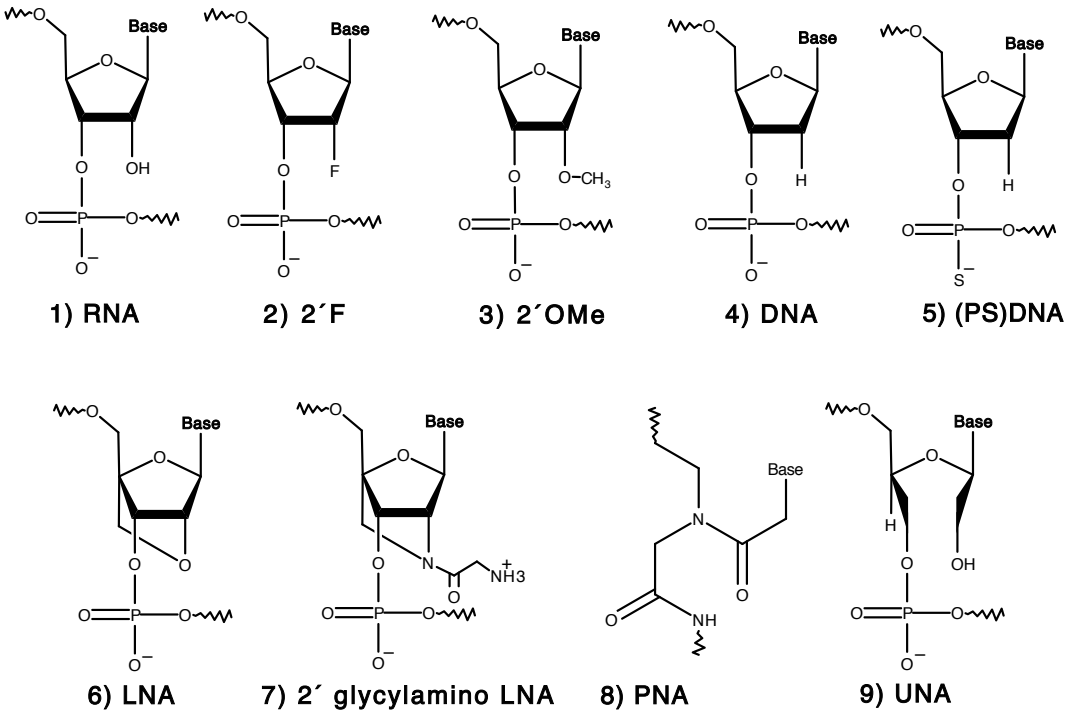


Figure 3. Structures of natural and modified nucleotides.

1.4 INTERCALATORS AND DNA LIGANDS

Intercalators, or molecules that can stabilize different nucleic acid structures non-covalently by insertion between two adjacent bases, constitute an efficient approach for the improvement of DNA targeting and binding (161). According to the first model proposed in 1961 based on acridine, the binding usually occurs non-specifically by hydrophobic and electrostatic π-π interactions, leading to a change in the double helix by decreasing the helical twist (162). Since then, many intercalators have been synthesized and several have even become drugs on their own as anti-cancer agents (163, 164), and when attached to ONs they get the target sequence-specificity otherwise missing.

One example of ONs combined with intercalators is the use of twisted intercalating nucleic acid (TINA). This compound can be incorporated at different positions in the ON, creating an increase in the thermal stability of both duplexes and triplexes (165). It stacks with the nucleobases by its aromatic ring and its stabilizing ability is much dependant on where it is placed in an ON sequence. For instance, when placed terminally, both ortho and para-TINA (figure 4E+F) gave higher stability for an antiparallel duplex compared to when placed at other positions (166).

A special case of DNA binding low-molecular weigtht (small) molecules is the G-quadruplex (G4) ligands, capable of stabilizing G4 DNA and RNA structures by groove binding and intercalation. These are typically planar, aromatic molecules and many can have selective binding of certain preferred G4 motifs (167). Some early-discovered G4 ligands are the macrocyclic compounds such as porphyrins and their derivatives (168). One of the most studied porphyrins is TMPyP4 (figure 4D), which was shown by Hurley *et al* to be capable of stabilizing G4 structures present in the telomeric DNA sequence and inhibit telomerase activity (169, 170). It was shown to stack to the G tetrads, and thereby stabilizing both intra and intermolecular G4 structures (171).

Another example of small molecules capable of stabilizing G4 structures is ellepticine and its derivatives (for examples, see figure 4A-C). Ellipticine has been investigated as a potent antitumoral agent, and it was also found to be able to intercalate into DNA (172). Soon it was realized that it could stabilize G4 structures, after which some derivatives were synthesized to enhance this interaction and create more specific stabilization of various motifs (173, 174).

The stoichiometry of G4 ligands and binding mode to their target can vary considerably. For instance, TMPyP has been shown to bind four, and even five molecules per G4 structure (175, 176). Thus, careful investigation of G4 ligand binding mode is an important parameter to assess, since any additional excess of ligands present can result in non-specific interactions (177).

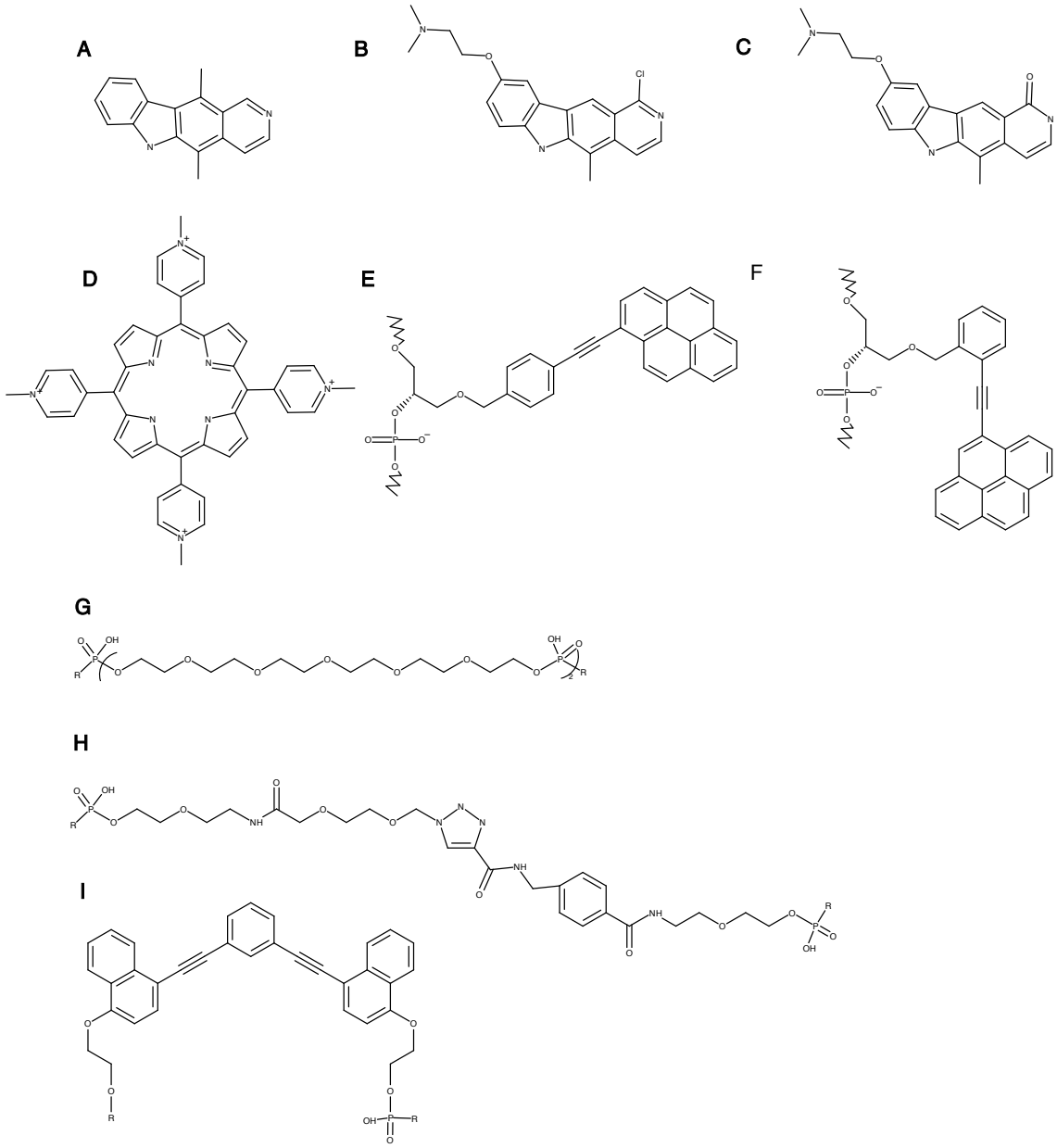


Figure 4. Different intercalators, G4 ligands and linkers. **A** Ellipticine and derivatives **B** 1-ChE and **C** 1-OxE, **D** TMPyP4, **E** ortho-TINA, **F** para-TINA, **G** 2xHEG linker used in some single stranded Zorros, **H** Click chemistry linker used in the novel Zorro-LNA constructs, **I** the M3-linker used in bis-LNA.

1.5 NON-VIRAL OLIGONUCLEOTIDE DELIVERY

Naked ONs are usually not suitable for *in vivo* cellular delivery, even though, as discussed above, the PS-containing ONs have quite high bioavailability due to their binding to serum proteins (178). The main concern lies in gaining high enough local accumulation for cell internalization to have the wanted biological effect. For this, normally a delivery platform is utilized which could aid in the uptake and at the same time give protection and/or stealth properties to the ONs during systemic circulation. Also of importance, this delivery vehicle should not stimulate immune responses, in order to promote multiple dosing and the efficacy of the ON drug.

Non-viral delivery methods can be divided into non-targeted and targeted approaches and they both include direct conjugation or formulation with various entities that can promote cell internalization. Conjugated ON approaches are often more defined and predicable in terms of chemical identity and size. Particle formulations on the other hand, show increased polydispersion and heterogeneity and are more prone to be affected by environmental parameters, such as storage, handling and mixing. This variability can be reduced by the use of microfluidic systems, which are capable of rapidly producing more defined particles and complexes (179).

1.5.1 Cellular entry

ONs and nanoparticles are normally entering cells by endosomal pathways. These are classically assigned to various receptors and entry mechanisms such as the clathrin-dependent and independent pathways, pinocytosis, phagocytosis, macropinocytosis and caveolin-dependent and independent uptake routes (180). Following encapsulation the cargo needs to have some ability to escape the endosomal entrapment, which otherwise could lead to its degradation after fusion with lysosomes, or export from the cell via late endosomal recycling and various vesicle trafficking (181). The endosomal environment undergoes a general reduction in pH during its maturation, and entrapped cargo can escape at certain time points, through mechanisms that are largely unknown.

In the context of lipoplex-ON formulations, some evidence characterize the endosomal escape of ONs as non-pH dependent, cation-induced disruption of the lipid membrane resulting in the displacement of anionic lipids from the cytoplasmic side to the inner core facing the lipoplex. This in turn drives the increased lateral diffusion and ion paring with the lipoplex cations finally releasing the cargo ON into the cytoplasm (182). The details regarding these mechanisms remain to be shown, and will depend on many different variables such as ON/vector chemistry, charge, particle size and the type of cell being studied. Attempts to enhance endosomal escape by construct design has been pursued, such as the recent chemically enhanced Endosomal Escape Domain (EED) platform which was shown to benefit the cytoplasmic delivery of therapeutic ONs (183).

1.5.2 Non-targeted delivery approaches

Agents that promote cellular entry in a non-specific manner to deliver ONs have been explored with increased intensity in the recent years. A major class of such non-viral delivery vectors is the nanocarriers. Cell-penetrating peptides (CPPs) constitute a subclass used for efficient delivery of nucleic acids to cells and tissues. By either direct cargo conjugation or as nanoparticle formulations, CPPs can typically carry large biopolymers and therapeutic ONs of different sizes into cells both *in vitro* and *in vivo* (184, 185). Since its first usage in conjugation with PNA, the CPP concept has been much further developed and is now being used in combinations with different therapeutic ONs.

The basis for the mode of action is a cationic peptide, under 30 amino acids in length, harboring varying degrees of amphiphatic properties that aid in the ON internalization through endocytotic pathways (186). ON chemistry compatible with CPP conjugation is in general restricted to charge-neutral chemistries such as PNA or morpholino nucleobases, otherwise charge interactions between the positive peptide and negative ON would complicate the synthesis and physiochemical properties of such entities. One example of CPP formulation, also used in paper II of this thesis, is the Pepfect14 (187), which is capable of forming nanocomplexes with ONs with the same efficiency as commercial lipoplex formulations for the delivery of ONs into a number of cell lines (188).

Another important nanocarrier is based on lipid nanoparticles. Cationic lipoplexes are efficiently forming nanoparticles with ONs by condensation, and this vehicle class has been used extensively for ON delivery (189-192). Cationic lipoplexes usually consist of a mix of polethylenimine (PEI) with lipids and other helper components that can add to the efficacy, such as cholesterol and 1,2-dioleoyl-3-trimethylammonium-propane (193).

However, the lipoplexes are known to induce various degrees of toxicity depending on the exact formulation composition, which is why they are generally not pursued as *in vivo* gene delivery vehicles. In comparison, neutral liposomes have a better cytotoxic profile and typically consist of charge-neutral lipids such as cholesterol that interact to a lesser extent with serum proteins. They are however, compared to the lipoplexes, more pH-dependent in order to achieve efficient endosomal escape (194). Recent advances for lipid nanoparticle formulations and composition have resulted in several ongoing clinical studies such as the stable nucleic acid lipid particles (SNALPs) by Tekmira, and the Smarticles by Marina Biotech (195).

1.5.3 Targeted delivery approaches

Strategies for targeted delivery of ONs rely on the attachment of molecules and ligands that promote specific uptake by cells and tissues, either directly to the ON itself or to the surface of a delivery vehicle. Specific uptake and effect in the liver following administration have been reported for cholesterol siRNA conjugates (196), also used for siRNA targeting *HTT* mRNA in an animal model for Huntington´s disease (197). By conjugating siRNA to alpha-tocopherol, tissue-specific targeting and reduction of ApoB production in the liver was

achieved (198). Another highly successful lever-targeting approach is based on the interaction between the asialoglycoprotein receptor (ASGR) and the multivalent N-acetylgalactosamine (GalNac) group (199), which has been successfully conjugated to siRNA in a trivalent form (200). The concept has been further developed by Alnylam Pharmaceuticals and the GalNac conjugates have now advanced to phase III in clinical trials and provide a promising and potent platform for specific targeting and delivery of therapeutic ONs to the liver.

Folate conjugated ONs and nanoparticles have been evaluated in different therapeutical settings. The concept is based on the specific recognition and internalization by binding to the folate receptor overexpressed on the surface of certain cancer cells that efficiently take up the folate conjugates, which normal cells do not since they have a much lower folate receptor density (201, 202). Another receptor overexpressed on tumor cells is the transferrin receptor, and by conjugating transferrin to nanoparticles containing siRNA against EWS-FL 11, Ewings sarcoma cells were efficiently targeted and knocked down *in vivo* following an intravenous dose of 2.5 mg/kg (203). Conjugating nanoparticles using monoclonal antibodies has been shown to be an efficient way to internalize ON cargo in a specific manner. For example, siRNA has been delivered using this strategy to melanoma cells (204), activated leukocytes (205), and malignant B-cells (206). Using aptamers for ON specific cell targeting and internalization constitutes a well-studied delivery platform, ranging from conjugates with ONs, small molecules, various nanoparticles and even antibodies (207). Typically, an overexpressed cell surface receptor is targeted through the use of various SELEX approaches.

1.5.4 AS1411 and oligonucleotide delivery

A notable exception from the use of SELEX in aptamer development is AS1411, an aptamer used extensively as a targeting moiety in the last decade. It was discovered when screening for antiproliferative effects of some guanine-rich TFOs in cancer cell lines using naked delivery (208). The control ON used in these experiments was AS1411, and it unexpectedly showed remarkable antiproliferative effects and resistance to degradation even though consisting of non-modified DNA with a 3´ protecting group. Later it was found that these properties were due to the formation of G-quadruplex structures and the aptamer´s ability to bind nucleolin (NCL) receptors overexpressed on the cell surface of many cancer cells. After this, it underwent extensive testing and even reached clinical phase II as an anti-turmoral agent, where it was later discarded due to lack of efficacy (209).

Since then, the interest in AS1411 has turned from a therapeutic to a delivery platform, since many cancer cells efficiently and selectively internalize it via the NCL receptor. Various attempts to shuttle cargo into cells have been made, including small molecules (210, 211), nanoparticles (212), antimiRs (213), and SSOs (214). In paper IV, the direct conjugation of LNA-ONs to AS1411 and their specific delivery to cancer cells is investigated (215).

1.6 DNA STRUCTURE AND ORGANIZATION

Since the famous description of the double helix structure by Watson and Crick in 1953 (216), in the close footsteps of Wilkins (217), and Franklin (218), much has been discovered related to DNA in terms of structure and function. The four DNA nts had already been uncovered together with Uracil in 1881 (219), but their true function and properties were described shortly after the seminal paper presented by Watson and Crick. Now, the landscape of the genome and its components are known in more detail, and additional discoveries are made each year.

1.6.1 The double helix

The DNA double helix consists of two polynucleotide strands containing the four bases cytosine (C), guanine (G), adenine (A) and thymine (T), organized in a dynamic helical spiral held together through non-covalent stabilizing and destabilizing forces. The helical shape is created when the polynucleotide strands with opposite polarity interact through their hydrophobic inner core, consisting of the nucleobases, and the hydrophilic outer surface, outlined by the phosphates of the strand backbone. These phosphate groups are electrostatically repelled by each other, both from groups on the same strand, and from the phosphate backbone of the opposite strand (220). This creates a destabilization of the helix, which is countered by the presence of shielding cations in the surrounding aqueous solution (221).

The higher the ion concentration the more of a shielding effect and stabilization of the double helix occurs. The nucleobases facing each other contribute to the helical stabilization by forming planar pase pairs (C-G with three and T-A with two hydrogen bonds) and, more importantly, by stacking through π-interactions between the aromatic rings of the bases. The stacking force is the major contributing factor to the observed 3.4 Å distance between the bases (222). Purines are able to stack with higher efficiency compared to pyrimidines due to their larger surface and polarization properties (220). As mentioned already, the DNA stability is also affected by the various metal ions and water molecules, which all contribute to the helix formation (220).

DNA can exist in different conformations depending on environmental factors and sequence. All double helical structures have in common the minor and major groove, which are pockets created due to the helical twist of the two antiparallel strands where the edges of the nucleobases are exposed. These grooves constitute the sites of sequence specific recognition and binding of proteins or nucleic acids, where the major groove has higher propensity for interactions due to a wider pocket (223). Three characteristic DNA conformations exist for the DNA double helix in the genome: B-DNA, A-DNA and Z-DNA. The shape is mainly determined by the sugar pucker conformation of the nts.

B-DNA is the most abundant DNA conformation found under physiological conditions and has the typical symmetrical right-handed turning shape with 10.5 bases per turn and 23.7 Å in diameter. The bases are perpendicular in respect to the helical axis, and the sugar pucker is in

a C2′-*endo* conformation which, together with the *anti*-conformation of the bases, give B-DNA its properties.

A-DNA is also right handed but with C3′-*endo* conformation of the ribofuranose ring, tilting the bases slightly with respect to the helical axis. This also affects the number of bases per turn, which are now 11 with a wider diameter of 25.5 Å. Under low hydration conditions the major groove becomes slightly wider and water molecules are less prone to interact with the DNA, resulting in the A-DNA conformation.

Z-DNA constitutes a much less organized form of DNA compared to A-DNA and B-DNA. Both C2′- and C3′-*endo* sugar puckers are present giving the structure a scattered impression. Z-DNA can be found in GC-rich regions and the helix is left-handed.

Under certain conditions, the DNA can adopt other specific conformations with different characteristics and function, some of which are important for this thesis, as discussed below.

1.6.2 The triple helix

Shortly after the double helix structure was reported, a paper describing the triple helix was published in 1957 (224). But it was Karl Hoogsteen that published the paper showing the structural differences of triplexes compared to WC base pairing (225). Following these initial discoveries, many therapeutic investigations using TFOs were published (226), such as the interactions and sequence specific binding of TFOs to a double helix (227, 228). More recently, evidence of triplex formation *in vivo* has also been reported (229). In particular, intramolecular triplex structures, so-called H-DNA, are associated with certain diseases (229-231).

Triplex formation in DNA occurs through HG or reverse-HG mode of binding (figure 5), and requires the duplex sequence to be composed of polypurine/polypyrimidine stretches. A pyrimidine TFO, or third strand, forms HG hydrogen bonds with the purine bases of the duplex (figure 6A+B), through a mechanism known as “nucleation zippering” starting from the 3′extremity where it initially binds to 2-3 nts followed by a zipper-like binding of the rest of the TFO (232, 233).

Atomistic data from X-ray crystallography, NMR and modeling suggest that triplexes adopt different forms than A-DNA or B-DNA. The major groove is wider in order to accommodate the third strand, and as a result, the minor groove becomes much more narrow (234, 235). The different modes of strand interactions in a triplex fall under three motif-categories depending on sequence and backbone orientation (figure 5A) (226, 236).

(T,C)-motifs, or pyrimidine motifs (i), form when the third strand binds in antiparallel with respect to the duplex. Some of the Cs in the TFO need to be protonated in order for an efficient triplex to be formed and as a consequence, these triplexes are unstable at neutral pH (237-239). Thus, this type of motif requires either low pH, or the incorporation of a modified base such as pseudoisocytidine, discussed above. (G,A)-motifs (ii) forms reverse-HG

hydrogen bonds in an antiparallel mode of binding, and (G,T)-motifs (iii) bind either in parallel or anti-parallel with respect to the purine strand of the duplex. Both (G,A)- and (G,T)-motifs are called purine-motifs and since they do not contain C, they can form stable triplexes at neutral pH. Due to the presence of G bases, these sequences can also adopt G-quadruplex conformations (240), discussed further below. Examples of WC and HG base pairing can be seen in figure 6A+B.

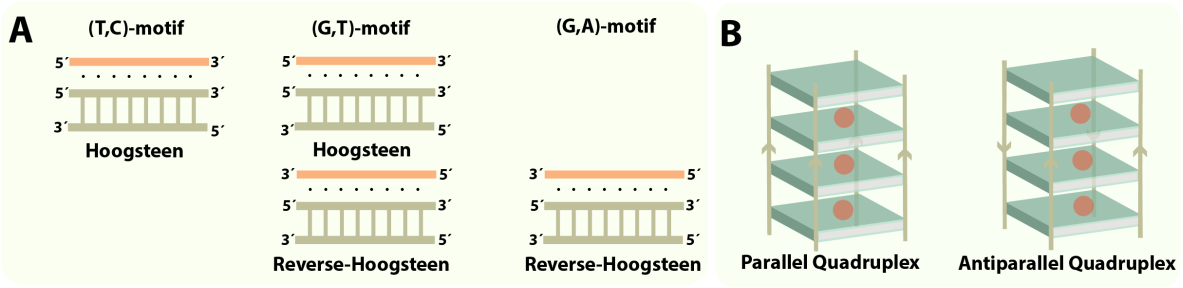


Figure 5. Different structural motifs involving **A** triplexes and **B** quadruplexes. B also shows the centrally placed stabilizing monovalent cation (potassium or sodium), represented as a red ball.

1.6.3 G-quadruplexes

In 1962 it was shown that the G bases of a guanylic acid (GMP) could form a helix consisting of linear aggregates due to base stacking and base pairing between four different Gs in a planar tetrad (241). This G-quadruplex (G4) helix conformation was later confirmed in other situations and have since then been extensively studied both structurally and functionally. The structural stability of the planar G-quartets originates in the HG hydrogen bonding between the Gs at positions N_1-O_6 and N_2-N_7, and from a centrally placed monovalent cation (mainly K^+, but also Na^+ or $NH4^+$), interacting with the O_6 electron lone pairs facing the inner space of the quartet (see figure 6C) (242). When an ON consists of continuous G-stretches (separated by 1-7 other bases), it can adopt stable helical G4 conformations where the G-quartets stack upon each other due to π-π interactions.

Depending on the polarity and the number of strands involved, G4 structures can form inter- or intramolecular parallel and antiparallel conformations (Figure 5B). For parallel strands, the sugar conformation of the Gs are in *anti*, and in antiparallel they adopt the *syn* conformation (243). The glycosidic angle will affect the phosphate backbone orientation, which in turn will affect the wideness of the groove, resulting in wide grooves for parallel strands and narrower grooves for antiparallel strands. Bases outside of the G-stretches will form loop regions that connect the structure together, and depending on how many strands are involved in forming the G4, and if they run parallel or antiparallel, the loops can make turns on the same side or may cross the structure to continue on the other side.

G4 sequences have been extensively studied *in vitro* to assess their conformational properties, interactions with proteins and ligands, as well as their therapeutic target potential. Both RNA and DNA are capable of adopting G4 conformations. Their stability is related to the number of G repeats in the sequence, the type of ion and its concentration in the buffer (244). For instance, a sequence capable of forming both a triplex and G4, can be shifted more to a G4 state if the K^+ concentration is raised in relation to Mg^{2+} (245). In contrast, Mg^{2+} can destabilize a G4 at high concentrations, and K^+ almost always give more stable structures compared to Na^+. Also, the stability of G4s is affected by the loop sequence size, where longer loops give lower stability (245). However, RNA G4 seem to be much less affected by loop size, indicating higher stability and less polymorphism (246).

The evidence for G4s *in vivo* and their potential biological role is under intensive investigation. Using bioinformatics and G4 sequencing methods, it has been estimated that potential G4 sequences in the human genome range from 300 000 to well above 700 000, depending on the number of other disrupting bases and loops in and between the G-stretches (247, 248). They seem to be distributed in a non-random fashion and are highly conserved between species, suggesting functional and evolutionary important roles for G4s (249). The G4 sequences are mostly found at telomeric regions followed by places such as promoters, intron/exon junctions, regions involving immunoglobulin class switching recombination, and the 5´- and 3´-UTR of mRNA (250). In addition, they have been shown to be associated with certain genetic diseases (251).

With the exception of mRNA and telomeric regions, the other G4 sequences are present in dsDNA well packaged and hidden in chromatin. However, during transcription and replication, these regions are accessible and transiently single stranded, making it possible for the G4 structures to form, and it is also for this reason many of their functional roles in the genome seem to be connected to these events (250), such as the involvement in the regulation of DNA replication and epigenetics (252). A key factor to the functional roles of G4s in the genome is the folding kinetics, which has been found to take between milliseconds, as in the case of telomeric G4 sequences, up to several minutes when the folding/unfolding is directed by certain helicases or chaperones (250).

To show that the folding of these structures exists *in vivo*, a few methods have currently been based on using antibodies capable of recognizing both G4 structural motifs (253), as well as selective ligands binding stabilizing G4 structures (254, 255). Also, very recently, a method to study the formation of RNA G4s in cellular extracts has been developed in which 7-deaza-G RNA is used instead of regular Gs (256). These cannot form the required HG bonds seen in G4s, making it possible to study the presence of G4 in long RNA using combined with footprinting and G4 selective antibodies.

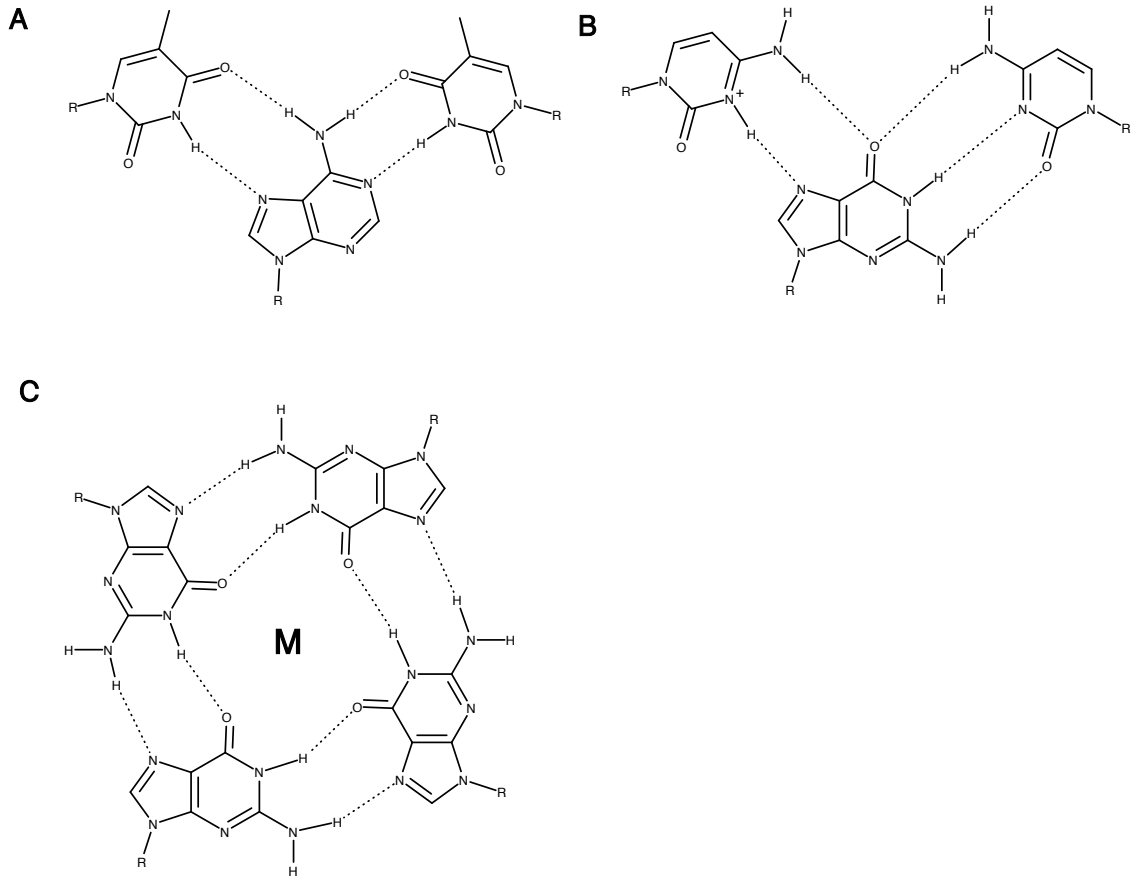


Figure 6. Examples of base pair interactions in different settings. A shows the Hoogsteen and Watson-Crick hydrogen bonding involving T*A-T, **B** shows the Hoogsteen and Watson-Crick hydrogen bonding involving C*G-C, **C** shows the Hoogsteen interactions in a G-quartet surrounding the central monovalent cation (M).

1.6.4 Higher order of DNA structures

DNA topology is highly regulated in the cellular environment. The genomic organization is a fundamental component of life and has direct impact on gene regulation and function. As outlined earlier in this chapter, the chemical surrounding of DNA constitutes the foundation for the organizational components in this complicated equilibrium. In addition, many proteins and enzymes are functional entities in this machinery and play major roles in the orchestration at both low and high hierarchical levels. The overall organization the genome are the chromosomes, which is comprised of the highly dynamic chromatin material, that can be further subcategorized in terms of DNA packaging.

First, the DNA helix is negatively supercoiled, meaning a thermodynamic strain is introduced between two points that create an underwined state where the DNA contains fewer helical turns compared to the ideal B-DNA. The degree of supercoiling can be changed by the introduction of a break, or the relaxation by some other means such as through an enzymatic action. Indeed, the topoisomerases in control of these events are important during packaging and replication of DNA (257). The supercoiled DNA is further organized with more dense twists in structural units called nucleosomes, which consist of 146 bp DNA wrapped around

an octamer of 4 histone proteins (H2A, H2B, H3 and H4). These multiple nucleosomal units form an 11 nm thick fiber of bead-like units of the histone core and 200 bp DNA each. The organization of DNA in the nucleosomes is not random and seems to be governed by the accessibility of regulatory proteins to certain sequences (258). Also, the N-terminal tail of the histone proteins can be modified with various functional groups, which have been shown to affect chromatin structure and gene regulation, one example being the methylation of lysines (K) and arginines (L) on histone 3 (H3) (259).

The DNA is compacted 7-fold when wrapped in the nucleosomal context, and about 100-fold when the nucleosomes are ordered in a 30 nm fiber, made possible by further coiling and loop formations. Since the overall compacting of DNA in the cell is greater than 10 000-fold, it clearly exist higher orders of DNA organization. Both 300 and 700 nm fibers have been defined in the 1400 nm dense structure of one mitotic chromosome, all made possible through the interaction and function of several proteins involved in chromatin organization and folding.

The order of DNA packaging and the supercoiling of DNA will directly affect ON binding and strand invasion. For instance, in plasmids, the circular DNA used in this thesis for assessing and ranking strand invasion capacity of different ONs, negative supercoiling and the resulting dynamic effects such as DNA breathing make ON binding possible. Thus as a result, supercoiled DNA will in certain situations favor ON binding, something especially clearly demonstrated when comparing to invasion of relaxed DNA, which is more difficult (71). In the cell, these “open regions” are much more regulated and differ between cells at different time points in association to events that involve chromatin remodeling, such as the events involved in actively transcribed genes (260).

1.7 HUNTINGTON´S DISEASE

Huntington´s disease (HD) is an autosomal dominant neurodegenerative, fatal disease, which is characterized by the progressive loss of motoric and cognitive functions. 5-10 in 100 000 are affected and symptoms usually starts at 15-20 years before death. There is no curative treatment, although symptomatic treatment exists for the involuntary movements, *chorea*, that most patients suffer from (261). The disease mechanism behind HD stems from a gradual expansion of the CAG trinucleotide repeat region in exon 1 of the *Huntingtin* (*HTT*) gene, giving rise to a mutated HTT protein (muHTT) where the many polyglutamine repeats give the protein its gain-of-function toxicity and propensity for aggregation (262). More recently it has also been suggested that the *muHTT* mRNA could contribute to the pathology to a certain extent by sequestering factors and regulatory elements (263). In normal, healthy individuals, the CAG repeats usually do not exceed 20, but in patients when the expansion reaches above 35 repeats, the symptom begin to appear and correlate well with the severity of the disease, as well as age of onset (261). Studies in mice have shown that HTT is important during development (264), but the function of HTT in the adult individual appears to be of less significance for normal physiological processes (265, 266).

The CAG repeat sequence in the gene has been shown to make intrastrand self-interactions, creating branches of many small hairpin structures (267-269), which is also present in the mRNA (270). These hairpins are more extensively formed in regions with higher number of repeats (271), and are directly involved in disease progression. They are mechanistically connected to the CAG expansion process during DNA repair and replication by the introduction of asymmetric replication biases, as the polymerase is stalled on one strand at the repeats but can continue on the other, leading to the inclusion of new repeat loops as the replication process is restarted (272).

1.7.1 Oligonucleotide treatment strategies

Ever since the finding that HD symptoms may be reversed when muHTT was knocked out in a mouse model (273), much attention has been directed towards the reduction of the toxic protein. At present, several ON based therapeutic options are under intensive investigation. These strategies fall into two main categories, i) non-allele specific reduction of total HTT or ii) the allele-specific reduction of muHTT. The advantage of the non-allele specific approach is that one ON drug can target all HD patients and not just a patient subpopulation. On an RNA level, both these approaches have been tested using siRNA (274), SSO (275), ASO (both RNase H mediated and steric blocking) (276, 277), ZFN (278), RNAi and RNase H mediated approaches (279), and one ASO designed for the reduction of total HTT is currently investigated in a phase I/II clinical trial by Ionis Pharmaceuticals (280).

ASO-mediated targeting of the repeats present in the mRNA has been evaluated and proven to give allele-specific effects due to the increased number of targets present on this allele (281). This strategy has been evaluated using different chemistries and can potentially target a

broader patient population compared to other allele-specific approaches targeting single-nt polymorphisms (SNPs) (282).

2 AIMS

The overall aim of this thesis is to optimize AGOs using novel chemical modifications and characterize their efficacy and delivery. More specifically this includes investigating AGOs with different modes of binding and the effect on specific uptake of AGOs with different chemical composition:

- Developing a fast and efficient method for the connection of the two ON arms in Zorro-LNA in a 3'-5'-5'-3' orientation using click chemistry, and evaluate their efficacy.
- Optimization of single stranded AGOs targeting the trinucleotide repeats in HD for the downregulation of HTT and investigation of their DNA binding and specificity.
- Characterization and DNA binding optimization of bisLNAs containing novel chemical modifications and intercalating molecules.
- Using a modified nucleolin receptor targeting aptamer connected to modified ONs to study the effect on the specific uptake of the constructs and their efficacy in cells.

3 METHODOLOGY

The following methods were used in this thesis to assess AGO effect, DNA binding or physiochemical characterization of the construct or its target sequence.

3.1 GEL ELECTROPHORESIS

In this thesis two types of gel electrophoreses are used to separate fragments of DNA and oligonucleotides, polyacrylamide gel electrophoresis (PAGE) and agarose gel electrophoresis. The separation in both cases depends on the size and structure of the fragments as they migrate through the polymer matrix at different rates. PAGE gives better resolution for smaller fragments (<1000 bp), while agarose gels are usually used for larger fragments. Also the percentage of the polymer in the gel, buffer composition and the time and magnitude of the supplied voltage will affect fragment migration along with any hybridized ON. These parameters need to be considered when assessing DNA binding of AGOs to target sequences as it can affect the result.

In addition, the electrophoresis can be performed using denaturing or non-denaturing conditions. Non-denaturing, or native, gel electrophoresis refers to conditions in which the chemical environment of analysis can preserve ON secondary structures and hybridization products. In denaturing conditions, the gel is supplemented with a chemical capable of maintaining the separation of two hybridized ON strands or internal structures. Typically, this is achieved using PAGE containing 7% UREA in combination with heating the sample before loading and the addition of 50% formic acid in the sample loading buffer. However, we have noticed that analyzing ON hybridizations with high LNA content using denaturing PAGE does not always result in denaturation using standard protocols. For instance, due to the stronger binding of the LNA-ON to its target it might not necessarily give more information analyzing the interactions with denaturing PAGE compared to native PAGE.

3.1.1 Electrophoretic mobility shift assay (EMSA)

To determine the binding of bisLNA to plasmids containing the target sequence, EMSA was used as has been described previously for other and similar applications (283). BisLNA and plasmid were hybridized together at 37°C using intranuclear pH and salt concentrations (Tris-acetate 50mM, [pH 7.4], 120mM KCl, 5mM NaCl, 0.5mM magnesium acetate) and treated with restriction enzymes for the generation of fragments of 150 bp consisting of bisLNA bound to the target sequence. The DNA fragments are then made visible using an intercalating dye (Ethidium Bromide or SybrGold) and analyzed using a gel documentation system with a camera. Depending on the chemical composition of the bisLNA, different constructs will bind to the target with different strength, and this was visualized and quantified using both PAGE and agarose gel electrophoresis. The plasmid linearization using the restriction enzymes relaxes the supercoiled DNA, which could result in detachment of the previously bound bisLNA. The ratio of bound bisLNA-bound target to the fraction of unbound target makes it possible to rank the compounds after binding efficacy. Since PAGE makes it harder for any fragment to retain a hybridized bisLNA, any bound bisLNA detected

after PAGE could be considered a strong binder. Agarose gels are used to compare and rank weaker binding bisLNAs, or to confirm findings obtained from the PAGE.

3.1.2 Proximity EMSA

As described for the bisLNA and EMSA in section 3.1.1, the EMSA was also used for assessing binding of the single stranded AGOs in paper II targeting the repeat regions in the *HTT* gene. Here, the same principle was used but the restriction enzymes were applied in a different way. Instead of generating fragments with restriction sites far from the target sequence, one restriction site was used located only one bp outside of the CAG repeats. When an AGO bind to the target, it will make it sterically more difficult for this restriction enzyme to cleave the DNA, which can be seen as a reduction in the fragment created from this site. Moreover, as AGOs with different lengths were tested, the longer AGO the higher the probability that a protruding unbound part of the AGO would interfere with this restriction enzyme, creating a length-dependent reduction in fragment intensity seen on the gel. See figure 7 below. This method has been used and described previously for PNA constructs and TFOs in assessing DNA binding (284, 285).

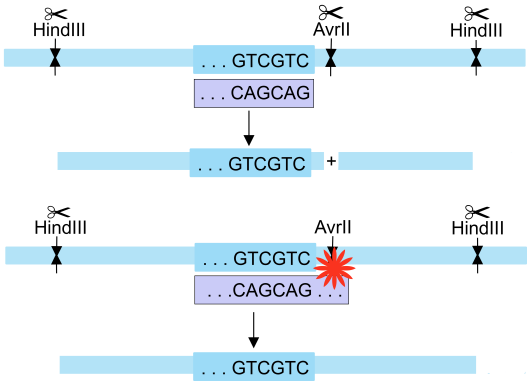


Figure 7. Schematic picture of the proximity EMSA used to detect ON binding to a target plasmid. The presence of an ON hinders the cleavage by restriction enzymes giving rise to a characteristic restriction fragment pattern.

3.2 S1 NUCLEASE ASSAY

One way to visualize and quantify dsDNA strand invasion by AGOs is to take advantage of the S1 nuclease enzyme, which is capable of recognizing and cleaving single stranded regions within supercoiled DNA (286, 287). We have been using plasmids containing binding sites for our constructs to assess DSI capacity and allow for construct ranking. When DSI occurs by an AGO, short stretches of single stranded regions are formed around the binding sites for the construct (figure 8A). These are then cleaved by the S1 nuclease, creating a nicked or linear relaxed plasmid with a different migration rate during gel electrophoresis compared to the supercoiled plasmid. This allows us to calculate the amount of nicked/linear plasmid in relation to the remaining uncleaved plasmid and the corresponding ratio gives the percentage of DSI by one particular AGO. Since the plasmid always give some background nicking after S1 treatment due to plasmid dynamic DNA breathing, the overall percentage of DSI is normalized to the mock treated plasmid.

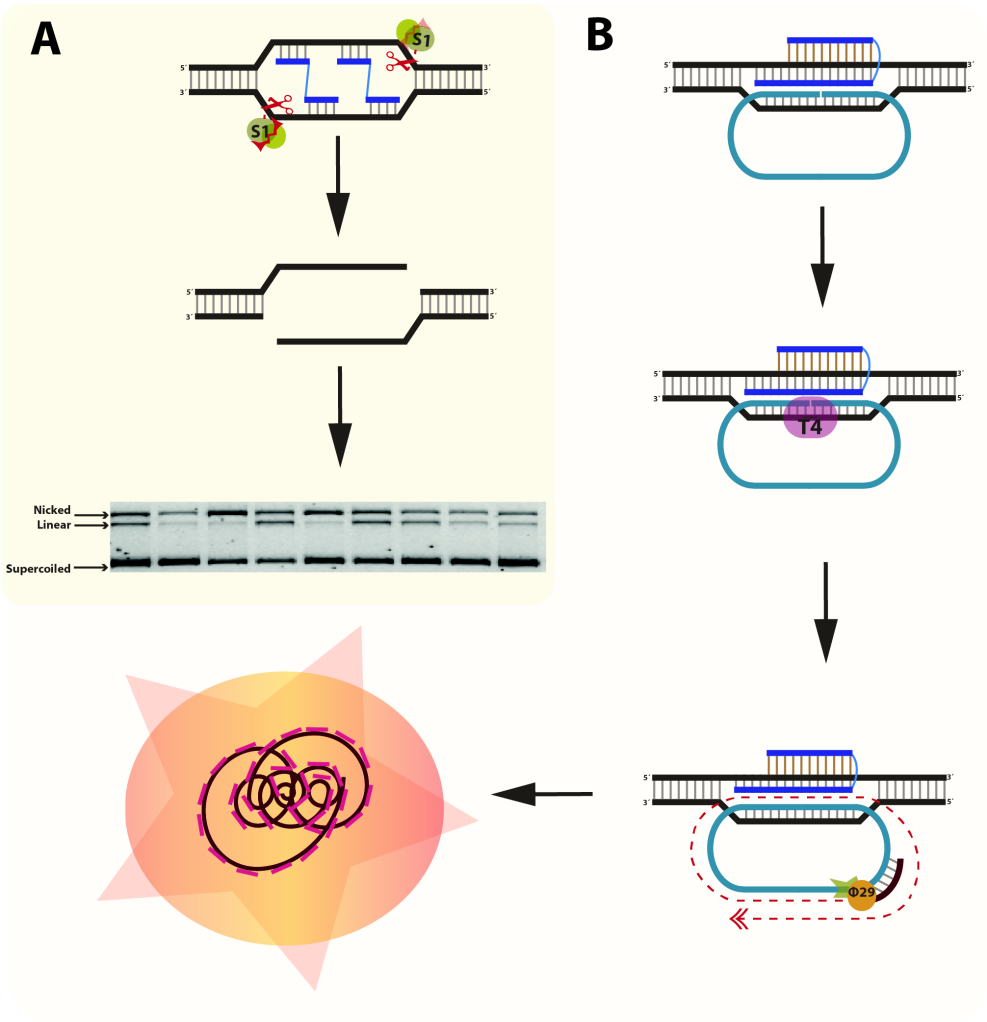


Figure 8. A show the S1 nuclease assay and the single strand specific cleavage of unprotected regions in the invaded plasmid, and the resulting gel image. **B** show rolling circle amplification on the displaced strand following bisLNA binding.

3.3 ROLLING CIRCLE AMPLIFICATION

Rolling circle amplification (RCA) is a versatile technique for the detection of single molecules in complex biological environments. With such high sensitivity it has been used in many different settings (288-290), such as for the detection of DNA (291, 292), DNA methylation (293), small molecules (294, 295), single nucleotide polymorphisms (296), mRNA (297-299), antigens in immunoassays (300), viral RNA (301), and proteins (302). These applications clearly show how RCA can be performed *in vitro*, in bacteria, and in cells and tissues.

The use of padlock probes (PLPs) in RCA (303, 304), where a hybridized targeting ON is circularized by ligation using the T4 ligase gives the technique the capacity to discriminate between single nucleotide mismatches (305). After the ligation, the circular PLP is amplified using primer extension creating a long, repetitive DNA sequence, which can be detected, by hybridization using small fluorescently labeled ONs.

In paper III RCA is used to show bisLNA binding to plasmids in solution and in bacteria. One challenge in this setting is the complexity of using hybridized DNA targets (see figure 8B). The RCA uses phi29 DNA polymerase for the primer extension, which has varying efficiencies depending on the PLP-target complex. For instance, it has been shown that single stranded linear targets give more RCA product compared to circular targets, owing to the greater ability of phi29 to unwind a single stranded target during replication as the 5′ and 3′ ends are free (306). Thus, careful titration of the experimental conditions is needed for the detection of bisLNA binding.

3.4 THERMAL UV-MELTING ASSAY

The way in which an ON interacts with itself, its target sequence or other molecules such as proteins or specific ligands are important parameters to consider when designing or optimizing anti-gene or antisense strategies. The sequence composition, type of chemical modifications or synthetic nucleotides used together with the chemical environment will affect ON behavior both *in vitro* and *in vivo* (307). UV spectroscopy to follow the thermal denaturation of DNA and RNA with or without the presence of ligands can be a useful tool for the stability assessment of a complex. In this thesis UV melting, or temperature of melting (T_m), is used not only to characterize AGO target binding, but also for structural assessment of the constructs. Usually when investigating DNA and RNA duplexes, one would follow the change in absorbance at 260 nm as the temperature decreases or increases from a state of denaturation to renaturation. ssDNA or RNA absorbs more light at 260 nm than a duplex due to the unstacked state of the bases. Therefore, hyperchromicity is seen when a duplex is melted and reversely, hypochromicity is seen during renaturation of the duplex (308).

To assess inter- or intramolecular structures, which could have important implications for ON efficacy, other wavelengths are often used to assess the T_m that better reflect the state transitions of interest. Complex structures such as a triplex or a G-quadruplex can be followed at 295 nm where they will show hypochromicity during the melting. It is often of interest to follow the melting curve at another wavelength in parallel for a better assessment of structure kinetics and behavior. Thus, for G-quadruplexes, 245 nm is often used in combination with 295 nm where the T_m and thermodynamic parameters should be the same for both wavelengths. Any deviations from these observations suggest more complex melting kinetics and imply a higher order of structural complexity (309).

In paper IV the structural properties of a G-quadruplex forming aptamer and its related versions with different chemical compositions were characterized by UV melting at 295 and 245 nm. These aptamers can adopt several conformations and show complex melting profiles. To further gain structural information that potentially could help understand the biological behavior of the aptamers, the T_m was also investigated in the presence of a G-quadruplex ligand capable of stabilizing certain conformations. Since G-quadruplexes can form in the presence of both sodium and potassium ions, but not in the presence of lithium ions, all ONs used were dissolved in lithium cacodylate buffer and then adjusted to suitable concentrations of potassium or sodium.

There are several eneral factors that affect the T_m measurement to take into consideration (307, 309). First, the purity of the ON analyzed should be high. Secondly, the buffer used should not absorb light in the far UV region or have a pKa that is affected by temperature. Generally, cacodylate (pKa 6.14), acetate (pKa 4.62) or phosphate (pKa 6.81) buffers are acceptable for this purpose. Thirdly, the increase in temperature per minute could also affect the reading, since structures such as G-quadruplexes form and reform at slower rates than a duplex. This can lead to hysteresis effects when reformation of a structure during cooling occurs, with different kinetics than during melting. The effect will lead to a shift in the melting curve or overestimation of the T_m. Thus, a slow change in temperature is preferred (0.1 - 0.2 °C/min) when investigating complex structures.

3.5 FOOTPRINTING

To identify the exact binding location of an AGO in the target plasmid, DNA footprinting can be used. There are several footprinting methods developed to determine the specific binding of a ligand to its target along with its affinity and binding kinetics (310). The principle relies on the protecting properties of the ligand bound to DNA when subjected to chemical or enzymatic treatments. Unprotected areas where no ligand is present will be subjected to DNA modification and cleavage, whereas the sequence with bound ligand will withstand this treatment creating a chemical footprint at this position.

The footprinting is visualized using radiolabeled or fluorescently tagged primers that are extended using the Sanger chain termination method (311), which will give rise to transcripts terminated at different positions. The sequence and the protected bases can be analyzed using dideoxy-nucleotides (ddNTPs) together with normal deoxy-nucleotides (dNTPs) during chain elongation. ddNTPs, which lack the 3´-hydroxyl group, block further elongation of the chain when incorporated and this allows for the specific assessment of what is protected from the footprinting after sequencing and alignment of fragments.

Classical footprinting is analyzed using denaturing gel electrophoresis and radiolabeled primers. In paper II footprinting with S1 nuclease and capillary electrophoresis detecting fluorescently labeled primers is used to assess binding of bisLNA compared to previously published results where the bisLNA was mapped using chloracetaldehyde treatment and radiolabeled primers (312).

3.6 CELLULAR ASSAYS

In this thesis, both AGO and aptamer effect and uptake are evaluated in cells. Culturing adherent cells in monolayer and using them in experimental settings such as those in the included papers require strict maintenance and awareness of important experimental parameters. To minimize irrelevant factors influencing the AGO evaluation, all cells used were tested for mycoplasma infection at regular intervals, and cells were grown in medium without any addition of antibiotics. Cells were split when reaching 90% confluency, and never used for more than 15 consecutive passages.

3.6.1 Transfections

Using various transfection agents to deliver genetic material into cells are today standard methods in many laboratories. Many chemical reagents can be used and all have their own advantages and disadvantages (313). In this thesis lipofection, or positively charged lipid complexes, and cell penetrating peptides (CPPs) capable of forming nanoparticle complexes with the ONs are used to deliver the material into the cells. Both approaches involve an incubation phase in which the ONs are formulated together with the transfection reagent. The nanoparticle complexes are then given to the cells after which a period of incubation follows, usually several days, before the cells are analyzed. Factors affecting transfection efficiency include the ON and transfection agent concentrations, the formulation of ON-nanoparticle complex, the number of cells in the well, the type of cells transfected and the composition of the medium. These parameters should be optimized during the design of the experimental protocol, since suboptimal use will result in lower transfection efficiency at one extreme end and cellular toxicity at the other end.

3.6.2 Gymnosis

Gymnosis, or the unaided, naked delivery of ONs to cells (314), is a useful delivery method to investigate ON or aptamer uptake after entering cells through various receptor-mediated pathways. The internalization process efficacy will depend on ON sequence, chemistry and secondary structure, and after uptake the ON needs to escape endosomal entrapment before it can be released and locate to the cytoplasm or the nucleus of the cell. Typically, a 10 to 100-fold higher concentration is needed for the ON to have effect after gymnotic delivery. However, as for transfections, delivery effect will also depend on factors such as ON concentration, total number of cells and cell type used.

3.6.3 Cellular uptake studies

The internal ON distribution as well as kinetic aspects of cellular uptake is a highly relevant experiment to perform. Depending on delivery method, chemistry and secondary structure, the ONs will show differences in cellular localization. This can be followed over time by using fluorescently labeled ONs, which can be imaged with a fluorescence microscope. Since AGOs are expected to have an effect on chromosomal DNA in the nucleus, ON accumulation in this compartment would be advantageous. However, the actual number of ONs needed to have an anti-gene effect may in some cases be lower than what can be detected with the fluorescence microscope, giving the false impression that no nuclear accumulation is present. Nuclear transporting could still be present where ONs could be transported from the cytoplasm to the nucleus in sufficient amounts to have an anti-gene effect.

3.6.4 Cell viability assay

As discussed above, cellular toxicity can be induced during the experimental conditions when studying ON effect and uptake. In certain cases, such as for the aptamer studied in paper IV, the expected effect after ON treatment is cytotoxicity which makes the elimination of non-

specific toxic parameters by assay optimization highly important. To assess cell viability in a quantitative manner, the WST-1 assay was used in this thesis. This assay is similar to the MTT assay and relies on the change in absorbance when tetrazolium salts are cleaved by mitochondrial dehydrogenases to formazan. This conversion can be detected using a spectrophotometer and correlates to the number of metabolically active cells in the well. Normally, to reduce experimental variations, these reactions are carried out in 96-well plates using triplicates or quadruplicates.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Fast and efficient synthesis of Zorro-LNA type 3'-5'-5'-3' oligonucleotide conjugates via parallel *in situ* stepwise conjugation

Following the first publication describing the Zorro-LNA strategy for gene targeting and transcriptional blocking (83), the focus was on finding more efficient Zorro-LNAs by optimizing their chemical composition. One of the most effective Zorro-LNA constructs uses two ON DNA/LNA mixmer arms connected by a flexible HEG-linker (84). Unlike the first generation Zorro-LNA (Z-2ON), that uses a nucleotide linker through which the two arms can hybridize to each other, the HEG construct (Z-2HEG) is smaller in size but requires the arms to be connected in a 5′-5′ orientation. This in turn requires the use of reverse unmodified and LNA phosphoroamidites during synthesis in order to switch strand polarity in the sequence. When many different compounds need to be generated for the screening of new targets, the use of reverse LNA amidites poses a problem, since they are not commercially available, therefore requiring in house synthesis of these building blocks.

In paper I we developed a more simple screening procedure that could be used when designing new Zorro-LNA constructs taking advantage of the smaller size of the Z-2HEG and at the same time abolish the need for reverse amidites. This was achieved using Cu(I)-mediated cycloaddition of a 5′-alkyne linked ON with a 5′-azide functionalized ON, a procedure called “click chemistry” used previously in related applications (315). Our “click chemistry” approach begins with the functionalization of two Zorro arms previously obtained commercially on a solid support. The arms are 5′-detritylated followed by the coupling of a 5′-amino modifier (2-(*N*-(4-monomethoxytrityl)aminoethoxy)ethyl H-phosphonate) (98). In parallel, the amino linker containing the azido or triple bond is detritylated and activated followed by their addition to the Zorro arms on support. The functionalized arms are then deprotected and cleaved from the support and either clicked together directly in aqueous solution or kept separately as “free arms”, followed by HPLC purification.

The new Zorro-LNAs and their corresponding “free arms” are shown in table 1 and figure 9 and were compared to the old constructs Z-2ON and Z-2HEG in their double strand invasion (DSI) capacity. These constructs were previously designed to bind two adjacent target sequences in the plasmid pN25-2BS, and tested using 0 or 1 nt overlap between the two arms. Now, we investigated the influence of having 1 or 4 overlapping bases in the constructs. Finally, using the knowledge gained from the pN25-2BS construct comparison, we designed click Zorro-LNA against a novel target in the plasmid pN25-i5. Although we could see efficient DSI with the 2BS target, two identical adjacent target sites are rarely found in the genome. With the new “i5” target we were able to show, for the first time, DSI using two different Zorro-LNAs.

Name	Sequence of Zorro arms	Crude intermediate
D1	CCCtCCtcTTtcTTCa	D1azido
D4	TgCcCCtCCtcTTTcTTCa	D4azido
U1	GgCACccATgCgcTgA	U1PATA
U3	cAacAaGcaCggCctC	U3PATA
D3	TtgCcAgaCtcTgCc	D3azido
D2	CccCagCcaCccTctG	D2azido
U2	GgaAacCtcCctAaG	U2PATA

Table 1. Name and sequence of the Zorro arms and the modifications added for the preparation of "clickable" intermediates. D1 or D4 was combined with U1 for the 2BS target and D2+U2 and D3+U3 were combined for the new i5 target. Azido = 2-(2-(2-azidoethoxy) ethoxy) acetate acid, PATA = (N-Propynoylamino)-p-toluic acid, LNA monomers in upper case, DNA in lower case. Reprinted from (95) with permission from the Royal Society of Chemistry.

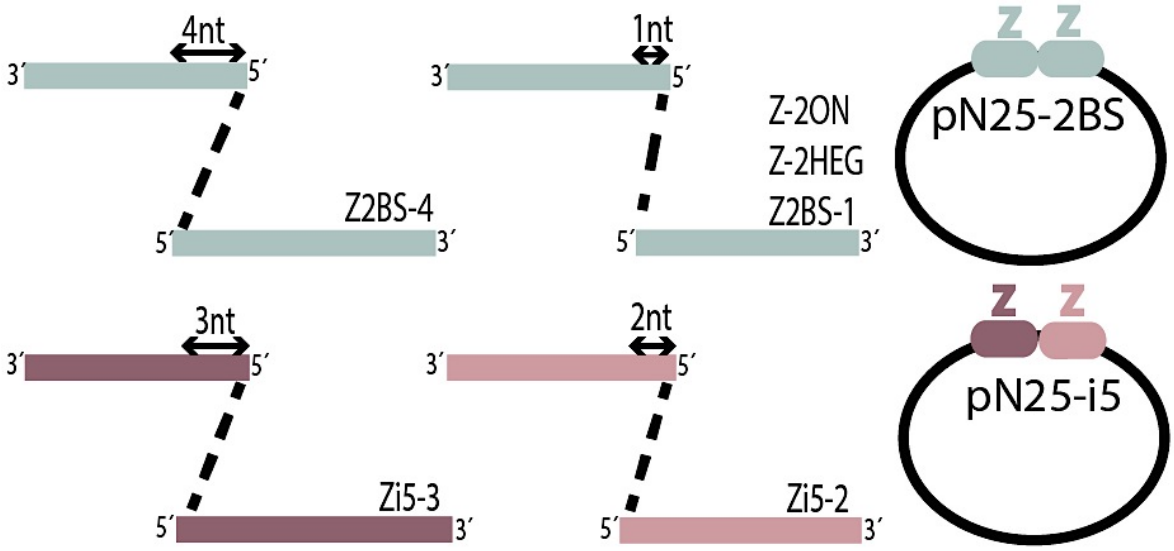


Figure 9. Zorro constructs used in the paper. Top panel shows the 2BS target sites in the plasmid beside the Zorro constructs compared along with their respective internal base overlap. Bottom panel shows the new i5 target and the two different Zorro constructs used together with their internal base overlap.

To compare the effect of having additional base overlap between the Zorro arms, the unmodified arms D1+U1 (1 nt overlap) and D4+U1 (4 nt overlap) were initially incubated together with the plasmid pN25-2BS at 37 °C using intranuclear salt conditions and pH for DSI assessment using the S1 nuclease assay. From these experiments we could see that the 4 nt extended overlap did not reduce the DSI capacity. Interestingly, when the arms were

incubated separately together with the plasmid, we noticed that the D4 could invade the plasmid as efficiently as the D1+U1 together, while the D1 and U1 alone performed very poorly. This effect has been noted before, and could be due to a part of the D4 sequence acting as a TFO, thus enhancing the DSI performance compared to the other sequences (84).

Next, we investigated the DSI of the clicked Zorros Z2BS-1 and Z2BS-4 and compared them to the previous constructs Z-2ON and Z-2HEG. Both click constructs showed time and dose dependent increase in DSI, but were not as efficient as the previous constructs, especially the Z-2HEG that showed almost double efficiency in comparison. We attributed these differences to the linker composition and the importance of using a flexible linker, such as the 2xHEG, when optimizing DSI for a lead Zorro construct after screening targets and sequences using the click chemistry approach.

Finally, we wanted to investigate the ability to target two different adjacent sequences in a gene using our click Zorro constructs. Since we already showed that up to 4 nt overlap between the arms could be used, the new constructs Zi5-2 and Zi5-3 were designed using 2 and 3 bases overlap respectively. These were then incubated with the pN25-i5 plasmid and we could demonstrate an increase in DSI over time using the tandem combination of Zi5-2 and Zi5-3. Even though the overall DSI was lower for the i5 constructs compared to the 2BS, DSI using two different Zorro-LNAs were shown for the first time, which could potentially be utilized as a promising anti-gene strategy in the future using more optimized constructs.

4.2 PAPER II

GTC repeat-targeting oligonucleotides for down-regulating Huntingtin expression

In paper II we developed modified ONs targeting the trinucleotide CAG repeat area in exon 1 of the *HTT* gene, responsible for the disease phenotype seen in HD. Our therapeutic approach is based on the non-allele specific binding of LNA-containing ONs to chromosomal DNA, which will block transcription and production of both the wild type and mutant HTT protein thus reducing total HTT which in turn would reduce or possibly eliminate disease pathology and patient symptoms. At present, different treatment strategies are investigated by others (277, 282), and when ONs are used they normally are designed to target the mRNA to either block translation or induce degradation of the transcripts. Our aim of this paper was to a) show reduction in HTT levels in patient-derived fibroblasts and b) provide evidence that the effect comes after ON binding to DNA and not to RNA.

First we designed CAG repeat DNA/LNA mixmer ONs with PS or PO backbone ranging in length from 10 up to 19 nts. These ONs were then tested in patient derived fibroblasts for the effect on HTT on both mRNA and protein levels using Q-PCR and Western Blotting. From these experiments it was shown that we could get stable and effective downregulation of mRNA and HTT levels, reaching as low as 35% remaining compared to untreated cells, at both 4 and 7 days after treatment. Moreover, we could show effect of the CAG-ONs at low nanomolar concentrations following transfection and low micromolar concentrations following gymnosis. The ONs with PS backbone all showed similar efficacy, while the PO

ONs did not give any effect on HTT levels following transfection. When following fluorescently labeled PS and PO CAG-ONs after transfection, we could detect high signal from the nucleus using PS ONs, while the PO ONs were mainly found in the cytoplasm. All ONs capable of downregulating HTT were able to do this regardless of ON length, with the exception of the 10-mers that were not effective at all, and the 12-mers that showed a reduced effect on mRNA after naked delivery. In addition, the 19-mer reduced the mutated HTT protein levels significantly more compared to the wild type HTT.

We also designed two CAG ONs with the DNA bases exchanged for 2´-*O*-Me, or with four LNA bases exchanged for 2´-glycylamino 5-methylcytosine LNA. Their effect on *HTT* mRNA was assessed using both transfection and gymnosis. However, no improvement over the regular CAG-ONs was seen suggesting no added benefit using these kinds of modifications.

To show that the CAG-ONs did not induce their effect by binding to *HTT* mRNA, we compared transcript levels after transfection with both CAG-ONs and CTG-ONs. The CTG-ONs are antisense ONs complementary to the *HTT* mRNA and have been reported to inhibit translation by steric blocking (281). Indeed, only the CAG-ONs showed reduced mRNA levels, with the exception of a 19-mer CTG-ON, which gave a slight reduction of the mRNA as well. The HTT protein levels were highly reduced using both CTG and CAG ONs, confirming the steric antisense mechanism for the CTG-ONs and points to a different mechanism for the CAG-ONs consistent with DNA binding.

To further study CAG-ON interactions with RNA we performed UV melting using CAG and CTG-ONs together with the RNA sequence (CAG). From these experiments it was evident that only the CTG-ON was able to give rise to a shift in T_m, while the CAG-ON did not. Thus, the CAG-ONs do not interact with the RNA, pointing to another mode of action in accordance with the observed results in cells.

A more direct proof of CAG-ON binding to DNA was obtained using a plasmid containing a 72 CAG repeat sequence. This plasmid was incubated together with the CAG-ONs, after which the restriction enzymes HindIII and AvrII were used to cleave near the ON binding site. HindIII cuts 40 bp 5´ upstream of the CAG sequence and 745 bp 3´of this region, while AvrII cuts just one bp outside. When analyzing the cleavage patterns using gel electrophoresis, it could be shown that AvrII had difficulties to cut the plasmid at this site. Moreover, the inhibition of cleavage was more pronounced using longer CAG-ONs, and this we interpreted as a steric blocking of the AvrII activity due to the presence of a CAG-ON. The longer CAG-ONs had a higher probability to interfere with AvrII cleavage, as seen by the weaker bands generated compared to the shorter CAG-ONs.

These results together strongly suggests direct ON binding to the *HTT* DNA, even though a full understanding of the cellular effect is missing. HTT levels were clearly reduced without the ONs binding to mRNA. Along providing a possible new treatment strategy for HD, this AGO approach opens for new possibilities to treat diseases associated with repeat regions in

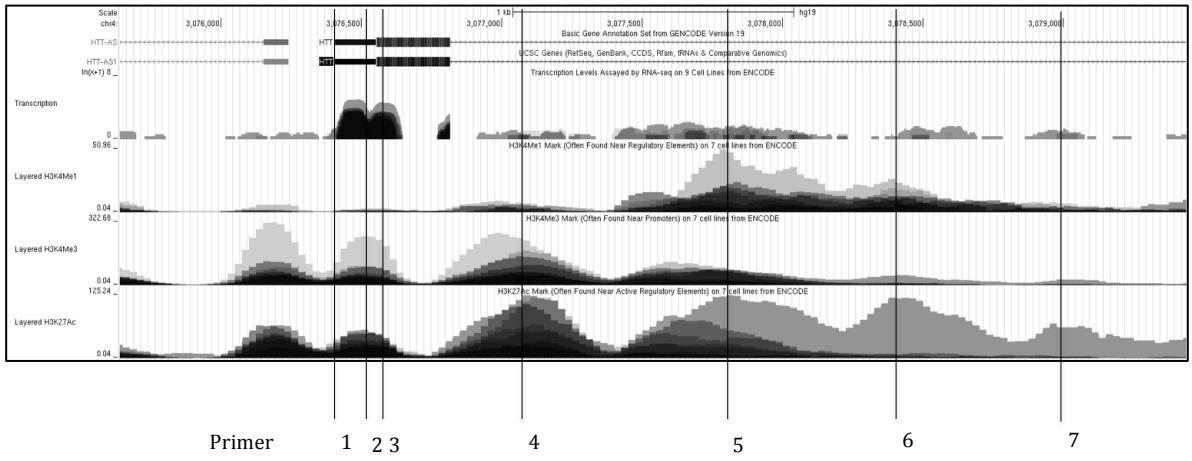
the gene. However, more studies are needed to closer evaluate the relationship between effect and DNA binding to exclude other possible mechanisms of *HTT* downregulation. Mainly, it has been suggested that ONs can have anti-gene effects by binding Antisense transcripts and affect gene expression by either sterically blocking or induce RNase H-mediated cleavage of the transcript (61).

Indeed, for the *HTT* gene an Antisense transcript has been reported, spanning the region of exon 1 and 2 (316). It has been thought to be involved in the regulation of the sense transcript; consequently, any downregulation of this transcript would in fact increase the level of the sense *HTT* transcript. Thus, it is unlikely that our ONs act by binding to this Antisense transcript, further supporting our hypothesis that the effect comes from binding to chromosomal DNA and blocking of the polymerase during transcription. Future studies could provide detailed proof of this mode of action by searching for specific changes reflected on a chromatin level, as well as evidence of polymerase stalling.

4.2.1 Preliminary results – Oligonucleotide chromosomal binding

In order to confirm our CAG-ONs binding to chromosomal DNA we set out to investigate whether histone modifications are changed when cells are treated with the ONs. We reasoned that the silencing effect following ON treatment could be associated with reduced gene activation at the chromatin level. For instance, epigenetic silencing could be a plausible effect at certain locations in the *HTT* gene. The state of methylation and acetylation of the promoter-associated H3 are two modifications that correlate with the activation level of gene transcription. Thus, the H3 tri-methylation Me3 of K4 together with acetylation (Ac) of K27 in this region is normally associated with active genes (317). As seen in figure 10, there was no loss of these markers, suggesting the ON effect to be independent from promoter activation and silencing.

A



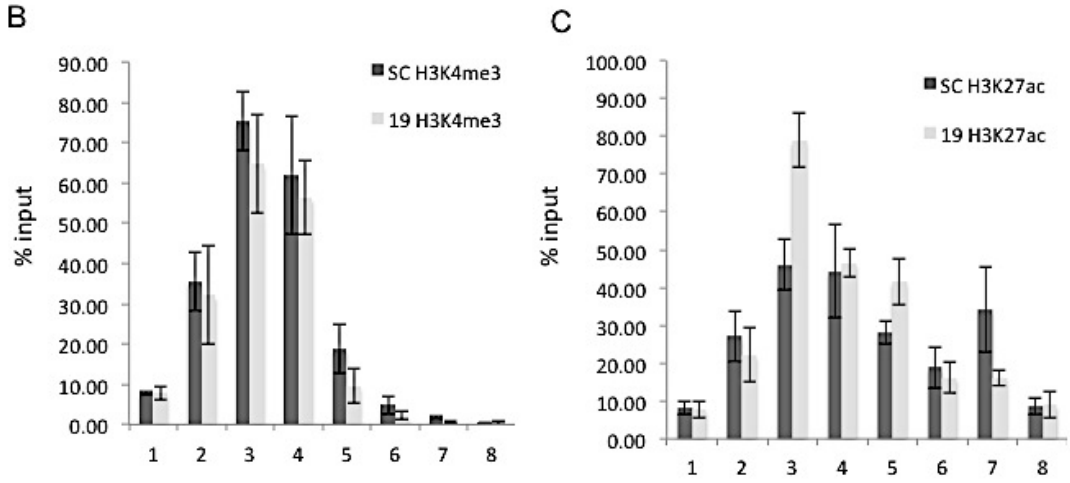


Figure 10. Active histone marks are maintained across the *HTT* gene TSS during *HTT* mRNA knockdown. (**A**) UCSC genome browser image demonstrating RNA abundance H3K4me3 and H3K27ac abundance across the *HTT* gene TSS region. Primer positions used for ChIP-qPCR are numbered 1-7. (**B**) H3K4me3 and (**C**) H3K27ac abundance measured by ChIP-qPCR across the HTT TSS region.

In addition, we also examined the ON effect on transcriptional elongation and RNA polymerase II serine 2 (S2) occupancy. Since the ON bind to exon 1 downstream of the polymerase initiation site, this might affect the elongation. The occupancy of S2 due to phosphorylation is correlated to gene expression and elongation rates, so that in 3´ exons of genes this occupancy will be higher compared to upstream (318). Any stalling of the polymerase in exon 1 due to ON blocking should therefore show lower occupancy at downstream exons while possibly have increased occupancy at or near exon 1, when comparing treated to untreated cells. As seen in figure 11, this is indeed the case, as the 3´ occupancy is reduced, and at the same time increased at exon 1 upstream of the CAG region, suggesting ON binding and stalling of polymerase elongation.

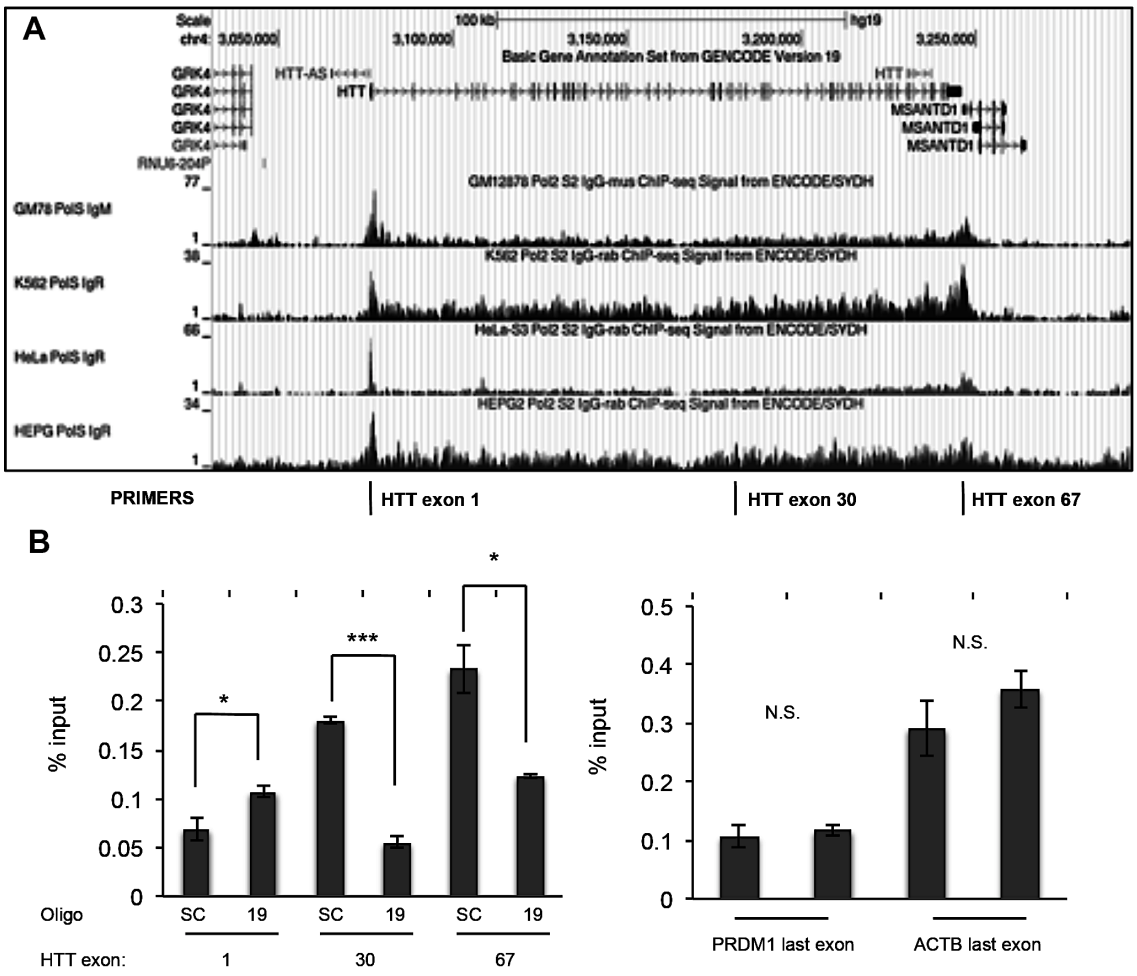


Figure 11. S2 phosphorylated RNA Pol II occupancy across the *HTT* gene is reduced upon transfection with HTT targeting ONs. (**A**) UCSC genome browser image demonstrating RNA Pol II S2 occupancy across the *HTT* gene. Primer positions at exon 1 upstream of the CAG repeat and at exons 30 and the last exon, 67, are indicated. (**B**) RNA pol II occupancy is significantly increased at exon 1 of the *HTT* gene immediately upstream of the CAG repeat (*p<0.05) and reduced at the 3' exons 30 and 67 (*p<0.05, ***p>0.001). (**C**) No difference is observed at control loci in the last exons of the *PRDM1* or *ACTB* genes.

Taken together these result clearly suggest the binding of the ONs to chromosomal DNA and that the effect on downregulation is indeed due to this binding. Further studies to show direct binding to chromosomal DNA using chromatin immunoprecipitation techniques or nascent RNA sequencing approaches could be performed in order to investigate this novel mode of action more thoroughly, especially to exclude the involvement of, or effect on, antisense transcripts spanning the region of binding.

4.3 PAPER III

Next-generation bis-locked nucleic acids with stacking linker and 2'-glycylamino-LNA show enhanced DNA invasion into supercoiled duplexes

This paper aims at investigating the influence of chemically modified nts and intercalators on DSI and DNA binding efficiency of different bisLNA constructs. It has previously been shown that the incorporation of LNA nts in the bis-LNA sequence has a positive effect on strand invasion, and that the binding is sequence-specific and using a linked clamp construct of two ONs for both WC- and HG-binding through a TFO is a feasible strategy (312). However, the mechanism of binding was not fully understood, as it was shown that using a single stranded ON binding via WC mode was as effective as the clamp construct, which also has the TFO arm. Thus, to fully take advantage of the different options for the enhancement of bisLNA constructs through chemically modified bases, a deeper understanding of the binding mechanism was needed.

To investigate this, we examined how both the WC and TFO arms contribute to strand invasion by incubating them together with a target plasmid, separately and in combination. Here we could see that the free TFO makes it harder for the WC arm to strand invade. At the same time, reducing the number of LNAs in the TFO arm makes strand invasion less efficient, possibly due to a higher kinetic rate of dissociation of the TFO. These findings led to the proposed mechanism of binding where the bisLNA first binds to the target sequence by forming a triplex involving its TFO. After this, DSI most probably begins from the tail of the WC arm. This is supported by several experimental observations such as tail containing bisLNAs are more efficient compared to clamp-only containing constructs, and the reduction of bases in the tail region, or mutations in the corresponding target sequence, reduce the invasion efficiency severely.

Intercalating molecules introduced in the bisLNA constructs were shown to affect the stabilization of the WC and HG arms after hybridization to the target DNA. The TINA intercalator gave very different results on the compound performance depending on the position where it was placed. BisLNAs carrying a TINA in the TFO part could stabilize the triplex formation, however, when placed in the WC arm at the junction, the final complex is destabilized even though overall strand invasion was improved.

One important finding we made was that the linker region connecting the TFO arm with the WC arm has a major influence on strand invasion efficiency of the bisLNA. By replacing the 5 nt DNA linker with an intercalating moiety called M3 (319), strand invasion was much improved as shown using both S1 nuclease assay and EMSA. This positive contribution to efficacy was attributed to the stacking properties and optimal length of the M3 linker due to increased initial triplex formation and enhanced cooperative effect between both arms. These observations were further highlighted by the fact that taking away the TFO arm diminished the advantages of having an M3 in the linker, and this effect was even more pronounced when using a scrambled TFO arm. M3 stacking and stability enhancement seems to require a

proper triplex formed. M3 linkers are of particular interest in bisLNA clamp constructs, which do not rely on any WC tail at all, since such constructs devoid of an intercalator showed very low DSI capacity.

We could also demonstrate that M3 molecules are beneficial for TFO stabilization not only in the linker region. When placed in the 5′ extrimity as well as in the linker for the clamp constructs, strand invasion was even higher. By testing strand invasion in plasmids containing mutated target sequences we could show that the specificity of M3 containing constructs was maintained, as the discriminatory ability of the TFO part of the bisLNA was much higher with the M3 constructs compared to the ones without, again stressing the accuracy of our mechanistic explanation that binding starts with the TFO formation.

Since the triplex is destabilized by the electrostatic repulsion of the phosphate backbone of the three strands, we sought to investigate if introducing LNA analogues carrying a positive charge into the constructs could reduce this repulsion. Indeed, both the 2′-aminoglycyl LNA modified clamp and tail bisLNAs showed great improvements in strand invasion, possibly due to an advantageous conformation favoring interactions between the positive charge and the phosphate group, reducing the overall electrostatic repulsion. When combined with M3 intercalating moieties, very efficient bisLNA constructs were demonstrated with high strand invasion capacity at low concentrations (< 500 nM).

The need for efficient and stable TFO formation for bisLNA constructs was further demonstrated when targeting different genomic locations. Here we found that strand invasion efficiency in plasmids was heavily influenced by the target sequence base composition. C-rich bisLNA constructs were found to be less efficient which is probably due to the need for C protonation in the TFO contexts, something that is not efficient at physiological pH.

Finally we wanted to test the bisLNA performance in a more complex context. *E.coli* bacteria containing target plasmids were grown on microscope slides after which the bisLNA was incubated with the bacteria on the slides and strand invasion assessed using RCA. We found that without bisLNA, very low rolling circle product and signal was detected, indicating bisLNA binding on target since high signal was detected in the bacteria where the bisLNA was present. We also concluded that RCA could not be induced from other bacterial components since bacteria containing control plasmid and bisLNA gave very little signal.

Taken together, we have successfully constructed very efficient and novel bisLNAs with high strand invasion capacity at low concentrations. We also present substantial evidence to the mechanistic model of bisLNA binding, starting at the TFO, and the constructs were applied in a biological context were they showed target specific binding and efficacy.

4.4 PAPER IV

Delivery, effect on cell viability, and plasticity of modified aptamer constructs

In paper IV we investigated the effect on cellular uptake and conformational plasticity of a set of aptamer-connected ONs. Aptamers are ONs capable of adopting secondary structures that are recognized by ligands, such as cell surface receptors that could enable specific uptake. The aptamer we have studied is called AS1411 and was developed some ten years ago as a potential treatment for tumors that overexpress the NCL receptor on the cell surface (208). AS1411 can adopt a G4 conformation and can bind and become internalized through NCL receptors in targeted cells, where it reduces viability. We wanted to use the targeting and internalization process to study the potential of the aptamer to deliver LNA containing cargo ONs into the cells. These ONs were previously tested elsewhere individually or combined as Zorro arms, and one of these (named Tox) was found to be highly cytotoxic. To test the combined aptamer + ON, they were synthesized as one chimeric construct and delivered to cells using both lipofection and by gymnotic delivery. We also wanted to investigate the use of UNA in the loop regions for the G4-forming aptamer sequence, which could potentially change the aptamer folding properties.

First we set out to confirm that the chimeric constructs maintained the cytotoxic effect seen using Tox alone and in combination with the second ON, noTox. This was shown by transfecting the constructs into A549 and U2OS cells at low nanomolar concentrations and assessing cell viability after 24h using the WST-1 assay. Since we were able to confirm the effect of Tox in all constructs and combinations, we concluded that there was no negative influence on efficacy of using Tox in a chimeric context.

Secondly, we wanted to investigate the effect on cell viability under gymnotic conditions. During 5 days, the constructs were incubated at 37 °C with A549 cells, and cell viability was determined again using the WST-1 assay. From these experiments we found that only the aptamer alone and the chimeric aptamer-Tox construct had an effect on cell viability at low micromolar concentrations. One construct, which carried the noTox hybridized to the 5´ end of aptamer-Tox, showed reduced toxicity. UNA substitutions in the loop region of the G4-forming aptamer did not influence the effect of AS1411 alone, but slightly reduced the effect in the chimeric construct connected to Tox.

The fact that the inherent toxicity of AS1411 alone a) disappeared when attached to noTox, b) was reduced when connected to a large cargo (Tox+noTox) and c) was reduced in the UNA chimeric construct, led us to suspect that the cargo ON by size or chemical composition could affect the conformational plasticity of the aptamer needed for effective binding and internalization. Thus we looked at the cellular uptake of AS1411 and the chimeric constructs in A549 cells by using fluorescently labeled ONs. Here it was evident that only the AS1411 and AS1411-Tox was internalized efficiently since very low signal was detected from the AS1411-noTox.

On the other hand, when the constructs were tested in U2OS cells, we noticed that only the cargo-less AS1411 with UNA substitution had any effect on cell viability after naked delivery. The overall effect on cell viability using this construct was also much reduced compared to A549 cells, and when following uptake in U2OS cells we could see that the signal was indeed very low. This led us to conclude that the aptamer does not enter U2OS as efficiently as A549 cells, possibly due to different number of expressed NCL receptors on the cell surface.

Since it was evident from the cell experiments that the cargo ON could affect the internalization properties of the chimeric constructs, we wanted to compare the folding and conformational profiles using UV-melting and gel shift electrophoresis. Since the AS1411 is known to adopt G4 conformations, we followed their melting profiles at 295 nm in the presence of KCl. Indeed the T_m transitions in all constructs were indicative of the presence of G4 structures, but no differences indicated by changes in T_m and folding kinetics were observed for the AS1411 constructs. Neither were any structural differences seen using gel electrophoresis. However, the UNA based chimeric constructs showed hysteresis effects during reformation of the G4, suggesting differences in folding kinetics. This was also reflected in the cellular experiments as these constructs showed reduced effect. The AS1411 alone did not suffer from hysteresis effects during UV melting, which was consistent with the higher effect seen in cells compared to the chimeric UNA constructs. From this we concluded that UNA substitutions in the loop region are acceptable in the aptamer alone, but give a negative contribution to the chimeric construct plasticity and cellular effect.

It has been previously reported that the AS1411 can adopt several G4 conformations, and it has been problematic to try to characterize these conformations using various techniques. Furthermore it is not known which conformation is biologically relevant. This led us to investigate a novel approach to characterize conformational changes in the AS1411 by using small molecule G4 ligands as probes. G4-binding molecules capable of stabilizing G4 structures have been used extensively in the literature for various purposes. Here we synthesized two novel ellepticine derivatives called 1-ChE and 1-OxE for the purpose of following their stabilizing effect on the conformations of the aptamer constructs during UV melting to assess any subtle differences not otherwise possible to detect.

From these experiments we could see a dose dependent stabilization of the G4 structure of AS1411 indicated by an increase in Tm for each fold of molar excess of the G4 ligand present. The 1-ChE was more effective compared to 1-OxE indicated by higher T_m shifts, however, no significant difference in the melting profiles was observed compared to the chimeric constructs. In contrast, when comparing the UNA containing aptamer alone to the corresponding chimeric construct carrying Tox, a dramatic hysteresis effect was observed in the presence of G4 ligand. This correlates well with the observed reduced effect of this construct in cells, suggesting a conformational difference of this construct.

Taken together, we showed that AS1411 could efficiently deliver toxic LNA ONs to cells. However, we provided evidence of the importance of aptamer cargo composition and the

effect it can have on plasticity of the construct and cellular uptake. These parameters need to be taken into consideration when designing future chimeric constructs for the specific delivery of cargo to targeted cells.

5 CONCLUDING REMARKS

This thesis describes different applications of gene targeting ONs and the use of aptamers for the specific delivery of ON cargo into cells. A useful screening strategy for Zorro-LNA optimization using click chemistry is presented, as well as investigations on ON effect following DNA binding or double strand invasion. Single stranded ONs targeting the *HTT* gene have been shown to downregulate HTT expression, and their binding to DNA and not RNA was demonstrated on several levels. BisLNAs were developed using various chemistries, and we could show that the most efficient constructs for strand invasion needed a stacking linker and aminoglycyl LNA, in combination with a stable TFO.

When using aptamer mediated delivery of ON cargo into cells, the chemical compostion of the ON is important since it can have a direct effect on cellular uptake. The structural plasticity of the chimeric constructs need to be carefully characterized, especially when incorporating modified bases such as UNA, which did not automatically correlate with greater activity.

Given the recent advances and success seen with ASO therapeutics, the future holds great promise also when it comes to AGO approaches. Still, several hurdles need to be overcome for this to become a reality. As for all ON therapeutics, the delivery is still the main issue for targeting disease tissues. So far, the most successful treatments have been locally administrated since systemically administered ONs mainly end up in either the liver or kidneys due to their pharmacokinetic properties, even when formulated with delivery vehicles.

On the other hand, ONs can have great effect in these organs, illustrated by the many therapeutics under investigation equipped with the GalNAc functional group for enhanced uptake in the liver. Thus, for CNS-active ONs, such as those used for investigative HD treatments, local administration to the brain through intracerebroventricular injections or related techniques seems likely to be the route of choice, since the blood brain barrier is not permeable for this class of therapeutics. Indeed, ON effect and exposure after single injections of therapeutic doses seem to be present several weeks in the brain (276).

To show DNA binding in a chromosomal context is the ultimate proof of the AGO concept. Our preliminary result on serine 2 phosphorylation on RNA polymerase II suggests ON binding to the DNA and the subsequent stalling of transcriptional elongation. Future experiments can give further insights to the complex assessment of such mechanisms. These would likely benefit from the use of many different assays in combination for a greater understanding of the basic principles associated with gene transcription during ON-binding. This thesis provides a starting point for such investigations, and hopefully these small steps could some day move the anti-gene field closer to treatments and benefits for the patient.

6 ACKNOWLEDGEMENTS

The scientific journey of my PhD studentship and writing of this thesis would not have been possible without the support from many different people. First of all I would like to thank my main supervisor **Edvard Smith** for giving me the opportunity to be part of the group and for giving me lots of freedom to operate and try new things in the various projects I have been involved in. It has been a pleasure working together and this would not have been possible without your devoted time and positive attitude. Thank you also **Karin Lundin** for your dedicated support as co-supervisor and for being there to give feedback on ideas and experimental design. I would also like to thank co-supervisor **Roger Strömberg** for your valued input from a synthesis aspect on things and for being there when questions needed answers. Also thank you co-supervisor **Rula Zain** for your commitment to my development and helpful feedback when problems arose, always available regardless of trivialities such as hour of the day or holidays. Many thanks **Johan Sandin** my former boss from AstraZeneca, for being my mentor and following me during this period of life, our lunches were much appreciated and helped me to navigate and get direction in future career strategies.

Thank you also fellow present and former colleagues of the MCG group, **Samir el Andaloussi** for your generous personality and helpful tips. **Vladimir** my Avaris partner-in-crime, thanks for the many, many laughs and your support during hard times and good times (tea times especially). **Sylvain**, the bis-Wiz, for sharing the passion for anti-gene targeting, knowing the true meaning of DSI (and Peter Gabriel), **Abdul** for always being helpful and kind, **Qing-Qing** for very good company and sharing Avaris together and **Yue** for nice conversations. **Burcu** thank you for the support in the lab and for great talks about life, the apocalypse and zombies. Thanks also to **Janne** for sharing your great scientific knowledge in splicing and your input in many projects, **Sofia** for nice company in the lab and during travel and help with the Q-PCR machine, **Lotta** for your kind help in many lab related issues, **Leonardo** for introducing the world of fluorescence microsopy to me and nice lunches, **Dara** – always helping out and sharing know-how, **Manuela** for your nice company in the lab and guidance in the administrative jungle of dissertation, **Cristina** for your firm lab guidance and nice company during the AFM time at KTH and your very helpful proof-reading of the thesis manuscript, **Oscar** for your nice company and help when needed during the HTT permit process, and **Joel** for great company both inside and outside the lab.

Thank you **Dhanu** for great company and always having and sharing the stuff needed at the right moment both in the lab and the office, **Kariem** – thank you so much for sharing your nanoparticle knowledge and awesome company within the ups and downs of the peptide project, as well as your views on the future of science, academia and industry, **Fiona** for your great personality and kind help in the lab, always providing sweets and candy in times of need, **Helena** thanks for sharing both good and hard parts of PhD studentship with interesting discussions over many cups of tea, **Giulia** for great company in the lab (and the subway!) always happy to help out with order "issues" and much more, **Kathrin** for your kind and extremely helpful nature from floor seven in desparate times of need in the lab, as well as

helping out with practical details such as malfunctioning shoe laces. **Hanna** for your superior support in administrative issues and your help in so many things, **Emelie** for your great company in the lab and your awesome assistance in administrative issues, **Kristi** for helping out with peptide orders. Many thanks also to **Anthony Wright** for interesting scientific discussions, **Gustav** for great company and travel tips on Gotland and for your efforts in getting me on the bicycle, **Amir** for your good company and generous nature, **Laia** and **Narmeen** for great company in the lab.

Thanks also **Taavi** for great company both inside and outside the lab and for your peptide knowledge which you shared, **Andre´** for your company and shared passion for CD133, **Eman** for your positive and very generous personality – thank you so much also for assistance in the lab and in our work with Zorros, you are one of the key persons contributing to my PhD from the very first day I arrived, **Anna** for your superior artistic input on many illustrations and slides, and for cheerful times in Avaris always making me jealous of your travels, **Niels** for your nice company in the lab and during recreation (Aifur!!), I am so glad you found the position you deserved finally, **Mamiko** for being a nice "room-mate" and great company, **Beston** and **Yuye** for great company in the lab, **Osama** for your positive personality and great company in the lab. **Alamdar** for great company in the lab, **Nawaz** for nice chats in the corridor, **Noriko** and **Ulrika** for pleasant conversations and nice company in the lab, **Abdi** for nice company in the lab, **Helen** for conducting the helicase experiments with me, **Antonio** for nice discussions both in the lab and outside and **Micke** at MTA and **Nelson** for technical assistance.

Thank you also my undergrad students **Izzy**, **Fulla**, **Negin** and **Tea** for helping me grow as a supervisor and good times in the lab. Thanks also **Fatin** for great company during your BSc stay. Thanks also to Roger Strömbergs group, **Malgo** for your support, hard work and helpful knowledge in chemical synthesis, **Martina** for your postitive attitude and great collaboration during the click chemistry work, **Alisha** for helping out when T_m problems arose and **Dmytro** for the nice CD introduction.

Others I would specially like to thank: **Tom Krzywkowski** from Scilifelab for outstanding help during the RCA project and for letting me learn the tools of RCA, **Ruben Alvarez Asencio** for great company and dedication during our AFM experiments at Alba Nova- KTH, **Jesper Wengel** for your great scientific input on the joint projects and hospitality during visits, **Per Trolle** for useful feedback on manuscripts and nice discussions, **Erik Pedersen** for your triplex knowledge and nice discussions, **Chenguang Lou** (Harvey) for your dedicated work and great collaborations, **Mats Nilsson** at Scilifelab for nice RCA discussions during our joint project and for letting me stay at your lab to learn the techniques and **Per Svenningsson** for helpful input in the HTT project design. Also many thanks to **Daniel Vare** and **Lee Siggens** for experimental support in the HTT project. Thank you also KI doctoral administrative staff for the support and help when needed, **Andrej Weintraub**, **Marita Ward**, **Arja Kramsu**, and many others. Thanks also to **Moustapha Hassan** for you endless patience during the permit application process.

Finally I would like to thank my all my family and friends, **Lina** for your love and understanding of this long journey – I love you so much! My children **Sebastian**, **Adrian** and **Gabriel** for bringing joy and meaning into all this, without you I would be nothing. A big thank you also to my parents **Sture** and **Viveka** for their support during this time and my sister **Karin** with family (**Asbjörn**, **Tora** and **Aksel**) for understanding and love. A special thanks to **Andrea**, my mother-in-law, who came to the rescue in times of need such as o pick up kids from daycare in lato hours during times when experiments disagreed. Thanks also to **Veronika** and **Kalle** with family (**Irma** and **Signe**), **Micke** and **Kaj**. Thank you **Lars Goldammer** for recreations and musical escapisms, a welcome break from science from time to time.

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