DEVELOPMENT OF ssRNAs FOR THERAPEUTIC GENE KNOCK-DOWN

By Paulina Klaudyna Powałowska, MSc

Thesis submitted to the University of Nottingham

for the degree of Doctor of Philosophy

September 2016

Acknowledgement

First of all, I would like to thank my supervisors, Professor David Brook and Professor Christopher Hayes, for giving me the opportunity to work on this project, their enthusiasm, encuragement and help. I would also like to thank the BBSRC and University of Nottingham for funding.

I would like to thank Susanne Kruse, who developed and synthesised the (*E*)-vinylphosphonate dimers, which I used in my work. I would also like to thank Anna Grabowska, for providing me with the MDA-MB 231 and fluc cell lines used for this study and Professor Neil Oldham for his help with obtaining MS data for ASOs I made.

During this four years of postgraduate studies I was lucky to be member of two labs which allowed me to meet a huge number of people. I would like to thank everyone for their help and friendship. They not only supported me, but also made this experience intresting and fun. Special thanks to Ami, who always believed that I can do it and who was not only a lab collegue, but a very good friend.

Apart from the people I have been working with, the biggest support I achived from my parents and brother. Barbara, Jerzy and Marcin I could never have done it without you and I am very thankful for your encouragement and support, it means a lot to me! And last but not least, John thank you for going through our PhD's together, maybe it was not always easy but it was an increadible experience. Thank you!

Pracę dedykuję moim rodzicom, i bratu,

którzy zawsze we mnie wierzyli i mocno mnie wspierali.

Abbreviations

Ab	Antibody
Ago	Argonaute
ASO	Antisense oligonucleotide
Bz	Benzoyl
CDK12 (CrkRS)	Cyclin dependent kinase 12
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
CUG-BP1	CUG-binding protein
DNA	Deoxyribonucleic acid
DM1	Myotonic Dystrophy type 1
DMD	Duchenne muscular dystrophy
DMPK	Dystrophia Myotonica Protein Kinase
DMTr	4,4′-Dimethoxytrityl
FDA	Food and Drug Administration (United States)
h	Hour(s)
HPF	HiPerFect
KD	Knock-down
LNA	Locked nucleic acid
MBNL1	Muscleblind-like
Me	Methyl
min	Minute(s)
MOE	Methoxyethyl
mRNA	Messenger ribonucleic acid
ORF	Open reading frame
PAZ	Piwi-Argonaute-Zwille
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine

PLB	Passive Lysis Buffer
PNA	Peptide nucleic acid
Pr	Propyl
RFU	Relative fluorescence units
RISC	Ribonucleic acid-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
rt	Room temperature
dsRNA(s)	Double-stranded ribonucleic acid(s)
siRNA(s)	Short-interfering ribonucleic acid(s)
SSO	Splice-switching oligonucleotides
ssRNA(s)	Single-stranded ribonucleic acid(s)
TBDMS	t-Butyldimethylsilyl
Tm	Melting temperature
UTR	Untranslated region

Abstract

Work presented in this thesis shows how chemical modifications of ssRNAs can increase their nuclease resistance, resulting in potent gene inhibition. The first part of thesis describes how a range of chemical modifications were introduced into the ssRNAs to test their effect on oligonucleotide stability and ability to inhibit gene expression. The ssRNAs were tested in a cancer cell line that stably expresses firefly luciferase. This work demonstrates that DNA nucleotides can be incorporated into ssRNA sequences without loss of potency in the RNAi mechanism. By using additional chemical modifications (2'-OMe and 2'-F) further improvement of ssRNA stability and a significant inhibition of firefly luciferase activity, reaching 91%, was achieved. Moreover, we show that the level of inhibition can be improved when the DNA dinucleotide linked by the *(E)*-vinylphosphonate is used as a protection of the 5'-end of the ASO (95% KD). The use of ssRNAs rather than dsRNAs is beneficial, since the possibility of off-target effects caused by the remaining sense strand is eliminated and the cost of production of such a medicine is reduced.

The second part of this thesis focuses on development of an experimental system that will allow detection of the inhibition of a disease relevant target. Several approaches were used in order to detect KD of the target by the unmodified dsRNAs. First a western blotting technique was employed for detection. Although different ASOs and concentrations were used no KD was observed. More attempts were taken in order to generate target KD, but unfortunately they were unsuccessful so far.

Table of content

1 Introduction

1.1 Therapeutic agents

The aim of medicine development is to find a molecule that is not only therapeutically effective but is also selective, does not cause off-target effects and can be produced at reasonable cost. Much has changed in the pharmaceutical industry from its early days in which research was focused on small molecule development. More recently alternative strategies have been investigated and introduced, including antibody and antisense therapeutics.

Traditionally, disease has been treated by using small molecule drugs that target proteins such as enzymes and receptors.[1] Small molecule development is a time consuming procedure and involves screening of hundreds of thousands of compounds before the best ones can be tested in clinical trials and released to the market. When designing traditional drugs, protein structure has to be carefully analysed so the potential binding sites can be found.[1] The structure of the protein depends on the unique sequence of amino acids, information that is encoded by deoxynucleic acid (DNA). Transcription of DNA results in the production of messenger RNA (mRNA) that is read during translation to produce a polypeptide chain and create the desired protein. Completed in 2003,[2-4] the Human Genome Project produced the first complete sequences of individual human genomes and provided the possibility of better understanding the genome and proteome. Even with the benefit of this knowledge, it is still very complicated to predict secondary and tertiary structures of proteins, and as a result, it is difficult to design small drug molecules that will interact with them. Therefore, alternative approaches to small molecule drugs were sought and two

of them, therapeutic antibodies and antisense therapeutics, are described in the following sections.

1.1.1 Therapeutic antibodies

Monoclonal antibody therapy is a form of therapy where appropriately designed monoclonal antibodies bind specifically to certain cells or proteins, to block function or to stimulate a patient's immune system to attack those cells.[5] An efficient procedure for the production of monoclonal antibodies was first described by Kohler and Milstein[6] in 1975 and the first therapeutic monoclonal antibody, to be used in the treatment of acute transplant rejection, Orthoclone OKT3, was approved by the American Food and Drug Administration (FDA) in 1986. The success of Orthoclone OKT3 was followed by the development and production of more than 20 molecules that are used in the clinic, mostly for cancer and immune disorders.[7] Although it has been widely reported that antibody therapeutics are ideal candidates for therapy, it has become apparent that their production and use as therapeutics faces serious problems. Their limitations include: high production costs, the pharmacokinetics of action, tissue penetration and mode of action. Chames *et al.* outlined the main problems in their publication.[7] To overcome these problems, the next generation of antibodies were developed and many are still being optimised to provide better results for use as drugs. The therapeutic antibodies are not a subject of this project and therefore more information about the mechanism, development, advantages and disadvantages can be found in following reviews.[7,8]

1.1.2 Antisense technology

An alternative method to target a protein is via the nucleic acid that codes for the protein, and this is where the antisense therapy comes to the fore. Antisense oligonucleotides (ASOs) are synthetic strands of nucleic acids, which are complementary to the mRNA produced by the target gene. Binding of the antisense oligonucleotide to the mRNA results in its blocking or cleavage, and as a result, silencing of the target (Figure 1.1).[9]

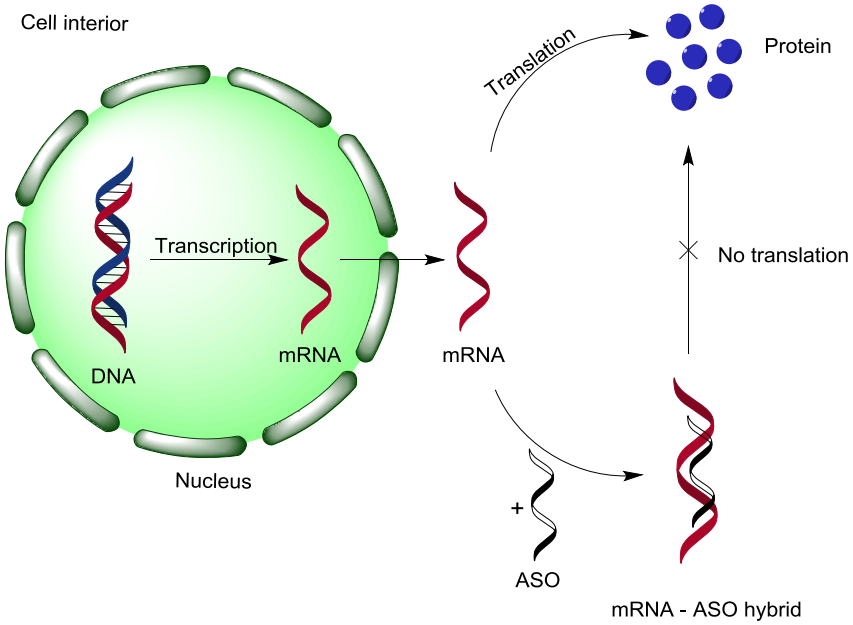


Figure 1.1 General mechanism of antisense oligonucleotide action. DNA that encodes the genetic information is stored in the cell nucleus. The information is transcribed from the DNA into mRNA, which is processed and exported from the nucleus to the cytoplasm where it is translated into protein (upper arrow). However, when an antisense oligonucleotide (ASO) complementary to the mRNA is present it binds to its target resulting in translation suppression.

Research into antisense drugs has been conducted for nearly 50 years. The first antisense oligonucleotides targeting defined gene sequences were synthesised in 1967.[10] In 1977 Paterson *et al.*[11] demonstrated that the synthetic ASO could inhibit mRNA translation in a cell-free system. The following year, Zamecnik *et al.*[12] designed a 13-mer ASO, which targeted the 3' terminal sequence of the *Rous sarcoma* virus and inhibited viral replication in an *in vitro* system. As with

every new technology, antisense oligonucleotides had their drawbacks and limitations. There were two aspects of ASO development that had to be addressed before the biological aspect could be fully investigated. First, oligonucleotides had to be made easily in a fast and automatic way. Secondly, due to the DNA and RNA instability to nucleases, chemical modifications of ASOs had to be developed. For that reason there were few publications regarding antisense oligonucleotides between 1970 and 1990, and those published were focused mostly on chemical development of ASOs.[13,14] Nevertheless, successful development of ASO chemistry in mid 1990s resulted in an increase in publications describing their biological applications. Hopes associated with therapeutic use of ASO were strengthened with the discovery of microRNAs (miRNAs). miRNAs were first described in *Caenorhabditis elegans* by Lee *et al*.[15] in 1993. However, it was in 2000 that their importance was fully recognised when miRNAs were reported in mammalian systems. miRNAs regulate gene expression by binding to the 3'-UTR of an mRNA target and directing the target for degradation or translational repression. They are involved in wide range of biological processes, including cell cycle control, apoptosis and developmental and physiological processes.[16-19]

ASOs have many potential advantages over traditional drugs such as lower toxicity, higher selectivity and ease of design. In the case of designing oligonucleotides, simple base-pair rules may be applied when a sequence of a drug target is known. In contrast to standard drugs, there is also no need for high-throughput screening or molecular modelling. Oligonucleotides can be produced faster and they have the potential to work with higher affinity,[14] selectivity and lower toxicity than conventional drugs. However, what seems to

be a big advantage of ASO's easy design, may also be seen as a drawback. Although ASOs target specific sequences it has to be remembered that due to the post-transcriptional modifications and alternative splicing more than one protein isoform can be present. Therefore if a researcher's aim is to inhibit one specific isoform, it may be a challenge, due to the large homology with others. For that reason it should be confirmed that the selectivity is retained and levels of all isoforms should be checked when the experiment is performed.

Another example of problems with ASO design is allele-selective inhibition. For instance, Huntingdon disease is an incurable neurological disorder,[20,21] caused by dominant heterozygous expansion of CAG trinucleotide repeats within the protein-encoding region of the huntingtin (HTT) gene. The disease is dominantly inherited with patients carrying both mutant- and wild-type alleles. It has been shown that reduction of the wild-type HTT allele may be detrimental.[22,23] Designing ASO that will selectively bind only to the mutant-allele is very challenging, but there have been attempts to do this and some of the results are very promising.[24-26]

ASO's versatility has resulted in an increased amount of research to develop antisense therapies, not only for genetic disorders (e.g. Huntington Disease, Duchenne Muscular Dystrophy, Myotonic Dystrophy) but also for cancers, diabetes, asthma etc.[9] Thus far, two antisense drugs have been approved by the FDA: Fomivirsen and Mipomersen. Fomivirsen was the first commercially available antisense oligonucleotide, approved in 1998 under the trade name Vitravene. It is a 21 nucleotide, phosphorothioate modified DNA oligonucleotide, which is used as a treatment for *cytomegalovirus retinitis* in immunocompromised patients. The oligonucleotide blocks translation of viral

mRNA by binding to the complementary sequence of human CMV immediate-early 2 (IE2) mRNA.[27] Although Vitravene was the first commercial antisense oligonucleotide it has been discontinued after ten years in the market, mostly due to its unpleasant delivery method (intraocular injection) and to insufficient numbers of recipients, making production unprofitable. Mipomersen, known under the trade name as Kynamro, was approved by FDA in 2013 and is a second generation, gapmer antisense oligonucleotide.[28] Kynamro is a cholesterol-reducing drug that targets apolipoprotein B (apoB100) RNA resulting in reduction levels of its protein.[29,30] The oligonucleotide consist of ten central DNA nucleotides "gap" and five 2'-Methoxyethyl (MOE) modified nucleotide "wings" on the 5' and 3' ends, it also has a fully sulphurised backbone.[29] This specific structure allows the ASO to work through the RNase H mechanism, which results in cleavage of the mRNA target. The drug is typically administrated subcutaneously once a week and is used in patients with Homozygous Familial Hypercholesterolemia (HoFH), which is a rare genetic condition.[31] Both Fomivirsen and Kynamro were developed by ISIS pharmaceuticals (currently Ionis pharmaceuticals), who are a leading company in the oligonucleotide filed with 31 drug candidates in clinical trials. Many more antisense oligonucleotides are in advanced clinical trials,[32-34] and some of Ionis' competitors include Alnylam, Prosensa (Biomarin), Sarepta Therapeutics, Sirnaomics etc. The big commercial interest in antisense oligonucleotide development shows that they are a promising potential alternative to other available therapeutics. The fact that two drugs have already been made available at the market shows that it is possible to produce an effective antisense

oligonucleotide. However more has to be done to overcome some issues related with their delivery, stability, pharmacokinetics etc.

1.2 Mechanisms of gene suppression by antisense oligonucleotides

Antisense oligonucleotides may prevent protein synthesis by binding to the mRNA and acting as steric blockers or by cleaving the template mRNA. Therefore oligonucleotides can be categorised by the outcome they have on the target, which includes:

- antisense oligonucleotides that do not cleave the target (steric blocking and splice-switching oligonucleotides),
- antisense oligonucleotides that cleave the target (RNase H dependent mechanism and RNA interference).

1.2.1 Antisense oligonucleotides that do not cleave the target

1.2.1.1 Steric blocking mechanism

In the steric blocking mechanism antisense oligonucleotides bind to the target mRNA without causing its degradation. Usually, the oligonucleotides are chemically modified to improve their affinity to the mRNA. For the target choice, most commonly the antisense oligonucleotide binds to regions close to the AUG start codon of mRNA. When the oligo is bound to the mRNA at this position it provides steric hindrance, and as a result the ribosome cannot translate the information coded by the mRNA (Figure 1.2).

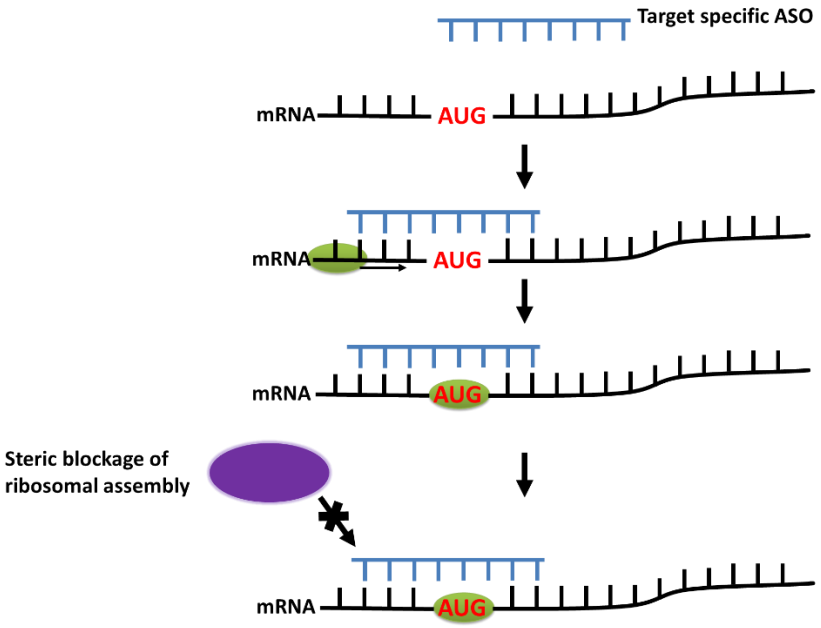


Figure 1.2. Steric blocking mechanism. Antisense oligonucleotide binds to the targeted mRNA close to the AUG start codon and small ribosomal subunit (green). This prevents the recruitment of the large ribosomal subunit (purple) and as a result blocks the translation of mRNA into protein.

It has been shown that with this type of inhibition, the best knock-down (KD) values can be achieved by targeting the 5'-untranslated region (5'-UTR) of a sequence.[35,36] In a study to evaluate ASO design, Doyle *et al.* analysed 27 PNAs (peptide nucleic acids, Figure 1.26) targeting locations along the luciferase mRNA sequence (5'-UTR, start site and coding regions).[35] PNAs designed to bind only within the 5'-UTR region showed good inhibitory activities whereas those complementary to other sites did not block gene expression.

1.2.1.2 Splice-switching oligonucleotides

Another type of antisense oligonucleotide that acts via steric hindrance, but does not block translation is exemplified by oligonucleotides that bind to the pre-messenger RNA (pre-mRNA) to interfere with the alternative splicing. Splicing is a modification or processing of the pre-mRNA transcript in which introns are removed and exons are joined.[37,38] Sometimes during splicing, exons can be extended or skipped (included or excluded from the mature transcript),

or introns may be retained. This event is called alternative splicing.[39] As a result of alternative splicing, more than one mRNA molecule can be produced from one gene, which results in protein variability. By using splice-switching oligonucleotides (SSO) pre-mRNA splicing can be altered or redirected and as an outcome, a novel splice variant is produced. A great amount of the research on the SSOs has been devoted to Duchenne muscular dystrophy (DMD), which affects 1 in 3500 boys. DMD is caused by a mutation in the dystrophin gene that codes for the protein dystrophin.[40] The dystrophin protein is crucial for sarcolemmal integrity.[41] Patients that are affected by DMD have multiple deletions of exons resulting in frameshifts and leading to premature termination and lack of synthesis of dystrophin. This then causes muscle degradation and as a consequence premature death due to the respiratory or cardiac failures.[42] Interestingly in the milder form of the disease known as Becker's muscular dystrophy, the same gene is affected, but despite the deletions the reading frame is preserved and the internally truncated, but partially functional, dystrophin protein is produced.[43] In the case of DMD, the use of an SSO allows restoration of the open reading frame by skipping mutation containing exons to restore the protein reading frame.[44] That allows the production of internally truncated but mostly functional dystrophin protein similar to observed in the Becker's muscular dystrophy, leading to milder symptoms in affected boys (Figure 1.3).

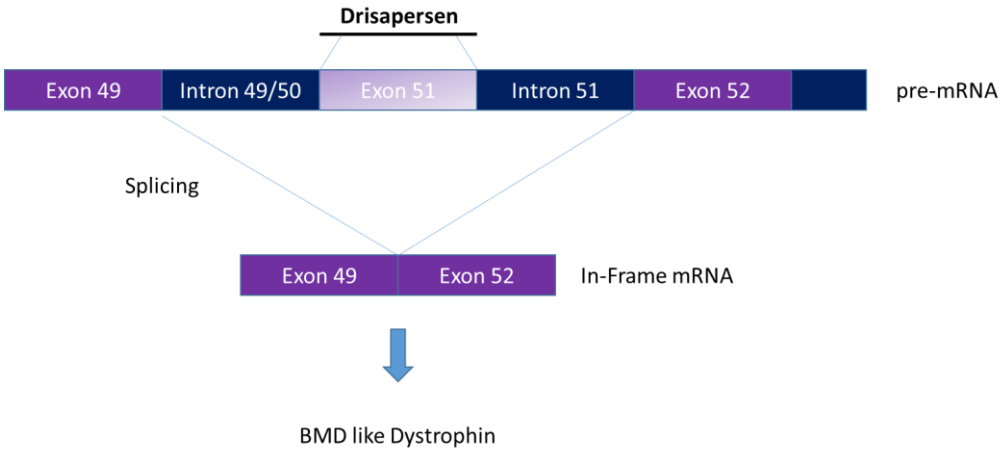


Figure 1.3 Splice switching oligonucleotide action. Drisapersen binds to exon 51 in pre-mRNA leading to an in frame mRNA transcript that produces shorter but functional protein like the one in Becker's muscular dystrophy.

There are two competing drugs, targeting exon 51, in development for DMD. Drisapersen, a 2'-OMe modified ribose oligonucleotide with phosphorothioate bonds, designed by Prosensa (currently BioMarin) and Eteplirsen, a morpholino antisense oligonucleotide, developed by Sarepta, both currently in clinical trials awaiting FDA's approval.

1.2.2 Antisense oligonucleotides that cleave the target

1.2.2.1 RNase H dependent mechanism

ASOs can also stimulate mRNA cleavage by another mechanism, that is by activating RNase H (Figure 1.4).[45] Mammalian RNase H1 is present in nuclei and mitochondria[46] and it is an endonuclease that hydrolyses RNA in RNA/DNA hybrids.[47] The crystal structure of catalytic domain mutant human RNase H complexed with an RNA/DNA hybrid substrate was reported by Nowotny *et al*.[48] As shown in Figure 1.5 the active site of human RNase H consist of the amino acids Asp145, Glu186, Asp 210 and Asp274. Nowotny *et al*. have shown that the RNA strand of the complex is recognised by the protein through its interaction with the 2'-OH group, and catalytic action occurs via a two-metal

ion mechanism.[48-50] In the presence of Mg^{2+} cations, RNase H causes hydrolysis of an internucleotide phosphate linkage in the RNA part of the hybrid to produce 3'-hydroxyl and 5'-phosphate terminated products (Figure 1.5c). As a result, the mRNA levels significantly decrease when compared to the initial state.

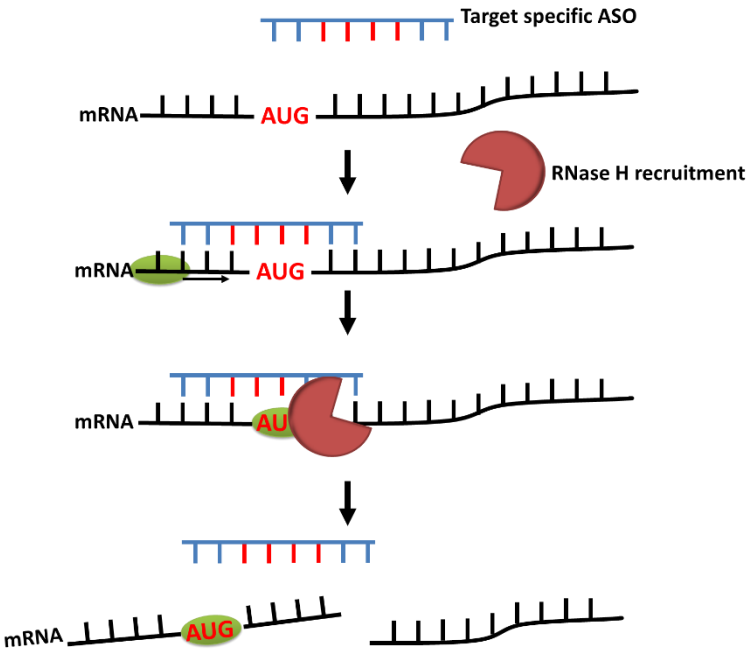


Figure 1.4. Gene silencing by activating RNase H endonuclease results in mRNA cleavage.

A specified structure of oligonucleotide has to be provided to activate the RNase H which involves 10 centrally located DNA nucleotides.[51] In most cases, the oligo backbones are fully sulphurised, which significantly increases nuclease resistance, still allowing RNase H mediated cleavage, but unfortunately it reduces binding capacity to complementary mRNA and increases the risk of non-specific binding to proteins which may cause toxic side effects. Therefore the DNA is surrounded by 5-6 chemically modified nucleotides which reduces negative effects of phosphorothioate backbones and additionally improve exonuclease resistance.[51] The modified tail might additionally improve binding

to the target mRNA.[51] Because of the design and the central DNA gap this type of ASOs is called a gapmer.

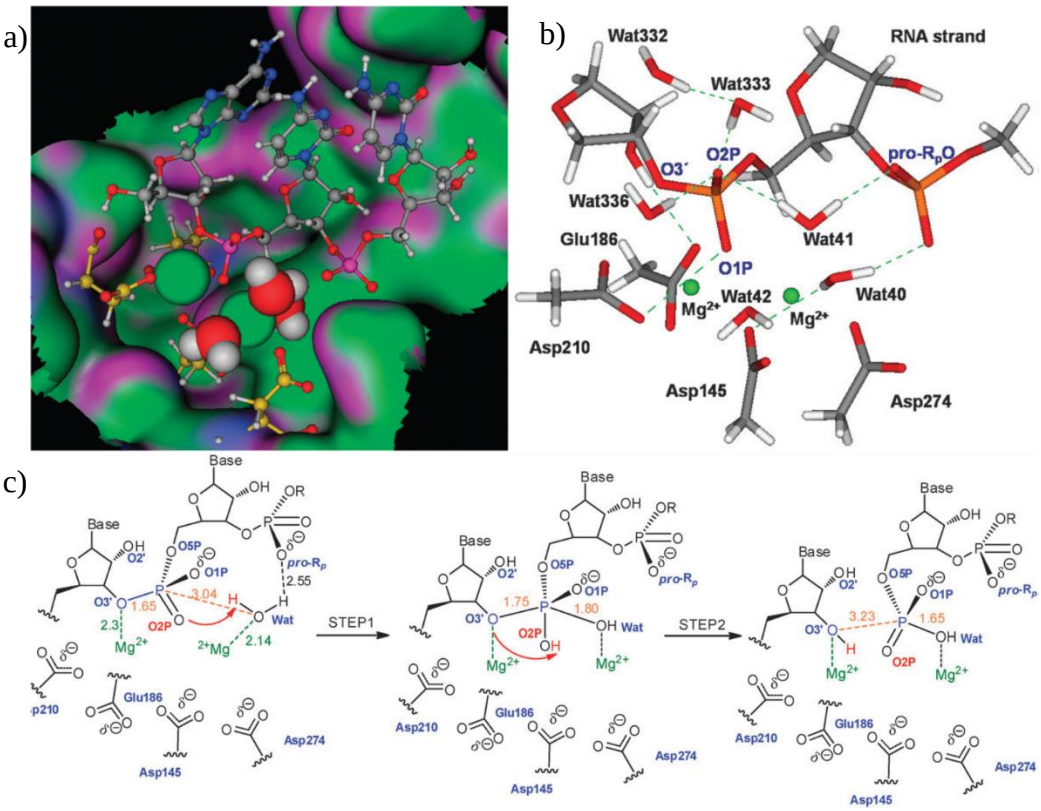


Figure 1.5. Catalytic pocket of human RNase H. a) Surface of the catalytic pocket showing the RNA strand of the RNA/DNA hybrid complex and the active site residues (balls and sticks). The magnesium ions (green) and the water residues are represented as space filling objects. b) Close up view of the catalytic pocket. Magnesium ions shown in green, protein residues, essential waters and RNA are represented as sticks. Dotted lines represent hydrogen bonds. c) Proposed reaction pathways for the O3'-P cleavage of human RNase H. Pictures taken from B. Elsässer *et al*.[52] Protein data bank code 2QKK.

In contrast to ASOs that work through steric blocking and usually target the 5' or the AUG codon region, gapmer oligonucleotides can be designed to target any area in the coding region, however differences in effectiveness against target inhibition may be observed.[53]

The use of gapmers for the potential treatment of DM1 has been reported in 2012 by Lee *et al.*, who used the 2'-MOE and LNA modified molecules to target CUG repeats.[54] An effective KD of mutant transcripts in both cell culture and DM1 mice (EpA960/HSA-Cre) was shown.[54] The gapmers were able to target

selectively and reduce levels of expanded CUG transcripts and as a result disrupt the toxic foci (more about foci in 1.3.1). The same year Wheeler *et al.* tested a 2'-MOE modified gapmer with a central 10 nucleotide DNA gap in a mouse model of Myotonic dystrophy (HSA^{LR}) with an expanded CTG repeat in the 3'-UTR of human skeletal actin (h*ACTA1*) transgene.[55] Three oligonucleotides were tested, of which two targeting the 3'-UTR were active and one targeting the 5'-UTR was not. After 4 weeks of subcutaneous injection, the most effective ASO (445236) inhibited the number of expanded repeat by 80% in the hindlimb muscle. The alternative splicing of exons was also tested (Serca1, Ttn, Zasp and Clcn1) and an improvement was observed. Additionally correction of myotonia was seen. Interestingly, the effect of treatment was prolonged and when the animals were examined again after 15 and 31 weeks after the last ASO injection, the mRNA levels were still low and splicing correction was evident as well. Even 1 year after the last gapmer dosage, the target was reduced by around 50% for the best working ASO 445236.

Human RNase H1 is located mostly in the nucleus and only a little presence in cytoplasm is observed.[56] The use of gapmer oligonucleotides to treat the Myotonic Dystrophy 1 seems to be a good choice due to the fact that the toxic repeats in DM1 are stuck in nucleus and therefore are a good target for RNase H cleavage.[55]

1.2.2.2 RNA interference

The discovery of small noncoding RNAs that regulate genes has been transformational for biology. There are three classes of small RNAs:

short-interfering RNAs, microRNAs (miRNAs) and piwi-interacting RNAs[57] (piRNAs). siRNAs have been used extensively and are the focus of this review.

In 1998 Fire and Mello showed that by introducing RNA into *Caenorhabditis elegans* the expression of the chosen gene can be manipulated.[58] Both double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) (antisense or sense strand) were tested, but only dsRNAs were able to silence expression of the targeted gene.[58] The discovery that siRNA can degrade a target mRNA seemed to be a very appealing alternative to the steric blocking and the RNase H dependent mechanisms resulting in the increased amount of research and publications focused on RNAi. Both RNase H and RNAi cause strand cleavage, however in contrast to gapmers the siRNA molecules do not need any chemical modifications to stabilise them or to increase binding affinity, making the design and synthesis easier. Moreover siRNAs are used catalytically, meaning one molecule can be reused by the silencing machinery and cleave more than one target, therefore low concentration of drug can be used. The first use of siRNA in cultured mammalian cells was shown by Elbashir *et al.* in 2001.[59] Only one year later the first successful use of siRNA *in vivo* in mice was published.[60] The importance of RNAi is emphasised by fact that in 2006, eight years after their discovery, Fire and Mello were awarded the Nobel Prize for their research on the discovery of RNA interference.

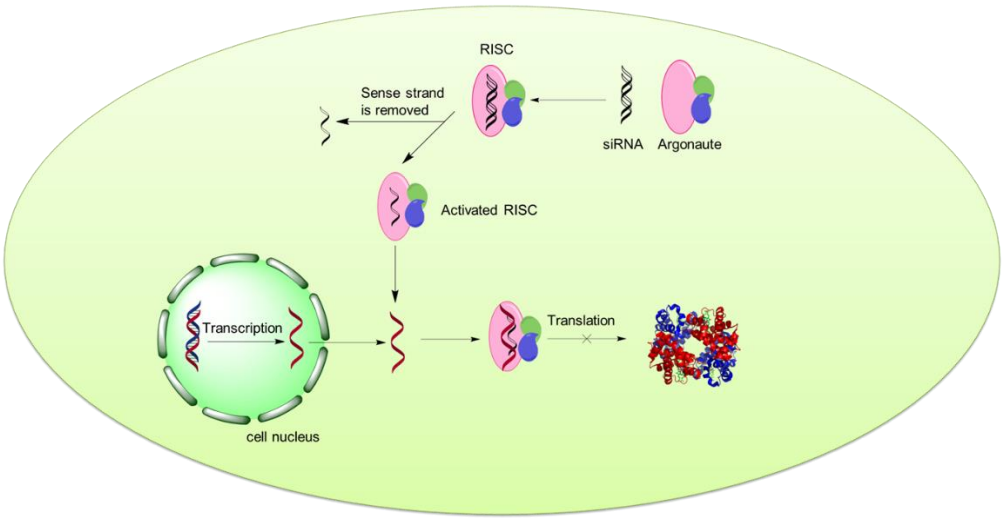


Figure 1.6. Gene silencing by the RNAi mechanism within the cell. The antisense strand from siRNA duplex is selected by the Argonaute protein and incorporated into the RISC complex. The activated RISC with bound ssRNA recognises mRNA, targets and cleaves them, resulting in efficient gene inhibition.

In nature RNA interference serves to protect the genome from invasion by transposons and viruses that produce abnormal RNA or dsRNA when they are active in the host cell.[61,62] RNAi is a mechanism (Figure 1.6) in which the dsRNA effectively inhibits gene expression by removing template mRNA. When long, dsRNAs enter the cytoplasm they are cleaved by the endonuclease Dicer, an RNase III family enzyme, into short-interfering dsRNAs (siRNAs), usually 21-25 nucleotides in length. siRNAs that are naturally processed by Dicer have a specific structure (Figure 1.7) including the presence of a 5'-phosphate, a 3'-hydroxyl group and two nucleotide 3' overhang.[63,64] This unique structure determines that siRNAs are recognised by the Argonaute protein and all these features have to be considered when synthetic dsRNAs are designed.

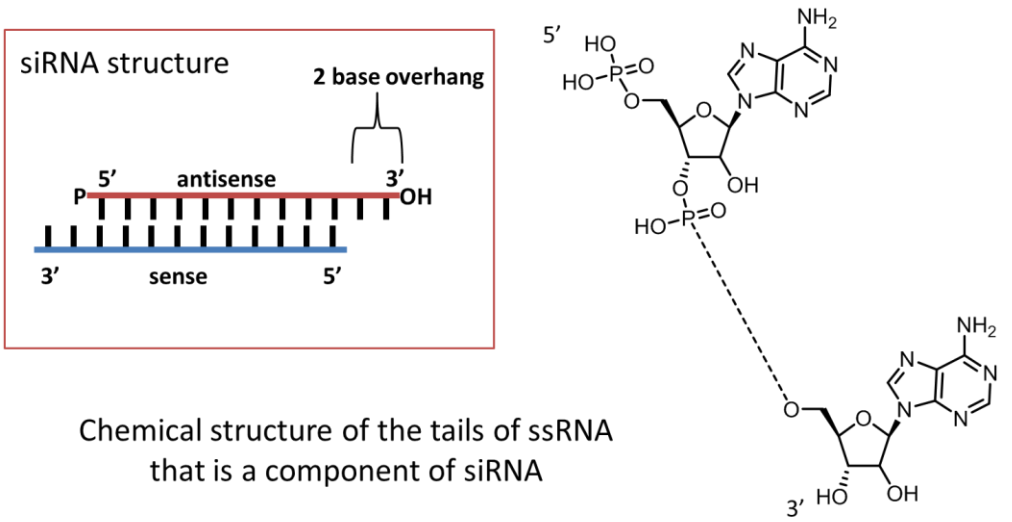


Figure 1.7. Standard dsRNA design. As shown above in the red box the siRNA that was processed by Dicer has a 2 base overhang on the 3'-end. On the right side the schematic structure of the strand is presented. The phosphate group is present at the 5'-end and the hydroxyl groups are present at the 3'-end.

The dsRNAs processed by Dicer are incorporated into a protein complex, which consist of Argonaute (Ago) and its binding partners (e.g. Pumilio, importin 8, UPF1),[65] to create the RNA-induced silencing complex (RISC). The strand whose 5'-end is less tightly bound to its complement is selected[66] and loaded into the active RISC (RISC*) when the remaining strand is degraded. The active RISC* with bound single-stranded RNA recognises complementary mRNAs and cleaves them, opposite the 10th and 11th nucleotide from the 5'-end of the antisense strand,[67] which subsequently results in significantly decreased levels of protein translation.[45,68]

The family of Argonaute proteins is identified by the presence of PAZ (Piwi-Argonaute-Zwille) and PIWI domains and was first discovered in plants.[69] The crystal structure of human Ago was published in 2012 (Figure 1.8).[70]

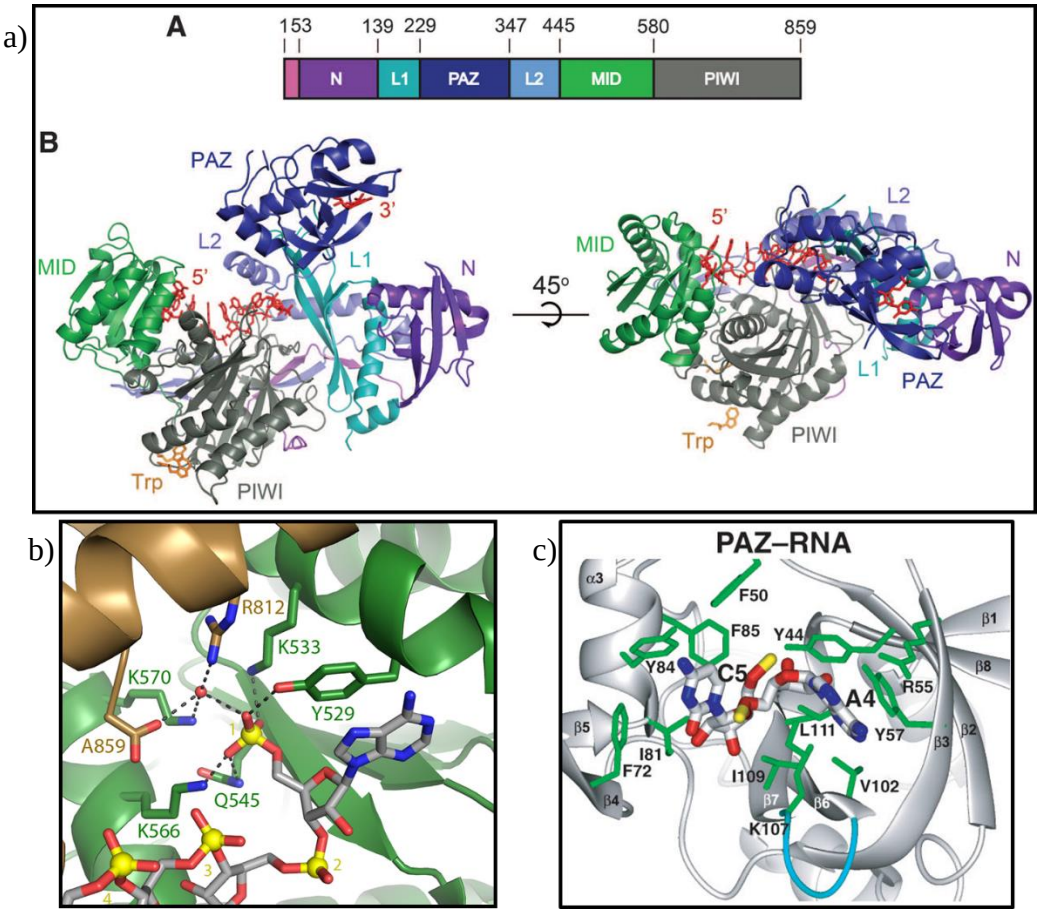


Figure 1.8 Structure of human Argonaute 2. a) Scheme of primary sequence (A) and front and top views (B) of Ago2. b) Conformation of 5'-phosphate of an antisense strand bound to MID and PIWI domains of human Ago2. An ordered water molecule is shown as a pink sphere. Hydrogen bonds are shown as dashed orange lines. R – arginine, K – lysine, Y – tyrosine, A –alanine, N – asparagine. Pictures taken from T. Schirle publication.[70] Coordinates of Ago2 and Ago2 bound tryptophan have been deposited in the Protein Data Bank (4EI1 and 4EI3). c) Details of two terminal 3' nucleotides of the RNA recognition by PAZ domain. Side chains of amino acid involved in nucleic acid recognition are green and labelled. Picture taken from A. Lingel publication.[71] Protein Data Bank accession number 1T2R.

Argonaute is a bilobed molecule composed of four globular domains: N-terminal (amino terminus), PAZ, Mid and PIWI connected through two linker domains (L2 and L2).[70,72] A PIWI domain is located at the amino terminus, a Mid domain and a PAZ domain are located at the C-terminus (carboxyl terminus),[71,73-75] to form a binding surface for the antisense strand of the duplex. As shown in Figure 1.8b the Mid and PIWI domains bind specifically to the 5' terminal phosphate and the backbone at the 5' pole of the antisense strand.[75] The two terminal nucleotides on the 3' end were shown to bind to the PAZ domain (Figure 1.8c).[71,76] An essential feature of Argonaute is its slicer activity. The

crystal structure of the Argonaute protein from *Pyrococcus furiosus* showed that the PIWI domain adopts a tertiary structure like the RNase H enzymes (accession number 1U04 in Protein Data Bank).[72] This RNase H like PIWI domain, results in the slicer activity of the Argonaute protein and its ability to cleave target RNA by the RISC complex.[72] In mammals, from the family of four Argonaute genes, only the Argonaute 2 (Ago2) has catalytic character and functions as an endonuclease.[75,77] Similar to the RNase H dependent mechanism, the products of RISC cleavage have 5'-phosphates and 3'-hydroxyl groups and the mechanism is dependent on the presence of Mg^{2+} cations (red ball in Figure 1.8b).[78,79]

Design of ssRNAs that activate RNAi

Although siRNAs are double-stranded structures it has been shown that once bound with the human Ago2 complex the sense strand is removed and the active RNA-induced silencing complex contains only the antisense strand.[80,81] At the current stage of development the use of siRNAs is limited by the requirement of simultaneous use of the lipid formulations to deliver siRNA to tissues.[82] Lipid delivery reagents for antisense oligonucleotides are expensive to manufacture and have been shown to be toxic.[83] Synthetic ssRNAs would be preferable to dsRNAs because of the reduced possibility of off-target effects caused by the remaining sense strand, reduced cost of production, and potentially easier delivery that does not involve the use of transfection reagent.[84] ssRNAs have been used to reduce complementary mRNA *in vitro*, however their efficacy was poor when compared to dsRNAs.[85-87] Low activity of ssRNAs most likely results from their metabolic instability.[88,87] Incorporation of chemical modifications to ssRNAs is crucial for making them nuclease resistant. However, the problem is

how to design a single-stranded RNA that is not only stable to nuclease degradation, but can also trigger RNAi. Not much research has been done to investigate what type of chemical modifications could increase ssRNA performance. In the early years of single- and double-stranded RNA development, Holen *et al.* showed that by using ssRNA with 2'-O-methylated nucleotides in Hela cells, the IC_{50} value is ~5 fold higher compared to corresponding dsRNA and most probably results from its lower intracellular stability.[89] More has been done on evaluation of chemical modifications in dsRNAs. In 2003 Chiu and colleagues demonstrated that the 2'-OMe (Figure 1.15) and 2'-F (Figure 1.17) modifications can be incorporated at multiple positions of siRNA to increase the stability, specificity and to reduce immunogenicity.[90] That was confirmed in 2005 by Prakash *et al.*, who evaluated the effect of 2'-sugar modifications of siRNAs *in vitro*.[91] The 2'-fluorinated nucleotides were well tolerated, irrespective of the position. More bulky modifications like 2'MOE were allowed, but only at the specific positions of the antisense strand.[91] Similarly the 2'-OMe modification showed a strong influence on siRNA activity depending on the position of modification.[91] The same year, Allerson (2005) demonstrated that by full incorporation of both 2'-F and 2'-OMe modifications into dsRNAs more than 500-fold improvement in potency can be seen when compared to unmodified dsRNAs.[92] All of above showed that commercially available and popular modifications like 2'-OMe, 2'-MOE and 2'-F are well tolerated in siRNAs.[93] For that reason similar patterns were used in ssRNAs, however they never worked as well as the corresponding dsRNAs.[89,94] It took more than 12 years, from when siRNAs were first used in mammalian cells, to see more optimistic reports regarding the use of ssRNAs.

In 2012 Haringsma *et al.* demonstrated that by using 2'-fluoro modification in combination with 5'-end phosphorylation in ssRNA, both *in vitro* and *in vivo* activity is observed.[95] However, the modified ssRNAs were still less potent than dsRNAs, which suggests that in the case of ssRNA, more research has to be done.

What are the challenges of making potent therapeutics from single-stranded RNAs? Two factors should be recognised. First the internal stabilisation of the ssRNA and protection from endonucleases, and second the protection of its tails from degradation by exonucleases (Figure 1.9). At the same time it has to be assured that the ssRNA is capable of activating the RNAi. The above review shows that a lot is known about how to protect the oligo internally, mostly by using 2'-sugar modifications. Maybe the ssRNAs success depends more on the incorporation into the active RISC complex and therefore it is also important to stabilise ssRNA ends, especially the 5'-end that is recognised by the Argonaute domains and activates the silencing mechanism.[74]

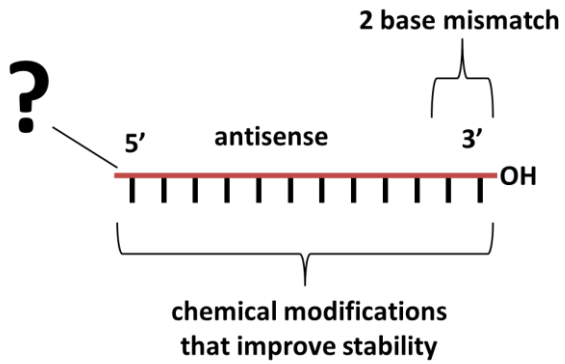


Figure 1.9. Proposed ssRNA design. Protection of the 3' and 5'-ends is necessary. For improvement of internal stability chemical modifications of nucleotides might be required.

As mentioned previously the siRNAs processed by the Dicer have 5' phosphates on their 5'-end[63,64] that bind specifically to the Mid and PIWI domains of the

Argonaute 2.[70] In 2005 Rivas *et al.* showed, that although phosphorylation of the 5' tail of siRNA is not essential, its presence significantly improves siRNA's performance and the gene inhibition is greater (in comparison to the 5'-hydroxyl).[81] It has also been demonstrated that a 5'-phosphate stabilises the Ago2-siRNA complex, and is responsible for accuracy of cleaving the target mRNA (Ago2 measures from the 5'-end of the siRNA to determine the cleavage site within the target).[80,81] Schwarz and colleagues performed experiments in Hela cells and found that siRNAs with terminal 5'-hydroxyl groups are phosphorylated by the cellular kinase and therefore activate the RNAi.[96] In contrast the siRNA with 5'-methoxy group, that could not be phosphorylated, did not cleave the target RNA.[96] The same experiments were performed for ssRNAs with identical results. This data showed that the 5'-phosphate is required to trigger RNAi in mammals and should be present in synthetic ssRNA. The problem, however is, that the synthetic single-stranded RNA needs a terminal 5'-group that is not only functional, but also stable to nucleases. The question is then, what chemical modification could meet these requirements?

In 2012[97] Lima *et al.* evaluated the abilities of chemically modified ssRNAs to bind human Ago2 and assist the cleavage activity. First it was established that introduction of phosphorothioates into the backbone improved the ssRNA's metabolic stability and hence activity. The ssRNA with all internucleoside linkages sulphurised was the most stable, but it was inactive in cells. Nevertheless, Lima established that by positioning seven continuous phosphorothioate backbones, counting from the 3' terminus, the ssRNA had increased stability while still being capable of efficient cleavage of the target.[97] To further protect the 3'-end, two adenosine nucleotides (2'-MOE modified)

were added, as the presence of a two purine overhang was previously reported to improve siRNAs *in vivo* potency.[98,76] A 4-fold enhancement in potency was detected for ssRNA containing two adenosines in comparison with shorter RNA.[97] However, none of the above alterations to the chemical structure of ssRNA have contributed to the enhancement in metabolic half-lives (~30 minutes). Additional experiments demonstrated that nuclease degradation takes place on the 5' part of the ssRNA's sequence, not on the 3' pole, indicating the necessity of incorporating further chemical modifications to improve stability. Lima and colleagues tested the inclusion of different combinations of 2'-F and 2'-OMe modifications into oligo sequences and concluded that by alternating every 2'-OMe with 2'-F gives the best results.[97] The half-lives reached more than 8 hours, and a 2-fold increase in potency was noted (compared to ssRNA that was fully 2' fluorinated) which indicates that active and metabolically stable ssRNA was made. Consistent with previous reports[96] Lima confirmed that presence of the 5'-phosphate improves ssRNA activity *in vitro*. Experiments performed in mice revealed that 5'-phosphate analogues are needed for ssRNA *in vivo* activity since ssRNAs with phosphorylated 5'-ends were inactive *in vivo* due to their degradation caused by nucleases. A 5'-methylenephosphonate (5'-MP) modification was tested first.[99] In this modification the oxygen atom, adjacent to 5'-carbon, is replaced with a carbon atom resulting in formation of a phosphorus-carbon bond in **2** instead of a phosphorus-oxygen bond in **1** (Figure 1.10), the new bond cannot be cleaved since there are no known enzymes capable of doing this.

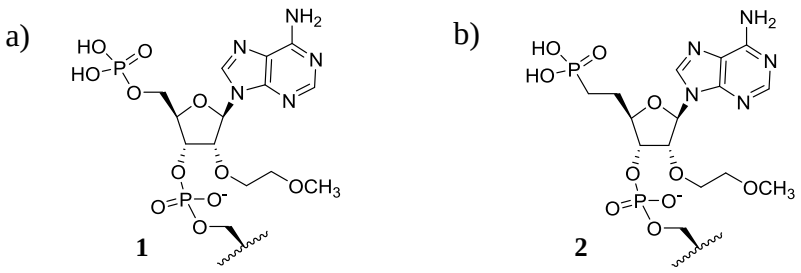
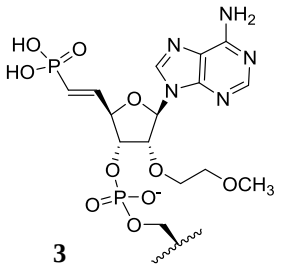


Figure 1.10. The standard 5'-phosphate (a) and the 5'-MP modification (b). The oxygen atom (a) is replaced with the carbon (b) resulting in formation of phosphorus carbon bond instead of phosphorus oxygen bond.

The ssRNAs with 5'-MP possessed greater *in vivo* stability but lower potency than 5'-phosphorylated ssRNAs. The crystal structure of Ago2 shows that the 5'-phosphate interacts with the amino groups of lysines K533 and K566 by the electrostatic and hydrogen-bond interactions (Figure 1.8b).[70] The precise positioning of 5'-P in ssRNA at the binding pocket is very important. When other analogues of the methylenephosphonate were tested, they did not improve ssRNAs effectivity, therefore conformational and stereo-electronic differences between them impact activity of ssRNA.[97] The extended screen of methylenephosphonate analogues highlighted the 5'-(*E*)-vinylphosphonate (5'-VP) as the most effective (Figure 1.11). In comparison to the 5'-MP the 5'-VP modification additionally contains a double bond between the 5' and 6' carbons in a trans configuration and, due to its conformation and stereoelectronic properties, better mimics natural phosphate. As a result an ssRNA with the 5'-VP modification is preorganised into the conformation that allows its docking into the Mid and PIWI domains (Figure 1.8b). The ssRNA with 5'-VP was as potent as the ssRNA with a 5'-phosphate and even 7-fold more potent that an ASO with a 5'-MP modification (*in vitro*). Also higher potency *in vivo* was observed compared to 5'-MP. Moreover it was demonstrated that despite all the

modifications in the final ssRNA the Ago2 involvement in target degradation was confirmed and no off-target effects were seen.



3

Figure 1.11. 5'-*(E)*-vinylphosphonate modification.

One of the disadvantages of Lima's work is the synthesis of phosphoramidite with the terminal 5'-(*E*)-vinylphosphonate that is shown in Figure 1.12.

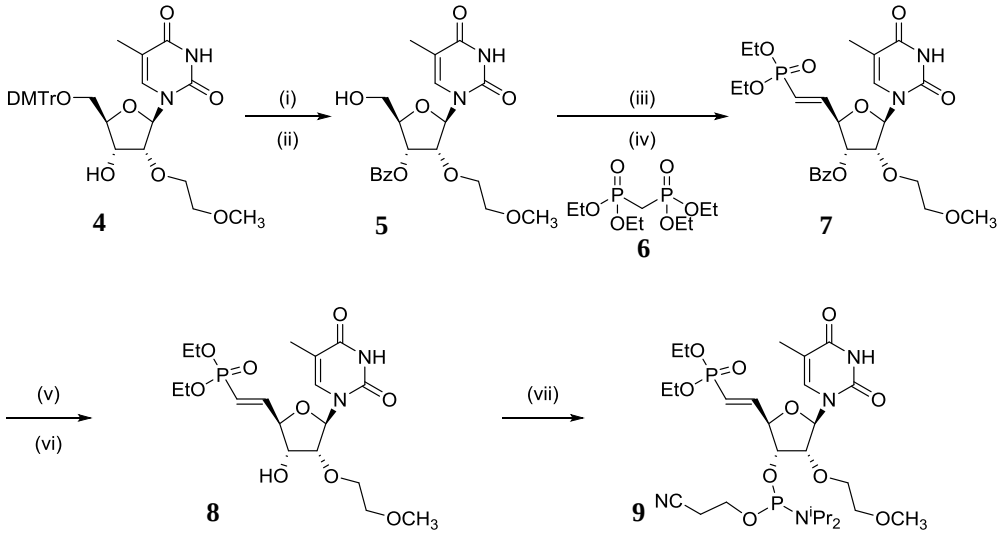


Figure 1.12. Synthesis of phosphoramidite with the terminal 5'-(*E*)-vinylphosphonate proposed my Ionis pharmaceuticals. Reagents: (i) BzCl, Py, 86%; (ii) TFA, CH_2Cl_2, $(C_2H_5)_3SiH$, 97%; (iii) DCC, DMSO, Py-TFA; (iv) potassium-tert-butoxide, THF, 68%; (v) Pd/C, H_2, MeOH, 88%; (vi) NH_3, MeOH, 82%; (vii) Phosphitylation, 76%.

The synthetic route developed by Ionis pharmaceuticals involves 7 steps and is protected by patent rights.[100] Additionally the starting material **4** is not commercially available and another 6-step synthesis is required before the phosphoramidite can be made. However the main drawback of the Ionis synthesis is the composition of the final product.

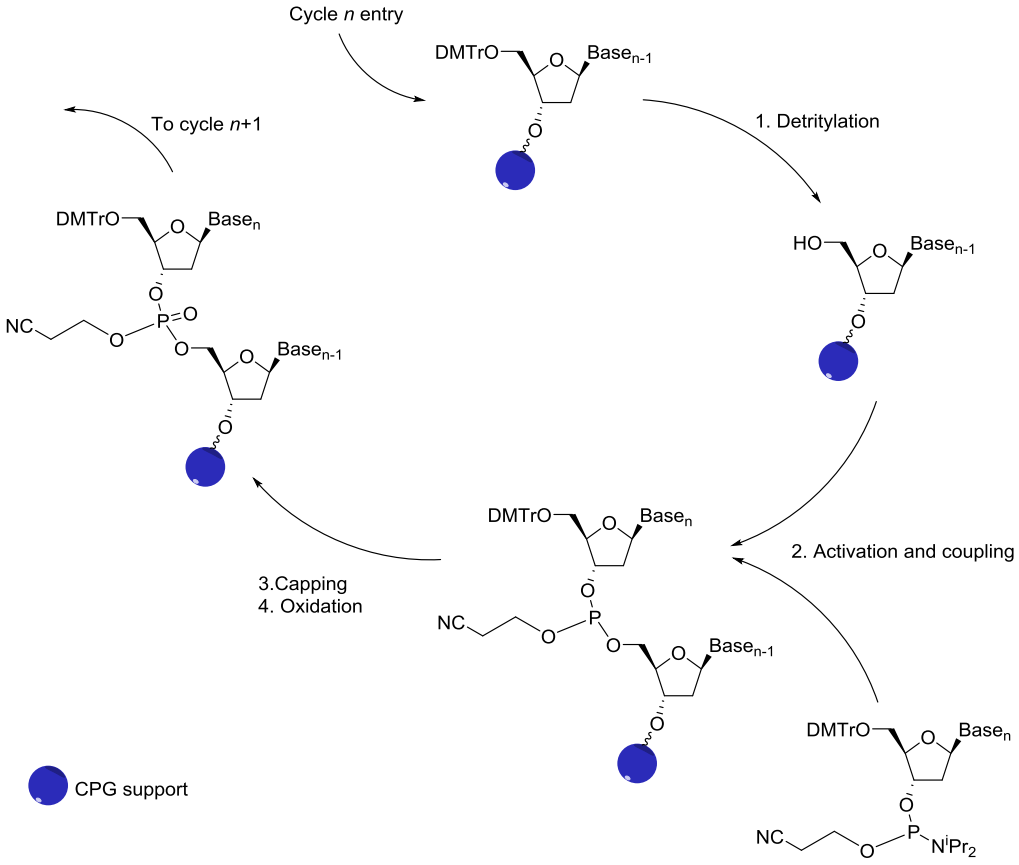


Figure 1.13 Scheme of oligonucleotide synthesis by the phosphoramidite method.

Solid-phase oligonucleotide synthesis by the phosphoramidite method (Figure 1.13) allows for monitoring of the coupling efficiency. When the DMTr group is cleaved from the oligo the resulting cation has an orange colour, which can be collected and the absorbance measured by the UV-Vis spectroscopy to quantify the coupling yield. In the Ionis synthesis, classic DMTr protecting groups were replaced with ethyl groups, so not only the synthesis cannot be monitored, but also a new deprotection protocol had to be developed as the standard would be ineffective.[99] Conventional deprotection conditions, using aqueous ammonia at 55 °C, was not able to hydrolyse the ethyl ester group used as a 5'-phosphate protecting group during ssRNA synthesis. To remove ethyl protecting groups a solid support containing ssRNA was treated with a solution of iodotrimethylsilane in dichloromethane containing pyridine for 30 minutes at

room temperature. To quench the reaction a solution of 50% trimethylamine in acetonitrile containing 2-mercaptoethanol (1M) was used. Finally to remove all protecting groups and cleave oligo from the support, the supernatant was decanted and the solid support bound ssRNA treated with aqueous ammonia (28-30 wt%) containing 2-mercaptoethanol (1M) and heated at 55 °C for 2 hours. The resulting solution was left for 24 hours. The unbound oligonucleotide was next filtered, washed and precipitated. The redissolved product was purified by HPLC. The developed protocol not only makes the synthesis and purification process longer, but also more complicated. The modified approach of removing the ethyl protecting groups, compared to the standard protocol, may affect the presence of other groups resulting in the wrong composition of the final oligonucleotide and necessity of repeating the synthesis.

1.3 Nucleic acid modifications

Unmodified DNA or RNA are unstable in biological systems, primarily due to nuclease activity, which cleaves the phosphodiester linkage. ASOs are usually single strands of chemically modified DNA or RNA, 17 to 25 nucleotides in length, which is based on a statistical evaluation of the length necessary to avoid random matches in the human genome or mRNA population. When oligonucleotides are designed not only stability but other aspects must be considered, for example: high affinity and specificity for the target, ability to affect the desired pharmacology, sufficient pharmacokinetic properties, an acceptable therapeutic index and acceptable manufacturing costs. For this reason many chemical modifications have been developed to improve ASOs *in vitro* and *in vivo* properties.[101-105] Depending on the type and positioning of chemical modification, different outcomes can be achieved (increased stability, higher T_m,

improved pharmacokinetics etc.). Parts of the oligonucleotide that can be modified include:

- nucleobase,
- sugar,
- sugar phosphate backbone,
- backbone modifications ((*E*)-vinylphosphonate modification).

Modifications can be used alone or in combination, and the number of modified nucleotides can vary.[101,102] The following chapter reviews some of the most commonly used chemical modifications and the ones that were used in this project. As nucleobase modifications are not as common as sugar or sugar phosphate backbone modifications, and were not used by us, we decided to exclude them from this review. However, a detailed overview of nucleobases modifications was conducted by Herdewijn in 2000 and can be found in his publication.[106]

1.3.1 Sugar modifications

The conformation of the sugar rings defines the structure of the nucleic acid helix, which is formed when an ASO binds to its target, and can be described using phase angle values, calculated from the dihedral angles of the furanose rings. These phase values can be characterised on a "pseudorotational wheel", that allows the description of the nucleoside sugar "puckering" or conformation. The sugar ring in natural DNA molecules can adopt two different conformations that are known as North (N- type, C3' endo) and South (S-type, C2' endo) (Figure 1.14).[107,108] As a consequence of pseudorotational movement of the sugar ring the secondary structure of DNA helix can adopt A, B or Z form. The

A-helix adopts North sugar conformation and B-helix adopts South helix conformation.

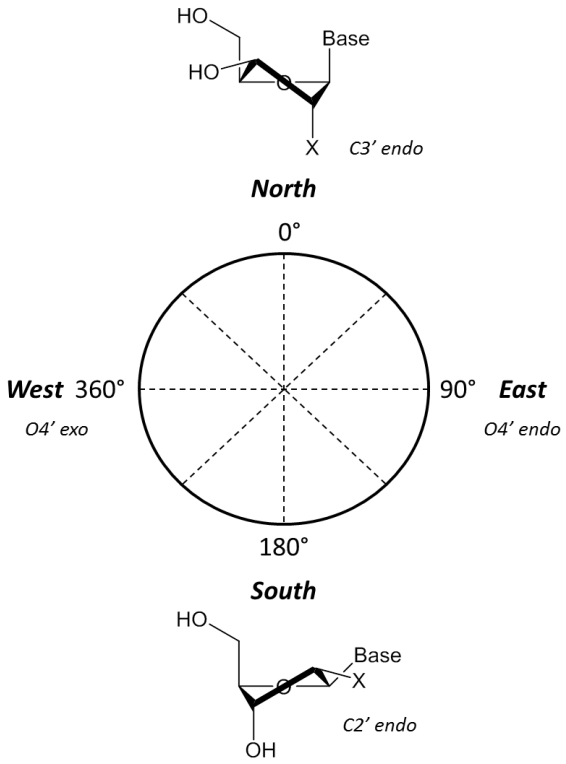


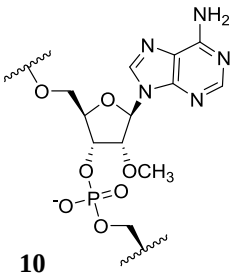
Figure 1.14. The pseudorotation cycle of the furanose ring and preferred conformation of deoxyribose nucleotides. Each point on the circle represents a specific value of pseudorotation angle.

Modifications of the 2'-position of the sugar moiety have provided one of the best ways to enhance ASOs drug properties. Preorganisation, by introduction of chemical modifications at the 2'-position, of the sugar into an RNA like 3'-endo pucker, or Northern conformation in the "pseudorotational wheel" results in increased binding affinity. Moreover, the close distance of the 2'-substituent to the 3'-phosphate enhances nuclease resistance. As a result this single 2'-modification adds two very important properties to an oligonucleotide.

2'-O-Methyl modification (OMe)

One of the most commonly used sugar modifications is the 2'-O-Methyl (2'-OMe) nucleoside (Figure 1.15). 2'-OMe ASOs usually adopt the North

sugar conformation, which makes them similar to RNA, and hence form A-form duplexes. When compared to a DNA strand, incorporation of 2'-OMe units within oligonucleotides increases binding affinity for RNA complements (increased T_m),[109] and increases nuclease stability.[110] Because of this feature 2'-OMe modified ASOs are suitable for employment into siRNA. This modification has also been well studied in the application of gapmers.[111]



10

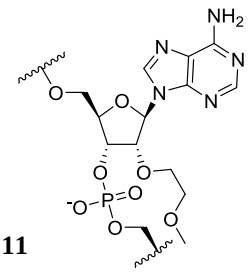
Figure 1.15. 2'-O-Methyl modification (2'-OMe)

As the 2'-OMe modification increases binding affinity of the oligonucleotide it seems reasonable to use it in the steric blocking applications. A type of antisense oligonucleotide that was predicted to work as steric blocker was developed for the treatment of Myotonic Dystrophy type 1 (DM1). DM1 is caused by the abnormal expansion of the CTG trinucleotide repeat in the 3' non-coding region of DM protein kinase (DMPK) gene on chromosome 19q13.3.[112-114] These extended repeats form hairpin-like structures, which accumulate within cell nuclei and deregulate the function of RNA-binding proteins. There are two most comprehensively studied proteins: Muscleblind-like (MBNL1, 2 and 3) proteins which are sequestered by the expanded RNA and together form toxic RNA aggregates[115,116] and CUG-binding protein (CUG-BP1).[117] The reduction of free MBNL1 in the cell interferes with RNA splicing and deregulates normal cellular function.[118,119] The overexpression of CUG-BP1 has been reported in DM1 patients and leads to missplicing and depletion of chloride channel (Clcn1).[120,121]

The abnormal alternative splicing of Clcn1 is one of the hallmarks of DM1 and is associated with myotonia. Mahadevan *et al.* showed in experiments conducted in DM1 mice models (expressing $(CUG)_n$ RNA repeat) that by reducing the level of mutant DMPK transcripts, features of RNA toxicity can be reversed.[122] For this reason, the most obvious of the possible approaches to treat DM1 is to interfere with the cause of the disease: the expanded CUG repeat. Mulders *et al.* described a fully 2'-O-methyl (2'-OMe) phosphorothioate modified ASO, called PS58, complementary to the $(CUG)_n$ repeat.[123] The oligo was expected to bind to the toxic $(CUG)_n$ repeat resulting in release of the MBNL1 protein what should improve the condition as the free MBNL1 could fulfill its usual functions. Interestingly the ASO did not act as steric blocker but significantly reduced levels of mutant transcripts. This data suggest that there is an unknown mechanism, or the result is specific to the particular condition, as it has not been reported previously that the 2'-OMe phosphorothioate modified ASO is capable of degrading RNA transcripts. PS58 was tested in both DM1 cells and mouse models (DM1 myoblasts, DM500 mice and HSA^{LR} mice) with the same result. Additionally in cultured human DM1 myoblasts, PS58 showed allele selectivity between expanded and normal-sized hDMPK transcripts.

2'-Methoxyethyl (MOE)

The 2'-Methoxyethyl (MOE) (Figure 1.16) modification increases ΔT_m by about +2° C per modification when compared to RNA. It reduces some nonspecific protein binding and greatly increases resistance to nucleases.[124] Like the 2'-OMe the 2'-MOE modification also adopts the North sugar conformation, which is additionally stabilised by hydration effects.[124] This modification has been successfully applied in gapmers[124] (terminal nucleotides) and siRNA.[91]

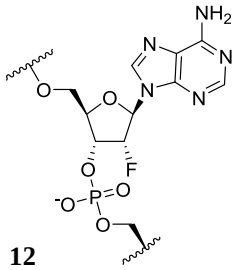


11

Figure 1.16. 2'-O-methoxyethyl modification (2'-MOE)

2'-Fluoro (F)

The 2'-Fluoro modification (2'-F) (Figure 1.17) imparts strong binding affinity for target RNA (ΔT_m about +2° C per modification when compared to DNA).[125] The 2'-F modification adopts the North sugar conformation.[126] It also improves stability relative to RNA. This modification is very well tolerated in siRNA applications.[127]



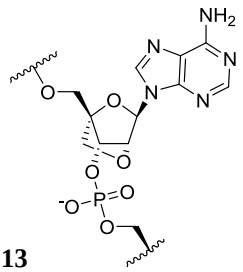
12

Figure 1.17. 2'-fluoro modification (2'-F)

Locked Nucleic Acid (LNA)

LNA (Figure 1.18) belongs to the group of 2',4'-bicyclic nucleic acids. LNA is a chemically modified RNA analogue containing a methylene bridge joining the 2'-OH to the C4', which forms a conformationally restricted bicyclic nucleoside.[128] The LNA modification adopts a Northern sugar conformation that closely mimics A-form RNA, preorganising LNA for RNA-binding.[129] This results in very strong binding properties for LNA and makes them especially useful in anti-mRNA applications, where short sequences might be necessary.

One modification can increase the melting temperature from 1.5 ° to 6.0 ° against RNA target.[130] The LNA modification improves nuclease resistance and stability and decreases immune stimulation.[131] Moreover, this modification is widely used in gapmer and siRNA applications.[131,88] LNA shows very high binding affinity to itself, higher than to the RNA target, and therefore when used at typical ASOs lengths, 21-23 nucleotides, with all nucleotides LNA modified, the LNA strand often forms stable hairpin and self-dimers what can affect its performance. Therefore most LNAs are commonly used together with other modifications like 2'-OMe or with unmodified DNA for example as gapmers.

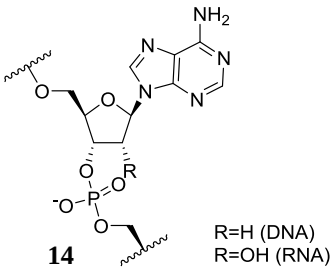


13

Figure 1.18. Locked Nucleic Acid (LNA)

1.3.2 Sugar phosphate backbone modifications

As mentioned previously, the internucleotide phosphodiester linkages of DNA and RNA (Figure 1.19) are negatively charged and can be readily cleaved by endo and exonucleases found *in vivo*.



14

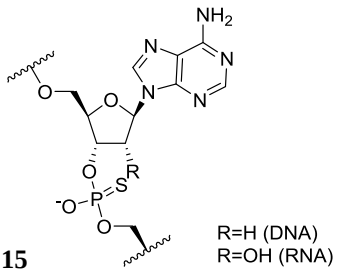
Figure 1.19. Phosphodiester backbone (PO)

For that reason chemical modifications of the phosphodiester bonds have been extensively studied. Several chemical strategies have been very successful for improving nuclease resistance.[132] One of the first to be developed, a phosphorothioate modification, is the subject of the following review.

Phosphorothioate (PS)

The phosphorothioate linkage (PS) (Figure 1.20), where sulphur is substituted for one of the nonbridging phosphate oxygens, is one of the most commonly used modifications and imparts significant resistance to nuclease degradation. The first example of increased stability of phosphorothioate modified polyribonucleotides, toward ribonucleases compared to unmodified parent nucleic acid was shown in 1969 by De Clercq *et al*.[133] The synthesis of phosphorothioate containing oligonucleotides, which has not changed in years, is straightforward and is performed by phosphoramidite chemistry, either by the conventional or Scaringe version.[134] In the synthesis cycle the iodine oxidation step is replaced by a sulphurisation step which most commonly uses Beaucage reagent.[135] By replacing the oxygen atom with sulphur the negative charge of the phosphate group is retained and the size of sulphur atom is similar to the oxygen atom. By the introduction of the sulphur the phosphorus centre becomes chiral resulting in presence of two diastereomers (Sp and Rp),[13] which can be separated by HPLC to be identified by ^{31}P-NMR spectroscopy or enzymatic digestion.[136] Pure Rp configuration ASOs have higher binding affinity (higher T_m) than pure Sp or mixed populations.[137] However, the Rp diastereomer is as nuclease sensitive as phosphodiester linkage and therefore using unseparated oligonucleotides with both Rp and Sp diastereomers is often the best option.[138] Phosphorothioate modified oligonucleotides are highly water soluble, which is

important for the biological use.[13] PS linkages in nucleic acids can enhance their affinity for binding with serum albumin, which can improve pharmacokinetics and circulation time.[111] PS oligodeoxynucleotides can efficiently elicit RNase H cleavage of the target RNA, which is crucial for the success of the majority of antisense drugs. PS linkages have been successfully incorporated into siRNAs without significant loss of potency, but can reduce activity in some cases. It has been reported that oligonucleotides with this modification can be used *in vitro* without the use of transfection or electroporation.[139] Both antisense oligonucleotide drugs (Vitravene and Kynamro), which have been licensed by the FDA, have phosphorothioate backbones.



15

Figure 1.20. Phosphorothioate backbone (PS)

1.3.3 Vinylphosphonate modification

Backbone and sugar modifications have been widely studied as they increase the thermal stability of oligonucleotides (conformational restriction of the sugar moiety) and nuclease resistance (phosphorothioate backbone). Little was known about conformational restrictions of the backbone of nucleic acid and the modifications that were developed were used mainly for the investigation of secondary structural elements of nucleic acids.[140,141] An example is work done by Harris and colleagues,[140] who developed two-, three- and four-carbon tethers to oligonucleotides and investigated they ability to bend DNA and subsequently

decrease replication by *E. coli* polymerases (Figure 1.21). All linkers blocked the replication process due to inhibited ability of nucleotides to adopt the correct conformation at the active site of polymerases. A lot of research on accessing dinucleotides with restricting cross-links has been done by Sekine, Vigoroux and Nielsen (reviewed by Kruse[142]), however they were found to be disadvantageous. For example, developed modifications added a steric factor introduced by restricting linker. Moreover, the cross-links not only added one or two stereocentres, which have to be controlled during synthesis, but also in some cases resulted in neutral backbones at the position of modification. All of which could interfere with outcomes of studies in which the modifications were used.

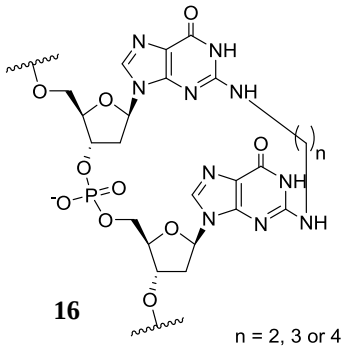


Figure 1.21 Dinucleotides with intrastrand cross-links.

An easier way to limit conformational freedom of the nucleic acid backbone is to introduce vinylphosphonate linkage between nucleosides. Moreover, as first shown by Zhao and Caruthers,[143] substitution of 5'-oxygen with carbon in **17** results in new 5'-phosphor-carbon bond, in **19,** that is stable toward hydrolysis (Figure 1.22).

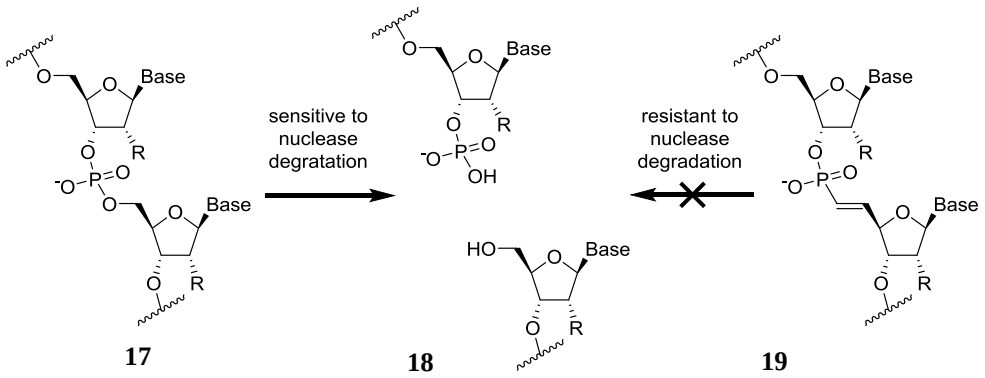


Figure 1.22. Difference in stability towards nuclease degradation of the natural and modified backbone of the nucleic acid.

The Hayes group has been working on the synthesis and biological applications of the vinylphosphonate linked nucleotides. First they have shown that the (*E*)-vinylphosphonate linkage in the backbone of the translocating strand inhibits activity of the 3'→5' DNA helicase.[144] However, the more interesting use of the aforementioned modification in the context of this project is its effect on the nucleases. Three DNA substrates were synthesised, N1 had no modifications, N2 had four (*E*)-vinylphosphonate modifications in the middle of the sequence and N7 had one (*E*)-vinylphosphonate backbone.[145] All three substrates were exposed to endonuclease III digestion and the products were run on a gel (Figure 1.23). No bands were observed for the unmodified substrate, meaning the DNA was completely degraded. However, for both substrates N2 and N7 undigested products were observed and they corresponded to the position where the (*E*)-vinylphosphonate modification was placed. This demonstrated that the modification alters cleavage specificity of exonuclease III.[145]

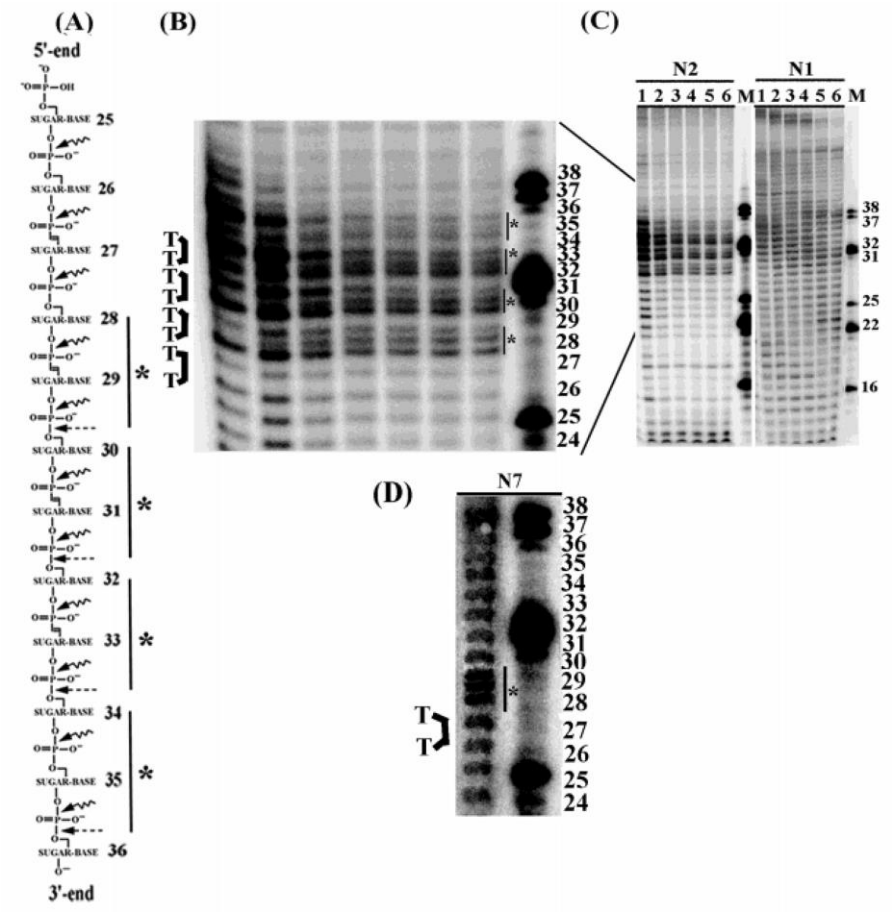


Figure 1.23 Time course experiments of exonuclease III digestion of substrate N1, N2 and N7. The precise positions of cleavages are shown by wiggly arrows, when the interrupted arrows show the vinylphosphonate-induced cleavages (A). The corresponding triplets are marked with asterisks in both A and B. The magnified region containing triplets is shown in B. The complete gels showing time course digestion with exonuclease III for substrates N1 and N2 are shown in C. D shows digestion for substrate N7. Figure taken from Doddridge *et al.* (2003).[145]

One of the problems with the synthesis of (*E*)-vinylphosphonate containing nucleotides is their compatibility with solid-phase oligonucleotide synthesis. Presence of a DMTr group as well as 3'-phosphoramidite functionality is essential for automatic synthesis on DNA synthesizers (Figure 1.13). All protecting group of nucleobases, riboses and phosphate backbone have to be not only stable during synthesis but also removable afterwards. Dinucleotides with the (*E*)-vinylphosphonate modification were obtained by the following methods: cross-coupling, cross-metathesis and Wittig reactions.[142,143,146-151] First the (*E*)-vinylphosphonate linked dimer was synthesised by Zhao *et al.*[143] using the

Witting reaction of aldehyde with ylide. The disadvantage of Zhao's approach was the use of *o*-chlorophenyl as a phosphate-protecting group, requiring an extra deprotection step after synthesis. Moreover the stereochemistry of phosphorus centre could not be controlled, which is important when the final oligonucleotide is modified at the non-bridging oxygens. An alternative route to access dinucleotide via a palladium(0)-mediated cross-coupling approach was proposed by the Hayes group.[146-148] Despite their best efforts, the final product was always a mixture of *E*- and *Z*- isomers, which proved to be inseparable. The trans geometry is necessary to mimic the natural phosphates hence synthesis of pure *E*- isomer was required. Further investigation of the problem by Collis[149] and Kruse[142] lead to the generation of phosphoramidites that are not only suitable for the solid-phase synthesis but also allow selective synthesis of the (*E*)-vinylphosphonate products. Moreover, Kruse showed that using the Wittig reaction gives the best results.[142] The synthesis route developed by Kruse is presented in Figure 1.24.

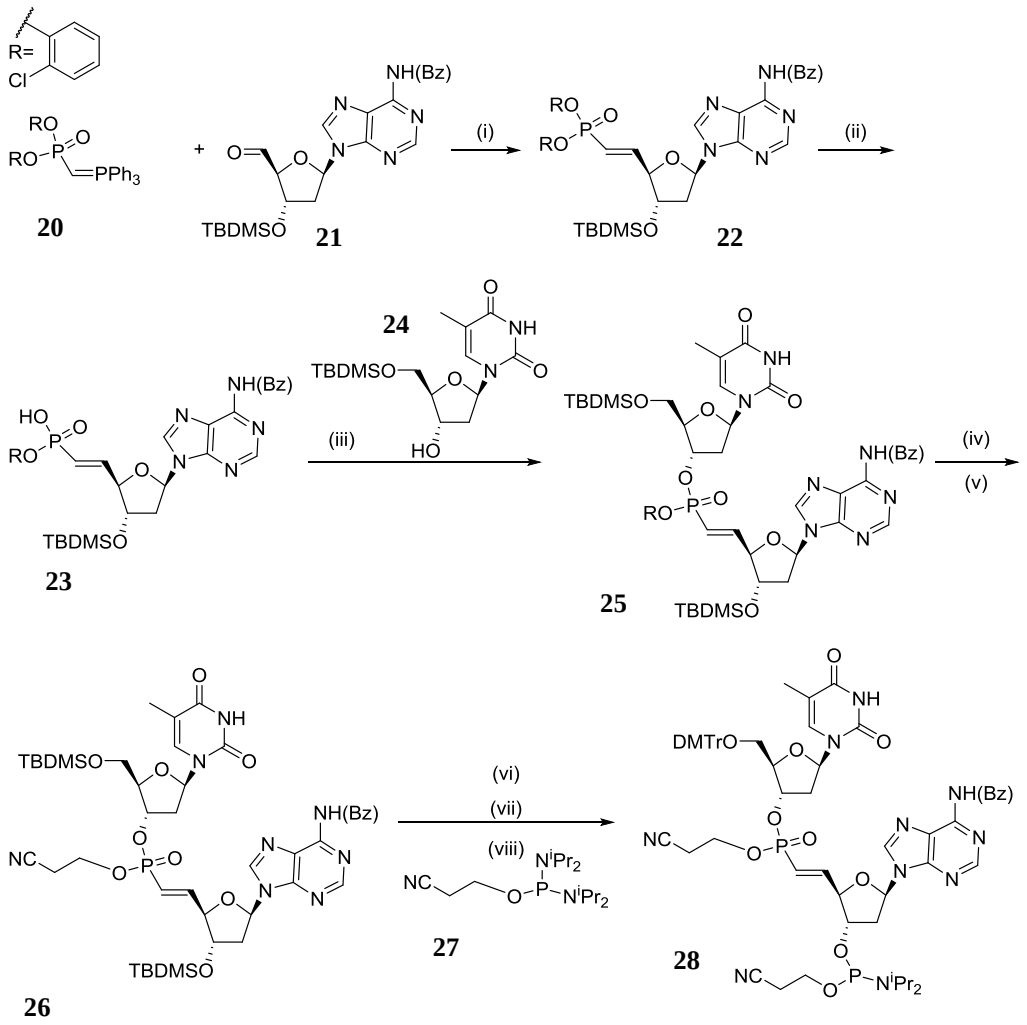


Figure 1.24. Hayes synthesis of the (*E*)-vinylphosphonate linked dinucleotides. Reagents: (i) CH_2Cl_2, 71%; (ii) 2-nitrobenzaldoxime, TMG, triethylamine, dioxane, 72%; (iii) 2,4,6-triisopropylbenzenesulfonyl chloride, NMI, pyridine, 77%; (iv) 2-nitrobenzaldoxime, TMG, TEA, dioxane, 85%; (v) 3-hydroxypropionitrile, 2,4,6-triisopropylbenzenesulfonyl chloride, NMI, pyridine, 83%; (vi) $NEt_3 \cdot 3HF$, THF, 79%; (vii) DMTrCl, pyridine, 52%; (viii) 5-methyl-1*H*-tetrazole, CH_2Cl_2, 85%.

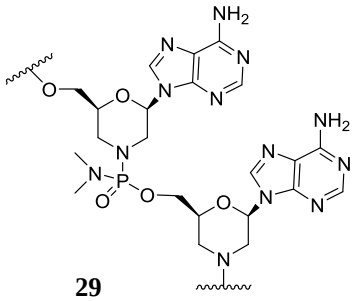
Reaction of ylide **20** and aldehyde **21** generated molecule **22** with double bond. One of the o-chlorophenyl groups was removed and resulting molecule **23** reacted with a secondary alcohol **24** to give the (*E*)-vinylphosphonate linked dinucleotide **25**. To incorporate functional and protecting groups to the dinucleotide, first the o-chlorophenyl group in **25** was removed and replaced with a cyanoethyl protecting group in **26**. That was followed by removal of silyl (TBDMSO) and introduction of DMTr group. Last step involved reaction of 3'-alcohol with phosphoramidite **27**. The synthesised dinucleotide is fully suitable for the solid-phase synthesis by the phosphoramidite coupling on the

standard DNA synthesizer. The presented synthesis is much easier than the one proposed by Ionis[99] and in contrast, the dimer can be incorporated at any position of the oligonucleotide sequence and the final ASO can be purified and deprotected with the use of a standard protocol, making it more accessible and preferable to use. Moreover, our oligonucleotides do not require HPLC purification, as they have very good purity, which also makes the synthesis process much faster and easier.

1.3.4 Sugar and backbone modifications

Morpholino

Morpholino phosphorodiamidates are neutral molecules, which are composed of a backbone modification that has a morpholine ring as a replacement for the furanose with a phosphorodiamidate linkage connecting the morpholine nitrogen atom with the hydroxyl group of the 3'-side residue (Figure 1.25). ASOs containing morpholino phosphorodiamidates have proven resistance to nuclease degradation.[152] Use of this modification in oligonucleotides disqualify them from use in gapmers as they do not activate RNase H, and they are therefore used in the steric blocking mechanisms.

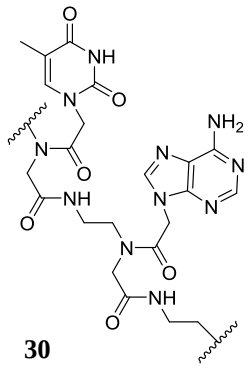


29

Figure 1.25. Morpholino

Peptide Nucleic Acid (PNA)

PNAs (Figure 1.26) are a class of neutral oligonucleotide analogues that contain peptide replacements for the sugar phosphate backbone, but maintain the ability to Watson-Crick base pair with complementary RNA or DNA. They are highly resistant to nuclease and protease degradation.[153] PNA modified ASOs can be used in the steric blocking mechanisms,[84] in addition to splice-switching applications.[154]



30

Figure 1.26. Peptide Nucleic Acid (PNA)

1.4 Delivery of oligonucleotides

All cell membranes, in living organisms, are composed of phospholipids and proteins. Phospholipids are amphipathic molecules that consist of two fatty acid chains, which are linked to the phosphate hydrophilic head group. Because their fatty acid tails are poorly soluble in water, phospholipids spontaneously form bilayers in aqueous solutions with the hydrophobic tails buried in the interior of the membrane and the polar head groups exposed on both sides in contact with water. This forms a stable barrier between two aqueous compartments and represents the basic structure of all biological membranes. The structure of the cell membrane is responsible for its primary function, control of the movement of substances in and out of the cells. Movement of substances through the

membrane can happen through passive diffusion or endocytosis. In passive diffusion, solutes with a low molecular weight diffuse through the membrane without the need for chemical energy. However, larger or charged molecules have to enter the cytoplasm and this can be done by endocytosis.

To have an effect on gene silencing ASOs have to pass through the cell membrane, escape from cytoplasmic vesicles and then reach their target in the cytoplasm (if the target is localised in the nucleus an ASO must also enter the nucleus). Oligonucleotides have high molecular weight and are negatively charged, hence are very unlikely to cross the membrane via passive diffusion. Thus, research suggests that transport of oligonucleotides takes place by endocytosis.[155-158] Endocytosis is the process in which a portion of the plasma membrane forms a vesicle that absorbs macromolecules and transports them to intracellular destinations.[159,160] Two factors of oligonucleotide internalisation should be considered: cellular uptake and release into the cytoplasm (or nucleus).[161] After entering cells, oligonucleotides accumulate in endosomes and lysosomes. Only a small proportion of them escape from the membrane, whereas the rest are likely to be degraded. Once the oligonucleotide has entered the cytoplasm it can stay there or move further and enter the cell nucleus. Nuclear penetration is due to the passive diffusion through nuclear pores or through active signal-mediated diffusion.[162,163] The biological activity that can be observed refers only to the small amount of ASO that was released from endosomes. The amount of ASO that has penetrated the cell membrane and reached its target is a determining factor for oligonucleotide activity and efficiency.

Many strategies have been developed to increase the rate and selectivity of cellular internalisation of ASOs. It has been proposed that delivery methods should be classified into two groups.[161] The first group focuses on the cellular uptake process and improving the binding properties of ASOs to the membrane. This group includes conjugation of various compounds (e.g. cholesterol), complexation with cationic compounds (e.g. cationic polymers) and encapsulation. The second group focuses on entry into the cytoplasm (and/or nucleus) and includes cytoplasmic transfer techniques (e.g. microinjection, electroporation) or endosomal membrane destabilising agents (e.g. viral peptides, fusogenic agents). These methods may be used alone or in combination, and each one has its own advantages.

1.4.1 Electroporation and injection

Electroporation

Electroporation is a method in which an electrical pulse is used to perturb the cell membrane and form temporary pores, which allow movement of the oligonucleotide through the membrane. Investigation of cell membrane response to electric fields started in 1960s.[164,165] When exposed to electric field, ions and soluble dipole molecules within cells reorganise themselves with respect to the field lines. Distribution of charged molecules becomes polarised resulting in one side of the cytoplasm being positively charged, relative to the other side. This results in alternations of the membrane and formations of pores, which allow transport through the cell membrane.[166] The first use of electroporation to deliver DNA into the mouse cells was reported by Neumann *et al.* in 1982.[167] Following this success, electroporation became a popular method for

transfecting animal and plant cells.[168-170] Although, this technique was initially developed for *in vitro* use, it has also been successfully employed in *in vivo* applications.[166,171-173] For example Mathiesen[171] showed that delivery of foreign DNA into rat muscle fibres is enhanced when muscles are simultaneously stimulated electrically. A 10 fold increase in expression of transfected DNA was observed compared to injection.[171] Another example of successful *in vivo* electropermeabilization-mediated delivery was presented by Heller *et al.*[173] In this study a rat model was employed to electroporate hepatic parenchyma. Plasmid was injected directly into liver, followed by administration of series of six 100-µs electric pulses, resulting in 25-30% hepatocyte transfection efficacy. The transfection efficacy was significantly higher than any *in vivo* transfection reported to date.[173]

As the probability of cell death is quite high when compared to other transfection methods, electroporation is most commonly used in tissue culture for cell lines that are difficult to transfect with other methods.

Injection

The form of injection that is used in tissue culture is called microinjection and it is a process in which an ASO is injected to a single cell using a micropipette. It has been shown that microinjection of an ASO into the cytosol leads to its rapid nuclear localisation,[174-176] which can be disadvantageous if the ASO's target is in the cytoplasm. Another disadvantage of microinjection is its complexity as the technique is very laborious and with this method only a limited number of cells can be processed at any time.

Injection is a preferred method of delivering naked ASOs *in vivo*. Delivery can be performed by subcutaneous, intravenous, intramuscular or intraocular injection. To produce a therapeutic effect, injection requires several doses.[177] Both of the FDA approved antisense drugs, Vitravene and Kynamro, are delivered by injection (intraocular and subcutaneous respectively).

1.4.2 Synthetic polymers

The majority of tissue culture transfections of ASOs are performed with the use of synthetic polymers, which include: cationic polymers (PEI,[178-180] dendrimers[181]), anionic polymers (poly(acrylic acid) hydrogel[182]), neutral polymers (polycyanoacrylates[183]), electrically conductive polymers[184-186] and synthetic peptides (polylysine,[187] polyornithine[188]). Polymers can be specially designed by choosing appropriate molecular weight, coupling cell or tissue specific targeting moieties or performing other modifications.[189] Polymers are relatively easy and inexpensive to produce, which may favour them over other transfection methods. As both cell membrane and ASOs are negatively charged, cationic polymers and cationic lipids have attracted the most attention as delivery reagents and are probably the most widely used.[190] Cationic polymers form nanocomplexes with oligonucleotides and as result compensate their negative charge.[191] Examples of popular commercial polymers used in laboratories include: Lipofectin, Lipofectamine, HiPerFect and ExGen 500. Although synthetic polymers show good potency, when used as delivery regents, they are not favourable tools for all cell lines as they can demonstrate high cytotoxicity.[192-194]

1.4.3 Oligonucleotide conjugates

A novel type of oligonucleotide delivery involves conjugation of the nucleic acid with a lipophilic group or a ligand that can be recognised by membrane proteins and receptors, resulting in the molecule being able to penetrate the cell membrane. Most conjugates aim to improve biological properties and cellular uptake, but some of them can target specific cell types. A number of different molecules have been attached to the ASOs to improve their delivery. The strategies include:

- attachment of the lipophilic group (e.g. cholesterol),[195,196]
- cell-penetrating peptides (CPPs) (like spermine and penetratin),[197,198]
- attachment to ligands of specific receptors (carbohydrate *N*-acetyl galactosamine (GalNAc)),[199,200]
- attachment of antibodies.[191,201,202]

Ligand-oligonucleotide conjugates offer the possibility of selective delivery to the cells or tissues via a receptor mediated mechanism.[191] This branch of ASO research is probably one of the fastest evolving, with new modifications being developed every year (e.g. dynamic polyconjugates[203]). The delivery-improving ligands are attached to the oligonucleotide in a chemical step by solid-phase approach, solution phase conjugation or "click chemistry".[204-206] Most commonly they are coupled to the 3' or 5'-hydroxyl group. These positions are technically easy to access and do not interfere (or interfere only a little) with base pairing, even when large ligands are used. Additionally the bioconjugate can be a part of the solid support that is used for oligonucleotide synthesis.[205] Another option is the attachment of ligands to the internal positions

(2'-O-hydroxy)[207] or to nucleobases.[208] Oligonucleotide conjugates provide an interesting alternative to other delivery methods, mostly because of their reduced toxicity.[191] Therefore they seem to be good candidates to improve ASO delivery in *in vivo* studies. Nonetheless, more understanding of uptake mechanisms to develop effective intracytoplasmic oligonucleotide delivery is still needed.

1.4.4 Gymnosis

Despite the fact that employment of transfection reagents enhances an ASO's uptake resulting in its increased activity, some of the cellular functions may be affected. Most transfection reagents interact with cell membranes and consequently receptors, hence signalling process and other cell functions might be affected. For that reason, research into the delivery of naked oligonucleotides has been conducted.

In 2009 Stein and colleagues described a method called gymnosis.[139] The name refers to the Greek word "gymnos" which means naked. In this work researchers characterised a process of oligonucleotide uptake by growing cells in tissue culture in which no serum additives were used. Variously composed LNA-DNA phosphorothioate gapmer oligonucleotides, targeting codons of the Bcl-2 protein, were tested. Stein reported that gymnotic delivery is slow when compared with other methods. Depending on the cell line, the optimal treatment time varied from 3 to 10 days. When the oligonucleotide was removed from the media, following 10 days of naked delivery, the protein levels returned to 100% of base line expression after 72 hours. Gymnosis was shown to take place in numerous cell lines, even some difficult to transfect with lipid formulations (LNCaP cells, Nmalwa B cells). The gymnotic delivery method was also

checked in respect of target-specific effects. The naked oligonucleotide targeting Bcl-2 showed inhibition of its target only, and none of the reference genes (Bcl-xL, p21, PKC-α, Mcl-1 and tubulin). In comparison it has been reported that transfection reagents, for example Lipofectin can affect random genes resulting in early cell apoptosis.[209,210] Another conclusion from Stein's work is that other modifications, not only LNA, can be used in oligonucleotides delivered to the cells by gymnosis. 2'-deoxy, 2'-fluoro, β-D-arabinonucleic acid (FANA) phosphorothioate gapmers[211] and 2'-OMe modified phosphorothioate oligomers all showed activity. In the initial studies no activity for neutral ASOs like morpholino or peptide nucleic acids was observed.

As has been shown above, numerous methods can be used to introduce oligonucleotides into cells, each of them having positives and negatives. Many reports suggest that delivery of ASOs in association with an adjuvant enhances its therapeutic effect, increases cellular uptake and acts as protection against degradation.[177,212-217] Nevertheless some carriers might increase size and complexity of the delivered molecules limiting their access to targets or even contributing to their degradation. Each decision about choosing one of the transfection methods depends on the system in which it will be used and the researchers' individual preferences. For example, the most successful delivery method in tissue culture involves the use of cationic polymers, which significantly improve oligonucleotide uptake, but are relatively toxic and therefore are not recommended for animal or human use. For that reason other delivery methods were needed, resulting in development of oligonucleotide conjugates. It has been also shown that ASO delivery *in vivo* is possible by local injection without any enhancer.[218] In some cases targeting a specific cell,

combined approaches are used. For example when oligonucleotide cholesterol-conjugated oligonucleotides are injected into mice.[196] As presented, many factors have to be taken into account when choosing the transfection method, with more than one technique being tested before the most effective is found.

2 Aims and objectives

The aim of this project is to develop stable oligonucleotides which can be used as therapeutic agents. As the results of *in vitro*/*ex vivo* studies are often different to *in vivo* outcomes, we decided to test our oligonucleotides in both systems. Therefore the aims of research project outlined in the following chapters are:

- identification of suitable sequences for KD of firefly luciferase target with the use of siRNAs,
- testing if unmodified ssRNAs can inhibit production of firefly luciferase,
- development and synthesis of nuclease stable, single-stranded, oligonucleotides that can inhibit gene expression *in vitro*/*ex vivo*,
- defining IC_{50} and melting temperature for successful ssRNAs,
- use of the developed single-stranded ASOs to regulate expression of chosen, disease related target *in vitro*/*ex vivo*,
- extending the study towards regulation of the same disease related target *in vivo*,
- examination of the mechanism through which newly developed ssRNAs work.

3 Results and discussion

3.1 Knock-down of firefly luciferase

3.1.1 Choice of mRNA target sequence and ASO design

In order to test the success of ASO inhibition of gene expression a luciferase target was chosen. Luciferase was selected for these experiments as it is a well-established reporter protein for which robust assays are commercially available. Experiments were conducted in an MDA-MB fluc cell line, which is a well-established human, breast carcinoma line that expresses firefly luciferase endogenously under the control of a CMV promoter. Moreover this cell line can be used in the mouse model that was developed by our collaborators. In order to produce gene expression KD it was necessary to identify suitable sequences to target. Primer3 software was used (http://primer3.ut.ee/) to analyse the luciferase DNA sequence. The 5'-UTR, open reading frame (ORF) and 3'-UTR of firefly luciferase were used as a source sequence (the sequence of plasmid used to generate the stable cell line can be found in 7.1). The standard parameters were kept (see 5.2.1 for details) and the downstream primer was chosen. Next the firefly luciferase mRNA was checked for secondary structures using mfold web server (http://unafold.rna.albany.edu/).

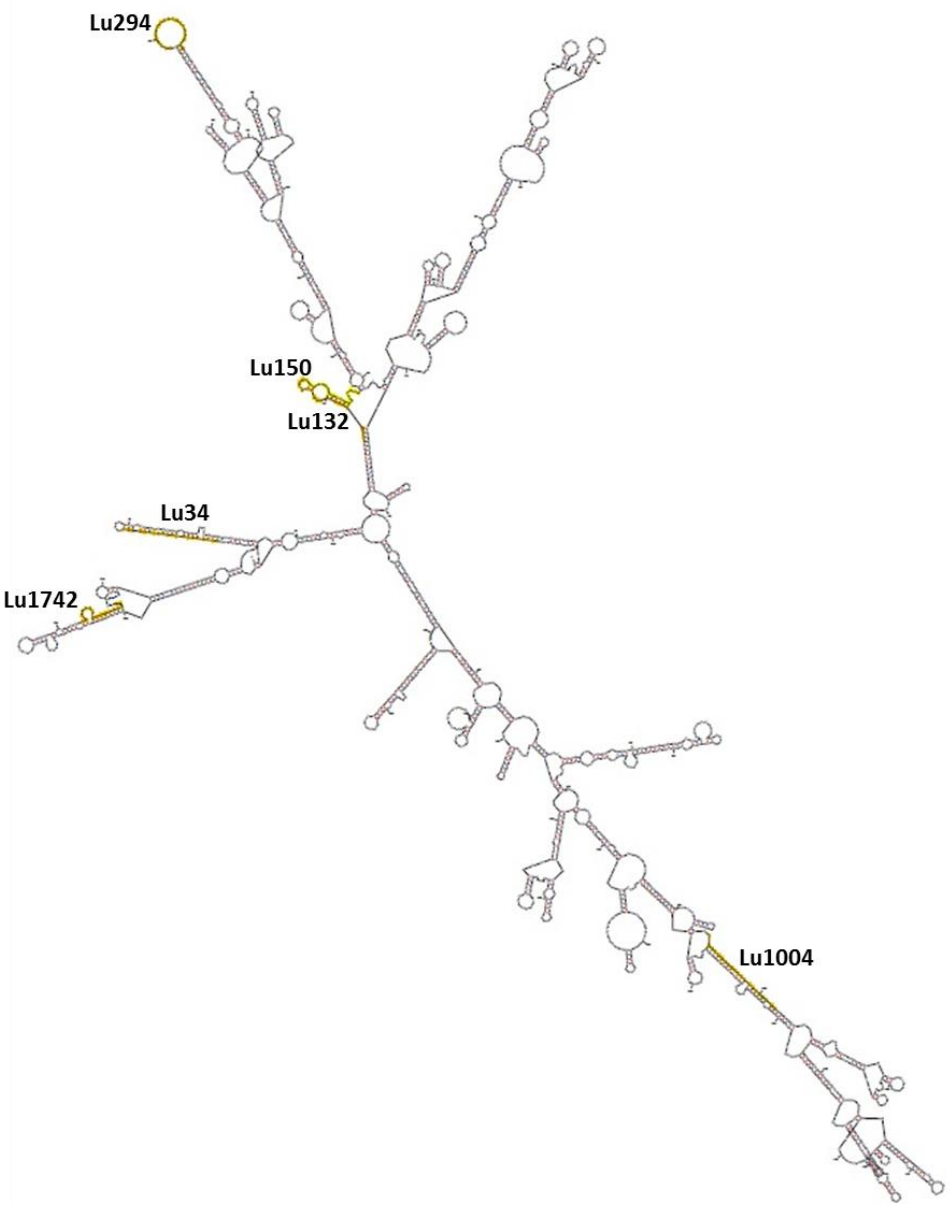


Figure 3.1 mfold prediction of secondary structure of the firefly luciferase mRNA. Oligonucleotide binding sites are highlighted in yellow.

We decided to choose oligos that potentially bind only to these regions of mRNA where, by the mfold assessment, the mRNA creates a loop (Figure 3.1). We reasoned that the ASO is likely to show better binding to the loops than to the regions where mRNA forms base pairing interactions within itself. Initially six different targets within the luciferase sequence were selected (Figure 3.2).

Figure 3.2. Location of the oligonucleotides relative to the sequence of firefly luciferase mRNA. Six target sites within firefly luciferase sequence were chosen using Primer3 software. Two targets are located in the 5'-UTR, one is spanning the ATG start codon of the ORF, and three more are located further in the ORF of the gene.

Three targets within the 5'-UTR region were picked since it has been reported[35,36] these are the best sites for steric blocking. Additionally, four downstream sequence targets were selected for comparison. The oligo sequence names refer to their target. "Lu" means that ASO targets firefly luciferase. The number in bracket relates to which position, relative to the start of 5' untranslated region of the targeted gene, the 5' end of the oligo binds. For example Lu(34) indicates the ASO that targets firefly luciferase with the first nucleotide of the oligo's 5'-end binding to the 34th base in the sequence of luciferase (sequence numbering starts from 5'-UTR).

Apart from siRNA obtained from Eurogentec (*Lu1742.com) all of the oligonucleotides were synthesised and purified in house. Syntheses were performed on an ABI synthesizer (0.2 µM scale) with phosphoramidite coupling on a CPG Solid Support. After synthesis the oligonucleotides were deprotected, purified and freeze dried to give white precipitate that was then desalted and resuspended in RNase free water. That was followed by determination of concentration of oligonucleotide that usually varied between 50 µM and 100 µM.

3.1.2 dsRNAs design

First we tested if targeting chosen firefly luciferase sequences produce gene expression KD. To do so, we decided to use siRNAs as they are a commonly used tool for gene silencing and provide good results. Three targets were used (Lu(132) and Lu(150)) and Lu(1742) (Figure 3.2). The single-strands of RNAs (Table 3.1) were synthesised on an ABI synthesizer by the phosphoramidite coupling method and annealed to give the desired double-stranded RNAs. To ensure that the oligos reached their target a transfection reagent was used for delivery. HiPerFect (HPF) transfection reagent was chosen and used subsequently in all RNA oligonucleotide experiments.

Table 3.1. The synthesised ssRNAs. Black stands for unmodified RNA, underlined – AA overhangs, "s" phosphorothioate linkage and "P" – phosphate group at the 5' position. Depending on the type of used modifications the ASO length varies from 19 to 23 nucleotides. *ASO from commercial source. Calculated and observed masses are presented in 5.3.2.

ASO ID	Structure 5' → 3'	Length
Lu1742.1	AAU CUC GCG GAU CUU GCG G	19
*Lu1742.com	AAU CUC GCG GAU CUU GCG GTT TT UUA GAG CGC CUA GAA CGC C	21
Lu1742.2	AAU CUC GCG GAU CUU GCG GAA	21
Lu1742.3	P-AAU CUC GCG GAU CU_sU_s $G_sC_sG_s$ G_sA_sA	21
Lu1742.sense	CCG CAA GAU CCG CGA GAU UAA	21
Lu132.1	P-CCA UGG UGG CUU UAC $C_sA_sA_s$ $C_sA_sG_s$ A_sA	23
Lu132.sense	CUG UUG GUA AAG CCA CCA UGG AA	23
Lu150.1	P-UAA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	23
Lu150.sense	UGG AAG AUG CCA AAA ACA UUA AA	23

To explain how ASOs were designed an oligo **Lu1742.1** (Table 3.1) is used as an example. The sequence of **Lu1742.1** is $^{5'}$AAU CUC GCG GAU CUU GCG G$^{3'}$ and it is fully complementary to the Lu(1742) mRNA target. Oligo **Lu1742.1** is 19 nucleotides long, has no modifications and is a framework for other ASOs that have the same target. Therefore all oligos that have any addition (or change) to **Lu1742.1** are considered modified ASOs. All of the modified RNAs have two additional purine overhangs on the 3'-ends (underlined in the sequence) since it has been reported to enhance their *in vitro* potencies,[101,219] and as a result are 21 nucleotides long, for example oligo **Lu1742.2**. As the aforementioned addition of an overhang was likely to be insufficient to improve resistance against nucleases another modification was introduced resulting in synthesis of oligo **Lu1742.3**, that has seven phosphorothioate bonds on the 3' tail. Moreover ssRNA **Lu1742.3** is phosphorylated on the terminal 5' nucleotide. These two

modifications were introduced based on information presented in Lima *et al.* publication (2012).[97]

3.1.3 Testing dsRNAs inhibition potency *ex vivo*

The inhibitory activities of oligonucleotides targeting Lu(1742) were measured. Two oligonucleotides, one purchased from a chemical supplier (Eurogentec) and one synthesised in the Hayes' group laboratory were compared. Both oligos had the same length and target sequence, however oligo **Lu1742.com** has two thymidine (TT) overhangs whereas that synthesised in our laboratory oligo **Lu1742.2** had two adenosine (AA) overhangs. Additionally **Lu1742.com** was already annealed while **Lu1742.2** was annealed in the laboratory to the sense strand **Lu1742.sense** that was also synthesised in house. As can be seen in Figure 3.3 (a) treatment with both dsRNAs resulted in more than 85% of inhibition for 5 nM concentrations. A similar decrease in luciferase activities was observed for higher concentrations: 10 nM and 50 nM. Another experiment was conducted for **Lu1742.2** annealed with **Lu1742.sense** to determine if lower concentrations have the same effect (Figure 3.3b). In the range of concentrations from 1 to 50 nM there was no obvious difference in oligonucleotide efficacy. The inhibition effectiveness varied from 79% for1 nM to 93% for 50 nM. Additionally repeating the experiment confirmed its reproducibility and helped to identify a positive control for further research. These results provided a very good starting point for development of single-stranded RNAs and proved that oligonucleotides synthesised in our laboratory have as good properties as commercial ones.

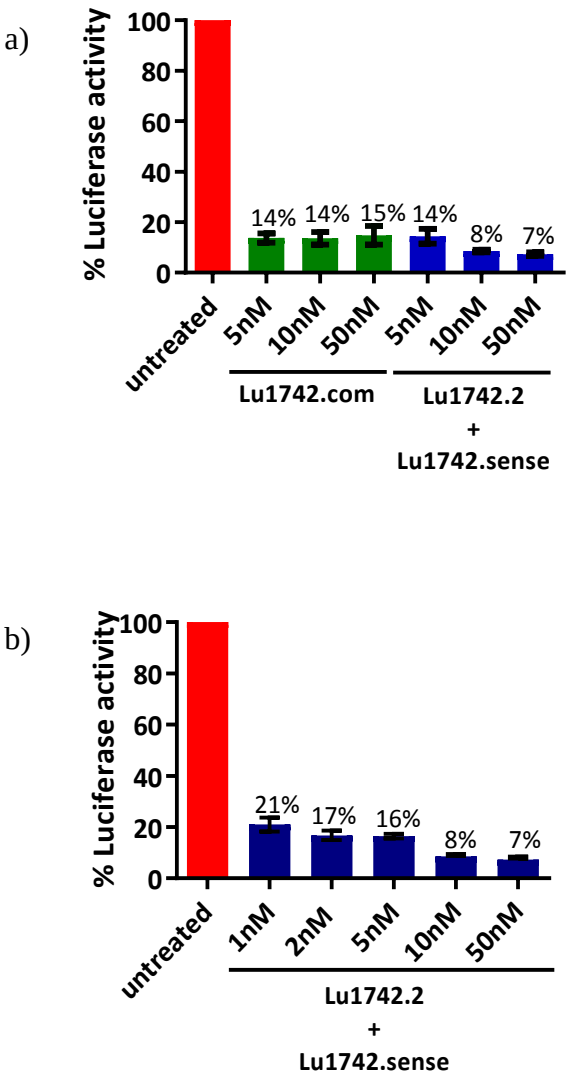


Figure 3.3. *Ex vivo* activity of dsRNAs targeting Lu1742 in MDA-MB fluc cell line. Graphs show potency of tested siRNAs in inhibiting firefly luciferase. a) Comparison of commercial siRNA (**Lu1742.com**) and siRNA synthesised in Hayes laboratory (**Lu1742.2**+**Lu1742.sense**). b) Further investigation of efficacy of the **"Lu1742.2**+**Lu1742.sense"** siRNA used at different concentrations. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows, panel a: Lu1742.com: 5 nM – 1.819; 10 nM – 2.492; 50 nM – 3.710. Lu1742.2+ Lu1742.sense: 5 nM – 2.865; 10 nM – 0.534; 50 nM – 0.727. Panel b: Lu1742.2+ Lu1742.sense: 1 nM – 2.677; 2 nM – 1.806; 5 nM – 0.884; 10 nM – 0.534; 50 nM – 0.727).

The next step was to check the potency of dsRNAs designed for two other sites Lu(132) and Lu(150). To do so, four single strands of RNAs were synthesised and annealed. This time both of the antisense strands had all three modifications used for Lu(1742) (AA overhangs, seven phosphorothioate bonds and phosphorylated terminal base). RNA targeting Lu(132) was obtained from the antisense strand **Lu132.1** and the sense strand **Lu132.sense**. The same was done

for Lu(150). The antisense strand **Lu150.1** was annealed with the sense **Lu150.sense**. The resulting duplexes were tested *ex vivo* together with ASO "**Lu1742.3**+**Lu1742.sense**", and showed respectively 55%, 80% and 68% inhibition (Figure 3.4).

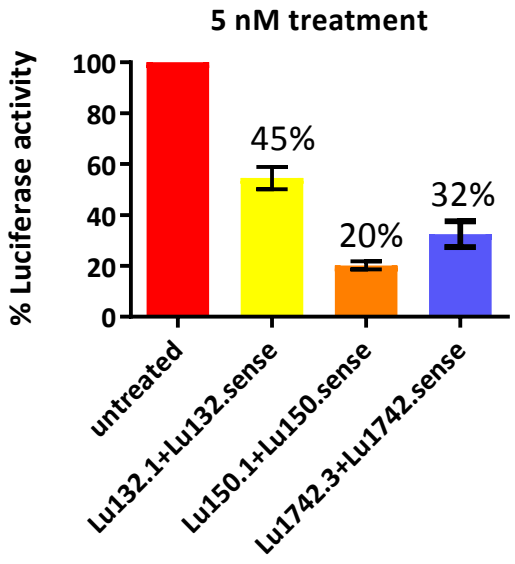


Figure 3.4. *Ex vivo* activity of chemically modified dsRNAs in MDA-MB fluc cell line. Graph shows potency in inhibiting firefly luciferase for tested dsRNAs targeting Lu132 (**Lu132.1**+**Lu132.sense**), Lu150 (**Lu150.1**+**Lu150.sense**) and Lu1742 (**Lu1742.3**+**Lu1742.sense**). Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: Lu132.1+Lu132.sense – 4.380; Lu150.1+Lu150.sense – 1.535; Lu1742.3+Lu1742.sense – 5.051).

The experiments, performed so far, show that when targeting firefly luciferase with dsRNAs at all designed sites (Lu(132), Lu(150) and Lu(1742)) the amount of produced protein is significantly reduced, but the KD value varies depending on the target and modifications that were incorporated within the oligo's sequence. For example oligo "**Lu1742.2**+**Lu1742.sense**" (Figure 3.3b) and "**Lu1742.3**+**Lu1742.sense**" (Figure 3.4) show that introduction of the chemical modifications reduces siRNA's target inhibition efficacy, as for 5 nM treatment the KD value goes down from 84% ("**Lu1742.2**+**Lu1742.sense**" Figure 3.3b) to 68% ("**Lu1742.3**+**Lu1742.sense**" Figure 3.4). Although the difference is

significant, the "**Lu1742.3**+**Lu1742.sense**" ASO still performs well, suggesting that the antisense strand will benefit from the chemical modifications should it be used as the ssRNA. Once the active double-stranded RNAs were defined the next step was to test their single-stranded analogues. Initially, we decided to focus on one target sequence and the Lu(1742) site was chosen.

The three oligos **Lu1742.1**, **Lu1742.2** and **Lu1742.3** were compared when used as single-strands and in duplexes with a sense strand. The relative amount of firefly luciferase for each treatment was calculated by dividing the value of the luciferase readout (luciferase assay) by the amount of total protein (Bradford assay) present in the sample. This ratio will be used to examine the effectiveness of ASOs in inhibiting firefly luciferase throughout the remainder of this thesis. No KD activity was observed for the ssRNAs, however when annealed with the sense strand **Lu1742.sense**, all of them showed a very good decrease in luciferase activity (Figure 3.5).

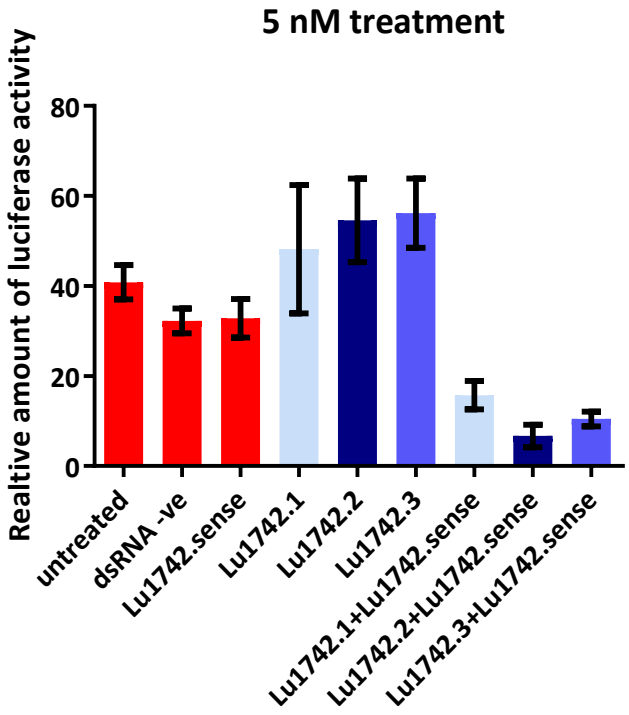


Figure 3.5. ***Ex vivo*** **inhibition of luciferase activity for treatments with ssRNAs in MDA-MB fluc cell line.** The relative amount of firefly luciferase is presented. Cells were treated with 5 nM of **Lu1742.1**, **Lu1742.2** and **Lu1742.3**. The double-stranded equivalent of each of the single-strands was used as a positive control. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: untreated – 3.081; dsRNA - 2.758; Lu1742.sense – 4.302; Lu1742.1 – 14.26; Lu1742.2 – 9.306; Lu1742.3 – 7.698; Lu1742.1+Lu1742.sense – 3.156; Lu1742.2+Lu1742.sense – 2.498; Lu1742.3+ Lu1742.sense – 1.628).

This experiment was repeated using different ASOs concentrations (up to 1 µM) without any success (data not shown). As ssRNAs are very prone to nuclease degradation, with very short half-lives that can be measured in seconds or minutes, more experiments were conducted to see if shorter treatment times might change these results. Unfortunately, none of the tested treatment times (5, 8, 16, 24 hours; data not shown) gave better results. The phosphodiester backbone is vulnerable to rapid degradation by nucleases and a lack of activity for

single-stranded ASOs is most likely due to the fact that, even if it contains a terminal 5'-phosphate and some phosphorothioate bonds, it provides the oligonucleotide with insufficient protection against degradation by nucleases.

The chemical modifications that were incorporated within the single-strands so far were not sufficient to make ASOs nuclease resistant. Thus different chemical modifications have to be applied to increase ssRNAs resistance.

3.1.4 2’-OMe modified ASO’s inhibition potency *ex vivo* by gymnosis

As we showed in the previous section, targeting the firefly luciferase with siRNAs at 50 nM gives up to 93% inhibition of the produced protein (Figure 3.3b). Attempts of the use of the ssRNAs were unsuccessful suggesting that chemical modifications increasing nuclease resistance are needed.

In this project we decided to inhibit protein production by cleaving the mRNA target rather than by stopping ribosome assembly. Interestingly in 2009 Mulders *et al.*[123] showed that the use of fully 2'-OMe modified ASOs caused degradation of a target, significantly reducing levels of mutant transcripts. This data suggested that there is an unknown mechanism, or the result is specific to the DM1 condition, as it has not been reported previously that the 2’-OMe phosphorothioate modified ASO is capable of degrading RNA transcripts. Therefore we decided to perform similar experiments in which we used the 2'-OMe modified oligonucleotides with phosphorothioate linkages to target firefly luciferase.

All treatments, unless stated otherwise, were for 48 hours. Three fully 2'-OMe modified oligonucleotides with phosphorothioate linkages were synthesised (Table 3.2) and tested at a range of concentrations. We decided to use these modifications as the 2'-OMe modification improves the binding affinity to the target whereas phosphorothioate linkages improve nuclease resistance. The initial experiments were performed in the absence of transfection reagents. This

was based on Stein's publication[139] showing that it is possible to take advantage of the cells' growth properties to efficiently deliver ASOs without the use of any extra components. This type of "naked" delivery is called gymnosis (1.4.4).

Table 3.2. Synthesised 2'-OMe modified oligonucleotides. All oligonucleotides are 21 nucleotides long, have 2'-OMe modification (blue) and phosphorothioate linkage ("s").

ASO ID	Structure 5'→ 3'	Length
Lu34.1	$G_sC_sU_s$ $C_sG_sA_s$ $G_sA_sU_s$ $C_sU_sG_s$ $A_sG_sU_s$ $C_sC_sG_s$ G_sU_sA	21
Lu132.2	$C_sC_sA_s$ $U_sG_sG_s$ $U_sG_sG_s$ $C_sU_sU_s$ $U_sA_sC_s$ $C_sA_sA_s$ C_sA_sG	21
Lu150.2	$U_sA_sA_s$ $U_sG_sU_s$ $U_sU_sU_s$ $U_sG_sG_s$ $C_sA_sU_s$ $C_sU_sU_s$ C_sC_sA	21
Lu294.1	$A_sC_sU_s$ $C_sG_sG_s$ $C_sG_sU_s$ $A_sG_sG_s$ $U_sA_sA_s$ $U_sG_sU_s$ C_sC_sA	21
Lu1004.1	$A_sA_sA_s$ $U_sA_sG_s$ $U_sG_sU_s$ $G_sG_sG_s$ $C_sA_sC_s$ $C_sA_sG_s$ C_sA_sG	21

The initial experiments were performed using ASOs at 100 nM. Due to the lack of luciferase inhibition more experiments were performed with increasing oligo concentration. As presented in Figure 3.6 even with very high 20 µM treatment, no KD of the target could be observed.

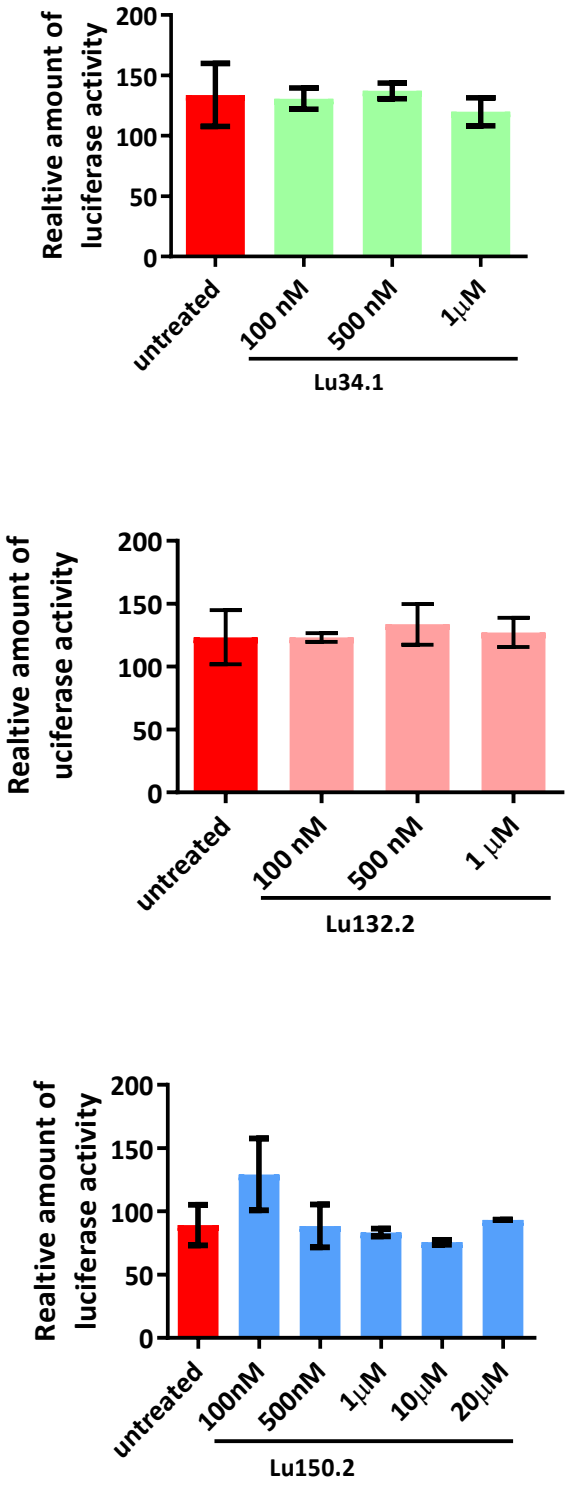


Figure 3.6. *Ex vivo* activity of the 2'-OMe modified ASOs in MDA-MB fluc cell line. A 48 hours treatment experiment was run for oligo **Lu34.1**, **Lu132.2** and **Lu150.2** to assess if any of the ASOs can down regulate amount of produced protein. The relative amount of firefly luciferase activity is presented for each ASO. Data is shown as mean with SD, number of biological replicas n = 3. (SD values as follows: Lu34.1: untreated – 26.05; 100 nM – 8.736; 500 nM – 6.508; 1 μM – 11.52. Lu132.2: untreated – 21.49; 100 nM – 3.426; 500 nM – 16.09; 1 μM – 11.67. Lu150.2: untreated – 16.05; 100 nM – 28.40; 500 nM – 16.95; 1 μM – 3.127; 10 μM – 1.897, 20 μM – 0.045). See 5.2.6 and 5.2.7 for experimental details.

The downstream sequence targeting oligos, **Lu294.1** and **Lu1004.1**, were also tested at the same concentrations. No down-regulation of the target protein could be observed (data not shown).

3.1.5 The effect of transfection reagent on 2'-OMe modified ASO's inhibition potency *ex vivo*

Lack of inhibition of the target raised a question of causation and it had been assumed that the MDA-MB fluc cells were able to uptake single-stranded ASOs. However, it is possible that the absence of KD was due to a lack of ASO delivery via gymnosis. Therefore repeat experiments were performed using transfection reagents. A polyethylenimine (PEI) transfection reagent called ExGen 500 was previously used to deliver 2'-OMe modified phosphorothioate oligonucleotides with good results[123] and as a result we decided to use it for our experiments.

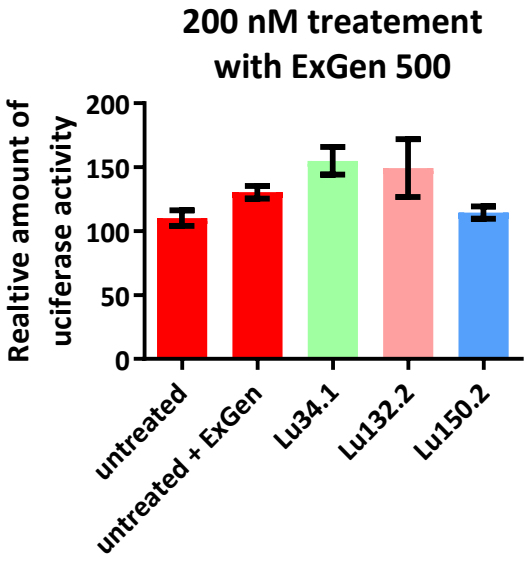


Figure 3.7. *Ex vivo* activity of the 2'-OMe modified ASOs transfected with ExGen500 in MDA-MB fluc cell line. Relative amount of firefly luciferase for 200 nM treatment of each oligo is presented. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: untreated – 6.091; untr.+ExGen – 4.908; Lu34.1 – 10.80; Lu132.2 – 22.57; Lu150.2 – 4.813).

Using ExGen 500 did not result in luciferase KD (Figure 3.7). Thus we investigated whether the oligonucleotides were being delivered to the interior of the cell. We compared three different oligo chemistries; 2'-OMe modified ASO, fully DNA substituted and fully morpholino substituted ASO, with Hex (Hexachloro-Fluorescein) and Cy3 (3'-Cyanine) fluorescent modification (see for 5.2.8 details). All three ASOs were not target-specific for firefly luciferase as they targeted the CTG expanded repeat (ASOs were made for DM1 studies). 2'-OMe and DNA ASOs are negatively charged and require the use of transfection reagents that will compensate for their charge, such as ExGen 500. The morpholino oligonucleotide is neutral and requires a different type of transfection method. For this reason the morpholino ASO is used as the negative control in this experiment. A typical 200 nM treatment was carried for 48 hours. After that time the medium containing ASOs was removed and cells were washed to remove any residual oligos. The cell nuclei were stained with Hoechst dye (blue stain Figure 3.8) and the fluorescence was measured on a Molecular Devices Micro plate reader. As can be seen in Figure 3.8 gymnosis barely produces any fluorescence in cells treated with 2'-OMe modified and DNA oligos and no fluorescence can be observed for morpholino. However, when the same treatment was performed with the addition of transfection reagent, ExGen 500, significant fluorescence was observed for both 2'-OMe and DNA ASOs and it is located mostly in the nuclei.

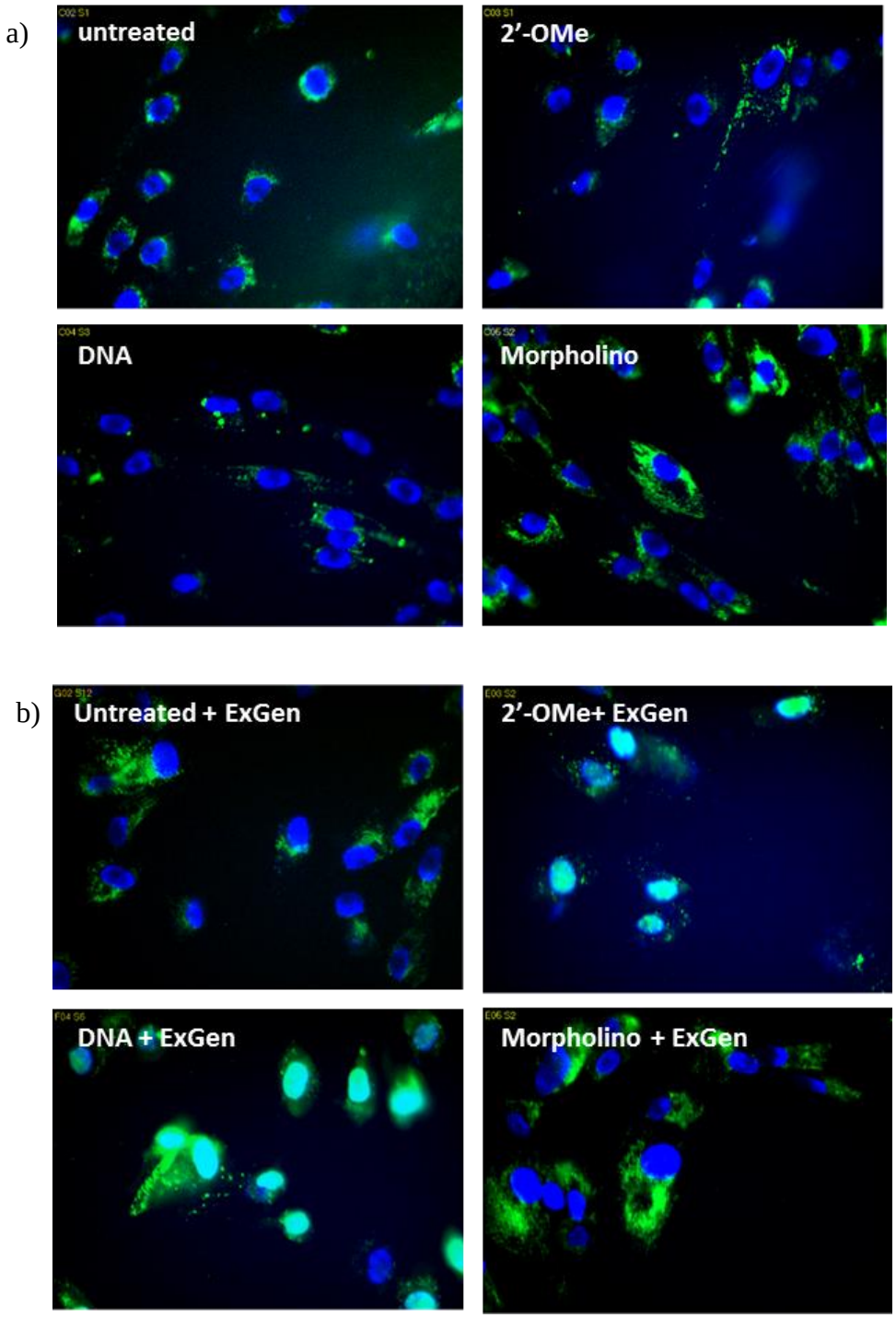


Figure 3.8. Distribution of fluorescently labelled oligonucleotides in MDA-MB fluc cell line. Two experiments were performed. Cells were incubated with ASOs for 48 hours followed by the detection of fluorescence. In first experiment (a) cells were transfected by gymnosis. Cell nuclei are stained in blue. In second experiment ExGen500 transfection reagent was used for the delivery (b). For the untreated cells and morpholino treated cells the green staining is due to the autofluorescence of cytoplasm.

To quantify the fluorescence, a modified version of the journal in MetaXpress software was run. Journal is a software module that performs automated analysis of the plate, according to user-specified parameters. 25 fields per well were

imaged and analysed. The nuclear area was identified by Hoechst stain and the intensity of fluorescence was determined by scoring adjacent pixels that were 80 greyscales or more above the background. Data were analysed in excel spreadsheet and are presented below (Figure 3.9).

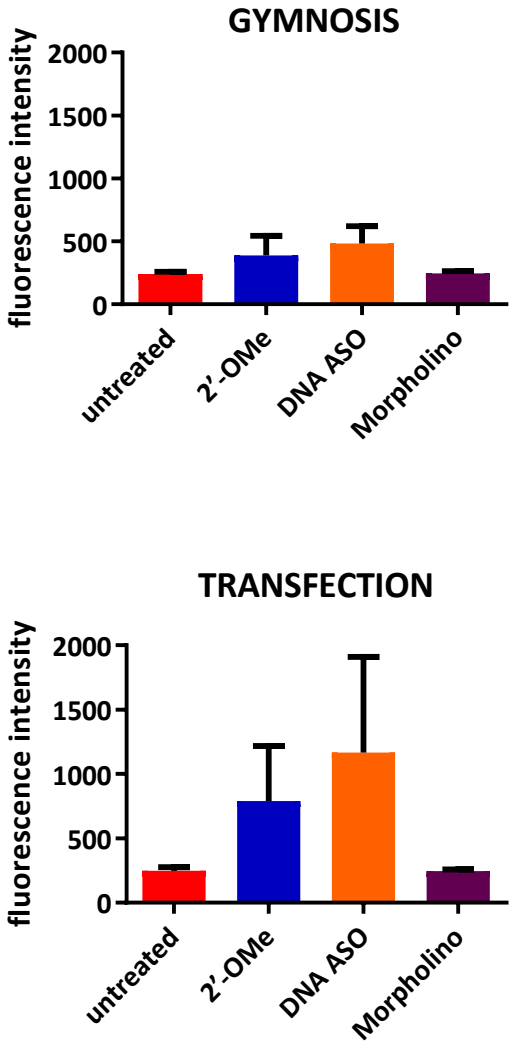


Figure 3.9. Quantification of the fluorescence measured across the cell. To confirm what was seen in photographs the Journal was run to quantify the intensity of fluorescence across the cells. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows, gymnosis: untr. – 18.09; 2'-OMe – 153.7; DNA – 136.1; Morpholino – 19.11. Transfection: untr. – 30.05; 2'-OMe – 428.4; DNA – 745.2; Morpholino – 12.69).

In the treatment using gymnosis, only 1.5-2.0 fold increase in the ASO uptake was observed, for both 2'-OMe and DNA oligos compared to untreated cells. However, when the transfection reagent was added to the treatment, the

fluorescence increased around 3.0 fold for 2'-OMe and 4.8 fold for DNA oligos. Results from journal quantification confirmed what was shown in Figure 3.8.

Thus although the ASOs are delivered into the cell they do not down-regulate expression of luciferase protein. For that reason it was decided to design, synthesise and test single-stranded ASOs that may work through another mechanism, the RNA interference (RNAi).

3.1.6 Design and development of vinylphosphonate modified single-stranded ASOs

As explained in 1.2.2.2 single-stranded RNAs have to be protected internally and on their tails to increase their nuclease resistance. 2'-OMe and 2'-F nucleotides provide good internal protection, but it is more difficult to find a 5'-end protection that will be not only stable, but will also ensure that the ssRNA is properly processed by RISC. Lima *et al.* showed in their publication[97] that 5'-*(E)*-vinylphosphonate (**3**) (Figure 3.10b) is more stable than a 5'-phosphate (**31**) (Figure 3.10a) modification. The reason for this is the introduction of a vinyl group in place of the C5'-O5' single bond. This is important because in contrast to the 5'-phosphate, the phosphonate group cannot be cleaved from the oligo. Additionally a new carbon-carbon double bond has a similar geometry to a natural phosphodiester linkage, so the conformation is not affected.

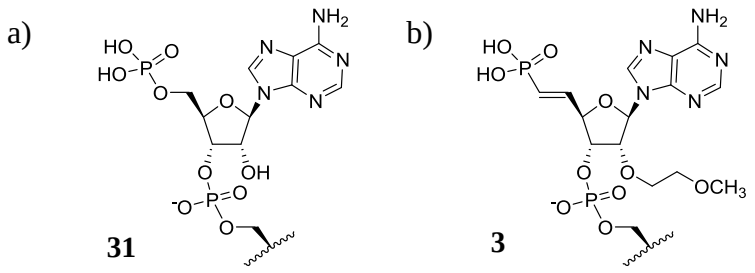


Figure 3.10. The 5' design of the ssRNAs. Naturally occurring ssRNAs are usually phosphorylated on their terminal nucleotide on the 5'-end (a). Lima proposed to use the 5'-*(E)*-vinylphosphonate modification (b), which mimics the phosphate group, to ensure protection to the nucleases resulting from the presence of the double bond.

As shown in section 1.2.2.2 synthesis of the phosphoramidite that was used in Lima's publication is rather complicated and the protecting groups of the 5'-hydroxyl group are not compatible with standard deprotection protocol.[99] That makes handling of the oligonucleotide harder compared to commercially available phosphoramidites. Moreover the phosphoramidite can only be coupled as the terminal nucleotide, due to the lack of a DMTr protecting group, that excludes its use in any other position. The Hayes group has been working on the synthesis of the *(E)*-vinylphosphonate modified DNA and RNA dimers[142, 147, 150] but has never used them as the protection of the ASOs. The advantage of the Hayes dimers (**35**) is that they are fully suitable for the solid phase synthesis by the phosphoramidite coupling and can be placed at any position of the oligonucleotide. With that knowledge we wondered if we could replace the 5'-*(E)*-vinylphosphonate proposed by Lima with the *(E)*-vinylphosphonate backbone modification moved one nucleotide down (**34**) in our vinylphosphonate linked dimer (with 5'-terminal hydroxyl group). As shown in Figure 3.11, our proposal was to use DNA nucleotides (**33**) instead of RNA (**32**) that would significantly simplify synthesis and handling.

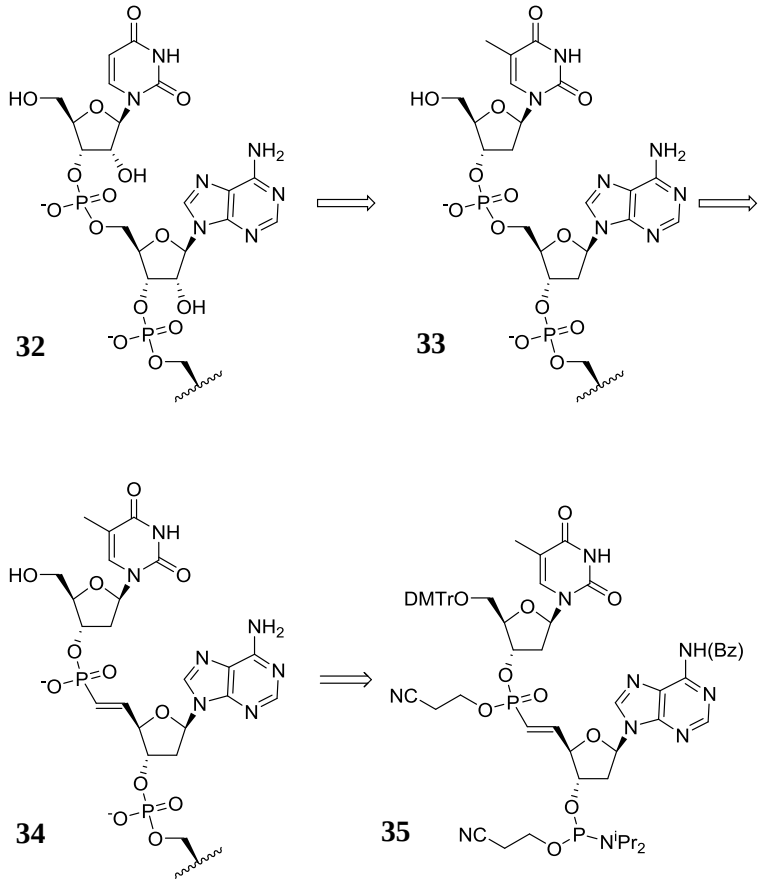


Figure 3.11. Schematic illustration of proposed pathway towards desired *(E)*-vinylphosphonate. Instead of using complicated synthesis of nucleotides with terminal 5'-*(E)*-vinylphosphonate, we suggested using dinucleotides linked by the vinyl group. First choice would be an RNA with incorporated *(E)*-vinylphosphonate, however it is difficult to make, therefore we proposed the use of easier accessible DNA dimer, that also contains vinyl group.

The synthesis of the phosphoramidite **35** is described in 1.3.3. **35** was coupled to the rest of the oligonucleotide in the last step of synthesis on the ABI DNA synthesizer. After deprotection and purification a ssRNA with dinucleotide **34** on its 5'-end was obtained. **34** is a vinylphosphonate-linked DNA dinucleotide and was predicted to increase ssRNAs stability and protect them from degradation by nucleases. Although the vinyl group is not present at the terminal nucleotide it is very likely that once the ssRNA is inside the cell, the terminal nucleotide with no protection on 5'-end would be cleaved by nucleases, resulting in the production of a terminal 5'-*(E)*-vinylphosphonate in the ssRNA that is one nucleotide shorter. Alternatively the 5'-hydroxyl group on the terminal

nucleotide can be phosphorylated inside the cell by the cellular kinases (Figure 3.12). A possibility to chemically phosphorylate the 5'-hydroxyl group of **34** using a phosphorylation reagent during oligo synthesis was considered, but eventually turned out to be unnecessary. Moreover, it has been shown that for the siRNAs the first three and last five nucleotides do not contribute much towards the binding to the target and the central regions are more important,[220] therefore even if one nucleotide is lost it should not affect effectiveness of the oligonucleotide.

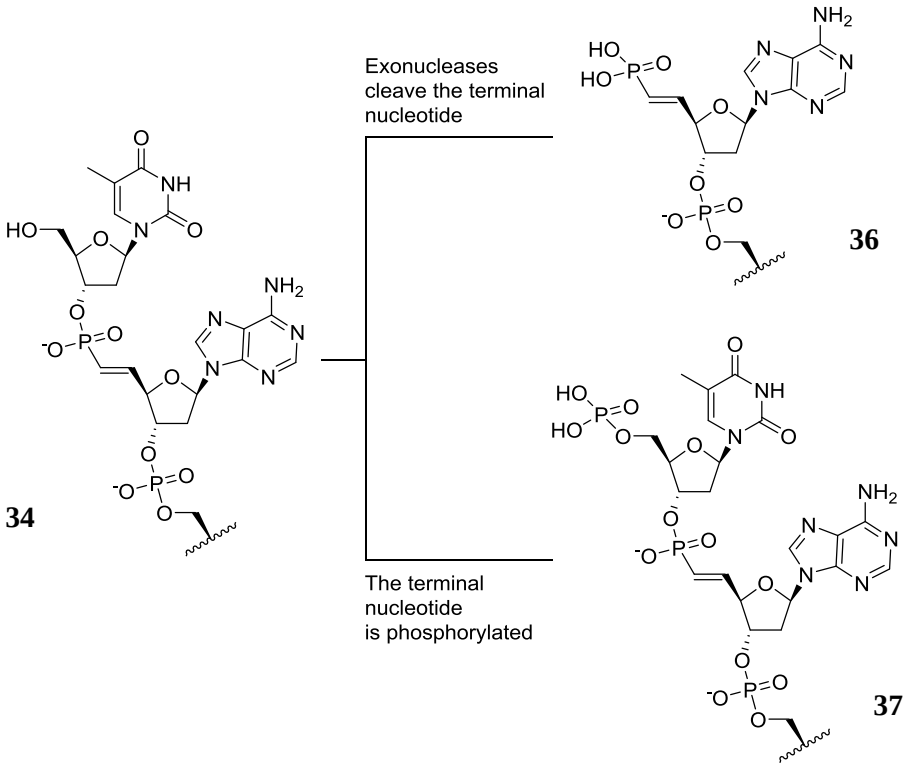


Figure 3.12. Schematic picture of how vinylphosphonate-linked DNA dinucleotide can be processed in the cell. We hypothesised that once the ASO is inside the cell the *(E)*-vinylphosphonate dinucleotide (**34**) will be either partially digested by nucleases what results in loss of the terminal nucleotide and production of ASO that is shorter, but now has the 5'-*(E)*-vinylphosphonate modification (**36**). Or the terminal nucleotide in dimer can be phosphorylated on its 5'-end (**37**).

As described in section 1.2.2.2 RNAi is triggered by siRNA, which consist of two complementary strands of RNA. The modification we want to introduce to our ASOs is a DNA dimer. For that reason it had to be tested first if the addition

of DNA at the 5'-end of the antisense strand is still applicable in the RNAi mechanism. Therefore a range of oligonucleotides was designed and synthesised. ASO **Lu150.3** is an unmodified RNA with a 2 base mismatch on the 3'-end. Oligo **Lu150.4** is similar to **Lu150.3**, with one alteration, the last two nucleotides on the 5'-end were changed from RNA to DNA. Comparison of treatments with **Lu150.3** and **Lu150.4** will provide the answer as to how addition of DNA to the RNA influences the KD efficiency. Finally ASO **Lu150.5** has the vinylphosphonate-linked DNA dinucleotide incorporated in its sequence. Two additional ASOs (**Lu150.6** and **Lu150.7**) were made and they are analogues of **Lu150.4** and **Lu150.5**, but have seven phosphorothioate backbones instead of having only phosphodiester bonds. Due to the restrictions of vinylphosphonate dimer sequences available, we had to abandon the Lu1742 site and choose other one. The last two nucleotides in the ASO targeting Lu150 site are UA and they are compatible with a dimer we had already made the T*A (**34**), therefore it was decided to work with this target.

Table 3.3. The synthesised single-stranded RNAs with gradual introduction of DNA nucleotides. Black stands for unmodified RNA, underlined – AA overhangs, "s" phosphorothioate linkage, DNA nucleotides are marked in red, DNA dinucleotide linked by the 5'-*(E)*-vinylphosphonate is labelled with * (T*A). All nucleotides are 23 bp long. Calculated and observed masses are presented in 5.3.2

ID	Structure 5' → 3'	Length
Lu150.3	UAA UGU UUU UGG CAU CUU CCA AA	23
Lu150.4	TAA UGU UUU UGG CAU CUU CCA AA	23
Lu150.5	T*AA UGU UUU UGG CAU CUU CCA AA	23
Lu150.6	TAA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	23
Lu150.7	T*AA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	23
Lu150.sense	UGG AAG AUG CCA AAA ACA UUA AA	23

3.1.7 *Ex vivo* inhibition potency of RNA with vinylphosphonate modified 5' head

The oligonucleotides listed in Table 3.3 were tested as double strands in the model system (Figure 3.13). As can be seen below all dsRNAs produced significant KD of the targeted firefly luciferase, which constituted a very encouraging starting point to test all of the prepared ASOs as single strands at a wide range of concentrations.

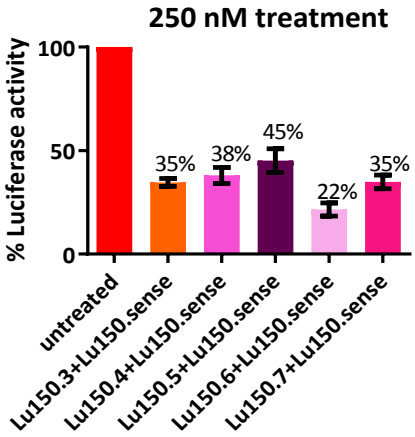


Figure 3.13. *Ex vivo* activity of modified dsRNAs at 250 nM. The inhibition of luciferase activity was measured for oligos consisting of the same sense strand and gradually modified antisense strands (ASOs are presented in Table 3.3). Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: Lu150.3+Lu150.sense – 1.887; Lu150.4+Lu150.sense – 3.859; Lu150.5+Lu150.sense – 5.682; Lu150.6+Lu150.sense – 3.179; Lu150.7+Lu150.sense – 3.275).

Of the oligonucleotides tested, the two containing *(E)*-vinylphosphonate modification (**Lu150.5** and **Lu150.7**), showed satisfactory KD levels of a target when used as double-stands. Although, they did not show the best efficacy among dsRNAs used, we expected that the modifications used would have a pronounced effect when used as single-strands. This assumption was based on Lima *et al*. observations.[97] Therefore ASOs **Lu150.5** and **Lu150.7** were tested as ssRNAs in a range of concentrations. Unfortunately, neither of them showed

any inhibition of luciferase (Figure 3.14). This suggested that additional modifications were required to stabilise the ssRNAs *ex vivo*.

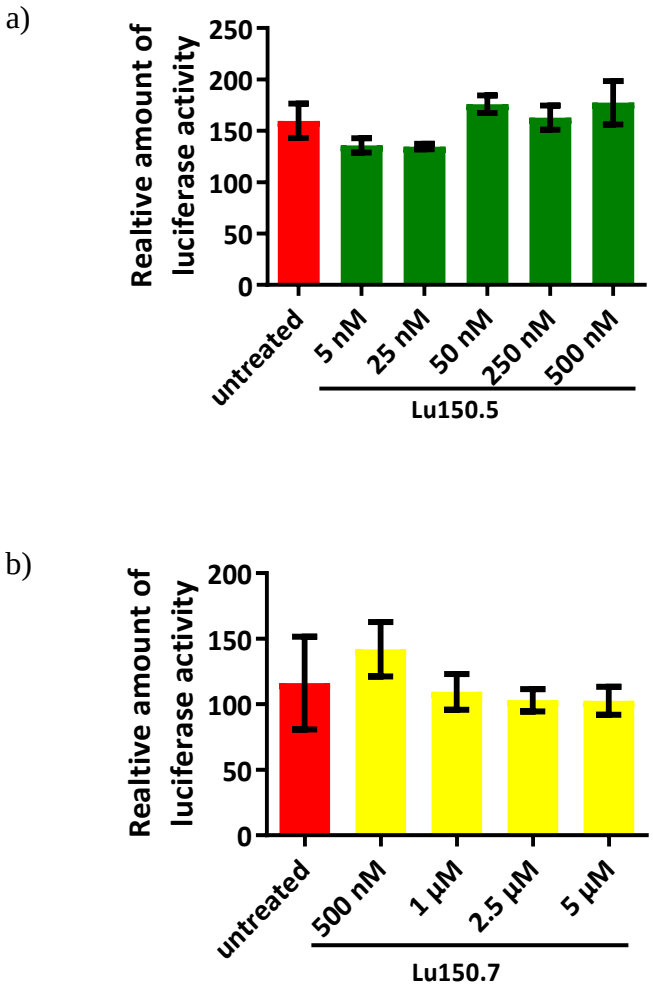


Figure 3.14. *Ex vivo* activity of ssRNAs containing the vinylphosphonate-linked DNA dinucleotide. Two ASOs containing the vinylphosphonate-linked DNA dinucleotide, **Lu150.5** (a) and **Lu150.7** (b), were tested in the range of concentrations and the firefly luciferase activity was measured. ASO **Lu150.7** was additionally modified with seven phosphorothioate backbones at the 3'-tail. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows, Lu150.5: untr. – 16.72; 5 nM – 7.024; 25 nM – 2.639; 50 nM – 8.499; 250 nM – 11.62; 500 nM – 21.00. Lu150.7: untr. – 35.26; 500 nM – 20.66; 1 µM – 13.61; 2.5 µM – 8.450; 5 µM – 10.62).

3.1.8 Introduction of further modifications towards effective silencing of ssRNAs

The above results showed that protecting ssRNA only on its ends might be not sufficient and an additional internal protection has to be used. It has been reported that 2'-OMe (Figure 1.15) and 2'-F (Figure 1.17) modifications are well

tolerated in ssRNAs and significantly improve their stability, while resulting in good KD of targets at very low concentrations.[80] Therefore another set of oligonucleotides (Table 3.4) was synthesised according to the design of Lima[97] and tested in cells. There are two easily accessible commercial, fluorinated RNA phosphoramidites: 2'-F-dU and 2'-F-dC, thus only these two could be used in our oligonucleotides. In the first attempt only 2'-F-dU was incorporated within the sequence. **Lu150.8** was designed so that every second nucleotide was 2'-F modified to include as many modified oligonucleotides as possible (6th, 14th, 16th, 18th and 20th position counting from oligo's 3'-end). Both treatments presented below were conducted for around 72 hours.

Table 3.4. The synthesised single-stranded RNAs with the introduction of DNA and fluorinated and methylated nucleotides. Black stand for unmodified RNA, green - 2'-F and blue - 2'-OMe modified nucleotides, underlined – AA overhangs, "s" phosphorothioate linkage, DNA nucleotides are marked in red, DNA dinucleotide linked by the *(E)*-vinylphosphonate is labelled with * (T*A)). Calculated and observed masses are presented in 5.3.2.

ID	Structure 5' → 3'	Length
Lu150.8	T*AA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	23
Lu150.9	T*AA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	23
Lu150.sense	UGG AAG AUG CCA AAA ACA UUA AA	23

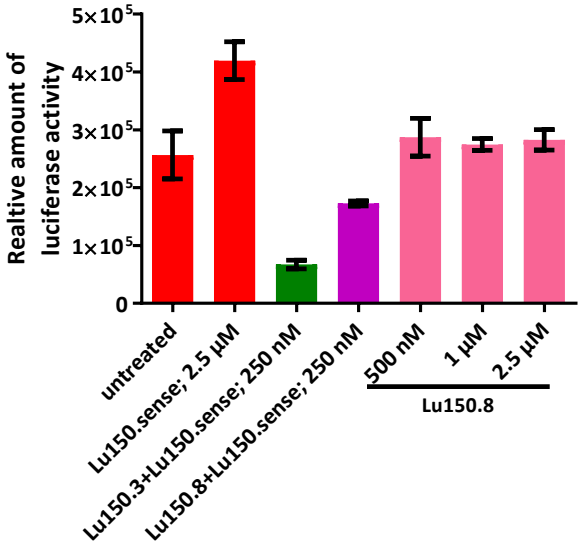


Figure 3.15. *Ex vivo* activity of the ssRNA Lu150.8 with the DNA *(E)*-vinylphosphonate dinucleotide and the 2'-F and 2'-OMe modifications (Table 3.4). The relative amount of firefly luciferase after treatment with single-stranded Lu150.8 is presented. Two siRNAs, one containing unmodified antisense strand (Lu150.3+Lu150.sense, Table 3.3) and one composed of tested modified antisense strand Lu150.8 (Lu150.8+Lu150.sense) were used as positive control. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: untr. - 41475; Lu150.sense - 32644; Lu150.3+Lu150.sense – 7452.0; Lu150.8+Lu150.sense – 4336.0; Lu150.8: 500 nM - 32457; 1 µM - 10261; 2.5 µM - 17708).

Introduction of five fluorinated nucleobases into ASO sequence was not successful, with no KD of the target being observed (Figure 3.15). It is interesting that when **Lu150.8** is annealed with its sense strand it gave less KD than a positive control "**Lu150.3**+ **Lu150.sense**" (unmodified sense and antisense strands used as a duplex).Therefore we further investigated whether this could be due to the motif of seven continuous nucleotides with a 2'-OMe modification. To identify whether interrupting this motif with additional fluorinated nucleotides could help efficacy, another ASO **Lu150.9** that has one more 2'-F modified nucleotide (9^{th} position counting from ASO's 3'-end) was synthesised and tested.

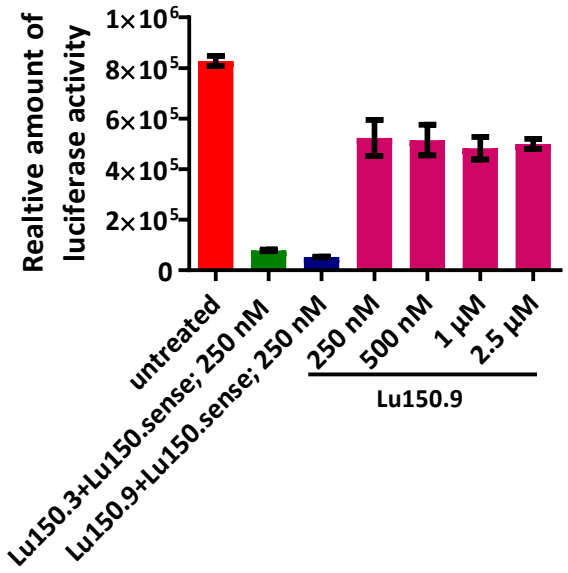


Figure 3.16. *Ex vivo* activity of the ssRNA Lu150.9 with the DNA *(E)*-vinylphosphonate dinucleotide and the 2'-F and 2'-OMe modifications. ASO **Lu150.9** with one more fluorinated nucleotide, compared to **Lu150.8**, was synthesised and tested. Two siRNAs, **Lu150.3+Lu150.sense** and **Lu150.9+Lu150.sense**, were used as positive control. Treatment with **Lu150.9** at 250 nM gave 37% KD of the firefly luciferase. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: untr. - 19549; Lu150.3+Lu150.sense – 2991.0; Lu150.9+Lu150.sense – 1267.0; Lu150.9: 250 nM - 71175; 500 nM - 60521; 1 µM - 44394; 2.5 µM - 19543).

When **Lu150.9** was annealed with its sense strand it produced a 94% KD of firefly luciferase. Additionally, for the first time, a single-stranded oligo showed inhibition of the target across the whole range of concentrations used (Figure 3.16). As can be seen there is not a significant difference in inhibition between 250 nM (37% KD) and 2.5 µM (40% KD) treatment. Most likely more transfection reagent has to be used for higher concentrations of ASOs to neutralise their negative charge.

Next we investigated whether the introduction of more 2'-fluorinated nucleotides improves the oligo performance. Therefore another ASO was synthesised with an additional two 2'-F deoxycytidines (Table 3.5).

Table 3.5. Additional ssRNA that was synthesised. Black stands for unmodified RNA, 2'-F and 2'-OMe modified nucleotides, underlined – AA overhangs, "s" phosphorothioate linkage, DNA nucleotides are marked in red, DNA dinucleotide linked by the 5'-*(E)*-vinylphosphonate is labelled with * (T*A)). Calculated and observed masses are presented in 5.3.2.

ID	Structure 5' → 3'	Length
Lu150.10	T*AA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	23
Lu150.sense	UGG AAG AUG CCA AAA ACA UUA AA	23
-ve.1	AAU UCU CCG AAC GUG $U_sC_sA_s$ $C_sG_sU_s$ A_sA	21

Our observation was that treatment with **Lu150.10** (Figure 3.17) gave similar KD to treatment with **Lu150.9** (Figure 3.17), both at 250 nM. ssRNA **Lu150.10** has two more fluorinated nucleotides than **Lu150.9**, but it did not improve its efficacy. Although fluorinated phosphoramidites are commercially available they are more expensive than the other modifications we used in this study and for that reason we decided to focus on ssRNA **Lu150.9**, that has six rather than eight fluorinated nucleotides in future experiments, to reduce the cost of synthesis.

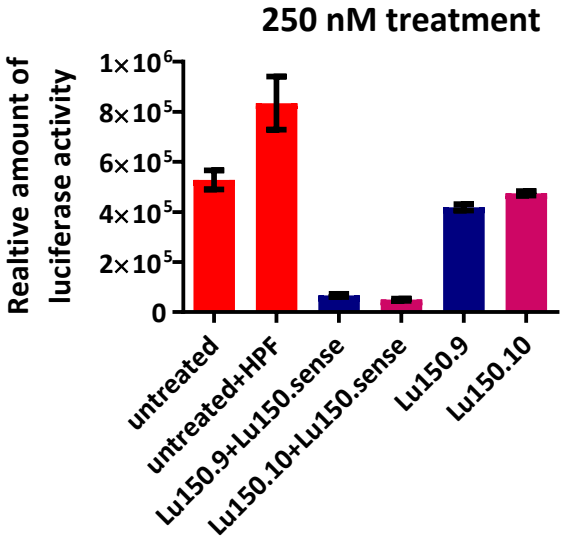
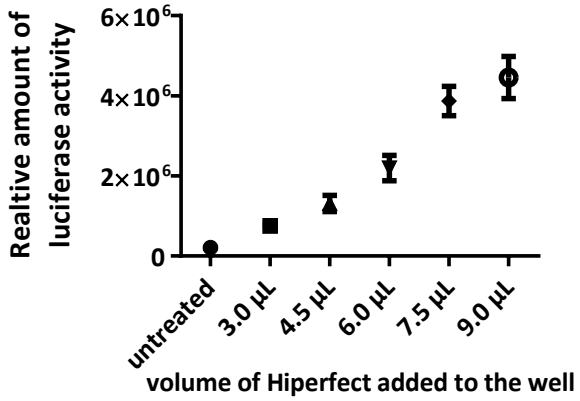


Figure 3.17. Comparison of firefly luciferase KD after treatment for Lu150.9 and Lu150.10 at 250 nM. Two single-stranded ASOs, **Lu150.9** and **Lu150.10** (**Table 3.4** and **Table 3.5**) were tested to check if adding two more fluorinated nucleotide, in ASO **Lu150.10** compared to **Lu150.9**, improves its activity. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: untr. – 38296; untr.+HPF - 106096; Lu150.9+Lu150.sense – 6044.0; Lu150.10+Lu150.sense – 4780.0; Lu150.9 - 13085; Lu150.10 – 8667.0).

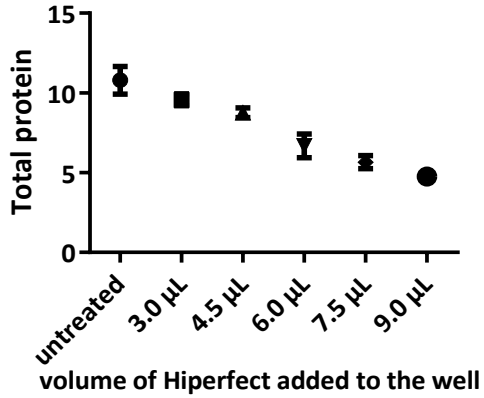
3.1.8.1 Effect of transfection methods on the luciferase expression

Interestingly in some experiments we observed that cells treated with transfection reagent only (no nucleic acid added) exhibit higher luciferase activity than untreated cells. To explore this further we decided to assess how different amounts of HPF influences luciferase activity. Therefore we set up an experiment where MDA-MB fluc cells were treated with different volumes of the aforementioned transfection reagent (Figure 3.18).

a)



b)



c)

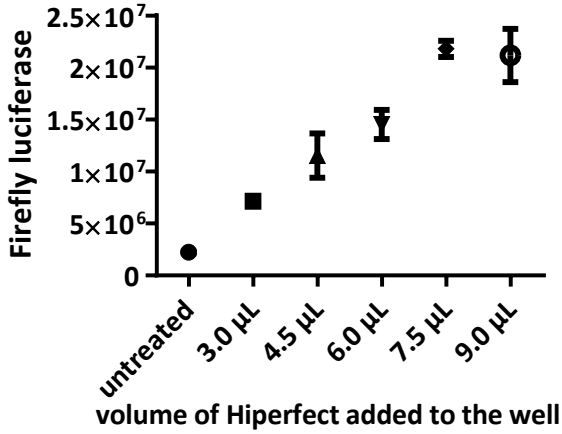


Figure 3.18. HPF influence on firefly luciferase activity. a) Relative amount of firefly luciferase for different volumes of HPF. This experiment suggests that the transfection reagent increases the luminescent signal from the firefly luciferase. b) Amount of total protein for all samples was also measured. Toxicity effect to the cells can be seen with increasing volume of HPF. c) Non normalised luciferase activity for all samples. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: panel a: untr. – 9708; 3.0 µL - 35620; 4.5 µL - 201220; 6.0 µL - 317343; 7.5 µL - 367279; 9.0 µL - 525495. Panel b: untr. – 0.8649; 3.0 µL – 0.3707; 4.5 µL – 0.306; 6.0 µL – 0.7367; 7.5 µL – 0.4143; 9.0 µL – 0.1457. Panel c: untr. – 75655; 3.0 µL - 487832; 4.5 µL - 2120562; 6.0 µL - 1399252; 7.5 µL - 781688; 9.0 µL - 2556333).

As can be seen above the transfection reagent we use significantly affects luciferase readout. The more reagent used the higher the luciferase signal. Even in the case of high doses of HPF, when fewer cells were present after treatment (Figure 3.18b shows that the amount of total protein decreases with increasing HPF dose), the luciferase activity (Figure 3.18c) still increases. We do not know what is responsible for this since we did not investigate it further, but we have found reports of similar problems in the literature. Raof *et al.* demonstrated in their study that by using the PEI transfection reagent a significant change in gene transcriptome was detected.[221] The HAoSMCs cells were treated either with PEI or PEI in combination with the control siRNA and in both cases alterations in gene expression was observed, mostly due to the inflammatory and immune responses.[221] Interestingly, it was also shown that polyamines significantly enhance the transcriptional activity of promoters and as the result the expression of the reporter genes is increased.[222] It is likely that the similar effect is caused by the use of the HPF in our study, suggesting that having appropriate controls is very important.

To overcome the issue connected with the use of transfection reagents, we decided to synthesise a scrambled oligonucleotide (**-ve.1**) to determine how treatment with "transfection reagent + modified ASO" affects luciferase activity. A negative control ASO was obtained from a commercial source (Qiagen, see 5.3). We predicted that when nucleic acid is present in cell culture the transfection reagent should be neutralised and no negative, artificial influence should be observed. That provides exactly the same conditions for all of our treatments and eliminates possible influence of transfection reagent on the results. Additionally cells treated with scrambled oligos are then a good

reference when a KD is measured. Another experiment was performed to assess our hypothesis (Figure 3.19).

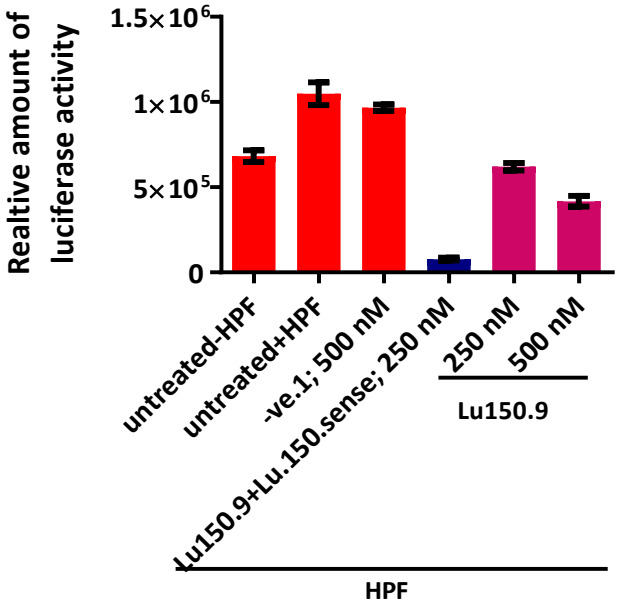


Figure 3.19. Effect of chemically modified scrambled oligonucleotide -ve.1 on firefly luciferase signal. Due to the effect of transfection reagent at luciferase signal a scrambled oligonucleotide was synthesised to provide a reference for comparison of different treatments. Oligo **-ve.1** and **Lu150.9** were tested and the relative amount of firefly luciferase is presented. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: untr.-HPF - 34752; untr.+HPF - 67024; -ve.1 - 19627; Lu150.9+Lu150.sense – 8044.0; Lu150.9: 250 nM - 21750; 500 nM - 31932).

As can be seen above the "relative amount of luciferase activity" is higher for cells that had only a transfection reagent or scrambled oligonucleotide **-ve.1**, when compared to untreated cells. However, cells that were treated with **-ve.1** or had only HPF on them show similar levels of relative luciferase. This shows that HPF has an effect on luciferase signal and it is necessary to use a reference that mimic the actual experiment more accurately than the untreated cells, in order to calculate the KD value. Therefore we decided that in subsequent experiments a "scrambled ssRNA treatment" should be used for comparison.

The amount of transfection reagent needed to get efficient KD for dsRNA at 250 nM had been previously optimised. We decided to perform our future

experiments for ssRNAs at 500 nM (which corresponds to the same amount of nucleic acid in 250 nM of dsRNA). Therefore the same amount of HPF could be used in both cases and any artificial effects from the transfection reagent are reduced.

To examine whether the observed effect is specific to HPF transfections two other methods were also tested. One of most widely used transfection reagents for delivery of both RNA and DNA is Lipofectamine 2000. In contrast to the HPF, which is a dendrimeric form of polyethylenimine, the Lipofectamine 2000 is blend of cationic liposomes. Although there is a difference in chemical composition both reagents work similarly, and they neutralise the negative charge of the ASO, make it more lipophilic and as a result transport the ASO through the cell membrane. To test whether Lipofectamine 2000 also interferes with the firefly luciferase expression, different volumes of the transfection reagent were added to the MDA-MB fluc cells and the plate was incubated for two days at 37 °C. After protein extraction and sample analysis the same result was observed. As shown in Figure 3.20 the higher the volume of Lipofectamine used, the higher the relative amount of detected luciferase.

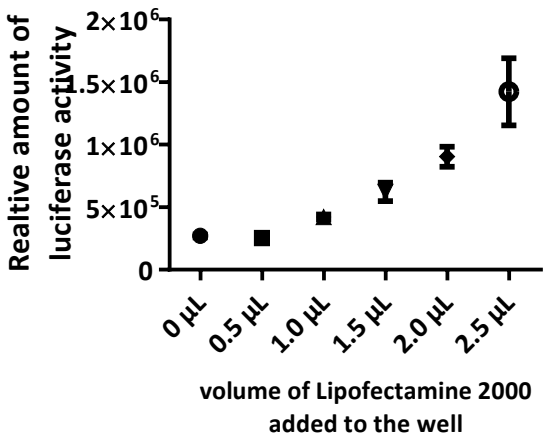


Figure 3.20. Lipofectamine 2000 influence on firefly luciferase activity. Relative amount of firefly luciferase for different volumes of reagent. This experiment suggests that the transfection reagent influences firefly luciferase signal. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: 0 µL - 12348; 0.5 µL - 14625; 1.0 µL - 20113; 1.5 µL - 73837; 2.0 µL - 80856; 2.5 µL - 267908).

Thus, we sought to examine the effect of other transfection reagents. We decided to test electroporation as a delivery method in which exposing cells to an electrical field the permeability of the cell membrane is increased, which allows the ASO to enter the cell. Electroporation does not use any carrier, the cells are only resuspended in salt solution and treated with an electric pulse. Surprisingly exactly the same phenomenon was observed and cells that had no nucleic acid added showed relative luciferase levels 68 times higher than untreated cells (Figure 3.21). Also, for cells with added scrambled dsRNA, the amount of luciferase was much higher compared to untreated cells. For cells treated with dsRNA to target firefly luciferase, the readout was much lower than for other samples. This suggests that when the level of KD is calculated it has to be compared to the "scrambled oligo + delivery reagent", and not untreated cells, since it mimics the experiment better and provides exactly the same conditions across the whole experiment.

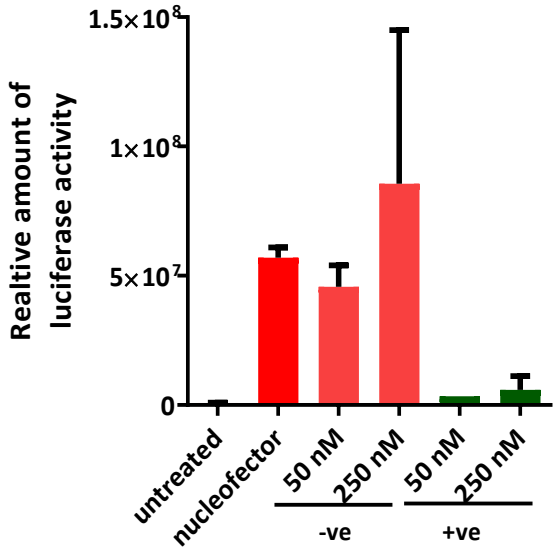


Figure 3.21. The effect of electroporation on firefly luciferase activity. Scrambled and luciferase targeting ASOs were tested at two different concentrations, 50 nM and 250 nM. The relative amount of firefly luciferase is presented. Data is shown as the mean with SD, number of biological replicas n = 2. (SD values as follows: untr. - 31742; nucleofector - 3966501; -ve: 50 nM - 8309424; 250 nM - 59366373; +ve: 50 nM - 2836; 250 nM - 5341763).

3.1.9 Firefly luciferase knock-down by chemically modified single-stranded ASOs

Thus, having established a significant KD with modified ssRNA, we set out to investigate which modification is responsible for this success; the 5'-*(E)*-vinylphosphonate modification or the absence of unmodified RNA in the ASO's sequence. To assess the effect of the aforementioned modifications we made stepwise changes to the ASO sequence (Table 3.6).

Table 3.6. Further analysis of ssRNAs tested for firefly luciferase KD. Black stand for unmodified RNA, 2'-F and 2'-OMe modified nucleotides, underlined – AA overhangs, "s" phosphorothioate linkage, DNA nucleotides are marked in red, DNA dinucleotide linked by the 5'-*(E)*-vinylphosphonate is labelled with * (T*A)). Calculated and observed masses are presented in 5.3.2.

ID	Structure 5' → 3'	Length
Lu150.3	UAA UGU UUU UGG CAU CUU CCA AA	23
Lu150.4	TAA UGU UUU UGG CAU CUU CCA AA	23
Lu150.11	TAA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	23
Lu150.12	TAA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	23
Lu150.9	T*AA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	23
Lu150.sense	UGG AAG AUG CCA AAA ACA UUA AA	23
-ve.2	AAU UCU CCG AAC GUG $U_sC_sA_s$ $C_sG_sU_s$ A_sA	23
-ve.sense	ACG UGA CAC GUU CGG AGA AUU AA	23

Initially we conducted a 3 day treatment experiment where all oligonucleotides were annealed with the same sense strand and tested as dsRNAs (Figure 3.22).

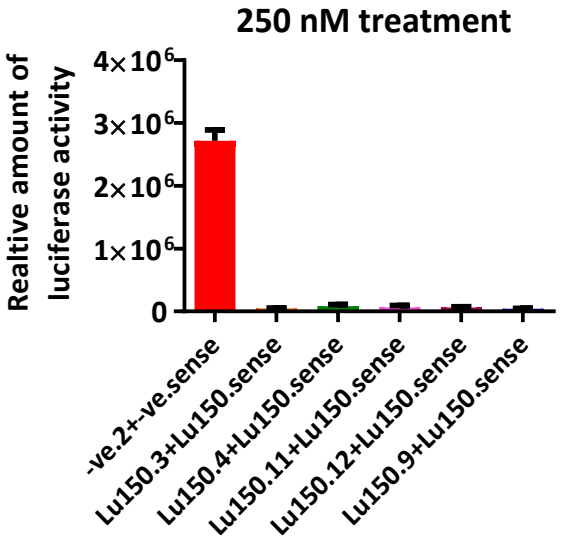


Figure 3.22. dsRNAs activity *ex vivo* in a 3 day treatment. ASOs presented in Table 3.6 were annealed with the sense strand and tested as dsRNAs to investigate the effect of chemical modifications on dsRNAs performance. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: -ve.2+-ve.sense - 171432; Lu150.3+Lu150.sense - 4150; Lu150.4+Lu150.sense - 24582; Lu150.11+Lu150.sense - 29398; Lu150.12+Lu150.sense - 9082; Lu150.9+Lu150.sense - 2950).

When oligonucleotides were tested as double strands, irrespective of the various chemical modifications used, we found they all significantly inhibited luciferase production compared to the negative control, giving 97%-98% KD ($F(5,12)=682.7$, $p<0.0001$). No significant difference between subsequent oligonucleotides was observed. To further investigate how chemical modifications improve ssRNAs activity and stability a 3 day treatment with ssRNAs at 500 nM concentration was performed (Figure 3.23).

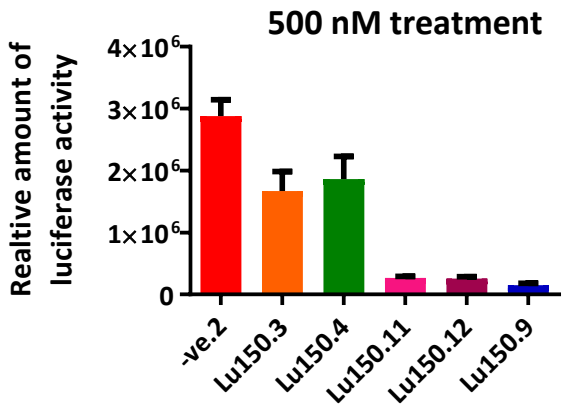


Figure 3.23. ssRNAs activity *ex vivo* in a 3 day treatment. ssRNAs from Table 3.6 were tested to determine whether chemical modifications change their effectiveness in knocking-down the target protein. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: -ve.2 - 265607; Lu150.3 - 314290; Lu150.4 - 365925; Lu150.11 - 25930; Lu150.12 - 27340; Lu150.9 - 33277).

As shown in Figure 3.23, an improvement in oligonucleotide efficacy can be seen when more chemical modifications are present in the sequence. Our first observation is that the biggest enhancement in ssRNAs activity results from the use of 2'-OMe and 2'-F modified nucleotides and this is most probably due to the increased stability of the oligonucleotide. This statement is based on the difference that can be observed in KD of protein activity for cells treated with **Lu150.3** and **Lu150.11**. Treatment with **Lu150.3** gave 42% KD whereas with **Lu150.11** the luciferase activity was reduced by 91% ($p<0.0001$). Oligonucleotide **Lu150.3** is an unmodified RNA with phosphodiester

backbones whereas **Lu150.11** has seventeen 2'-OMe modified nucleotides, six fluorinated uridines and both phosphodiester and phosphorothioate backbones. This difference in structure of both oligonucleotides influences their stability and also potency to be more effective gene inhibitors. Next, the effect of the addition of DNA with or without the *(E)*-vinylphosphonate modification was investigated. The exchange of two 2'-OMe RNA-like bases for two DNA bases (thymidine and deoxyadenosine instead of uridine and adenosine) did not interfere with RNAi and did not decrease effectiveness. ssRNA **Lu150.4** gave 35% of KD whereas **Lu150.3** gave 42%. However when one-way ANOVA and a *post-hoc* test was applied no significant difference was found. The same happened when treatments with **Lu150.11** and **Lu150.12** were compared, both inhibited luciferase production by 91%. **Lu150.11** and **Lu150.12** have the same modifications apart from the last two bases on the 5' end are DNA not 2'-OMe modified. This indicates that DNA can be used in single-stranded oligonucleotides without any loss of efficacy. The main aim of this study was to evaluate whether the incorporation of the DNA dinucleotide, linked by the *(E)*-vinylphosphonate, to the ssRNA sequence improves its activity. Therefore, treatment with **Lu150.9** was compared to the treatment with **Lu150.12**. Treatment with **Lu150.9** gave 95% KD when treatment with **Lu150.12** gave 91% KD, however no significant difference was observed ($p>0.05$). Although the difference is not big and not significant, it has to be considered that those oligos are designed to be used in the *in vivo* system. As showed by Lima stable phosphate analogues at the 5'-end were essential for the ssRNA performance in mice, not in the tissue culture.[97] Therefore we hypothesise that greater difference between ASOs containing the *(E)*-vinylphosphonate should be observed when

oligos will be tested in animal model. Moreover the difference in KD values may increase when a longer treatment time is used.

To test the above hypothesis a 6 day treatment experiment was performed for both dsRNA and ssRNAs (Figure 3.24).

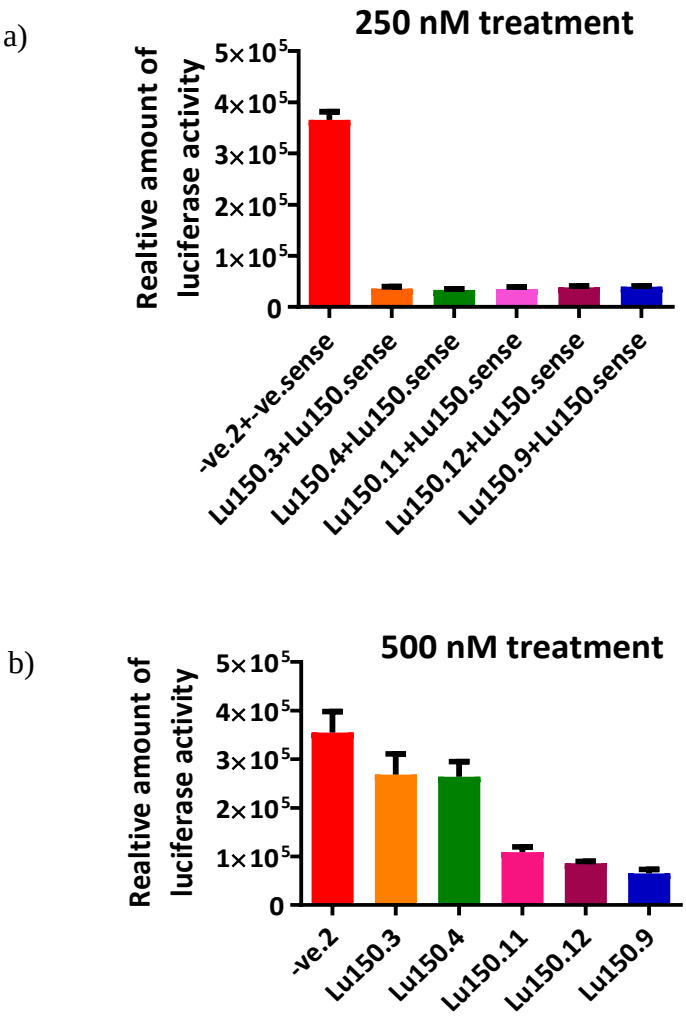


Figure 3.24. dsRNAs (a) and ssRNAs (b) activity *ex vivo* in a 6 day treatment. Longer treatment was performed to evaluate if addition of chemical modifications increase the stability when compared to unmodified RNA. ASOs design shown in Table 3.6. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: panel a: -ve.2+-ve.sense - 15881; Lu150.3+Lu150.sense - 3687; Lu150.4+Lu150.sense - 1867; Lu150.11+Lu150.sense - 3465; Lu150.12+Lu150.sense - 2684; Lu150.9+Lu150.sense – 1402. Panel b: ve.2 - 43169; Lu150.3 - 42472; Lu150.4 - 31073; Lu150.11 - 10599; Lu150.12 - 4170; Lu150.9 – 8356).

dsRNAs preserved their potency in the longer, 6 day treatment (89%-91% KD; significant difference between the control and dsRNAs, $F(5,12)=1120$, $p<0.0001$; Figure 3.24a) and no difference between individual treatments can be

observed. However, for a single-stranded treatment (Figure 3.24b) this difference is more apparent. Treatment **Lu150.11**, **Lu150.12** and **Lu150.9** gave respectively 69%, 76% and 82% of KD, when treatment with **Lu150.3** and **Lu150.4** gave 24% and 26% KD (one-way ANOVA followed by the *post-hoc* test showed significant difference between these two groups with $p<0.0003$). This shows that unmodified ssRNA are most likely being degraded faster that their modified analogues and therefore they lose effectiveness (KD reduced from 42% at 3 days for **Lu150.3** to 24% after 6 days) or because following degradation less oligo is present in cells, so the KD is lower. However, once chemical modifications are present the oligos maintain their properties and very good levels of KD were observed: **Lu150.11** – 69%, **Lu150.12** – 76% and **Lu150.9** – 82%. Still no significant difference was observed for the treatment with **Lu150.9** compared to treatment with **Lu150.12** ($p>0.05$).

In summary these results demonstrate that DNA nucleotides can be incorporated into ssRNA sequences without loss of potency. By using additional chemical modifications (2'-OMe and 2'-F) that improve ssRNA stability a significant inhibition of firefly luciferase activity (91%) was achieved. Moreover, we showed that the level of inhibition can be improved when the DNA dinucleotide linked by the *(E)*-vinylphosphonate is used as a protection at the 5'-end of the ASO.

3.1.10 Determination of IC_{50} for single-stranded oligonucleotide Lu150.9 and its double-stranded counterpart

In section 3.1.9 we have shown that specifically designed and modified ssRNA give KD values of luciferase target similar to those achieved by dsRNA (95%

for ssRNA in Figure 3.23 compared to 98% for dsRNA in Figure 3.22). Thus we decided to investigate further the activity of ASO **Lu150.9** and to test its inhibition potency in a range of concentrations to define the IC_{50} value. Defining the IC_{50} value for both ssRNA and its double-stranded equivalent allows comparison of their activity and assessment of whether it is reasonable to use ssRNA instead of dsRNA for gene KD. Performing experiments at different concentrations requires different volumes of transfection reagent. As the amount of transfection reagent influences firefly luciferase activity (3.1.8.1) we decided to use the same conditions applied previously when the successful KD experiments were performed with the same volume of HPF across all concentrations. Previously we found satisfactory conditions for 500 nM concentration and thus we decided to adapt the IC_{50} experiment to the same conditions. To do so, we kept the total amount of nucleic acid in each sample constant and therefore with decreasing concentration of ASO **Lu150.9** the amount of nucleic acid is compensated by adding the scrambled oligonucleotide **-ve.2** to the solution. The dose response curve has eight different data point (500, 250, 125, 62.5, 31.2, 15.6, 7.81, 0.00 nM). For the single-stranded treatment the 500 nM sample has only ASO **Lu150.9**, but the 250 nM sample has 250 nM of ASO **Lu150.9** and 250 nM of ASO **-ve.2** whereas the 0 nM sample has only 500 nM of ASO **-ve.2**. The same was applied to the double-stranded experiment, however the concentration refers to the concentration of the antisense strand of the duplex, not to the duplex concentration. Moreover the scrambled oligonucleotide used in this experiment was double-stranded.

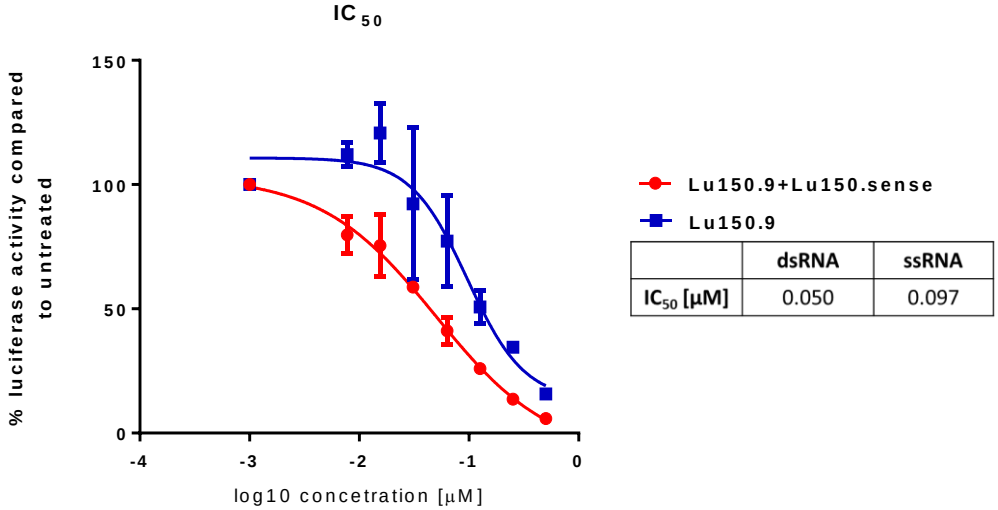


Figure 3.25. IC_{50} data. MDA-MB cells were treated for three days with eight different concentrations of either single stranded oligo (Lu150.9) or the same single-strand annealed with the complementary sense strand (Lu150.9+Lu150.sense). In both cases a dose response was observed and the IC_{50} values were calculated. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows, ds: 500 nM – 0.344; 250 nM – 1.803; 125 nM – 1.061; 62.5 nM – 5.532; 31.2 nM – 1.068; 15.6 nM – 12.613; 7.81 nM – 7.468; 0.00 nM – 0.000; ss: 500 nM – 1.031; 250 nM – 1.772; 125 nM – 6.714; 62.5 nM – 18.465; 31.2 nM – 30.623; 15.6 nM – 11.730; 7.81 nM – 4.854; 0.00 nM – 0.000).

The IC_{50} data presented in Figure 3.25 shows that both single and double-stranded treatment gave a dose response in the tested range of concentrations. The double-stranded (**Lu150.9+Lu150.sense**) oligo produced an IC_{50} value 50 nM, whereas the IC_{50} for the single-stranded ASO is around two fold higher (97 nM). Importantly as mentioned previously the concentration value refers to the amount of the antisense strand and therefore the actual amount of dsRNA used to give 50% inhibition is around 100 nM, equal to the concentration of ssRNA used to get the same KD. We considered this to be a very encouraging result because the difference in IC_{50} of ds and ssRNA is small. This suggests that significant inhibition of the target can be achieved using a single-stranded ASO at relatively low concentration, which may make it favourable for use as a treatment since the possibility of off-target effects caused by the sense strand is eliminated.

3.1.11 Viability assay

The effect of ASOs on cell viability was also investigated for each of the oligonucleotides in Table 3.6. Cells were treated with the specified concentration of either single or double stranded oligos and incubated for three days. After incubation, the cell viability assay reagent resazurin was added to the cells and after three hours incubation the fluorescence was measured. Resazurin is a nontoxic, cell permeable compound that is blue in colour and nonfluorescent. After entering cells, resazurin is reduced to resorufin, which produces very bright red fluorescence (Figure 3.26). The reduction process of resazurin to resorufin is continuous in viable cells and therefore is a quantitative method of measuring viability and cytotoxicity.

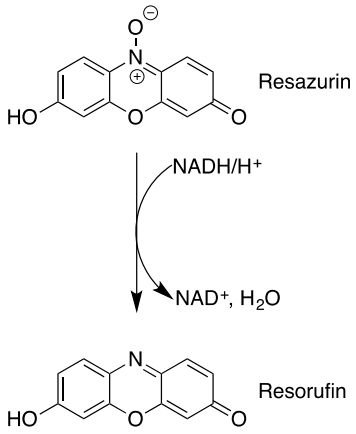


Figure 3.26 Reduction of resazurin to resorufin in living cells.

The viability assay data are presented in Figure 3.27.

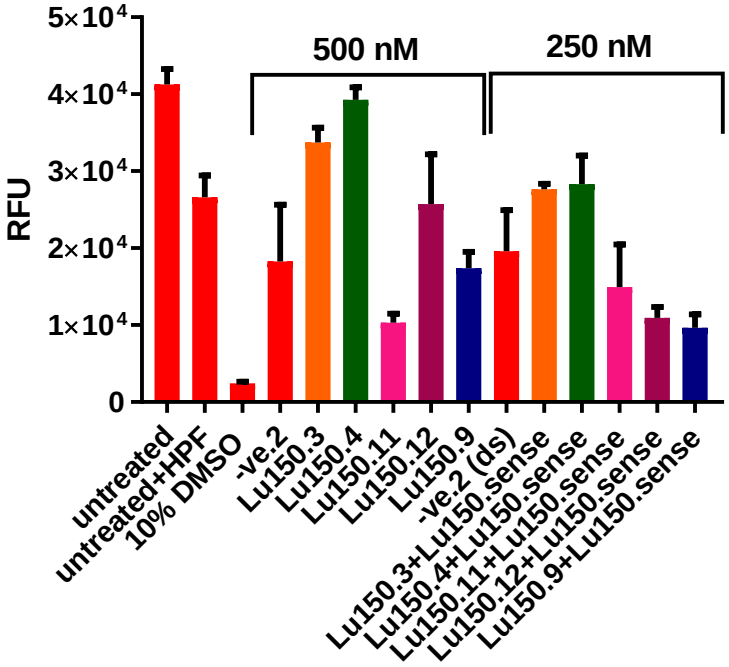


Figure 3.27. Cell viability assay. Cells viability was evaluated after 3 days treatment with either single (500 nM) or double-stranded oligonucleotides (250 nM). Controls are marked in red, all oligonucleotides are colour-coded according to experiments in chapter 3.1.9. 10% DMSO was used as positive control for cytotoxicity. RFU – relative fluorescence units. See 5.7 for experimental details. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: untr. – 1995; untr.+HPF – 2832; 10% DMSO – 248.9; -ve.2 – 7360; Lu150.3 – 1889; Lu150.4 – 1598; Lu150.11 – 1157; Lu150.12 – 6445; Lu150.9 – 2105; -ve(ds) – 5325; Lu150.3+Lu150.sense – 714.9; Lu150.4+Lu150.sense – 3694; Lu150.11+Lu150.sense – 5577; Lu150.12+Lu150.sense – 1404; Lu150.9+Lu150.sense – 1747).

Data were analysed with one-way ANOVA and found to be statistically significant (F (14,29)=26.72, p<0.0001). To compare single-stranded group to double-stranded group a multiple comparison test was performed. All controls are marked in red. As expected cells that were treated only with the HPF transfection reagent showed decreased fluorescence compared to untreated cells (p=0.0038). 10% DMSO was used as a positive cytotoxicity control and as can be seen, only a little fluorescence was detected (RFU at the level of background) which means that cells were dead. The viability across each treatment varied, however when looking at our most chemically modified oligo, **Lu150.9,** the viability compared to "untreated+HPF" is lower, but the difference is not significant (p=0.3956). It is important to compare to that control, because

addition of transfection reagent has a clear effect on cell viability and we want to determine whether addition of the ASO magnifies this effect. We see some reduction of fluorescence, but it is not statistically meaningful. Interestingly the viability is lower for all double-stranded oligos than for single-stranded oligos, which might be due to the presence of the non-targeting sense strand.

Although this assay showed some reduction in cell viability due to the use of ASOs it appears the effect is smaller when single-stranded oligonucleotides are used. That observation emphasises that the use of single-stranded ASOs over double-stranded ASOs is beneficial. Moreover as the toxicity is associated with the use of transfection reagents it may be possible to further optimise the amount of transfection reagent required for effective delivery or utilise a different transfection reagent. The transfection reagent is required because experiments were performed in tissue culture, however for the *in vivo* experiments it is not always necessary to use chemical transfection reagent. Most often the injection method is used in animal models and in patients.[216,217]

3.1.12 Melting temperature studies

The melting temperature (T_m) of a DNA duplex is defined as the temperature at which half of the strands are in a single-stranded state and half are in a duplex. The formation of stable duplexes is determined by the T_m, so the higher the T_m of an ASO the stronger it will bind to its target and the therapeutic effect should be more pronounced. As a follow up study the impact of chemical modifications on the melting temperature of the ASOs was evaluated. Our ASOs are designed to work through RNAi. To mimic these conditions for the melting temperature data we used unmodified RNA as the complementary strand in duplexes. Five

ASOs (Table 3.7) were annealed with the unmodified sense strand **Lu150.sense**, heated at 90 °C for 2 minutes, left to cool down at rt and then the samples were placed in the DU 800 UV/Vis spectrophotometer. Samples were heated to 85 °C, and the absorbance was recorded every 0.5ºC. When the temperature reached 85 °C samples were gradually cooled down to 20 °C. The reverse T_m curve was recorded with the absorbance measurement taken every 0.5 °C. The absorbance was plotted against the temperature and the melting temperature curves are presented in Figure 3.28. T_m values were calculated from the first derivative of the melt curves and are shown in Table 3.7.

Table 3.7. Melting temperature data. Both melting (Tm) and cooling curve (Tm reverse) were calculated from the first derivative and are presented below. Black stand for unmodified RNA, green - 2'-F and blue - 2'-OMe modified nucleotides, underlined – AA overhangs, "s" phosphorothioate linkage, DNA nucleotides are marked in red, DNA dinucleotide linked by the *(E)*-vinylphosphonate is labelled with * (T*A)).

ID	Structure 5' → 3'	Tm (°C)	Tm Reverse (°C)
Lu150.3	UAA UGU UUU UGG CAU CUU CCA AA	52.3	52.0
Lu150.4	TAA UGU UUU UGG CAU CUU CCA AA	50.8	50.0
Lu150.11	TAA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	61.2	60.3
Lu150.12	TAA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	59.3	57.8
Lu150.9	T*AA UGU UUU UGG CAU $C_sU_sU_s$ $C_sC_sA_s$ A_sA	58.8	57.8
Complementary strand (Lu150.sense)	UGG AAG AUG CCA AAA ACA UUA AA	-	-

The first observation is that replacement of two terminal RNA nucleotides with the DNA decreases T_m of **Lu150.4** by 1.5 °C when compared to **Lu150.3**. The same applies when oligos **Lu150.11** and **Lu150.12** are compared. Exchange of two 2'-OMe to two DNA nucleotides, on the 5'-end, decreases T_m of **Lu150.12** by 1.9 °C in comparison to **Lu150.11**. When the two terminal DNA nucleotides

are linked by the *(E)*-vinylphosphonate the T_m is even lower and is reduced by another 0.5 °C for ASO **Lu150.9**. Nonetheless, the decrease of T_m caused by addition of DNA is not that crucial, because of the presence of 2'-fluorinated and 2'-methylated nucleotides the melting temperature is increased by 8.9 °C (when **Lu150.3** and **Lu150.11** are compared), so the overall T_m raised (for **Lu150.9** T_m is higher by 6.5 °C in comparison to **Lu150.3**). Both melting and cooling curves have an undulating shape which suggests possible secondary structures of the ASO. However this was not investigated further. To conclude, we showed that the chemical modifications used in our final ASO, **Lu150.9**, did not decrease thermal stability of the duplexes but improved it by increasing the melting temperature.

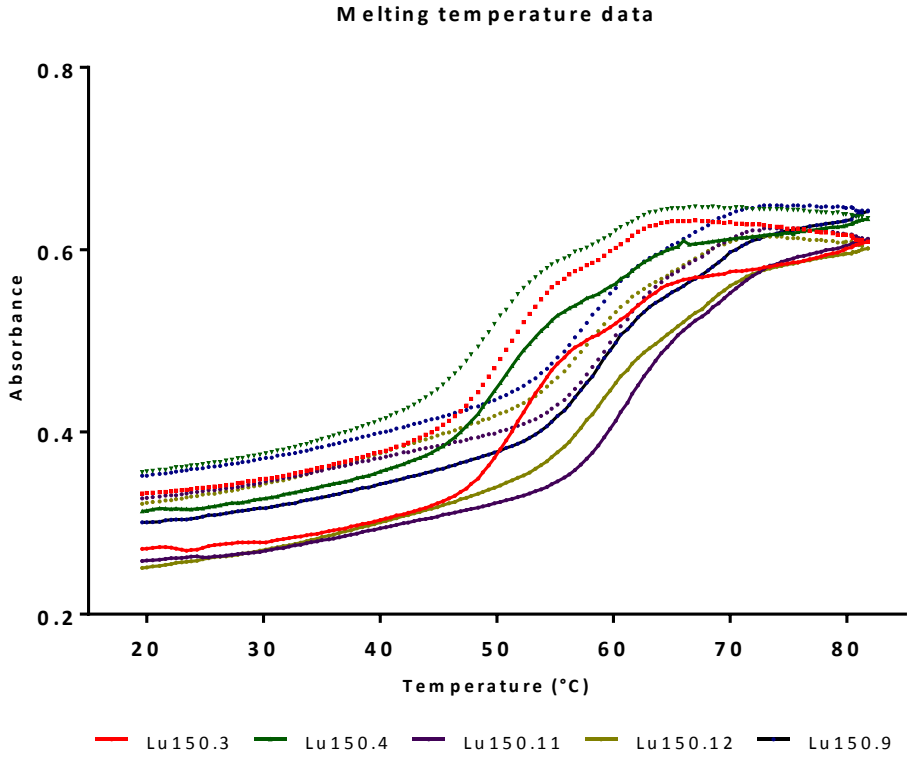


Figure 3.28. UV-melting curves for oligonucleotides Lu150.3, Lu150.4, Lu150.11, Lu150.12 and Lu150.9 (3 µM). Each of the tested strands was annealed with the complementary strand (UGG AAG AUG CCA AAA ACA UUA AA) and the absorbance was measured. Dotted lines represent cooling curves.

3.2 CDK12 knock-down

3.2.1 Introduction to CDK12

All previous experiments were performed in MDA-MB fluc, a well-established human breast carcinoma cell line which expresses firefly luciferase gene endogenously as a genetic reporter. One of the reasons we chose this cell line is that it was suitable to inject into the mouse in xenograft experiments, and a luminescent protein (firefly luciferase) can be detected with the use of a *in vivo* imaging system (Caliper). This provides a good model to test the activity of synthesised ssRNAs *in vivo*. We could proceed with luciferase as our target and test the oligonucleotides *in vivo* in mice. We decided that it would be better to look at a disease relevant target to apply the knowledge gained from performing the experiments to KD firefly luciferase. If successful that can be then followed by *in vivo* studies.

Cyclin-dependent kinases (CDKs) and their partner cyclins are involved in cell division, transcription and RNA splicing and are often deregulated in cancer.[223] There are more than 20 types[224] of CDKs and we decided to focus on CDK12 (CrkRS), a 1490 amino-acid protein. CycK/CDK12 complexes have been shown to protect cells from genomic instability by the regulation of DNA damage response (DDR).[225] Deregulation of CDK12 has been found in many cancers, for example it is one of the most frequently mutated genes in ovarian cancer.[226,227] CDK12 is interesting not only because of its relation to cancer, but also because of a connection with Myotonic Dystrophy 1 (DM1) a disease that is of interest to our research group. DM1 is an inherited autosomal-dominant disease and its main features include muscle wasting, cataracts, heart conduction

defects and myotonia. Currently there is no treatment available for DM1 patients. DM1 is caused by the abnormal expansion of a CTG trinucleotide repeat in the 3' untranslated region of the DM protein kinase (DMPK) gene in chromosome 19q13.3.[112-114] In unaffected individuals the repeat length is between 5-37 CTG repeats, while in DM1 patients the size of repeat is above 50 and can reach thousands of repeats. These extended repeats form hairpin-like structures which accumulate within cell nuclei and deregulate the function of RNA-binding proteins. There are two most comprehensively studied proteins: Muscleblind-like (MBNL1) protein which together with expanded RNA form toxic RNA aggregates[115,116] and CUG-binding protein (CUG-BP1).[117] The reduction of free MBNL1 in the cell interferes with RNA splicing and deregulates normal cell function.[118,119] In unpublished work Ketley *et al.* showed in a nuclear foci assay that by inhibiting CDK12 a reduction of toxic foci was observed. This was done with the use of small molecule kinase inhibitors, for example Dinaciclib. It was confirmed in an *in vivo* assay using HSA^{LR} mice treated with the aforementioned drug to produce a reduction of DM1 symptoms compared to vehicle treated animals. Dinaciclib is a drug that is currently tested in clinical trials for various cancer treatments. Due to its cytotoxicity and lack of selectivity for the CDK12 protein in experiments performed in our laboratory, other analogues have to be found. A new project aiming on developing small molecules that inhibit CDK12 is being currently conducted by the Brook and Hayes research groups. At the same time we decided to try to inhibit CDK12 expression using ssRNAs since they are less toxic and provide better selectivity than small molecules.

3.2.2 CDK12 knock-down quantification by western blotting

The experiments described in following sections were conducted in more than one cell line. To avoid confusion all cell lines are characterised in Table 3.8.

Table 3.8 Description of cell lines and tissue samples used to perform experiments described in this and following sections.

Cell line name	Description
MDA-MB 231	Human breast adenocarcinoma cell line.
MDA-MB fluc	Cell line generated from the MDA-MB 231 stably transfected with a pLVX-Luc plasmid, which expresses firefly luciferase.
KB TeloMyoD	Fibroblast cell line generated from DM1 patient.
SB TeloMyoD	Fibroblast cell line generated from healthy volunteer.
Hela	Cervical cancer cell line.
DM 18.1 and DM 23.1	Protein extracted from DM1 muscle patient biopsies.

As a first step to prepare for KD the presence of CDK12 protein in MDA-MB fluc cell line was analysed. To do so cells were plated in tissue culture flasks and after 4 days protein was extracted and a western blot was performed to detect CDK12. Different amounts of protein were loaded onto the gel, so the best loading amount could be determined. The predicted molecular weight of the CDK12 is 164 kDa, however due to post-transcriptional modifications i.e. phosphorylation, the real mass is higher. Strong bands corresponding to the sought protein were observed for all of samples at the size of around 220 kDa (Figure 7.1 in Appendix). It was decided to use 10 µg of protein in subsequent experiments. To obtain clear blots, the amount of primary and secondary antibody was optimised. To confirm that the top band corresponds to CDK12

another western blot (Figure 7.2 in Appendix) was performed. This time, to compare with a sample we knew to contain CDK12 protein, a DM 23.1 sample was run alongside the MDA-MB fluc sample. Both samples have the same size bands, confirming that CDK12 is present in the cells we plan to use for future experiments.

Once the presence of CDK12 was confirmed, the CDK12 KD experiments could be performed. We decided to use dsRNAs first and only if they worked would we use their single-stranded modified equivalents. Two targets were chosen (Table 3.9), CDK12(4697) was published by Cheng *et al.*[228] and the second target designed using Primer3 software.

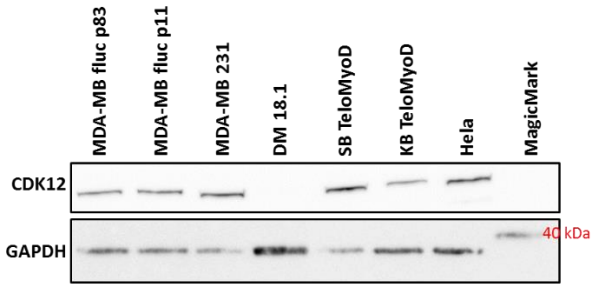
Table 3.9. CDK12 oligonucleotides. Black stands for unmodified RNA, underlined – AA overhangs.

ASO ID	Structure 5' → 3'	Length
CDK12(4697).1	AAC UGG UGC CAA CAU CAU UAU AA	23
CDK12(4697).sense	AUA AUG AUG UUG GCA CCA GUU AA	23
CDK12(306).1	UAG UUU GAA GGC CAU GUC AUC AA	23
CDK12(306).sense	GAU GAC AUG GCC UUC AAA CUA AA	23

Cells were plated shortly before transfection of siRNAs-HPF complexes and incubated for the specified time. The protein lysate was generated by lysing cells with Ripa buffer and samples were run on western blots for CDK12 detection. One, two and three day experiments were conducted with no KD of CDK12 observed (Figure 7.3 in Appendix). Apart from the ASOs presented in Table 3.9 there is another treatment shown on the blots, marked as "**CDK12(4697)*** 800 nM", and it represents a positive control, the dsRNA that we knew was capable of reducing target levels (this is the same batch of dsRNA that A. Ketley showed

successful KD of CDK12 in her project). This additional dsRNA was used to make sure that any apparent lack of KD does not result from poor quality of oligos, but other factors.

We could not demonstrate inhibition of CDK12, even with dsRNA that worked before. It has to be mentioned that our cell line is different to the cell line that was used for Ketley's KD (KB TeloMyoD), so they should be considered as two different systems. It is possible that the amount of endogenous CDK12 in these two systems is different and therefore the siRNA that worked in Ketley's experiments might not work in our cancer cell line. Consequently we decided to compare levels of CDK12 across different cell lines available in our laboratory. Seven protein samples from different origins were chosen for test: KB TeloMyoD (KB), Hela, SB TeloMyoD (SB), DM 18.1, MDA-MB 231 and MDA-MB fluc (at high and low passage). 10 µg of each protein lysate was loaded onto the gel and the western blot technique was used for detection. The level of CDK12 was normalised against the reference gene GAPDH. The results are presented in Figure 3.29.



Cell line	Relative amount of CDK12
KB TeloMyoD	1.00
Hela	2.13
SB TeloMyoD	6.86
DM 18.1	0.01
MDA-MB 231	4.62
MDA-MB fluc p11	2.05
MDA-MB fluc p83	1.66

Figure 3.29. Comparison of CDK12 levels in different cell lines and biopsy sample. The total protein was extracted from different samples to access if the amount of endogenous CDK12 depends on the cell line used for experiments.

The KB cell line serves as reference and therefore the amount of CDK12 in KB sample is 1.00. Both MDA-MB 231 and MDA-MB fluc (passage 11) have

shown elevated levels of CDK12 when compared to KB and were accordingly 4.62 and 2.05 fold higher. Interestingly protein extracted from MDA-MB fluc at high passage (p83) showed lower levels of CDK12 than protein extracted from lower passage (p11), 1.66 compared to 2.05. The SB sample showed the highest level of the CDK12 6.86 when compared to the KB. The DM 18.1 biopsy sample showed no CDK12. This may be due to the fact that it was not extracted from the cells, or the sample was not of good quality, because the other biopsy that was tested before has shown higher levels of CDK12 (Figure 7.2). As can be seen the amount of CDK12 across different samples is uneven and the protein expression is higher in the cell line we chose for our experiments. The greater amount of CDK12 in the MDA-MB fluc cell line might be a reason why no reduction of target was achieved in KD experiments. First there is more to inhibit and second with such a high levels of CDK12 even a small change might not be observable on western blots especially as we have shown before that, due to the high molecular weight of CDK12 protein, transfer from gel to membrane might be problematic. Taking all factors into account we decided that the system we chose might not be the best one to use and we should to try to use another system for the CDK12 KD.

3.2.3 Generation of the new CDK12pLVX-Luc system

The majority of this study focused on developing ASOs for firefly luciferase inhibition which resulted in a good understanding of the MDA-MB fluc model system. Thus we decided to adopt this system and use it to investigate CDK12 KD.

The MDA-MB fluc cell line was generated by stable transfection of wild type MDA-MB 231 breast cancer cells with a pLVX-Luc plasmid that encodes firefly luciferase (Figure 3.30a). We decided to generate new plasmid that expresses a hybrid protein consisting of CDK12 and firefly luciferase (Figure 3.30b).

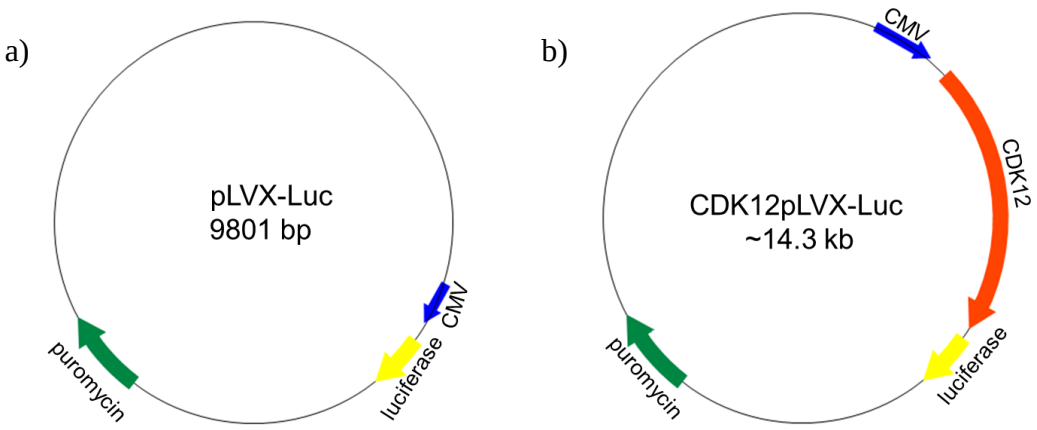


Figure 3.30. Schematic diagram of a) pLVX-Luc and b) CDK12pLVX-Luc plasmids. The pLVX-Luc plasmid was used as a template to create the CDK12pLVX-Luc plasmid that was used for the generation of cell line that expresses hybrid protein. See Appendix 7.1 and 7.2 for details of plasmid constructs.

The advantage of the resultant plasmid is that the CDK12 is 5' to the luciferase gene (Figure 3.31), so when the CDK12 expression is suppressed (by oligonucleotide) protein translation is stopped and no luciferase signal will be observed. This creates a system with indirect quantification of CDK12 inhibition that can be measured on a luminometer. Moreover siRNAs or ssRNAs targeting firefly luciferase, used in 3.1 can serve as a positive control to ensure that the system works. To obtain a CDK12pLVX-Luc plasmid the purified CDK12 sequence was inserted into pLVX-Luc plasmid using the Gibson assembly (plasmid generated in Brook laboratory according to Figure 3.31).

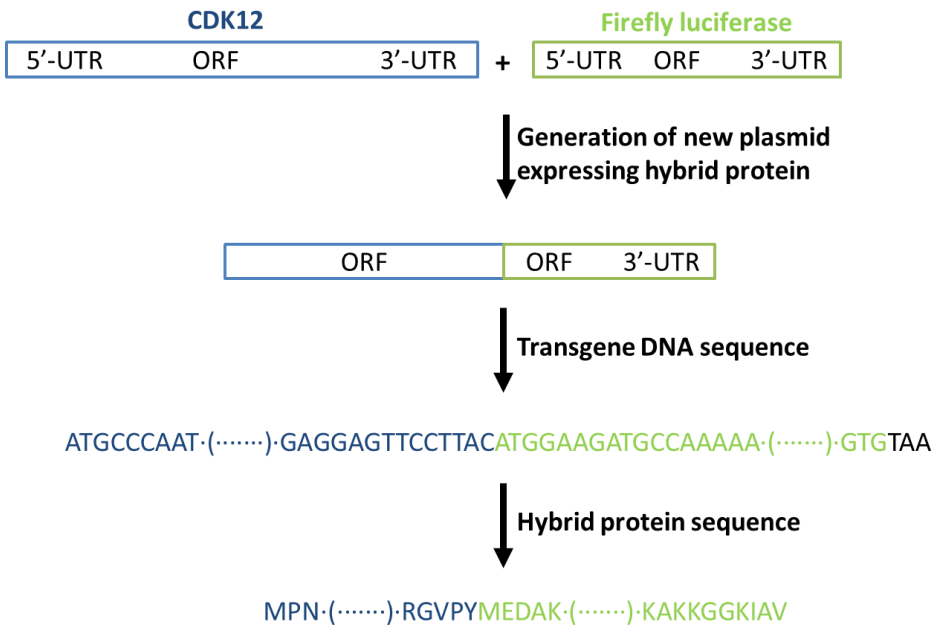


Figure 3.31 Scheme of CDK12pLVX-Luc plasmid generation. The ORF of CDK12 was placed 5' to the luciferase gene. This design allows production of transgene mRNA and its translation into hybrid protein.

The resultant plasmid can be used in two ways. It can be co-transfected together with an ASO into a cell line, for example Hela, or a new cell line that expresses the hybrid protein can be generated by stable transfection of the CDK12pLVX-Luc into the wild type cell line. In both cases the measurement of inhibition of target is easier when quantified with luminometer rather than by western blots which may be inconsistent.

Thus co-transfection of the CDK12pLVX-Luc plasmid and dsRNA against firefly luciferase (**Lu150.3+Lu150.sense**) into Hela cells was carried out. A significant 86% KD of target was observed compared to only plasmid transfected cells. However, when the experiment was repeated sometimes we did not observe any luciferase signal for control cells which means that the transfection of firefly luciferase did not work. Due to the lack of reproducibility we have decided to generate a stable cell line that expresses the hybrid protein. The CDK12pLVX-Luc plasmid was linearised and transfected into the

MDA-MB 231 cell line. Cells were incubated in complete growth medium for 3 days. On the third day medium with a selective antibiotic, puromycin, was introduced and cells were examined daily for visual toxicity. After 14 days, surviving cells that underwent plasmid integration were gradually transferred to different plates/flask formats to allow them to expand. Three colonies were tested for luciferase presence and only two clones 231 PP02 and 231 PP03 were positive (Figure 3.32).

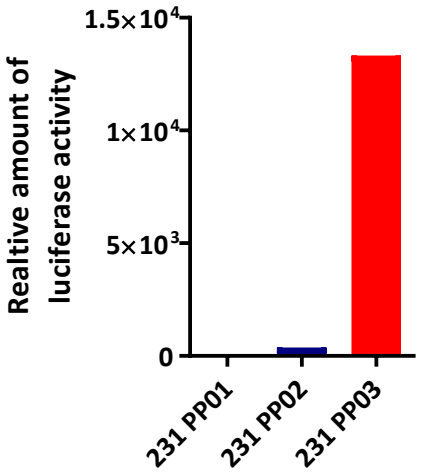


Figure 3.32. Quantification of firefly luciferase levels in three new cell lines. Three of the cell lines were tested for firefly luciferase presence. Two cell lines 231 PP02 and 231 PP03 gave a positive result, but the latter one had much higher expression and therefore was chosen for further work. Cell line 231 PP01 was negative for firefly luciferase presence.

The 231 PP03 cell line showed the highest luciferase expression and was chosen for further work. Initially 231 PP03 was tested for its response to dsRNA targeting firefly luciferase. An oligo **Lu150.3** was annealed with **Lu150.sense** to give a siRNA that the cells were treated with at the concentration of 250 nM. As shown in Figure 3.33 a significant 95% of inhibition of target was recorded.

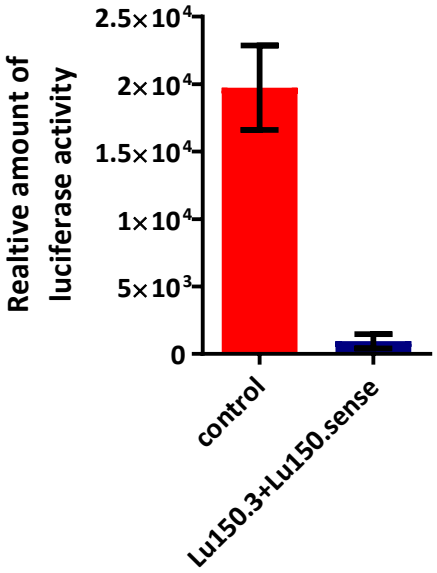


Figure 3.33. Firefly luciferase KD in the newly generated 231 PP03 cell line. 3 day treatment at 250 nM was performed to see if the new cell line was still responsive to the dsRNAs that were used previously (Table 3.3). A significant down-regulation of a target was achieved. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: control - 3130; Lu150.3+Lu150.sense – 526.3).

Of the two target sequences that were chosen for CDK12 KD (Table 3.9), only one could be used for the hybrid protein work. That is because only the open reading frame (ORF) of CDK12 was inserted into the plasmid that already contained the firefly luciferase gene (Figure 3.31). Therefore ASO targeting CDK12 4697 cannot be used, because it targets the 3'-UTR of the sequence, which is no longer present in the hybrid protein. As a result only ASO CDK12 306 could be used or a new ASOs have to be designed and synthesised.

First a previously made dsRNA (**CDK12(306).1+ CDK12(306).sense**) against CDK12 306 was tested together with a positive control dsRNA that targets firefly luciferase. Unexpectedly the relative amount of luciferase activity for **CDK12(306).1+ CDK12(306).sense** treatment increased and is almost four times higher than that of the scrambled treated cells (red in Figure 3.34), whereas the positive control against luciferase gave 90% inhibition.

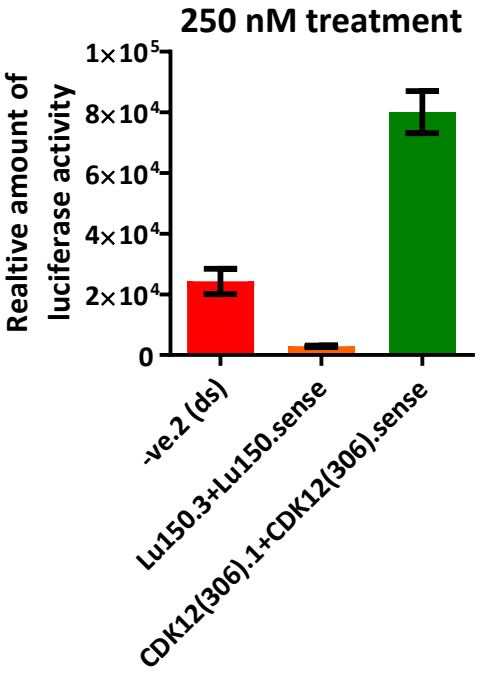


Figure 3.34. Targeting CDK12 in the 231 PP03 cell line. The dsRNA (Table 3.9) targeting CDK12 was tested for its efficacy. No repression of target was seen for the CDK12 oligo when the positive control targeting firefly luciferase gave 90% KD. Data is shown as the mean with SD, number of biological replicas n = 3. (SD values as follows: -ve.2 (ds) - 4200; Lu150.3+Lu150.sense – 251.8; CDK12(306).1+CDK12(306).sense - 6937).

We are unable to explain readily this odd result, but it might be associated with the transfection reagent that was used, since we have shown earlier that HPF influences luciferase activity (Figure 3.18). Another possibility is that the protein expressed by the transgene does not contain CDK12, but expresses only luciferase, and therefore cannot be suppressed by the siRNA to CDK12. We know that the CDK12pLVX-Luc plasmid was, at least partially, integrated into MDA-MB 231 cells because the firefly luciferase assay gives positive result (Figure 3.32). However it is possible that either the CDK12 sequence deleted from the plasmid or the mRNA corresponding to CDK12 is not translated into the protein and therefore its KD cannot be achieved. To check these hypotheses, two separate experiments were designed. First the presence of the transgene

mRNA was inspected by polymerase chain reaction (PCR) and second the presence of protein product was investigated by performing western blotting.

3.2.3.1 Confirmation of the CDK12pLVX-Luc hybrid protein presence in the 231 PP03 cell line

The presence of endogenous CDK12 in cell lines was confirmed previously and the result is shown in Figure 3.29. By introducing the CDK12pLVX-Luc transgene plasmid an exogenous form of CDK12 was introduced into the resulting 231 PP03 cell line. Thus for that particular cell line two different types of CDK12 target have to be distinguished. The difference between endogenous and exogenous CDK12 is that the exogenous CDK12 is part of a hybrid protein with the firefly luciferase resulting in the bigger size, by 61 kDa.

For PCR different sets of primers had to be design to distinguish between endo and exogenous CDK12's mRNA. To confirm the presence of firefly luciferase primers, called "total fluc", were designed and both forward and reverse primers are located in the ORF of the gene (Figure 3.35a). These primers will amplify any luciferase present in the mRNA extract. Thus they represent total firefly luciferase. To quantify total CDK12 (the sum of the endo and exogenous) primers for the ORF of CDK12 were designed and are called "total CDK12" (Figure 3.35 b and c). To demonstrate that exogenous CDK12 is present, unique sites had to be chosen for primer design. For the endogenous CDK12 "endo CDK12", primers are located in the 3'-UTR of CDK12, because as shown in Figure 3.35 (b and c) it is present in complete CDK12 sequence but is not present anymore in the CDK12 insert that was cloned into the CDK12pLVX-Luc plasmid that was used for stable cell line generation. As shown in Figure 3.35

(c) the transgene mRNA consist of the ORF of the CDK12 and the ORF and 3'-UTR of firefly luciferase. Therefore the forward primer is placed in the ORF of the CDK12 and the reverse is placed in the ORF of the firefly luciferase, this junction is unique to the exogenous CDK12. Thus if the PCR product for the "exo CDK12" primers is observed it will prove that the 231 PP03 cell line expresses hybrid protein. For each of the four sites two sets of primers were designed, which in total gives eight different sets of primers that were tested (primers sequence and product size are presented in 5.12.3).

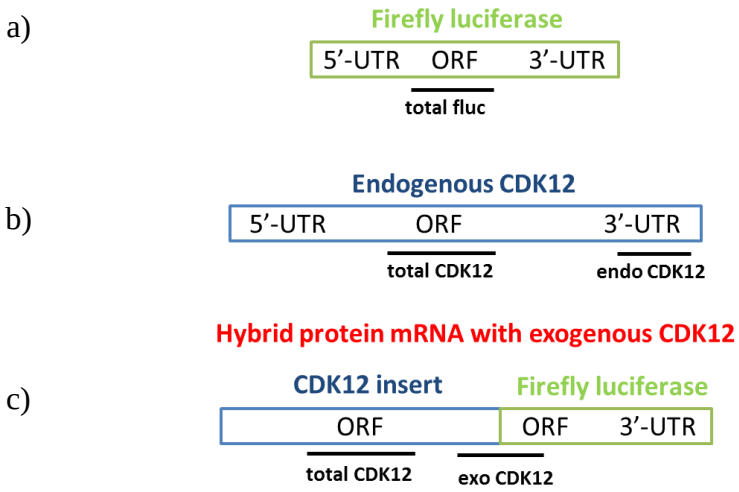


Figure 3.35. Schematic picture of design of primers used to detect endogenous and exogenous CDK12. a) "Total fluc" primers are placed in the ORF of the firefly luciferase. b) To detect endogenous CDK12 primers were located in the 3'-UTR of the CrkRS sequence. c) To identify any CDK12 present in the sample the "total CDK12" primers were designed and they are placed in the ORF of the CrkRS. And finally to check the presence of exogenous CDK12 coming from the CDK12pLVX-Luc plasmid the "exo CDK12" primers, spanning ORF of CDK12 and ORF of firefly luciferase, were designed.

After total RNA was extracted from the 231 PP03 cells, the cDNA was synthesised and PCR was performed. Samples were electrophoresed to visualise products. Bands of the expected size were observed for all of primers used (Figure 3.36). A 35 PCR cycles had to be undertaken so the "exo CDK12" product could be seen, whereas for all other primers 30 cycles were sufficient. Therefore this experiment should be considered only as qualitative not

quantitative analysis. Nonetheless we have shown that the transgene mRNA is present in the 231 PP03 cell line.

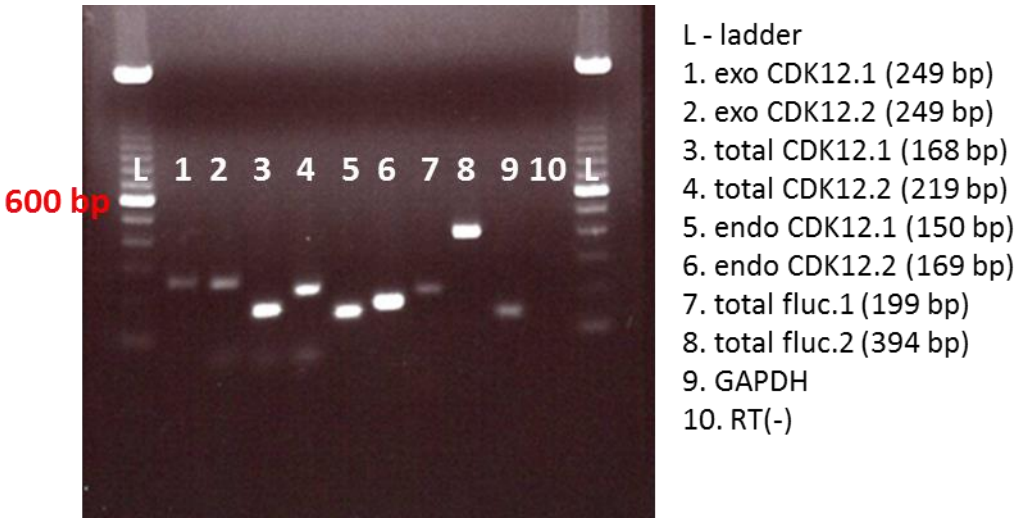


Figure 3.36. PCR products. The PCR reactions for all of the designed primers were electrophoresed. As shown above all of the samples gave products proving that both endogenous and exogenous CDK12 are present in the 231 PP03 cell line.

Our concern was that although the transgene mRNA is present in 231 PP03 cells it might not be translated into the complete protein. We do know that firefly luciferase is produced because the luciferase assay gave a strong positive result (Figure 3.32). However it is possible that the ribosome binds internally to the mRNA upstream of the luciferase gene and as a result only luciferase is translated (hybrid protein sequence can be found in 7.2). To investigate the presence of the complete hybrid protein western blotting was used.

CDK12 is a large protein which generates problems when it is subjected to western blotting mainly related to the transfer from gel to the membrane. This might result in incomplete transfer or loss of the protein. As a consequence CKD12 band sometimes “disappears” or is only partially visible, which makes the analysis difficult. In the new 231 PP03 cell line the product, a hybrid protein, is even larger as it consist of fused CDK12 and firefly luciferase. As such it is

harder to identify by western blotting. Moreover, the difference in size between endogenous CDK12 and the hybrid protein is only 61 kDa, due to the luciferase. Thus it might be hard to see any difference in band position on the blot due to the similar mass of the two proteins. For that reason we decided to use the anti-firefly luciferase antibody to detect the hybrid protein. In this case the firefly luciferase band expected mass is around 61 kDa whereas the hybrid protein's band will be located above it at 281 kDa, so we should be able to distinguish the two. If the hybrid protein is completely translated we should observe only one band at the top of the blot for the 231 PP03 cell line of around 281 kDa molecular weight. If only the luciferase part of the transgene mRNA is translated a lower product should be observed at 61 kDa.

First the MDA-MB fluc and MDA-MB 231 cell lines were used to define the firefly luciferase band. Cells were plated and incubated until confluence was reached. The total protein was extracted and samples were run on the gel. The expected band size of around 61 kDa is present in the MDA-MB fluc cell line protein, indicating that the firefly luciferase is expressed (Figure 3.37a). The wild type cell line MDA-MB 231, from which MDA-MB fluc was derived, does not show luciferase presence and serves as negative control.

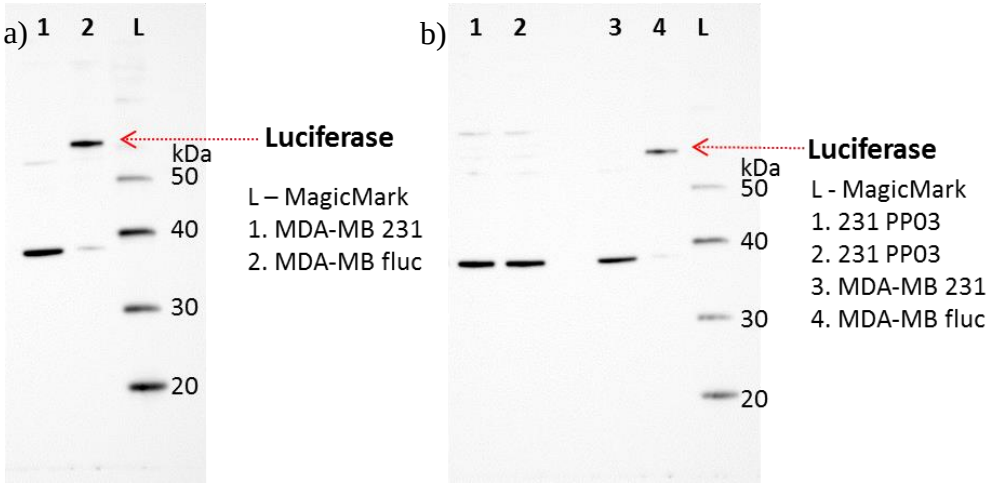


Figure 3.37. Western blot showing the firefly luciferase band. Protein lysate from the MDA-MB fluc cell line was processed by western blotting to visualise the firefly luciferase band (a). A wild type cell line sample (MDA-MB 231) was run alongside and served as a negative control. Newly established cell line 231 PP03 was tested for the presence of firefly luciferase (b).

After establishing the firefly luciferase band size and optimal conditions for western blotting, protein samples from the 231 PP03 cell line were tested. No band at the predicted size, corresponding to the hybrid protein (~281 kDa) was observed, and no band for firefly luciferase at 61 kDa was observed either (Figure 3.37b). It is striking that we could not see the presence of luciferase on the western blot but we can detect it in the luciferase assay (Figure 3.32). One of the concerns is that the hybrid protein's expression is much lower than CDK12 or firefly luciferase and therefore it might not be detected on the western blot. To test this hypothesis an immunoprecipitation technique was used to isolate the protein of interest from the total protein mixture.

Immunoprecipitation (also called pull down) is a method of precipitating a protein by adding the antibody specific for the protein of interest and incubating it with cell extract, which enables the antibody to bind to the protein in solution. The antibody/antigen complex is pulled out of the solution by using protein A/G agarose beads. The complex binds to the agarose beads when the mixture is incubated. After incubation, samples are centrifuged and the supernatant is

removed. The remaining beads are gently washed with lysis buffer and after the final wash the beads are resuspended in the electrophoresis sample buffer and boiled for 10 minutes followed by electrophoresis.

Three different cell lines were used for comparison and four samples were generated from the pull down experiment for analysis on western blots (half of the lysate was loaded onto gel in one run). The MDA-MB 231 cell line (wild type) was pulled down with the anti-CDK12 antibody to determine whether CDK12 could be detected and to serve as a control (Table 3.10). The MDA-MB fluc cells were immunoprecipitated with the anti-firefly luciferase antibody and 231 PP03 cells were pulled down with either the anti-CDK12 or the anti-firefly luciferase antibody to generate two separate samples. All four samples were run on the gel, transferred to the membrane and probed with either anti-firefly luciferase or anti-CDK12 antibody (Table 3.10).

Table 3.10 Samples generated in pull down experiment were visualised with two antibodies (firefly luciferase or CDK12).

Sample	Pull down Ab	Visualisation Ab
231 PP03	Firefly luciferase	Firefly luciferase
231 PP03	CDK12	
MDA-MB fluc	Firefly luciferase	
MDA-MB 231	CDK12	
231 PP03	Firefly luciferase	CDK12
231 PP03	CDK12	
MDA-MB fluc	Firefly luciferase	
MDA-MB 231	CDK12	

The first blot, probed with the anti-firefly luciferase antibody, is shown in Figure 3.38. The MDA-MB 231 sample, showed no bands since it does not express firefly luciferase (it is our negative control). For the MDA-MB fluc samples a clear band at around 61 kDa is observed and shows that the pull down experiment worked well. Unfortunately no bands can be seen for 231 PP03 samples, irrespective of whether they were immunoprecipitated with the CDK12 or the firefly luciferase antibody. If the band were to be visible, in both cases it should appear at the top of the blot at around 281 kD (220 kDa from CDK12 and 61 kDa from firefly luciferase).

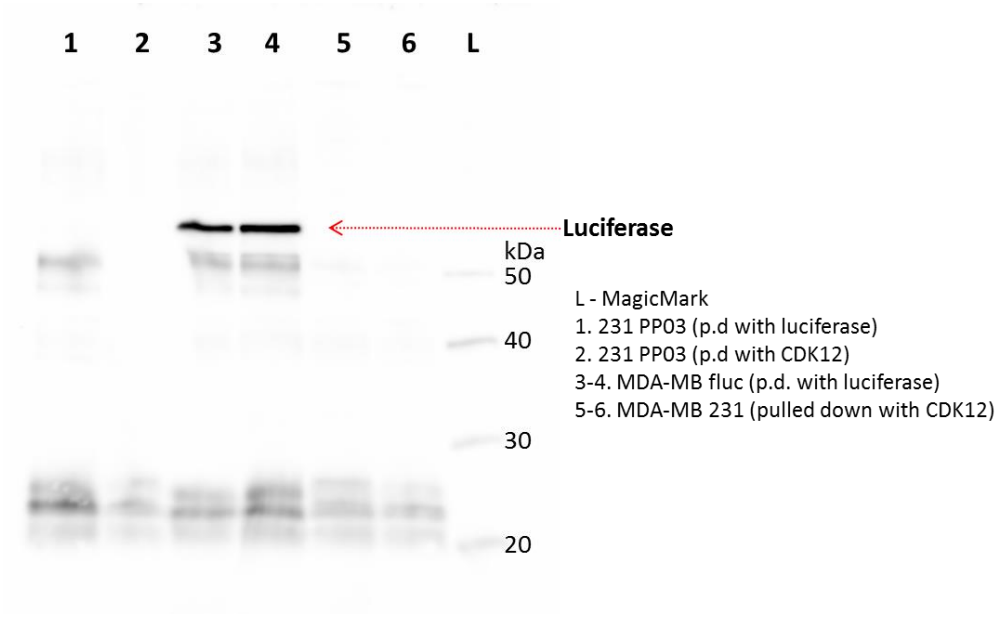


Figure 3.38. Firefly luciferase immunoprecipitation blot. Samples were run on the gel and probed with the anti-firefly luciferase antibody. A firefly luciferase band was observed only for the MDA-MB fluc samples. No luciferase band was seen at 61 kDa or 281 kDa in the 231 PP03 cell line.

The remaining half of the lysate was processed on a second western blot, but this time the anti-CDK12 primary antibody was used as a probe. Additionally on this blot two more samples, neither of which were immunoprecipitated, were loaded (MDA-MB fluc and DM 23.1, lane 1 and 2 in Figure 3.39) to localise the CDK12 band. Unfortunately, no bands of the appropriate size were detected.

The MDA-MB 231 (pulled down with CDK12, lane 7 and 8 in Figure 3.39) should show the same band as the MDA-MB fluc or DM 23.1 samples in lane 1 and 2, but it did not. This suggests that there is an issue during immunoprecipitation as the pulldown does not work, or the product is lost or that there is a problem with protein transfer from the gel to the membrane caused by the large mass of the transferred protein. For the 231 PP03 samples (lane 3 and 4), regardless which antibody was used for pull down, the expected band should be 61 kDa higher than the CDK12 band in lane 1.

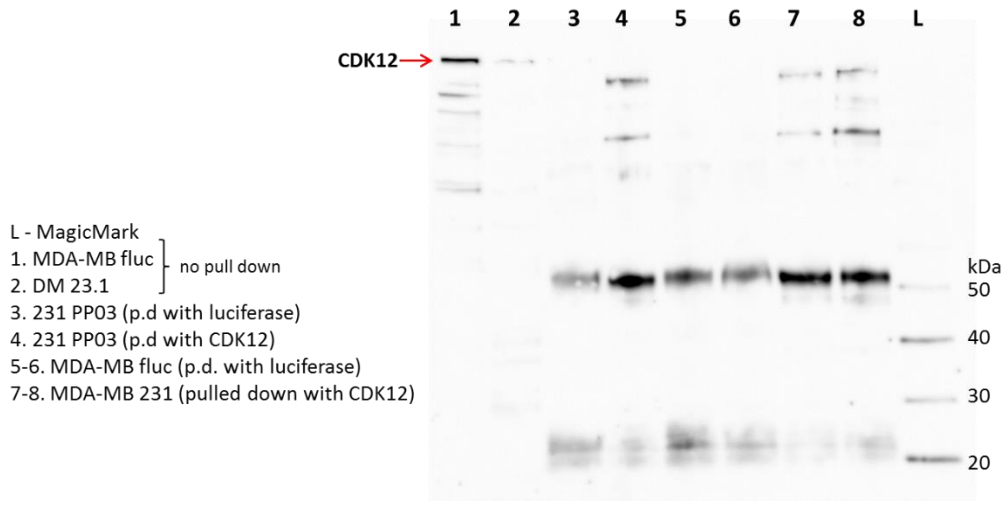


Figure 3.39. CDK12 immunoprecipitation blot. Samples were run on the gel and probed with the anti-CDK12 luciferase antibody. None of the pulled-down samples showed the CDK12 protein or hybrid protein. Two samples, that were not precipitated (the far left on the blot), were run on the same blot for easier localisation of the CDK12 band.

It is hard to understand why a band corresponding to the hybrid protein is not visible. It may be due to the large mass of the protein, since we have experienced problems with transfer of the CDK12 before and as the hybrid protein has a greater mass the transfer from gel to membrane might be less effective. Furthermore, the relative amount of luciferase in the MDA-MB fluc cell line is at the level of hundreds of thousands or even millions whereas for the 231 PP03 cell line it is much lower and therefore it might not be detectable on a western

blot, even when the protein of interest is isolated from the total protein mixture. On a positive note, a lower mass band of luciferase is absent from the 231 PP03 cell line suggesting translation via internal RES (ribosome entry site) is unlikely. To summarise it has been demonstrated by PCR that the transgene mRNA is produced in the 231 PP03 cell line. Using the luciferase assay to screen new cell lines (Figure 3.32), we showed that the firefly luciferase is produced. Unfortunately attempts at visualising the hybrid protein on a western blot were unsuccessful.

More experiments will have to be performed to overcome the above issues. A different cell line could be used to repeat the KD experiments with dsRNAs. Our suggestion is to use the SB or KB cell line. The CDK12 level in SB cells is high compared to other cells that were tested (Figure 3.29), but it might be a good choice because the cell line was derived from a healthy individual, so it is potentially easier to work with. The KB cells have much less of the CDK12, but are DM1 cells so other problems related to the condition could occur. Moreover we have tested only two double-stranded RNAs and it might be that oligos targeting different sites have to be synthesised and tested. As mentioned earlier the CDK12 is a relatively large protein, much bigger than firefly luciferase and might be hard to inhibit. Therefore another option is to use a combination of more than one dsRNAs, targeting different sites, to produce KD of the target.

4 Conclusions and Future Work

In the first part of this thesis we tested if targeting firefly luciferase sequences with siRNAs allows inhibition of protein production. As the positive results were obtained the 2'-OMe modified oligonucleotides were synthesised and checked for their efficacy in KD experiments. No inhibition of the target protein could be observed. It was demonstrated that ASOs were delivered to the cells and lack of gene-silencing is most probably due to insufficient binding properties of the tested oligonucleotides.

As fully 2'-OMe modified ASOs were unable to KD production of firefly luciferase we decided to introduce more chemical modifications within sequence of oligonucleotides. We decided to try to down-regulate the gene expression by activating the RNAi mechanism. First chemically modified dsRNAs were tested to check if are able to activate RNAi. All of tested ASOs were able to KD the target protein, so consequently the ssRNAs containing 5'-phosphate modification, AA overhangs and motifs of 7 phosphorothioate linkages were evaluated. None of the ssRNAs worked, so other modifications had to be introduced. It was decided to use an analogue of the 5'-*(E)*-vinylphosphonate modification (Figure 1.11), the vinylphosphonate-linked DNA dinucleotide (**34** in Figure 3.11). This work demonstrates that DNA nucleotides can be incorporated at the 5'-end of RNAs without significant loss of inhibition potency when double-stranded RNAs are used for treatment. Next a range of single-stranded oligonucleotides were synthesised and tested in the model system. We have also demonstrated that DNA nucleotides can be incorporated into ssRNA sequences without loss of potency and a significant inhibition of target can be achieved. By using additional chemical modifications (2'-OMe and

2’-F) ssRNA’s stability was improved. We show that *(E)*-vinylphosphonate DNA dinucleotide improves protection of the 5’-end of the ssRNA. We did not look at the mechanism by which the gene inhibition was triggered, however some publications suggest that not only RNAi but also other non-RNAi related mechanisms are involved.[229] It would be very interesting to perform similar experiments in our laboratory. Establishing which mechanism is activated by presented here ASOs is important and therefore it will be one of future experiments performed in the Hayes and Brook labs.

The second part of this thesis applies the knowledge we have gained to KD a disease relevant target *ex vivo* before *in vivo* mouse studies would be accomplished. We chose the CDK12 protein as our target. Several approaches were tried to detect CDK12 KD by the unmodified dsRNAs. The dsRNAs were used to inhibit the protein production in the MDA-MB fluc cell line and western blotting was employed for detection. Unfortunately, although different ASOs and concentrations were used no KD was observed. More attempts were taken in order to generate the CDK12 inhibition, but they were unsuccessful so far. The cell line that was used for the experiments is derived from cancer cells and therefore it is possible that some of the problems we faced are due to its nature. Therefore it might be worth repeating the experiments in a cell line obtained from a healthy volunteer, for example the SB cell line we have in our laboratory. Although the SB cells have shown elevated levels of CDK12 (Figure 3.29) when compared to other cell lines, the fact that they are not burdened with any disease might be beneficial in this case.

5 Experimental

5.1 Cell Culture Techniques

The human cancer cell lines MDA-MB 231 fluc and MDA-MB 231, used for cell culture work, were obtained from The Division of Pre-Clinical Oncology, School of Clinical Sciences, University of Nottingham, Nottingham.

5.1.1 Media and Solutions

5.1.1.1 Growth Media

Dulbecco's modified Eagle medium (DMEM) (Gibco) containing D-glucose (4.5g/L), L-glutamine and free of pyruvate. Media used for growing cells is supplemented with 10% (v/v) foetal bovine serum (FBS) EU approved origin (Sigma) and 1% (v/v) Pencicillin-Streptomycin (Sigma-Aldrich).

231 clones growth medium is DMEM supplemented with 10% (v/v) FBS, 1% (v/v) pencicillin-streptomycin and 5µg/mL of puromycin dihydrochloride (Sigma-Aldrich).

5.1.1.2 Additional Solutions

Dulbecco's phosphate buffered saline (DPBS) (Gibco) free of $CaCl_2$ and $MgCl_2$. DPBS is used for washing cells and to prevent cells from dying during the incubation phase of the different processes.

0.05% (v/v) Trypsin-EDTA solution (Gibco) used to passage the cell culture and to remove adherent cells from the surface of tissue culture treated flasks and plates.

Oligo treatment preparation media. Dulbecco's modified eagle medium supplemented with 1% (v/v) pencicillin-streptomycin.

5.1.2 Cell lines and plasmids

5.1.2.1 MDA-MB 231 cell line

The MDA-MB 231 line is a human breast adenocarcinoma cell line that was originally derived from metastatic site: pleural effusion.[230]

5.1.2.2 MDA-MB 231 fluc cell line

The MDA-MB 231 was stably transfected with a pLVX-Luc plasmid (sequence in Appendix 7.1) expressing firefly luciferase (*Photinus pyralis*) to give a final MDA-MB 231 fluc cell line (called MDA-MB fluc in this report) that is used in experiments. MDA-MB 231 fluc cells are easy to grow and maintained, which makes them a valuable tool for studies of gene KD.

5.1.2.3 CDK12pLVX -Luc plasmid

A CDK12pLVX-luc plasmid that is used for stable cell line generation was made in the laboratory by others in another project. It is a plasmid that expresses a hybrid protein consisting of CDK12 and firefly luciferase. To make a plasmid the CDK12 was amplified by PCR, isolated and extracted with QIAquick gel extraction kit (Qiagen) following the manufacturer's protocol. The purified CDK12 construct was inserted into pLVX-Luc plasmid, expressing firefly luciferase, using Gibson Assembly (NEB) following the manufacturer's protocol.

Plasmid sequence can be found in Appendix.

5.1.2.4 231 PP03 and 231 PP02 cell lines

5.1.2.4.1 Determining optimal selection antibiotic concentration

Prior to the MDA-MB 231 cell transfection with plasmid, an optimal antibiotic concentration for selecting cell colonies was determined. The MDA-MB 231 cells are plated in 24-well plates at 40'000 cells/well. One day after seeding the cells, puromycin dihydrochloride (Sigma-Aldrich) selection antibiotic is added to the wells in three replicates. An increasing concentration of antibiotic, including a non-antibiotic control, was used to determine the kill curve (0 µg/mL, 0.0625 µg/mL, 0.125 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 1.0 µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL). Cells were examined every 2-3 days for visual toxicity of antibiotic. The optimal dose was the one at which all cells were dead after one week of antibiotic selection. It is 0.5 µg/mL for this cell line.

5.1.2.4.2 231 PP03 and 231 PP02 cell lines generation

The MDA-MB 231 cells were seeded at a density 40,000 in a 24-well plate format one day before transfection. On the next day 1.5 µg (7.4 µL) of a linearised (digested with FseI Restriction Endonuclease, New England BioLabs) CDK12pLVX-Luc plasmid was mixed with 12 µL of HiPerFect and 380.6 µL of serum free DMEM. 100 µL of aforementioned mixture is added to each one of four replica wells. The plate was incubated for 3 days at 37 ºC in a 5% CO_2 atmosphere. 72 hours post transfection cells were exposed to the selection antibiotic, puromycin dihydrochloride (Sigma-Aldrich), at 0.5 µg/mL. The selection media was changed every 3-4 days and cells examined for visual toxicity daily. After 14 days surviving cells that undergo plasmid integration were transferred to 96-well plate, and then gradually expanded to a 24-well plate,

6-well plate and a tissue culture treated flasks to allow them to expand. Each of the obtained colonies was tested for luciferase presence and only two clones 231 PP02 and 231 PP03 were scored as positive.

5.1.2.5 KB Telo MyoD, SB Telo MyoD and Hela cell lines

KB Telo MyoD is a human fibroblast cell line from DM1 patient and has been established in Brook laboratory. SB Telo MyoD is a human fibroblast cell line from a healthy individual and was also established in Brook laboratory. Hela cell line is derived from cervical cancer cells.[231]

5.1.3 Maintenance of cell lines

5.1.3.1 Defrosting cell lines

A vial containing frozen cells was quickly thawed in a 37 °C water bath. Cells were transferred to a sterile tube containing 9 mL of a DMEM growth medium and centrifuged for 5 minutes at 1000 rpm. The supernatant was removed and the pellet resuspended in 10 mL of fresh DMEM and centrifuged again. Next the pelleted cells were resuspended in 10 mL of DMEM and transferred to a tissue culture flask containing 10 mL of pre-warmed medium. Cells were incubated at 37 °C in a 5% CO_2 incubator.

5.1.3.2 Passaging the adherent cells

MDA-MB 231 fluc cells were cultured in tissue culture grade plastic flasks in an incubator at 37 °C and 5% CO_2. The cells required passaging before they reached 100% confluence. Old media was removed and cells washed with 10 mL of DPBS pre-warmed to 37 °C. 3 mL of trypsin-EDTA was added and the flask incubated at 37 °C in a 5% CO_2 incubator for 5 minutes. This detached

cells from the surface of tissue culture flask, which then allowed resuspension in 10 mL of media. Cells were mixed thoroughly by pipetting up and down around 10 times. 1/4 of a mixture was then transferred to a fresh flask containing 20 mL of media. Cells reached confluence within 2-3 days at which point they again required passaging.

5.1.3.3 Counting cells

A haemocytometer was used to measure cell number following standard methodology. 500 µL of cell suspension was placed into the haemocytometer. The haemocytometer contains 2 sets of 9 squares, each of which is divided into 16 smaller squares. Cell number was counted within 3 of these 9 squares and an average taken to calculate the number of cells per 2 x 10^{-4} mL. To estimate the number of cells per mL of suspension the average number of cells was multiplied by 5000.

5.1.3.4 Cryopreservation of cells

Cells were trypsinised as described in 5.1.3.2, resuspended in 10 mL of DMEM growth medium and pelleted by centrifugation at 1000 rpm for 10 minutes. Pelleted cells were then resuspended in 1 mL of DMEM growth medium containing 5% (v/v) DMSO, transferred to a cryogenic vial (Thermo Scientific) and stored at -80 °C.

5.2 *Ex vivo* experiments with ASOs

The fluorescence of oligonucleotides in cells was measured on a wide-field system, the Molecular Devices Micro plate reader. Two filters were used to collect emission light from the samples corresponding to DAPI and TRITC. DAPI has an excitation and emission wavelength of 345 nm and 455 nm,

respectively. TRITC has an excitation and emission wavelength of 547 nm and 572 nm, respectively.

The luciferase assay was performed using the Turner Designs TD-20/20 Luminometer and the GloMax 20/20 single tube Luminometer (Promega).

The absorbance for the Bradford assay was measured at 595 nm on the Bio-Rad SmartSpec 3000 spectrophotometer.

5.2.1 Firefly luciferase target choice

Primer3 software was used (http://primer3.ut.ee/) to analyse the luciferase DNA sequence. The 5'-UTR, open reading frame (ORF) and 3'-UTR of firefly luciferase were used as a source sequence. The parameters below were used and the downstream primer was chosen.

	Minimum	**Optimum**	**Maximum**
Primers Size	18	20	23
Primer T_m	57.0	59.0	62.0
Primer GC%	30.0	50.0	70.0

5.2.2 Statistical analysis

Results for ASOs were processed with the GraphPad Prism 6 software that also performed statistical calculations. The data were analysed using one-way ANOVA test and were corrected for multiple comparisons by using Sidak's test.[232] The values in figures with individual samples and grouped controls were presented as the mean with SD. Every assay was performed in triplicate.

5.2.3 Protein extraction

Cells were washed with DPBS. To extract protein 100 µL of Passive Lysis Buffer (PLB) (1X) was added to the cells which were then scraped from the surface of the plate with mini cell scrapers and transferred to an Eppendorf tube. Tubes were left on ice for 30 minutes followed by two freeze-thaw cycles in -80 °C and 4 °C. The protein lysate was collected by centrifuging at 13,200 rpm for 25 minutes at 4 °C and the supernatant was stored at -80 °C.

5.2.4 Protein quantification

20 µL of protein sample was used for quantification following manufacturer's Instruction and using a Quick Start Bradford Protein Assay (Bio Rad). A bovine serum albumin standard set (0.125, 0.25, 0.5, 0.75 and 1.0 mg/mL) was used to generate a standard curve. The absorbance of the protein samples was measured using a photospectometer set to 595 nm.

5.2.5 Luciferase quantification

10 µL of protein sample was used for quantification. 50 µL of Luciferase Assay Reagent (Promega) was added to protein lysate, two components were mixed and the light intensity measured in a GloMax 20/20 single tube Luminometer (Promega).

5.2.6 *Ex vivo* potency of 2'-OMe modified oligonucleotides – gymnosis

Ex vivo potency experiments were performed in 24-well plate format. 100,000 cells per well were plated 1 day before transfection. The next day cells were washed with PBS, and new medium was supplemented. The 2'-OMe oligonucleotide was made up in DEPC water so it could be added at its indicated

concentration to the wells. The ASO was added drop-wise to each well and gently mixed. The plate was incubated for 48 hours, unless stated otherwise, at 37 °C in the incubator with a constant level of 5% CO_2. Cells were lysed with the Passive Lysis Buffer (PLB) and the reduction of target protein measured on the luminometer by luciferase assay and normalised against the amount of total protein, which was measured by performing the Bradford assay.

5.2.7 *Ex vivo* potency of 2'-OMe modified oligonucleotides – ExGen 500

Experiments were performed in a 24-well plate format. 100,000 of cells per well were plated 1 day before transfection. The next day cells were washed with PBS, and new media was supplemented. The 2'-OMe oligonucleotide solution (at the volume which gives the indicated concentration) was mixed with the transfection reagent (ExGen 500) and incubated for 10 minutes at room temperature. The final concentration of ExGen was 75 nM. Next, the formed complex was added drop-wise to each well and gently mixed. The plate was incubated for 48 hours, unless stated otherwise, at 37 °C in the incubator with a constant level of 5% CO_2. Cells were lysed with the PLB and the reduction of target protein was measured on the luminometer by luciferase assay and normalised against the amount of total protein, which was measured by performing the Bradford assay.

5.2.8 Determination of distribution of fluorescently labelled oligonucleotides

To obtain fluorescently labelled oligonucleotides a 5'-Hexachloro-Fluorescein-CE phosphoramidite was coupled to the desired oligo in the last step of the synthesis. The coupling time of the 2'-OMe modified oligo was preserved. The

products were purified using the Oligonucleotide Purification Cartridges (OPC®). The 2'-OMe modified and DNA oligos were synthesised in the Hayes laboratory by another student and had the following sequences:

2'-OMe: HEX-$C_sA_sG_s$ $C_sA_sG_s$ $C_sA_sG_s$ $C_sA_sG_s$ $C_sA_sG_s$ $C_sA_sG_s$ C_sA_sG

DNA: HEX-$C_sA_sG_s$ $C_sA_sG_s$ $C_sA_sG_s$ $C_sA_sG_s$ $C_sA_sG_s$ $C_sA_sG_s$ C_sA_sG

* "s" stands for phosphorothioate linkage, blue – 2'-OMe modification, black – DNA.

The morpholino oligonucleotide was obtained from Gene Tools, had 3'-Carboxyfluorescein modification and the following sequence:

Morpholino: CAG CAG CAG CAG CAG CAG CAG CAG C

The experiment was conducted in a 96-well plate format. 12,000 cells per well were plated 1 day before transfection. The next day cells were washed with PBS, and new medium was put on. The appropriate oligonucleotide solution, at desired concentration, was mixed with the transfection reagent (ExGen 500) and incubated for 10 minutes at rt. The final concentration of ExGen was 75 nM. The formed complex was added drop-wise to each well and gently mixed. The plate is incubated for 48 hours, at 37 °C in the incubator with a constant level of 5% CO_2. After that time cells are washed and their nuclei are stained with Hoechst dye (5µL in 50 mL of PBS) for 10 minutes. Next the stain was removed, cells are washed with PBS twice and imaged in PBS. The fluorescence was measured on a Molecular Devices Micro plate reader. To quantify the fluorescence a modified version of the journal in MetaXpress software was used. 25 fields per well were imaged and analysed. The nuclear area was identified by

Hoechst stain and the intensity of fluorescence was determined by scoring adjacent pixels that were 80 greyscales or more above the background. Data were analysed in excel spreadsheet and the means and standard deviations were calculated for the all samples.

5.2.9 Firefly luciferase knock-down with ssRNAs and dsRNAs

All of the experiments are performed in 24-well plate format. 40,000-50,000 of cells per well are plated 1 hour before transfection. ssRNAs or dsRNAs, in the volume to give the indicated concentration, are mixed with the transfection reagent (Table 5.1) and cell culture media with no serum and incubated for 10 minutes at rt. Afterwards the formed complex was added drop-wise to each well containing cells and gently mixed. The plate was incubated for 48 hours, unless stated otherwise, at 37 °C in the incubator with a constant level of 5% CO_2. Cells are lysed with the PLB and the reduction of target protein was measured on the luminometer by luciferase assay and normalised against amount of total protein, which was measured performing the Bradford assay.

Table 5.1. The volume of HiPerFect used for different oligo concentration in firefly luciferase KD experiments.

Final oligo concentration [nM]		**Volume of HPF used for transfection per well [µL]**
dsRNA	1 2 5 10 50	3
	250	6
ssRNA	250 500	6
	1000	6
	2500	8
	5000	12

5.2.10 CDK12 knock-down with dsRNAs and ssRNAs

For all of the CDK12 work, the CDK12 sequence was obtained from the Ensembl genome browser website (Genome assembly: GRCh38.p3). The transcript used was the CDK12-001, ENST00000447079, 8336 bp, 1490aa.

All of the experiments are performed in 24-well plate format with 70,000 of cells per well plated 1 hour before transfection. dsRNAs are mixed with the transfection reagent (Table 5.2) and cell culture media with no serum and incubated for 10 minutes at rt. The formed complex was added drop-wise to each well containing cells and the plate was incubated at 37 °C in the incubator with a constant level of 5% CO_2. When the treatment was finished the protein was extracted as described in 5.11.1. The amount of total protein was quantified in the Bradford assay (5.11.2). The target protein was detected on a western blot (5.11.3). The intensity of bands corresponding to desired protein was quantified with the use of Aida Image Analyzer and normalised against of CDK4 protein, which was also quantified by Aida Image Analyzer.

Table 5.2. The volume of HiPerFect used for different oligo concentration in firefly CDK12 KD experiments.

Final oligo concentration [nM]		**Volume of HPF used for transfection per well [µL]**
dsRNA	800	9
	1000	12

5.2.11 CDK12 knock-down in the 231 PP03 cell line

The experiment was performed as described in 5.2.9.

5.3 General procedures for ASOs synthesis, purification and analysis

The T*A (*E*)-vinylphosphonate-linked DNA dinucleotide (5´-O-(4,4´-Dimethoxytrityl)-thymidinyl-3´-O-(2-cyanoethyl)-phosphono-[3´(O)→5´(C)]-3´-O-(*tert*-butyldimethylsilyl)-5´-deoxy-5´-methylidene-2-deoxyadenosine-3´-O-(2-cyanoethyl)-phosphoramidite), which was used in this project was synthesised in Hayes laboratory by former PhD student Susanne Kruse.[142]

394 and 3400 ABI (Applied Biosystems) DNA synthesizers were used for oligonucleotide synthesis. For the negative control - scrambled oligonucleotide - a sequence was obtained from the Human Kinase siRNA set V4.1 (Qiagen), ID-SI04026960.

CPG Solid Supports (SynBase™ by Link Technologies), the 2'-OMe, RNA, DNA and 2'-F-phosphoramidites and reagents for the synthesizer were purchased from Link Technologies Ltd. NH_4OH (35%, Fischer) and $MeNH_2$ (33 wt.% in ethanol) was purchased from Fluka, $NEt_3 \cdot 3HF$ and *N*-methylpyrrolidinone (NMP) was purchased from Sigma-Aldrich, Oligonucleotide Purification Cartridge (OPC®) were obtained from Applied Biosystems, illustra NapTM5 columns were purchased from GE Healthcare Europe GmbH. Water used for oligonucleotides analysis and purification was a commercial nuclease-free water. MeCN HPLC grade was used for oligos synthesis and purification.

5.3.1 Synthesis of oligonucleotides

ASOs were synthesised on an ABI synthesizer (0.2 µM scale) by the phosphoramidite coupling method on a CPG Solid Supports. The following solutions were used:

- 3% trichloroacetic acid in dichloromethane (Deblock Mix)
- Anhydrous 0.3M 5-Benzylthio-1-H-tetrazole in acetonitrile (BTT activator)
- THF/pyridine/acetic anhydride (8:1:1) (Cap Mix A)
- 10% Methylimidazole in THF (Cap Mix B)
- 3H-1,2-Benzodithiol-3-one-1,1-dioxide (sulphurising reagent)
- 0.1M Iodine in THF/pyridine/water (78:20:2) (oxidiser)
- 0.1 M solutions of phosporamidites in MeCN

5.3.1.1 Synthesis and purification of 2'-OMe modified oligonucleotides

2'-OMe modified oligonucleotides were synthesised using the standard, manufacturer's 0.2 μM phosphorothioate DNA synthesis cycle with coupling time increased to 360 s.

After the desired sequence was synthesised the column was dried under vacuum for 20 minutes. The content of the synthesis column was transferred to the vial, 1 mL of ammonia solution (35%) was added to cleave the oligo from the column and the mixture was incubated at rt overnight. The next day the synthesised products were purified using OPC® and by following the manufacturer's protocol. MeCN HPLC Grade, TEAA (2 M), NH_4OH (1.5 M), 3% TFA, Milli-Q water and 20% MeCN were used as stock solutions in the purification protocol. Oligo samples were diluted with Milli-Q water (1 mL) prior to OPC® and loaded twice on the cartridge. Detritylation occurred using 3% TFA, DMTr-off samples are eluted upon addition of 20% MeCN and freeze-dried prior to further purification/analysis. The resultant oligonucleotide was desalted using a Nap-5 column and supplied protocol.

5.3.1.2 Synthesis and purification of RNA oligonucleotides

RNA oligonucleotides were synthesised using the standard, manufacturer's 0.2 μM RNA synthesis cycle with coupling time increased to 600 s.

The post-synthesis cycle consist of following steps:

- Cleavage and deprotection of cyanoethyl and base protecting groups:

The polymer-bound oligonucleotide was transferred from the synthesis column to a falcon tube and suspended in aqueous $MeNH_2$ (33 wt.% in ethanol, 1 mL). The solution was heated at 65°C for 10 minutes and cooled on ice to rt. The methylamine solution containing the oligonucleotide was separated from the CPG beads by pipetting to the Eppendorf tube. The beads were washed with RNase free water (2×500 μL), all supernatants were combined and dried under nitrogen for 2 hours, and then freeze-dried.

- Deprotection of 2'-OTBDMS groups and detritylation:

The dry oligonucleotide was resuspended in anhydrous $NEt_3 \cdot 3HF/NEt_3$/NMP solution (250 μL of a solution of 1.5 mL NMP, 750 μL NEt_3 and 1.0 mL $NEt_3 \cdot 3HF$), heated to 65°C for 1.5 hour, ensuring that the entire sample had dissolved. Next the mixture was allowed to cool to room temperature and quenched with 3M NaOAc solution (25 μL).

- Butanol precipitation:

n-butanol (1 mL) was added to the mixture, which was thoroughly mixed to produce a white precipitate. The sample was cooled to -80 °C for 2 hours, to encourage further precipitation. The precipitate was pelleted at 4 °C in a

centrifuge for 30 minutes (13,000 g). The supernatant was removed, and the pellet washed twice with 70% ethanol (0.5 mL). Finally the pellet was freeze-dried for 30 minutes. The dry precipitate was dissolved in RNase free water (0.5 mL) and desalted using Nap-5 column following the manufacturer's protocol.

5.3.1.3 Synthesis and purification of chemically modified ssRNAs with two DNA nucleotides or the vinylphosphonate dimer

The procedure described in 5.3.1.2 was used. For the chemically modified ssRNAs that contain a vinylphosphonate dimer in a last step of synthesis, three 10-minute couplings are performed to ensure sufficient coupling of the dimer to the rest of the oligo. The (*E*)-vinylphosphonate dimer was made by Susanne Kruse and the synthesis is presented in thesis.[142]

5.3.1.4 Synthesis and purification of fluorinated (2'-F) and methylated (2'-OMe) ssRNAs

For the sequences that contain both phosphodiester and phosphorothioate backbones the synthesis was divided into two cycles. First the 0.2 µM phosphorothioate DNA synthesis (5.3.1.1) cycle was selected with the coupling step extended to 360 seconds, all other steps in the protocol were not modified. For the phosphodiester backbones the standard 0.2 µM DNA synthesis cycle was selected with the coupling step extended to 360 seconds, all other steps remained unmodified. For the chemically modified ssRNAs that contain a vinylphosphonate dimer in a last step of synthesis three 10-minute couplings were performed to ensure sufficient coupling of the dimer to the rest of the oligo. The post-synthesis procedure that was used is described in 5.3.1.1.

5.3.1.5 HPLC purification

Sample analysis to check oligonucleotides purity was performed by reverse phase (RP) HPLC on the Phenomenex Calrity Oligo-RP column (50 x 4.6 mm) at rt. Two different sets of conditions are applied.

For unmodified RNAs: buffer A: 0.05 M TEAA, buffer B: MeOH. A gradient of 10-45% MeOH over 30 minutes was used, with a run rate of 1.0 mL/min. UV absorption at 260 nm and 290 nm.

For oligonucleotides with 2'-OMe and 2'-F modifications: buffer A: 0.1M TEAA, buffer B: CH_3CN. The following gradient of CH_3CN (B) was used: 0 min – 5%, 5 min – 5%, 20 min – 30%, 30 min – 30% with a run rate of 1.0 mL/min. UV absorption at 260 nm and 290 nm.

5.3.2 MS data

Electro-spray negative ionisation (ESI, M^-) mass spectrometry data was obtained using the Thermo LTQ FT Ultra to confirm masses of synthesised ssRNAs. Samples for MS analysis were made up in MeOH-H2O buffer (2:1 with 0.1% of ammonia v/v%) at 2 µM final oligo concentration.

Table 5.3. Examples of MS data.

ID	Ion	Calculated	Observed
Lu1742.1	$[M-4H^+]$	6708.10	6707.96
Lu1742.sense	$[M-4H^+]$	6714.12	6712.95
Lu150.sense	$[M-5H^+]$	7404.61	7404.05
Lu150.3	$[M-5H^+]$	7226.27	7225.90
Lu150.4	$[M-5H^+]$	7208.30	7206.95
Lu150.7	$[M-5H^+ + Na^+]$	7339.73	7338.80
Lu150.9	$[M-9H^+]$	7535.07	7535.04
Lu150.11	$[M-8H^+]$	7586.09	7585.04
Lu150.12	$[M-8H^+]$	7540.06	7538.96
-ve.2	$[M-8H^+]$	7586.15	7586.08
-ve.2.sense	$[M-5H^+]$	6731.06	6729.90
CDK12(4697).1	$[M-9H^+]$	7290.39	7290.00
CDK12(4697).sense	$[M-9H^+]$	7347.39	7347.06
CDK12(306).1	$[M-9H^+]$	7323.37	7323.03
CDK12(306).sense	$[M-9H^+]$	7329.43	7329.06

5.4 Annealing ASOs

ASOs were diluted in DEPC water (0.1% v/v diethylpyrocarbonate treated) to give a 100 µM concentration. 15 µL of each of the strand was placed in a sterile Eppendorf tube and 15 µL of annealing buffer (50 mM Tris, pH 7.5-8.0, 100 mM NaCl in DEPC H_2O) was added together with 30 µL of DEPC water. The final volume was 75 µL and the final concentration of siRNA duplex was 20 µM. Some oligonucleotides have the stock solution concentration higher or lower than 100 µM. For these the appropriate amount needed for annealing was calculated and the correct adjusted volume of each of the strand was added together with 15 µL of annealing buffer and the solution was made up with

DEPC water up to 75 µL. In both cases the final solution was mixed and centrifuged to collect all the liquid at the bottom. Next the mixture was heated at 90 ºC for 2 minutes, centrifuged again and allowed to cool to room temperature on a lab bench, for around 45 minutes. Annealed siRNAs are stored on ice if used on the same day or stored at -80 ºC for later use.

5.5 Determination of melting temperature (T_m)

The melting temperature data was obtained on a DU 800 Series UV/Vis Spectrophotometer (Beckman Coulter).

The antisense and the sense strand were mixed in equimolar ratios in a 20 mM NaCl and 10 mM phosphate buffer (pH 7.0) to give 3 µM of total duplex concentration. The samples were heated at 90 °C for 2 minutes and then allowed to cool down at rt for 45 minutes. To determine T_m, the duplexes were exposed to increasing temperature and the UV absorbance measurement (260 nm) was recorded every 0.5 °C until the 85 °C temperature was reached. The cooling curve was also recorded. T_m values were calculated from the first derivative of the melting curves.

5.6 Determination of IC_{50}

IC_{50} assays are performed in triplicate using 24-well plates, each well receiving 500 µL of media, containing 50,000 cells and are plated for 1 hour before transfection.

The amount of transfection reagent was kept the same for all oligonucleotide concentrations. The total amount of nucleic acid was kept constant at (500 nM for single-stranded ASO and 1 µM for double-stranded ASO), for all

concentrations of firefly luciferase ASO by the addition of a scrambled oligonucleotide; see Table 5.4 and Table 5.5.

The oligonucleotides were mixed with the transfection reagent and diluted with culture medium with no serum to give a final volume of 300 µL. The solution was incubated for 15 minutes at rt. After incubation, 100 µL of the mixture was added to each of the three replica wells and mixed. The plates were incubated for 72 hours at 37 °C with a constant 5% CO_2 atmosphere. Cells were lysed with the PLB and the reduction of target protein was measured on the luminometer by luciferase assay and normalised against amount of total protein, which was measured by performing the Bradford assay.

The IC_{50} curves are plotted using GraphPad Prism 6 software that also performs statistical calculations. Nonlinear regression with variable slope (four parameters) was used to obtain IC_{50} values.

Table 5.4. Preparation of solutions for IC_{50} study for single-stranded treatment. The table shows how the individual solutions at stated concentrations are prepared. Calculations are made to obtain the master mix for three replicas.

Volume of reagents used to prepare the oligo-transfection reagent complex	**Final luciferase oligonucleotide concentration [nM]**							
	500	**250**	**125**	**62.5**	**31.25**	**15.63**	**7.813**	**0**
Volume of luciferase oligo (67 µM stock solution) [µL]	13.43	6.72	3.36	1.68	0.84	0.42	0.21	0
Volume of scrambled oligo (75 µM stock solution) [µL]	0	6.0	9.0	10.5	11.25	11.62	11.81	12.0
Volume of HiPerFect [µL]	18	18	18	18	18	18	18	18
Serum free media used for oligo-transfection reagent incubation [µL]	268.6	269.3	269.6	269.8	269.9	270	270	270
Volume of media used to plate the cells [µL]	1500	1500	1500	1500	1500	1500	1500	1500

Table 5.5. Preparation of solutions for IC_{50} study for double-stranded treatment. The table shows how the individual solutions at stated concentrations are prepared. Calculations are made to obtain the master mix for three replicas.

Volume of reagents used to prepare the oligo-transfection reagent complex	Final luciferase oligonucleotide concentration [nM]							
	500	**250**	**125**	**62.5**	**31.25**	**15.63**	**7.813**	**0**
Volume of luciferase oligo (20 µM stock solution) [µL]	45	22.5	11.25	5.58	2.82	1.41	0.69	0
Volume of scrambled oligo (20 µM stock solution) [µL]	0	22.5	33.75	39.42	42.18	43.59	44.31	45
Volume of HiPerFect [µL]	18	18	18	18	18	18	18	18
Serum free media used for oligo-transfection reagent incubation [µL]	237	237	237	237	237	237	237	237
Volume of media used to plate the cells [µL]	1500	1500	1500	1500	1500	1500	1500	1500

5.7 Viability assay

Viability assays were performed in triplicate using 96-well black plates with clear bottom, each well receiving 100 µL of media, containing 4,000 cells and plated for 1 hour before transfection. ssRNAs or dsRNAs, in the volume to give the indicated concentration, were mixed with the transfection reagent (1 µL of HiPerFect per well) and cell culture media with no serum and incubated for 10 minutes at rt. The formed complex was added drop-wise to each well containing cells and gently mixed. The plate was incubated for 72 hours at 37°C in the

incubator with a constant level of 5% CO_2. 10 µL of alamarBlue (ThermoFisher Scientifiec) was added to each of the wells, the plate was vigorously shaken and the plate was incubated for 3 hours at 37 °C in the incubator. The fluorescence was measured on Infinite F200Pro plate reader (Tecan) at $535_{Ex}/590_{Em}$ nm. The relative fluorescence units (RFU) values were plotted using GraphPad Prism 6 software that also performs statistical calculations.

5.8 Effect of HiPerFect on firefly luciferase expression

The experiment was performed in 24-well plate format. 50,000 of cells per well were plated 1 hour before transfection. Increasing volumes (3.0 µL, 4.5 µL, 6.0 µL, 7.5 µL, 9.0 µL) of the HiPerFect were added to the cell culture media with no serum and incubated for 10 minutes at rt. The mixture was added drop-wise to each well containing cells and gently mixed. The plate was incubated for 72 hours at 37 °C in the incubator with a constant level of 5% CO_2. Cells were lysed with the PLB and luciferase signal measured on the luminometer by luciferase assay normalised against amount of total protein, which was measured by performing the Bradford assay.

5.9 Effect of Lipofectamine 2000 on firefly luciferase expression

The experiment was performed in 24-well plate format. 50,000 cells per well were plated 1 day before transfection in DMEM medium without serum and antibiotic. Increasing volumes (0.5 µL, 1.0 µL, 1.5 µL, 2.0 µL, 2.5 µL) of the Lipofectamine 2000 (Thermo Fisher Scientific) were added to the Opti-Mem medium with no serum or antibiotic and incubated for 20 minutes at rt. The mixture was added drop-wise to each well containing cells and gently mixed. The plate was incubated for 48 hours at 37 °C in the incubator with a constant

level of 5% CO_2. Cells were lysed with the PLB and luciferase signal was measured on the luminometer by luciferase assay and normalised against the amount of total protein, which was measured performing the Bradford assay.

5.10 Effect of electroporation on firefly luciferase expression

For the electroporation the Amaxa Nucleofector™ II device was used (Lonza).

The experiment was performed in 6-well plate format. 1 mL of pre-warmed DMEM medium was put into the wells of the plate. 1,000,000 cells were placed in a vial and resuspended in 100 µL of Nucleofector (Lonza) solution. The cell suspension was mixed with the siRNA (oligo concentration was calculated for 100 µL) and the mixture was transferred into the cuvette. The X-013 program was selected and the cuvette was inserted into the Nucleofector Cuvette Holder and the X-button pressed to execute the selected program. The cuvette was removed from the holder and 500 µL of DMEM medium was added immediately and the whole mixture was gently transferred into prepared 6-well plate. The plate was incubated for 48 hours at 37 °C in the incubator with a constant level of 5% CO_2. Cells were lysed with the PLB and the luciferase signal was measured on the luminometer by luciferase assay and normalised against the amount of total protein, which was measured by performing the Bradford assay.

5.11 Western blotting

Detection of bands on the membrane was performed on the LAS-3000 (Fujifilm) Imaging System or the SRX-201 (Konica Minolta) X-Ray film processor.

The intensity of bands corresponding to the desired protein was quantified with the use of Aida Image Analyzer.

For all of the CDK12 work the CDK12 sequence was obtained from the Ensembl genome browser website (http://www.ensembl.org/). The transcript used was the CDK12-001, ENST00000447079, 8336 bp, 1490aa.

5.11.1 Protein extraction from tissue culture

Cells were washed with DPBS followed by an addition of 100 µL of trypsin-EDTA solution. When the cells were detached from the surface of the plate 1 mL of medium was added and the whole mixture was transferred to a sterile tube and centrifuged for 5 minutes at 1000 rpm to obtain a pellet. The supernatant was discarded and the pellet was resuspended in DPBS and centrifuged again for 5 minutes at 1000 rpm to remove any residue of medium. To extract protein 100 µL of Ripa buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% (v/v) NP-40, 0.1% (v/v) SDS, 0.5% (w/v) Deoxycholic acid, Protease inhibitor cocktail) was added and cells left on ice for 30 minutes followed by two freeze-thaw cycles at -80 °C and at 4 °C. The protein lysate was collected by centrifuging at top speed for 25 minutes at 4°C and the supernatant was collected and stored at -80 °C.

5.11.2 Protein quantification

10 µL of protein sample was used for protein quantification. Bovine serum albumin was used at the concentration 1.0 mg/mL to produce a standard curve. Protein concentration was established using the BioRad DC Protein assay. The absorbance of the protein samples was measured using a Bio-Rad SmartSpec 3000 Spectrophotometer set to 750 nm.

5.11.3 Detection of CDK12 on western blot

All reagents for western blotting were obtained from Novex (Life Technologies). Protein samples were prepared as follows: 10 or 5 µg of cell lysate (unless otherwise stated), 2.5 µL NuPAGE LDS sample buffer, 1 µL NuPAGE reducing agent and sterile distilled water to a final volume of 10 µL. The amount of protein sample added was adjusted to ensure equal loading amounts, depending on sample concentration. The reaction was heated to 70 °C for 15 minutes and next run on a NuPage 10% Bis-Tris Gel for 1 hour. Protein was transferred to an Invitrolon PVDF membrane for 1.5 hour at 20 V, following the Invitrogen protocol and using an Xcell Blot Module. The membrane was washed twice in distilled water and blocked for 2 to 4 hours with 5% blotto in TBS-Tween (50 mM Tris HCl, 150 mM NaCl, 0.05% Tween 20; pH adjusted with HCl to 7.6). The membrane was washed in TBS-Tween and incubated in the 10 µL of primary antibody anti-CrkRS (Abcam, ab57311) diluted in 3% BSA in TBS-Tween (8 mL) overnight at 4 °C. The membrane was washed in TBS-Tween and incubated in the secondary antibody (1 µL), conjugated to horseradish peroxidase (HRP), for 30 minutes at rt. The membrane was washed again before being exposed using ECL detection reagents (Thermo Scientific) following the manufacturers' protocol. The developed membrane was then exposed using X-Ray film processor or LAS-3000 Imaging System.

5.11.4 Detection of reference genes on western blot

To ensure that protein loading was the same across the gel a CDK4 or GAPDH expression was used as internal control.

For CDK4:

After CDK12 detection the membrane was washed in TBS-Tween for 2 hours, and washing buffer was renewed every 30 minutes. Next, the membrane was incubated in 10 µL of primary antibody CDK4 (Santa Cruz, sc-260) diluted in 3% BSA in TBS-Tween (8 mL) at room temperature for 1.5 hour. The membrane was washed in TBS-Tween and incubated in the secondary antibody (1 µL), conjugated to horseradish peroxidase (HRP), for 30 minutes at rt. The membrane was washed again before being exposed using ECL detection reagents (Thermo Scientific) following the manufacturers' protocol. The developed membrane was then exposed using X-Ray film processor or LAS-3000 Imaging System.

For later western blots there are two alterations to the above protocol, which eliminates background smear:

- The membrane was blocked overnight at 4 °C with 5% blotto in TBS-Tween (50 mM Tris HCl, 150 mM NaCl, 0.05% Tween 20; pH adjusted with HCl to 7.6)
- The membrane was washed in TBS-Tween and incubated in the 5 µL of primary antibody anti-CrkRS (Abcam, ab57311) diluted in 3% BSA in TBS-Tween (8 mL) for 2 hours at rt.

For GAPDH:

The protocol 5.11.3 was used with one change that was a 30 minutes incubation time in primary antibody at rt. The membrane used for CDK12 detection was cut in half. The top part was used for CDK12 detection and the bottom part was used for GAPDH detection. The Anti-GAPDH Mouse mAb (6C5) was used

(Merck Millipore) at the 5 µL (of the 1 in 5000 dilution) in 8 mL of 3% (w/v) BSA in TBS-Tween.

5.11.5 Detection of firefly luciferase on western blot

All reagents for western blotting were obtained from Novex (Life Technologies). Protein sample reactions are prepared as follows: 5 µg of cell lysate (unless otherwise stated), 2.5 µL NuPAGE LDS sample buffer, 1 µL NuPAGE reducing agent and sterile distilled water to a final volume of 10 µL. The amount of protein sample added was adjusted to ensure equal loading amounts, depending on sample concentration. The reaction was heated to 70°C for 15 minutes and was then run on a NuPage 10% Bis-Tris Gel for 1 hour. Protein was transferred to an Invitrolon PVDF membrane for 1.5 hours at 20V, following the Invitrogen protocol and using an Xcell Blot Module. The membrane was washed twice in distilled water and blocked overnight at 4°C with 5% blotto in TBS-Tween (50 mM Tris HCl, 150 mM NaCl, 0.05% (v/v) Tween 20; pH adjusted with HCl to 7.6). The membrane was washed in TBS-Tween and incubated in the 4 µL of primary anti-firefly luciferase antibody (Abcam, ab21176) diluted in 3% BSA in TBS-Tween for 2 hours at rt. The membrane was washed in TBS-Tween and incubated in the secondary antibody (1 µL), conjugated to horseradish peroxidase (HRP), for 30 minutes at rt. The membrane was washed again before being exposed using ECL detection reagents (Thermo Scientific) following the manufacturers' protocol. The developed membrane was then exposed using X-Ray film processor or LAS-3000 Imaging System.

5.11.6 Immunoprecipitation

The Protein A/G Plus-Agarose (Santa Cruz) was used for protein precipitation following the manufacturer's Instruction. The isolated protein (half of the sample – 20 µL) was visualised by performing the western blot described in 5.11.3 and 5.11.5.

5.12 Detection of mRNA of hybrid protein

5.12.1 RNA extraction

Cells were trypsinised (description in 5.1.3.2), washed with DPBS and pelleted prior to homogenisation with TRI reagent (Sigma-Aldrich) and the manufacturer's protocol was followed to isolate RNA.

5.12.2 cDNA preparation

1 µg RNA was used as an input in cDNA synthesis. The reaction was performed using iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions.

5.12.3 PCR

Primers for PCR were designed using: Primer3web (http://primer3.ut.ee/). Primers sequences are presented in Table 5.6.

Table 5.6. Primers used for quantification of mRNA

Name	Primer	Sequence (5'→3')	Product length
endo CDK12.1	Fw	TGTGTACCCTGCCAGTTCTT	150 bp
	Rv	TTATCCCATCCTGACAGCCC	
endo CDK12.2	Fw	GTGGCCTGGCTGATTTGAAT	169 bp
	Rv	CAGGGAGAAGGGCGTATCTT	
total CDK12.1	Fw	TAGGCCTTGCTGACATCACA	168 bp
	Rv	TGATGGATTGGTTCAGGGCT	
total CDK12.2	Fw	GGGACAAGTACTGAGCCTGT	219 bp
	Rv	CTCTGGGTTGGAGTGGATGT	
exo CDK12.1	Fw	GGAAGAGGGAGAGGAGTTCC	249 bp
	Rv	ATATGTGCGTCGGTAAAGGC	
exo CDK12.2	Fw	ATTCTGCAGTCGACGGTACC	249 bp
	Rv	ACTCGGCGTAGGTAATGTCC	
total fluc.1	Fw	GAGGCTAAGGTGGTGGACTT	199 bp
	Rv	CCACGATGAAGAAGTGCTCG	
total fluc.2	Fw	TTCGAATTCTGCAGTCGACG	394 bp
	Rv	CACAGCCACACCGATGAAC	

For PCR, 1 µL of cDNA sample was used. The reaction mixture was made with 17 µL of MegaMix (Microzone limited) (1X), 1 µL of forward primer and 1 µL of reverse primer. The following program that was designed with the use of Optimase ProtocolWriter™, and samples were run on a standard thermal cycler:

Step 1: 95.0 ºC, 2 min.

Step 2: 95.0 ºC, 30 sec.

Step 3: 63.3ºC decrease 0.5 ºC per cycle, 30 sec.

Step 4: 72.0 ºC, 30 sec.

Step 5: Repeat steps 2-4 14 more times.

Step 6: 95.0 ºC, 30 sec.

Step 7: 56.3 ºC, 30 sec.

Step 8: 72.0 ºC, 30.0 sec.

Step 9: Repeat steps 6-8 19 more times.

Step 10: 72.0 ºC, 5 min.

Step 11: 4.0 ºC.

Obtained products are visualised on 1.0% (w/v) agarose gel and their size was estimated using a DNA ladder (Invitrogen).

6 Bibliography

1. Doweyko, A. M.; Doweyko, L. M., What is next for small-molecule drug discovery? *Future Medicinal Chemistry* **2009,** *1* (6), 1029-36.

2. Collins, F. S.; Morgan, M.; Patrinos, A., The Human Genome Project: lessons from large-scale biology. *Science (New York, N.Y.)* **2003,** *300* (5617), 286-90.

3. Initial sequencing and analysis of the human genome. *Nature* **2001,** *409* (6822), 860-921.

4. Venter, J. C.; Adams, M. D.; Myers, E. W.; Li, P. W.; Mural, R. J.; Sutton, G. G. *et al*. The sequence of the human genome. *Science (New York, N.Y.)* **2001,** *291* (5507), 1304-51.

5. Golay, J.; Introna, M., Mechanism of action of therapeutic monoclonal antibodies: Promises and pitfalls of in vitro and in vivo assays. *Archives of Biochemistry and Biophysics* **2012,** *526* (2), 146-153.

6. Kohler, G.; Milstein, C., Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **1975,** *256* (5517), 495-497.

7. Chames, P.; Van Regenmortel, M.; Weiss, E.; Baty, D., Therapeutic antibodies: successes, limitations and hopes for the future. *British Journal of Pharmacology* **2009,** *157* (2), 220-33.

8. Beck, A.; Wurch, T.; Bailly, C.; Corvaia, N., Strategies and challenges for the next generation of therapeutic antibodies. *Nature Reviews Immunology* **2010,** *10* (5), 345-352.

9. Saonere, J. A., Antisense therapy, a magic bullet for the treatment of various diseases: Present and future prospects. *Journal of Medical Genetics and Genomics* **2011,** *3* (5), 77-83.

10. Belikova, A. M.; Zarytova, V. F.; Grineva, N. I., Synthesis of ribonucleosides and diribonucleoside phosphates containing 2-chloro-ethylamine and nitrogen mustard residues. *Tetrahedron Letters* **1967,** *8* (37), 3557-3562.

11. Paterson, B. M.; Roberts, B. E.; Kuff, E. L., Structural gene identification and mapping by DNA-mRNA hybrid-arrested cell-free translation. *Proceedings of the National Academy of Sciences of the United States of America* **1977,** *74* (10), 4370-4.

12. Zamecnik, P. C.; Stephenson, M. L., Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proceedings of the National Academy of Sciences of the United States of America* **1978,** *75* (1), 280-284.

13. Eckstein, F., Nucleoside phosphorothioates. *Annual Review of Biochemistry* **1985,** *54*, 367-402.

14. Crooke, S. T., Therapeutic applications of oligonucleotides. *Annual Review of Pharmacology and Toxicology* **1992,** *32*, 329-76.

15. Lee, R. C.; Feinbaum, R. L.; Ambros, V., The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **1993,** *75* (5), 843-54.

16. Cai, Y.; Yu, X.; Hu, S.; Yu, J., A brief review on the mechanisms of miRNA regulation. *Genomics, Proteomics & Bioinformatics* **2009,** *7* (4), 147-54.

17. Carthew, R. W.; Sontheimer, E. J., Origins and Mechanisms of miRNAs and siRNAs. *Cell* **2009,** *136* (4), 642-55.

18. Pasquinelli, A. E., MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nature Reviews. Genetics* **2012,** *13* (4), 271-82.

19. Ha, M.; Kim, V. N., Regulation of microRNA biogenesis. *Nature Reviews Molecular Cell Biology* **2014,** *15* (8), 509-524.

20. Walker, F. O., Huntington's disease. *Lancet (London, England)* **2007,** *369* (9557), 218-28.

21. Finkbeiner, S., Huntington's Disease. *Cold Spring Harbor Perspectives in Biology* **2011,** *3* (6).

22. Nasir, J.; Floresco, S. B.; O'Kusky, J. R.; Diewert, V. M.; Richman, J. M.; Zeisler, J.; Borowski, A.; Marth, J. D.; Phillips, A. G.; Hayden, M. R., Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. *Cell* **1995,** *81* (5), 811-23.

23. Huang, K.; Sanders, S. S.; Kang, R.; Carroll, J. B.; Sutton, L.; Wan, J.; Singaraja, R.; Young, F. B.; Liu, L.; El-Husseini, A.; Davis, N. G.; Hayden, M. R., Wild-type HTT modulates the enzymatic activity of the neuronal palmitoyl transferase HIP14. *Human Molecular Genetics* **2011,** *20* (17), 3356-65.

24. Yu, D.; Pendergraff, H.; Liu, J.; Kordasiewicz, H. B.; Cleveland, D. W.; Swayze, E. E.; Lima, W. F.; Crooke, S. T.; Prakash, T. P.; Corey, D. R., Single-stranded RNAs use RNAi to potently and allele-selectively inhibit mutant huntingtin expression. *Cell* **2012,** *150* (5), 895-908.

25. Ostergaard, M. E.; Southwell, A. L.; Kordasiewicz, H.; Watt, A. T.; Skotte, N. H.; Doty, C. N. *et al*. Rational design of antisense oligonucleotides targeting single nucleotide polymorphisms for potent and allele selective suppression of mutant Huntingtin in the CNS. *Nucleic Acids Research* **2013,** *41* (21), 9634-50.

26. Hu, J.; Matsui, M.; Corey, D. R., Allele-Selective Inhibition of Mutant Huntingtin by Peptide Nucleic Acid (PNA)-Peptide Conjugates, Locked Nucleic Acid (LNA), and siRNA. *Annals of the New York Academy of Sciences* **2009,** *1175*, 24-31.

27. Mulamba, G. B.; Hu, A.; Azad, R. F.; Anderson, K. P.; Coen, D. M., Human Cytomegalovirus Mutant with Sequence-Dependent Resistance to the Phosphorothioate Oligonucleotide Fomivirsen (ISIS 2922). *Antimicrobial Agents and Chemotherapy* **1998,** *42* (4), 971-3.

28. Crooke, S. T.; Geary, R. S., Clinical pharmacological properties of mipomersen (Kynamro), a second generation antisense inhibitor of apolipoprotein B. *British Journal of Clinical Pharmacology* **2013,** *76* (2), 269-76.

29. Crooke, R. M.; Graham, M. J.; Lemonidis, K. M.; Whipple, C. P.; Koo, S.; Perera, R. J., An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis. *Journal of Lipid Research* **2005,** *46* (5), 872-884.

30. Merki, E.; Graham, M. J.; Mullick, A. E.; Miller, E. R.; Crooke, R. M.; Pitas, R. E.; Witztum, J. L.; Tsimikas, S., Antisense oligonucleotide directed to human apolipoprotein B-100 reduces lipoprotein(a) levels and oxidized phospholipids on human apolipoprotein B-100 particles in lipoprotein(a) transgenic mice. *Circulation* **2008,** *118* (7), 743-53.

31. Wong, E.; Goldberg, T., Mipomersen (Kynamro): A Novel Antisense Oligonucleotide Inhibitor for the Management of Homozygous Familial Hypercholesterolemia. *Pharmacy and Therapeutics* **2014,** *39* (2), 119-22.

32. Watts, J. K.; Corey, D. R., Silencing disease genes in the laboratory and the clinic. *The Journal of Pathology* **2012,** *226* (2), 365-79.

33. Pirollo, K. F.; Rait, A.; Sleer, L. S.; Chang, E. H., Antisense therapeutics: from theory to clinical practice. *Pharmacology & Therapeutics* **2003,** *99* (1), 55-77.

34. Szymkowski, D. E., Developing antisense oligonucleotides from the laboratory to clinical trials. *Drug Discovery Today* **1996,** *1* (10), 415-428.

35. Doyle, D. F.; Braasch, D. A.; Simmons, C. G.; Janowski, B. A.; Corey, D. R., Inhibition of gene expression inside cells by peptide nucleic acids: effect of mRNA target sequence, mismatched bases, and PNA length. *Biochemistry* **2001,** *40* (1), 53-64.

36. Braasch, D. A.; Liu, Y.; Corey, D. R., Antisense inhibition of gene expression in cells by oligonucleotides incorporating locked nucleic acids: effect of mRNA target sequence and chimera design. *Nucleic Acids Research* **2002,** *30* (23), 5160-7.

37. Roca, X.; Krainer, A. R., RNA Splicing A2 - Maloy, Stanley. In *Brenner's Encyclopedia of Genetics (Second Edition)*, Hughes, K., Ed. Academic Press: San Diego, 2013; pp 292-294.

38. Grainger, R. J.; Beggs, J. D., Pre-mRNA Splicing A2 - Maloy, Stanley. In *Brenner's Encyclopedia of Genetics (Second Edition)*, Hughes, K., Ed. Academic Press: San Diego, 2013; pp 442-445.

39. Lynch, K, Motta-Mena, L (2013). Alternative Splicing. Encyclopaedia of Biological Chemistry Second Edition: 75-80.

40. Muntoni, F.; Torelli, S.; Ferlini, A., Dystrophin and mutations: one gene, several proteins, multiple phenotypes. *The Lancet. Neurology* **2003,** *2* (12), 731-40.

41. Koenig, M.; Beggs, A. H.; Moyer, M.; Scherpf, S.; Heindrich, K.; Bettecken, T. *et al*. The molecular basis for Duchenne versus Becker muscular dystrophy: Correlation of severity with type of deletion. *American Journal of Human Genetics* **1989,** *45* (4), 498-506.

42. Moser, H., Duchenne muscular dystrophy: pathogenetic aspects and genetic prevention. *Human Genetics* **1984,** *66* (1), 17-40.

43. England, S. B.; Nicholson, L. V.; Johnson, M. A.; Forrest, S. M.; Love, D. R.; Zubrzycka-Gaarn, E. E.; Bulman, D. E.; Harris, J. B.; Davies, K. E., Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. *Nature* **1990,** *343* (6254), 180-2.

44. van Deutekom, J. C.; Bremmer-Bout, M.; Janson, A. A.; Ginjaar, I. B.; Baas, F.; den Dunnen, J. T.; van Ommen, G. J., Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. *Human Molecular Genetics* **2001,** *10* (15), 1547-54.

45. Lima, W., Wu, H. & Crooke, S. T., *Antisense Drug Technology: Principles, Strategies, and Applications, Second Edition*, Chapter 2 (CRC Press, Boca Raton, Florida, 2007).

46. Cerritelli, S. M.; Frolova, E. G.; Feng, C.; Grinberg, A.; Love, P. E.; Crouch, R. J., Failure to produce mitochondrial DNA results in embryonic lethality in Rnaseh1 null mice. *Molecular Cell* **2003,** *11* (3), 807-15.

47. Stein, H.; Hausen, P., Enzyme from calf thymus degrading the RNA moiety of DNA-RNA Hybrids: effect on DNA-dependent RNA polymerase. *Science (New York, N.Y.)* **1969,** *166* (3903), 393-5.

48. Nowotny, M.; Gaidamakov, S. A.; Ghirlando, R.; Cerritelli, S. M.; Crouch, R. J.; Yang, W., Structure of Human RNase H1 Complexed with an RNA/DNA Hybrid: Insight into HIV Reverse Transcription. *Molecular Cell* **2007,** *28* (2), 264-276.

49. Nowotny, M.; Yang, W., Stepwise analyses of metal ions in RNase H catalysis from substrate destabilization to product release. *The EMBO Journal* **2006,** *25* (9), 1924-33.

50. Yang, W.; Lee, J. Y.; Nowotny, M., Making and breaking nucleic acids: two-Mg2+-ion catalysis and substrate specificity. *Molecular Cell* **2006,** *22* (1), 5-13.

51. Lima, W., Wu, H. & Crooke, S. T., *Antisense Drug Technology: Principles, Strategies, and Applications, Second Edition*, 47-74 (CRC Press, Boca Raton, Florida, 2007).

52. Elsasser, B.; Fels, G., Atomistic details of the associative phosphodiester cleavage in human ribonuclease H. *Physical Chemistry Chemical Physics* **2010,** *12* (36), 11081-11088.

53. Dean, N. M.; McKay, R.; Condon, T. P.; Bennett, C. F., Inhibition of protein kinase C-alpha expression in human A549 cells by antisense oligonucleotides inhibits induction of intercellular adhesion molecule 1 (ICAM-1) mRNA by phorbol esters. *The Journal of Biological Chemistry* **1994,** *269* (23), 16416-24.

54. Lee, J. E.; Bennett, C. F.; Cooper, T. A., RNase H-mediated degradation of toxic RNA in myotonic dystrophy type 1. *Proceedings of the National Academy of Sciences of the United States of America* **2012,** *109* (11), 4221-6.

55. Wheeler, T. M.; Leger, A. J.; Pandey, S. K.; MacLeod, A. R.; Nakamori, M.; Cheng, S. H.; Wentworth, B. M.; Bennett, C. F.; Thornton, C. A., Targeting nuclear RNA for in vivo correction of myotonic dystrophy. *Nature* **2012,** *488* (7409), 111-115.

56. Suzuki, Y.; Holmes, J. B.; Cerritelli, S. M.; Sakhuja, K.; Minczuk, M.; Holt, I. J.; Crouch, R. J., An upstream open reading frame and the context of the two AUG codons affect the abundance of mitochondrial and nuclear RNase H1. *Molecular and Cellular Biology* **2010,** *30* (21), 5123-34.

57. Iwasaki, Y. W.; Siomi, M. C.; Siomi, H., PIWI-Interacting RNA: Its Biogenesis and Functions. *Annual Review of Biochemistry* **2015,** *84*, 405-33.

58. Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C., Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **1998,** *391* (6669), 806-11.

59. Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T., Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **2001,** *411* (6836), 494-498.

60. Song, E.; Lee, S. K.; Wang, J.; Ince, N.; Ouyang, N.; Min, J.; Chen, J.; Shankar, P.; Lieberman, J., RNA interference targeting Fas protects mice from fulminant hepatitis. *Nature Medicine* **2003,** *9* (3), 347-51.

61. Ratcliff, F. G.; MacFarlane, S. A.; Baulcombe, D. C., Gene silencing without DNA. rna-mediated cross-protection between viruses. *The Plant Cell* **1999,** *11* (7), 1207-16.

62. Tabara, H.; Sarkissian, M.; Kelly, W. G.; Fleenor, J.; Grishok, A.; Timmons, L.; Fire, A.; Mello, C. C., The rde-1 gene, RNA interference, and transposon silencing in C. elegans. *Cell* **1999,** *99* (2), 123-32.

63. Elbashir, S. M.; Lendeckel, W.; Tuschl, T., RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes & Development* **2001,** *15* (2), 188-200.

64. Lau, N. C.; Lim, L. P.; Weinstein, E. G.; Bartel, D. P., An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science (New York, N.Y.)* **2001,** *294* (5543), 858-62.

65. Meister, G., Argonaute proteins: functional insights and emerging roles. *Nature reviews. Genetics* **2013,** *14* (7), 447-459.

66. Schwarz, D. S.; Hutvagner, G.; Du, T.; Xu, Z.; Aronin, N.; Zamore, P. D., Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **2003,** *115* (2), 199-208.

67. Lima, W. F.; Wu, H.; Nichols, J. G.; Sun, H.; Murray, H. M.; Crooke, S. T., Binding and Cleavage Specificities of Human Argonaute2. *Journal of Biological Chemistry* **2009,** *284* (38), 26017-26028.

68. Davidson, Beverly L.; Monteys, Alex M., Singles Engage the RNA Interference Pathway. *Cell 150* (5), 873-875.

69. Bohmert, K.; Camus, I.; Bellini, C.; Bouchez, D.; Caboche, M.; Benning, C., AGO1 defines a novel locus of Arabidopsis controlling leaf development. *The EMBO Journal* **1998,** *17* (1), 170-80.

70. Schirle, N. T.; MacRae, I. J., The crystal structure of human Argonaute2. *Science (New York, N.Y.)* **2012,** *336* (6084), 1037-40.

71. Lingel, A.; Simon, B.; Izaurralde, E.; Sattler, M., Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. *Nature Structural & Molecular Biology* **2004,** *11* (6), 576-7.

72. Song, J. J.; Smith, S. K.; Hannon, G. J.; Joshua-Tor, L., Crystal structure of Argonaute and its implications for RISC slicer activity. *Science (New York, N.Y.)* **2004,** *305* (5689), 1434-7.

73. Ma, J. B.; Yuan, Y. R.; Meister, G.; Pei, Y.; Tuschl, T.; Patel, D. J., Structural basis for 5'-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. *Nature* **2005,** *434* (7033), 666-70.

74. Parker, J. S.; Roe, S. M.; Barford, D., Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex. *Nature* **2005,** *434* (7033), 663-6.

75. Liu, J.; Carmell, M. A.; Rivas, F. V.; Marsden, C. G.; Thomson, J. M.; Song, J. J.; Hammond, S. M.; Joshua-Tor, L.; Hannon, G. J., Argonaute2 is the catalytic engine of mammalian RNAi. *Science (New York, N.Y.)* **2004,** *305* (5689), 1437-41.

76. Lima, W. F.; Murray, H.; Nichols, J. G.; Wu, H.; Sun, H.; Prakash, T. P.; Berdeja, A. R.; Gaus, H. J.; Crooke, S. T., Human Dicer binds short single-strand and double-strand RNA with high affinity and interacts with different regions of the nucleic acids. *The Journal of Biological Chemistry* **2009,** *284* (4), 2535-48.

77. Meister, G.; Landthaler, M.; Patkaniowska, A.; Dorsett, Y.; Teng, G.; Tuschl, T., Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Molecular Cell* **2004,** *15* (2), 185-97.

78. Martinez, J.; Tuschl, T., RISC is a 5' phosphomonoester-producing RNA endonuclease. *Genes & Development* **2004,** *18* (9), 975-80.

79. Schwarz, D. S.; Tomari, Y.; Zamore, P. D., The RNA-induced silencing complex is a Mg2+-dependent endonuclease. *Current biology : CB* **2004,** *14* (9), 787-91.

80. Lima, W. F.; Wu, H.; Nichols, J. G.; Sun, H.; Murray, H. M.; Crooke, S. T., Binding and cleavage specificities of human Argonaute2. *The Jornal of Biological Chemistry* **2009,** *284* (38), 26017-28.

81. Rivas, F. V.; Tolia, N. H.; Song, J. J.; Aragon, J. P.; Liu, J.; Hannon, G. J.; Joshua-Tor, L., Purified Argonaute2 and an siRNA form recombinant human RISC. *Nature Structural & Molecular Biology* **2005,** *12* (4), 340-9.

82. Vaishnaw, A. K.; Gollob, J.; Gamba-Vitalo, C.; Hutabarat, R.; Sah, D.; Meyers, R.; de Fougerolles, T.; Maraganore, J., A status report on RNAi therapeutics. *Silence* **2010,** *1* (1), 14.

83. Tao, W.; Mao, X.; Davide, J. P.; Ng, B.; Cai, M.; Burke, P. A.; Sachs, A. B.; Sepp-Lorenzino, L., Mechanistically probing lipid-siRNA nanoparticle-associated toxicities identifies Jak inhibitors effective in mitigating multifaceted toxic responses. *Molecular Therapy : the Journal of the American Society of Gene Therapy* **2011,** *19* (3), 567-75.

84. Bennett, C. F.; Swayze, E. E., RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annual Review of Pharmacology and Toxicology* **2010,** *50*, 259-93.

85. Martinez, J.; Patkaniowska, A.; Urlaub, H.; Luhrmann, R.; Tuschl, T., Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **2002,** *110* (5), 563-74.

86. Schwarz, D. S.; Hutvagner, G.; Haley, B.; Zamore, P. D., Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. *Molecular Cell* **2002,** *10* (3), 537-48.

87. Wu, H.; MacLeod, A. R.; Lima, W. F.; Crooke, S. T., Identification and partial purification of human double strand RNase activity. A novel terminating mechanism for oligoribonucleotide antisense drugs. *The Journal of Biological Chemistry* **1998,** *273* (5), 2532-42.

88. Braasch, D. A.; Jensen, S.; Liu, Y.; Kaur, K.; Arar, K.; White, M. A.; Corey, D. R., RNA Interference in Mammalian Cells by Chemically-Modified RNA. *Biochemistry* **2003,** *42* (26), 7967-7975.

89. Holen, T.; Amarzguioui, M.; Babaie, E.; Prydz, H., Similar behaviour of single-strand and double-strand siRNAs suggests they act through a common RNAi pathway. *Nucleic Acids Research* **2003,** *31* (9), 2401-2407.

90. Chiu, Y. L.; Rana, T. M., siRNA function in RNAi: a chemical modification analysis. *RNA (New York, N.Y.)* **2003,** *9* (9), 1034-48.

91. Prakash, T. P.; Allerson, C. R.; Dande, P.; Vickers, T. A.; Sioufi, N.; Jarres, R.; Baker, B. F.; Swayze, E. E.; Griffey, R. H.; Bhat, B., Positional effect of chemical modifications on short interference RNA activity in mammalian cells. *Journal of Medicinal Chemistry* **2005,** *48* (13), 4247-53.

92. Allerson, C. R.; Sioufi, N.; Jarres, R.; Prakash, T. P.; Naik, N.; Berdeja, A.; Wanders, L.; Griffey, R. H.; Swayze, E. E.; Bhat, B., Fully 2'-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA. *Journal of medicinal Chemistry* **2005,** *48* (4), 901-4.

93. Watts, J. K.; Deleavey, G. F.; Damha, M. J., Chemically modified siRNA: tools and applications. *Drug Discovery Today* **2008,** *13* (19–20), 842-855.

94. Hall, A. H.; Wan, J.; Shaughnessy, E. E.; Ramsay Shaw, B.; Alexander, K. A., RNA interference using boranophosphate siRNAs: structure-activity relationships. *Nucleic Acids Research* **2004,** *32* (20), 5991-6000.

95. Haringsma, H. J.; Li, J. J.; Soriano, F.; Kenski, D. M.; Flanagan, W. M.; Willingham, A. T., mRNA knockdown by single strand RNA is improved by chemical modifications. *Nucleic Acids Research* **2012,** *40* (9), 4125-36.

96. Schwarz, D. S.; Hutvágner, G.; Haley, B.; Zamore, P. D., Evidence that siRNAs Function as Guides, Not Primers, in the Drosophila and Human RNAi Pathways. *Molecular Cell* **2002,** *10* (3), 537-548.

97. Lima, W. F.; Prakash, T. P.; Murray, H. M.; Kinberger, G. A.; Li, W.; Chappell, A. E.; Li, C. S.; Murray, S. F.; Gaus, H.; Seth, P. P.; Swayze, E. E.; Crooke, S. T., Single-stranded siRNAs activate RNAi in animals. *Cell* **2012,** *150* (5), 883-94.

98. Rose, S. D.; Kim, D.-H.; Amarzguioui, M.; Heidel, J. D.; Collingwood, M. A.; Davis, M. E.; Rossi, J. J.; Behlke, M. A., Functional polarity is introduced by Dicer processing of short substrate RNAs. *Nucleic Acids research* **2005,** *33* (13), 4140-4156.

99. Prakash, T. P.; Lima, W. F.; Murray, H. M.; Li, W.; Kinberger, G. A.; Chappell, A. E.; Gaus, H.; Seth, P. P.; Bhat, B.; Crooke, S. T.; Swayze, E. E., Identification of metabolically stable 5'-phosphate analogs that support single-stranded siRNA activity. *Nucleic Acids Research* **2015,** *43* (6), 2993-3011.

100. Patent WO2011139702 A2.

101. Chen, X.; Dudgeon, N.; Shen, L.; Wang, J. H., Chemical modification of gene silencing oligonucleotides for drug discovery and development. *Drug Discov Today* **2005,** *10* (8), 587-93.

102. Bell, N. M.; Micklefield, J., Chemical modification of oligonucleotides for therapeutic, bioanalytical and other applications. *Chembiochem : a European Journal of Chemical Biology* **2009,** *10* (17), 2691-703.

103. Lennox, K. A.; Behlke, M. A., Chemical modification and design of anti-miRNA oligonucleotides. *Gene Therapy* **2011,** *18* (12), 1111-20.

104. Deleavey, G. F.; Damha, M. J., Designing chemically modified oligonucleotides for targeted gene silencing. *Chemistry & Biology* **2012,** *19* (8), 937-54.

105. Verma, S.; Eckstein, F., Modified oligonucleotides: synthesis and strategy for users. *Annual Review of Biochemistry* **1998,** *67*, 99-134.

106. Herdewijn, P., Heterocyclic modifications of oligonucleotides and antisense technology. *Antisense & Nucleic Acid Drug Development* **2000,** *10* (4), 297-310.

107. Saenger, W. Principles of nucleic acid structure; Springer: New York, 1984; p556.

108. Altona, C., Conformational analysis of nucleic acids. Determination of backbone geometry of single-helical RNA and DNA in aqueous solution. *Recueil des Travaux Chimiques des Pays-Bas* **1982,** *101* (12), 413-433.

109. Majlessi, M.; Nelson, N. C.; Becker, M. M., Advantages of 2'-O-methyl oligoribonucleotide probes for detecting RNA targets. *Nucleic Acids Research* **1998,** *26* (9), 2224-9.

110. Rettig, G. R.; Behlke, M. A., Progress toward in vivo use of siRNAs-II. *Molecular therapy : the journal of the American Society of Gene Therapy* **2012,** *20* (3), 483-512.

111. Monia, B. P.; Lesnik, E. A.; Gonzalez, C.; Lima, W. F.; McGee, D.; Guinosso, C. J.; Kawasaki, A. M.; Cook, P. D.; Freier, S. M., Evaluation of 2'-modified oligonucleotides containing 2'-deoxy gaps as antisense inhibitors of gene expression. *The Journal of Biological Chemistry* **1993,** *268* (19), 14514-22.

112. Brook, J. D.; McCurrach, M. E.; Harley, H. G.; Buckler, A. J.; Church, D.; Aburatani, H.; Hunter, K.; Stanton, V. P.; Thirion, J. P.; Hudson, T.; et al., Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell* **1992,** *68* (4), 799-808.

113. Fu, Y. H.; Pizzuti, A.; Fenwick, R. G., Jr.; King, J.; Rajnarayan, S.; Dunne, P. W.; Dubel, J.; Nasser, G. A.; Ashizawa, T.; de Jong, P.; et al., An unstable triplet repeat in a gene related to myotonic muscular dystrophy. *Science (New York, N.Y.)* **1992,** *255* (5049), 1256-8.

114. Mahadevan, M.; Tsilfidis, C.; Sabourin, L.; Shutler, G.; Amemiya, C.; Jansen, G.; Neville, C.; Narang, M.; Barcelo, J.; O'Hoy, K.; et al., Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene. *Science (New York, N.Y.)* **1992,** *255* (5049), 1253-5.

115. Mankodi, A.; Urbinati, C. R.; Yuan, Q. P.; Moxley, R. T.; Sansone, V.; Krym, M.; Henderson, D.; Schalling, M.; Swanson, M. S.; Thornton, C. A.,

Muscleblind localizes to nuclear foci of aberrant RNA in myotonic dystrophy types 1 and 2. *Human Molecular Genetics* **2001,** *10* (19), 2165-70.

116. Miller, J. W.; Urbinati, C. R.; Teng-Umnuay, P.; Stenberg, M. G.; Byrne, B. J.; Thornton, C. A.; Swanson, M. S., Recruitment of human muscleblind proteins to (CUG)(n) expansions associated with myotonic dystrophy. *The EMBO Journal* **2000,** *19* (17), 4439-48.

117. Wheeler, T. M.; Thornton, C. A., Myotonic dystrophy: RNA-mediated muscle disease. *Current Opinion in Neurology* **2007,** *20* (5), 572-6.

118. Kanadia, R. N.; Johnstone, K. A.; Mankodi, A.; Lungu, C.; Thornton, C. A.; Esson, D.; Timmers, A. M.; Hauswirth, W. W.; Swanson, M. S., A muscleblind knockout model for myotonic dystrophy. *Science (New York, N.Y.)* **2003,** *302* (5652), 1978-80.

119. Ho, T. H.; Charlet, B. N.; Poulos, M. G.; Singh, G.; Swanson, M. S.; Cooper, T. A., Muscleblind proteins regulate alternative splicing. *The EMBO Journal* **2004,** *23* (15), 3103-12.

120. Philips, A. V.; Timchenko, L. T.; Cooper, T. A., Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy. *Science (New York, N.Y.)* **1998,** *280* (5364), 737-41.

121. Charlet, B. N.; Savkur, R. S.; Singh, G.; Philips, A. V.; Grice, E. A.; Cooper, T. A., Loss of the muscle-specific chloride channel in type 1 myotonic dystrophy due to misregulated alternative splicing. *Molecular Cell* **2002,** *10* (1), 45-53.

122. Mahadevan, M. S.; Yadava, R. S.; Yu, Q.; Balijepalli, S.; Frenzel-McCardell, C. D.; Bourne, T. D.; Phillips, L. H., Reversible model of RNA toxicity and cardiac conduction defects in myotonic dystrophy. *Nature Genetics* **2006,** *38* (9), 1066-70.

123. Mulders, S. A.; van den Broek, W. J.; Wheeler, T. M.; Croes, H. J.; van Kuik-Romeijn, P.; de Kimpe, S. J.; Furling, D.; Platenburg, G. J. *et al*. Triplet-repeat oligonucleotide-mediated reversal of RNA toxicity in myotonic dystrophy. *Proceedings of the National Academy of Sciences of the United States of America* **2009,** *106* (33), 13915-20.

124. Manoharan, M., 2'-carbohydrate modifications in antisense oligonucleotide therapy: importance of conformation, configuration and conjugation. *Biochimica et Biophysica Acta* **1999,** *1489* (1), 117-30.

125. Kawasaki, A. M.; Casper, M. D.; Freier, S. M.; Lesnik, E. A.; Zounes, M. C.; Cummins, L. L.; Gonzalez, C.; Cook, P. D., Uniformly modified 2'-deoxy-2'-fluoro-phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets. *Journal of Medicinal Chemistry* **1993,** *36* (7), 831-841.

126. Ikeda, H.; Fernandez, R.; Wilk, A.; Barchi, J. J., Jr.; Huang, X.; Marquez, V. E., The effect of two antipodal fluorine-induced sugar puckers on the

conformation and stability of the Dickerson-Drew dodecamer duplex [d(CGCGAATTCGCG)]2. *Nucleic Acids Research* **1998,** *26* (9), 2237-44.

127. Deleavey, G. F.; Watts, J. K.; Damha, M. J., Chemical Modification of siRNA. In Current Protocols in Nucleic Acid Chemistry, John Wiley & Sons, Inc.: 2001

128. Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J., LNA (Locked Nucleic Acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. *Tetrahedron* **1998,** *54* (14), 3607-3630.

129. Petersen, M.; Bondensgaard, K.; Wengel, J.; Jacobsen, J. P., Locked nucleic acid (LNA) recognition of RNA: NMR solution structures of LNA:RNA hybrids. *Journal of the American Chemical Society* **2002,** *124* (21), 5974-82.

130. Petersen, M.; Wengel, J., LNA: a versatile tool for therapeutics and genomics. *Trends in Biotechnology* **2003,** *21* (2), 74-81.

131. Wahlestedt, C.; Salmi, P.; Good, L.; Kela, J.; Johnsson, T.; Hökfelt, T.; Broberger, C.; Porreca, F.; Lai, J. *et al*. Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. *Proceedings of the National Academy of Sciences* **2000,** *97* (10), 5633-5638.

132. De Mesmaeker, A.; Altmann, K. H.; Waldner, A.; Wendeborn, S., Backbone modifications in oligonucleotides and peptide nucleic acid systems. *Current Opinion in Structural Biology* **1995,** *5* (3), 343-55.

133. De Clercq, E.; Eckstein, F.; Merigan, T. C., [Interferon induction increased through chemical modification of a synthetic polyribonucleotide]. *Science (New York, N.Y.)* **1969,** *165* (3898), 1137-9.

134. Scaringe, S. A.; Wincott, F. E.; Caruthers, M. H., Novel RNA Synthesis Method Using 5‘-O-Silyl-2‘-O-orthoester Protecting Groups. *Journal of the American Chemical Society* **1998,** *120* (45), 11820-11821.

135. Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L., The automated synthesis of sulfur-containing oligodeoxyribonucleotides using 3H-1,2-benzodithiol-3-one 1,1-dioxide as a sulfur-transfer reagent. *The Journal of Organic Chemistry* **1990,** *55* (15), 4693-4699.

136. Slim, G.; Gait, M. J., Configurationally defined phosphorothioate-containing oligoribonucleotides in the study of the mechanism of cleavage of hammerhead ribozymes. *Nucleic Acids Research* **1991,** *19* (6), 1183-8.

137. Koziolkiewicz, M.; Krakowiak, A.; Kwinkowski, M.; Boczkowska, M.; Stec, W. J., Stereodifferentiation--the effect of P chirality of oligo(nucleoside phosphorothioates) on the activity of bacterial RNase H. *Nucleic Acids Research* **1995,** *23* (24), 5000-5.

138. Dias, N.; Stein, C. A., Antisense oligonucleotides: basic concepts and mechanisms. *Molecular Cancer Therapeutics* **2002,** *1* (5), 347-55.

139. Stein, C. A.; Hansen, J. B.; Lai, J.; Wu, S.; Voskresenskiy, A.; Høg, A.; Worm, J. *et al*. Efficient gene silencing by delivery of locked nucleic acid antisense oligonucleotides, unassisted by transfection reagents. *Nucleic Acids Research* **2010,** *38* (1), e3-e3.

140. Kowalczyk, A.; Carmical, J. R.; Zou, Y.; Van Houten, B.; Lloyd, R. S.; Harris, C. M.; Harris, T. M., Intrastrand DNA cross-links as tools for studying DNA replication and repair: two-, three-, and four-carbon tethers between the N(2) positions of adjacent guanines. *Biochemistry* **2002,** *41* (9), 3109-18.

141. Seio, K.; Wada, T.; Sakamoto, K.; Yokoyama, S.; Sekine, M., Chemical Synthesis and Properties of Conformationally Fixed Diuridine Monophosphates as Building Blocks of the RNA Turn Motif. *The Journal of Organic Chemistry* **1998,** *63* (5), 1429-1443.

142. Kruse, S., Thesis. *University of Nottingham* **2009**.

143. Zhao, Z.; Caruthers, M. H., Synthesis and preliminary biochemical studies with 5′-deoxy-5′-methylidyne phosphonate linked thymidine oligonucleotides. *Tetrahedron Letters* **1996,** *37* (35), 6239-6242.

144. Bertram, R. D.; Hayes, C. J.; Soultanas, P., Vinylphosphonate internucleotide linkages inhibit the activity of PcrA DNA helicase. *Biochemistry* **2002,** *41* (24), 7725-31.

145. Doddridge, Z. A.; Bertram, R. D.; Hayes, C. J.; Soultanas, P., Effects of vinylphosphonate internucleotide linkages on the cleavage specificity of exonuclease III and on the activity of DNA polymerase I. *Biochemistry* **2003,** *42* (11), 3239-46.

146. Abbas, S.; Hayes, C. J., A Novel Palladium-Catalysed Coupling Strategy for the Rapid Synthesis of Nucleic Acid Analogues Bearing Modified Backbones. *Synlett* **1999,** *1999* (07), 1124-1126.

147. Abbas, S.; Hayes, C. J., An improved procedure for the synthesis of vinylphosphonate-linked nucleic acids. *Tetrahedron Letters* **2000,** *41* (22), 4513-4517.

148. Abbas, S.; Bertram, R. D.; Hayes, C. J., Commercially Available 5‘-DMT Phosphoramidites as Reagents for the Synthesis of Vinylphosphonate-Linked Oligonucleic Acids. *Organic Letters* **2001,** *3* (21), 3365-3367.

149. Collis, A. E., Thesis. *University of Nottingham* **2007**.

150. Lera, M.; Hayes, C. J., An Olefin Cross-Metathesis Approach to Vinylphosphonate-Linked Nucleic Acids. *Organic Letters* **2001,** *3* (17), 2765-2768.

151. Solesbury, N. J., Thesis. *University of Nottingham* **2005**.

152. Summerton, J.; Weller, D., Morpholino antisense oligomers: design, preparation, and properties. *Antisense & Nucleic Acid Drug Development* **1997,** *7* (3), 187-95.

153. Demidov, V. V.; Potaman, V. N.; Frank-Kamenetskil, M. D.; Egholm, M.; Buchard, O.; Sönnichsen, S. H.; Nlelsen, P. E., Stability of peptide nucleic acids in human serum and cellular extracts. *Biochemical Pharmacology* **1994,** *48* (6), 1310-1313.

154. Nielsen, P. E., Peptide nucleic acids (PNA) in chemical biology and drug discovery. *Chemistry & Biodiversity* **2010,** *7* (4), 786-804.

155. Juliano, R. L.; Alahari, S.; Yoo, H.; Kole, R.; Cho, M., Antisense pharmacodynamics: critical issues in the transport and delivery of antisense oligonucleotides. *Pharmaceutical Research* **1999,** *16* (4), 494-502.

156. Loke, S. L.; Stein, C. A.; Zhang, X. H.; Mori, K.; Nakanishi, M.; Subasinghe, C.; Cohen, J. S.; Neckers, L. M., Characterization of oligonucleotide transport into living cells. *Proceedings of the National Academy of Sciences of the United States of America* **1989,** *86* (10), 3474-8.

157. Wu-Pong, S.; Bard, J.; Huffman, J.; Jimerson, J., Oligonucleotide biological activity: relationship to the cell cycle and nuclear transport. *Biology of the cell / under the auspices of the European Cell Biology Organization* **1997,** *89* (4), 257-61.

158. Bennett, R. M., As nature intended? The uptake of DNA and oligonucleotides by eukaryotic cells. *Antisense Research and Development* **1993,** *3* (3), 235-41.

159. Mukherjee, S.; Ghosh, R. N.; Maxfield, F. R., Endocytosis. *Physiological Reviews* **1997,** *77* (3), 759-803.

160. Doherty, G. J.; McMahon, H. T., Mechanisms of endocytosis. *Annual Review of Biochemistry* **2009,** *78*, 857-902.

161. Liang, E.; Ajmani, P. S.; Hughes, J. A., Oligonucleotide delivery: a cellular prospective. *Die Pharmazie* **1999,** *54* (8), 559-66.

162. Leonetti, J. P.; Degols, G.; Clarenc, J. P.; Mechti, N.; Lebleu, B., Cell delivery and mechanisms of action of antisense oligonucleotides. *Progress in Nucleic Acid Research and Molecular Biology* **1993,** *44*, 143-66.

163. Mahato, R. I.; Takakura, Y.; Hashida, M., Development of targeted delivery systems for nucleic acid drugs. *Journal of Drug Targeting* **1997,** *4* (6), 337-57.

164. Coster, H. G. L., A Quantitative Analysis of the Voltage-Current Relationships of Fixed Charge Membranes and the Associated Property of "Punch-Through". *Biophysical Journal* **1965,** *5* (5), 669-86.

165. Sale, A. J. H.; Hamilton, W. A., Effects of high electric fields on microorganisms. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1967,** *148* (3), 781-788.

166. Somiari, S.; Glasspool-Malone, J.; Drabick, J. J.; Gilbert, R. A.; Heller, R.; Jaroszeski, M. J.; Malone, R. W., Theory and in vivo application of

electroporative gene delivery. *Molecular Therapy : the Journal of the American Society of Gene Therapy* **2000,** *2* (3), 178-87.

167. Neumann, E.; Schaefer-Ridder, M.; Wang, Y.; Hofschneider, P. H., Gene transfer into mouse lyoma cells by electroporation in high electric fields. *The EMBO Journal* **1982,** *1* (7), 841-5.

168. Potter, H.; Weir, L.; Leder, P., Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. *Proceedings of the National Academy of Sciences of the United States of America* **1984,** *81* (22), 7161-5.

169. Fromm, M.; Taylor, L. P.; Walbot, V., Expression of genes transferred into monocot and dicot plant cells by electroporation. *Proceedings of the National Academy of Sciences of the United States of America* **1985,** *82* (17), 5824-5828.

170. Tur-Kaspa, R.; Teicher, L.; Levine, B. J.; Skoultchi, A. I.; Shafritz, D. A., Use of electroporation to introduce biologically active foreign genes into primary rat hepatocytes. *Molecular and Cellular Biology* **1986,** *6* (2), 716-718.

171. Mathiesen, I., Electropermeabilization of skeletal muscle enhances gene transfer in vivo. *Gene therapy* **1999,** *6* (4), 508-14.

172. Wells, J. M.; Li, L. H.; Sen, A.; Jahreis, G. P.; Hui, S. W., Electroporation-enhanced gene delivery in mammary tumors. *Gene Therapy* **2000,** *7* (7), 541-7.

173. Heller, R.; Jaroszeski, M.; Atkin, A.; Moradpour, D.; Gilbert, R.; Wands, J.; Nicolau, C., In vivo gene electroinjection and expression in rat liver. *FEBS Letters* **1996,** *389* (3), 225-228.

174. Leonetti, J. P.; Mechti, N.; Degols, G.; Gagnor, C.; Lebleu, B., Intracellular distribution of microinjected antisense oligonucleotides. *Proceedings of the National Academy of Sciences* **1991,** *88* (7), 2702-2706.

175. Fisher, T. L.; Terhorst, T.; Cao, X.; Wagner, R. W., Intracellular disposition and metabolism of fluorescently-labeled unmodified and modified oligonucleotides microinjected into mammalian cells. *Nucleic Acids Research* **1993,** *21* (16), 3857-65.

176. Spiller, D. G.; Tidd, D. M., Nuclear delivery of antisense oligodeoxynucleotides through reversible permeabilization of human leukemia cells with streptolysin O. *Antisense Research and Development* **1995,** *5* (1), 13-21.

177. Hughes, M. D.; Hussain, M.; Nawaz, Q.; Sayyed, P.; Akhtar, S., The cellular delivery of antisense oligonucleotides and ribozymes. *Drug Discovery Today* **2001,** *6* (6), 303-315.

178. Fischer, D.; Bieber, T.; Li, Y.; Elsasser, H. P.; Kissel, T., A novel non-viral vector for DNA delivery based on low molecular weight, branched

polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharmaceutical Research* **1999,** *16* (8), 1273-9.

179. Marschall, P.; Malik, N.; Larin, Z., Transfer of YACs up to 2.3 Mb intact into human cells with polyethylenimine. *Gene Therapy* **1999,** *6* (9), 1634-7.

180. Ferrari, S.; Moro, E.; Pettenazzo, A.; Behr, J. P.; Zacchello, F.; Scarpa, M., ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. *Gene Therapy* **1997,** *4* (10), 1100-6.

181. Tang, M. X.; Redemann, C. T.; Szoka, F. C., In Vitro Gene Delivery by Degraded Polyamidoamine Dendrimers. *Bioconjugate Chemistry* **1996,** *7* (6), 703-714.

182. Riessen, R.; Rahimizadeh, H.; Blessing, E.; Takeshita, S.; Barry, J. J.; Isner, J. M., Arterial gene transfer using pure DNA applied directly to a hydrogel-coated angioplasty balloon. *Human Gene Therapy* **1993,** *4* (6), 749-58.

183. Chavany, C.; Le Doan, T.; Couvreur, P.; Puisieux, F.; Helene, C., Polyalkylcyanoacrylate nanoparticles as polymeric carriers for antisense oligonucleotides. *Pharmaceutical Research* **1992,** *9* (4), 441-9.

184. Livache, T.; Roget, A.; Dejean, E.; Barthet, C.; Bidan, G.; Teoule, R., Preparation of a DNA matrix via an electrochemically directed copolymerization of pyrrole and oligonucleotides bearing a pyrrole group. *Nucleic Acids Research* **1994,** *22* (15), 2915-21.

185. Piro, B.; Pham, M. C.; Ledoan, T., Electrochemical method for entrapment of oligonucleotides in polymer-coated electrodes. *Journal of Biomedical Materials Research* **1999,** *46* (4), 566-72.

186. Balint, R.; Cassidy, N. J.; Cartmell, S. H., Conductive polymers: Towards a smart biomaterial for tissue engineering. *Acta Biomaterialia* **2014,** *10* (6), 2341-2353.

187. Rubini, J. R.; Stahmann, M. A.; Rasmussen, A. F., Jr., Agglutination of red cells by synthetic lysine polypeptides. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)* **1951,** *76* (4), 659-62.

188. Koch, G.; Bishop, J. M., The effect of polycations on the interaction of viral RNA with mammalian cells: Studies on the infectivity of single- and double-stranded poliovirus RNA. *Virology* **1968,** *35* (1), 9-17.

189. Merdan, T.; Kopeček, J.; Kissel, T., Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Advanced Drug Delivery Reviews* **2002,** *54* (5), 715-758.

190. Chirila, T. V.; Rakoczy, P. E.; Garrett, K. L.; Lou, X.; Constable, I. J., The use of synthetic polymers for delivery of therapeutic antisense oligodeoxynucleotides. *Biomaterials* **2002,** *23* (2), 321-342.

191. Juliano, R. L., The delivery of therapeutic oligonucleotides. *Nucleic Acids Research* **2016**.

192. Yin, L.; Zhao, X.; Cui, L.; Ding, J.; He, M.; Tang, C.; Yin, C., Cytotoxicity and genotoxicity of superporous hydrogel containing interpenetrating polymer networks. *Food and Chemical Toxicology : an International Journal Published for the British Industrial Biological Research Association* **2009,** *47* (6), 1139-45.

193. Wong, S. Y.; Pelet, J. M.; Putnam, D., Polymer systems for gene delivery—Past, present, and future. *Progress in Polymer Science* **2007,** *32* (8–9), 799-837.

194. Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T., In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* **2003,** *24* (7), 1121-31.

195. Akhtar, S.; Juliano, R. L., Cellular uptake and intracellular fate of antisense oligonucleotides. *Trends in Cell Biology* **1992,** *2* (5), 139-44.

196. Wolfrum, C.; Shi, S.; Jayaprakash, K. N.; Jayaraman, M.; Wang, G.; Pandey, R. K.; Rajeev, K. G.; Nakayama, T.; Charrise, K.; Ndungo, E. M.; Zimmermann, T.; Koteliansky, V.; Manoharan, M.; Stoffel, M., Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nature Biotechnology* **2007,** *25* (10), 1149-57.

197. Said Hassane, F.; Saleh, A. F.; Abes, R.; Gait, M. J.; Lebleu, B., Cell penetrating peptides: overview and applications to the delivery of oligonucleotides. *Cellular and Molecular Life Sciences : CMLS* **2010,** *67* (5), 715-26.

198. Lindberg, S.; Copolovici, D. M.; Langel, U., Therapeutic delivery opportunities, obstacles and applications for cell-penetrating peptides. *Therapeutic Delivery* **2011,** *2* (1), 71-82.

199. Khorev, O.; Stokmaier, D.; Schwardt, O.; Cutting, B.; Ernst, B., Trivalent, Gal/GalNAc-containing ligands designed for the asialoglycoprotein receptor. *Bioorganic & Medicinal Chemistry* **2008,** *16* (9), 5216-31.

200. Akinc, A.; Querbes, W.; De, S.; Qin, J.; Frank-Kamenetsky, *et al*. Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Molecular therapy : the Journal of the American Society of Gene Therapy* **2010,** *18* (7), 1357-64.

201. Yu, B.; Zhao, X.; Lee, L. J.; Lee, R. J., Targeted Delivery Systems for Oligonucleotide Therapeutics. *The AAPS Journal* **2009,** *11* (1), 195-203.

202. Baumer, N.; Appel, N.; Terheyden, L.; Buchholz, F.; Rossig, C.; Muller-Tidow, C.; Berdel, W. E.; Baumer, S., Antibody-coupled siRNA as an efficient method for in vivo mRNA knockdown. *Nat. Protocols* **2016,** *11* (1), 22-36.

203. Rozema, D. B.; Blokhin, A. V.; Wakefield, D. H.; Benson, J. D.; Carlson, J. C.; Klein, J. J. *et al*. Protease-triggered siRNA delivery vehicles. *Journal of Controlled Release* **2015,** *209*, 57-66.

204. Winkler, J., Oligonucleotide conjugates for therapeutic applications. *Therapeutic Delivery* **2013,** *4* (7), 791-809.

205. Lonnberg, H., Solid-phase synthesis of oligonucleotide conjugates useful for delivery and targeting of potential nucleic acid therapeutics. *Bioconjugate Chemistry* **2009,** *20* (6), 1065-94.

206. Juliano, R. L.; Ming, X.; Nakagawa, O., The chemistry and biology of oligonucleotide conjugates. *Accounts of Chemical Research* **2012,** *45* (7), 1067-76.

207. Winkler, J.; Giessrigl, B.; Novak, C.; Urban, E.; Noe, C. R., 2′-O-Lysylaminohexyladenosine modified oligonucleotides. *Monatshefte für Chemie - Chemical Monthly* **2010,** *141* (7), 809-815.

208. Willibald, J.; Harder, J.; Sparrer, K.; Conzelmann, K.-K.; Carell, T., Click-Modified Anandamide siRNA Enables Delivery and Gene Silencing in Neuronal and Immune Cells. *Journal of the American Chemical Society* **2012,** *134* (30), 12330-12333.

209. Akhtar, S.; Benter, I., Toxicogenomics of non-viral drug delivery systems for RNAi: potential impact on siRNA-mediated gene silencing activity and specificity. *Advanced Drug Delivery Reviews* **2007,** *59* (2-3), 164-82.

210. Omidi, Y.; Hollins, A. J.; Benboubetra, M.; Drayton, R.; Benter, I. F.; Akhtar, S., Toxicogenomics of non-viral vectors for gene therapy: a microarray study of lipofectin- and oligofectamine-induced gene expression changes in human epithelial cells. *Journal of Drug Targeting* **2003,** *11* (6), 311-23.

211. Ferrari, N.; Bergeron, D.; Tedeschi, A. L.; Mangos, M. M.; Paquet, L.; Renzi, P. M.; Damha, M. J., Characterization of antisense oligonucleotides comprising 2'-deoxy-2'-fluoro-beta-D-arabinonucleic acid (FANA): specificity, potency, and duration of activity. *Annals of the New York Academy of Sciences* **2006,** *1082*, 91-102.

212. Rojanasakul, Y., Latest developments in modern drug deliveryAntisense oligonucleotide therapeutics: drug delivery and targeting. *Advanced Drug Delivery Reviews* **1996,** *18* (2), 115-131.

213. Weyermann, J.; Lochmann, D.; Zimmer, A., Comparison of antisense oligonucleotide drug delivery systems. *Journal of controlled release : official journal of the Controlled Release Society* **2004,** *100* (3), 411-23.

214. Lebedeva, I.; Benimetskaya, L.; Stein, C. A.; Vilenchik, M., Cellular delivery of antisense oligonucleotides. *European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V* **2000,** *50* (1), 101-19.

215. Lambert, G.; Fattal, E.; Couvreur, P., Nanoparticulate systems for the delivery of antisense oligonucleotides. *Advanced Drug Delivery Reviews* **2001,** *47* (1), 99-112.

216. Garcia-Chaumont, C.; Seksek, O.; Grzybowska, J.; Borowski, E.; Bolard, J., Delivery systems for antisense oligonucleotides. *Pharmacology & Therapeutics* **2000,** *87* (2-3), 255-77.

217. Akhtar, S.; Hughes, M. D.; Khan, A.; Bibby, M.; Hussain, M.; Nawaz, Q.; Double, J.; Sayyed, P., The delivery of antisense therapeutics. *Advanced Drug Delivery Reviews* **2000,** *44* (1), 3-21.

218. Akhtar, S.; Agrawal, S., In vivo studies with antisense oligonucleotides. *Trends in Pharmacological Sciences* **1997,** *18* (1), 12-18.

219. Bell, N. M.; Micklefield, J., Chemical Modification of Oligonucleotides for Therapeutic, Bioanalytical and other Applications. *ChemBioChem* **2009,** *10* (17), 2691-2703.

220. Haley, B.; Zamore, P. D., Kinetic analysis of the RNAi enzyme complex. *Nature Structural & Molecular Biology* **2004,** *11* (7), 599-606.

221. Raof, N. A.; Rajamani, D.; Chu, H. C.; Gurav, A.; Johnson, J. M.; LoGerfo, F. W.; Pradhan-Nabzdyk, L.; Bhasin, M., The effects of transfection reagent polyethyleneimine (PEI) and non-targeting control siRNAs on global gene expression in human aortic smooth muscle cells. *BMC Genomics* **2016,** *17*.

222. Bryans, M.; Harley, E.; Gilmour, S. K., Elevated cellular polyamine levels enhance promoter activity in vivo. *Biochemical and Biophysical Research Communications* **1996,** *226* (3), 618-25.

223. Bruyere, C.; Meijer, L., Targeting cyclin-dependent kinases in anti-neoplastic therapy. *Current Opinion in Cell Biology* **2013,** *25* (6), 772-9.

224. Malumbres, M.; Harlow, E.; Hunt, T.; Hunter, T.; Lahti, J. M.; Manning, G.; Morgan, D. O.; Tsai, L. H.; Wolgemuth, D. J., Cyclin-dependent kinases: a family portrait. *Nature Cell Biology* **2009,** *11* (11), 1275-6.

225. Blazek, D.; Kohoutek, J.; Bartholomeeusen, K.; Johansen, E.; Hulinkova, P.; Luo, Z.; Cimermancic, P.; Ule, J.; Peterlin, B. M., The Cyclin K/Cdk12 complex maintains genomic stability via regulation of expression of DNA damage response genes. *Genes & Development* **2011,** *25* (20), 2158-72.

226. Integrated genomic analyses of ovarian carcinoma. *Nature* **2011,** *474* (7353), 609-15.

227. Ekumi, K. M.; Paculova, H.; Lenasi, T.; Pospichalova, V. *et al*. carcinoma CDK12 mutations misregulate expression of DNA repair genes via deficient formation and function of the Cdk12/CycK complex. *Nucleic Acids research* **2015,** *43* (5), 2575-89.

228. Cheng, S. W.; Kuzyk, M. A.; Moradian, A.; Ichu, T. A.; Chang, V. C.; Tien, J. F.; Vollett, S. E.; Griffith, M.; Marra, M. A.; Morin, G. B., Interaction of cyclin-dependent kinase 12/CrkRS with cyclin K1 is required for the

phosphorylation of the C-terminal domain of RNA polymerase II. *Molecular and Cellular Biology* **2012,** *32* (22), 4691-704.

229. Liu, J.; Yu, D.; Aiba, Y.; Pendergraff, H.; Swayze, E. E.; Lima, W. F.; Hu, J.; Prakash, T. P.; Corey, D. R., ss-siRNAs allele selectively inhibit ataxin-3 expression: multiple mechanisms for an alternative gene silencing strategy. *Nucleic Acids Research* **2013,** *41* (20), 9570-83.

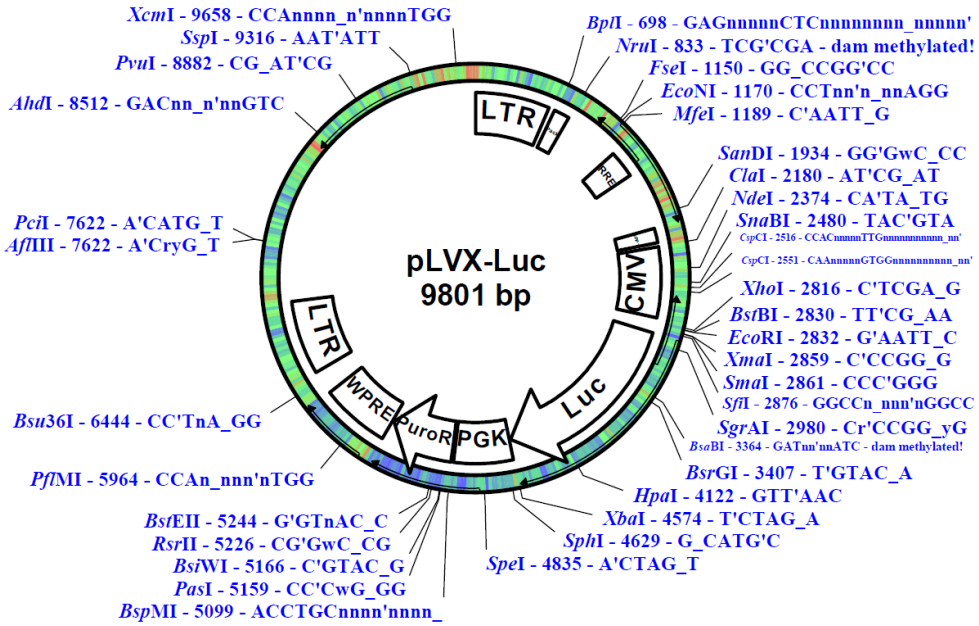
230. Cailleau, R.; Young, R.; Olivé, M.; Reeves, J. W. J., Breast Tumor Cell Lines From Pleural Effusions2. *JNCI: Journal of the National Cancer Institute* **1974,** *53* (3), 661-674.

231. Scherer, W. F.; Syverton, J. T.; Gey, G. O., Studies on the propagation in vitro of poliomyelitis viruses: IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain hela) derived from an epidermoid carcinoma of the cervix. *The Journal of Experimental Medicine* **1953,** *97* (5), 695-710.

232. Sidak, Z., Rectangular Confidence Regions for the Means of Multivariate Normal Distributions. *Journal of the American Statistical Association* **1967,** *62* (318), 626-633.

7 Appendix

7.1 The pLVX-Luc plasmid sequence



Green(dark)=CMV promoter

Turquoise=Luciferase ORF

Teal=sequencing primers

Pink=PGKprom

Green(light)=PuroR and WPRE

Blue= packaging signal, RREreg and cPPT elements

Purple=LTRs

TGGAAGGGCTAATTCACTCCCAAAGAAGACAAGATATCCTTGATCT
GTGGATCTACCACACACAAGGCTACTTCCCTGATTAGCAGAACTAC
ACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGATGGTGCT
ACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATA
AAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGGAT
GGATGACCCGGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCG
CCTAGCATTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTC
AAGAACTGCTGATATCGAGCTTGCTACAAGGGACTTTCCGCTGGGG
ACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGAG
CCCTCAGATCCTGCATATAAGCAGCTGCTTTTTGCCTGTACTGGGTC
TCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTA
GGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTC
AAGTAGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCC

CTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGTGGCGCCC
GAACATGGCGCCCGAACAGGGACTTGAAAGCGAAAGGGAAACCAG
AGGAGCTCTCTCGACGCAGGACTCGGCTTGCTGAAGCGCGCACGGC
AAGAGGCGAGGGGCGGCGACTGGTGAGTACGCCAAAAATTTTGAC
TAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTAT
TAAGCGGGGGAGAATTAGATCGCGATGGGAAAAAATTCGGTTAAG
GCCAGGGGGAAAGAAAAAATATAAATTAAAACATATAGTATGGGC
AAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAA
ACATCAGAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCC
TTCAGACAGGATCAGAAGAACTTAGATCATTATATAATACAGTAGC
AACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAGACACCAAG
GAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGACC
ACCGCACAGCAAGCGGCCGGCCGCTGATCTTCAGACCTGGAGGAG
GAGATATGAGGGACAATTGGAGAAGTGAATTATATAAATATAAAG
TAGTAAAAATTGAACCATTAGGAGTAGCACCCACCAAGGCAAAGA
GAAGAGTGGTGCAGAGAGAAAAAAGAGCAGTGGGAATAGGAGCTT
TGTTCCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGC
GTCAATGACGCTGACGGTACAGGCCAGACAATTATTGTCTGGTATA
GTGCAGCAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAACAGC
ATCTGTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGGCAAG
AATCCTGGCTGTGGAAAGATACCTAAAGGATCAACAGCTCCTGGGG
ATTTGGGGTTGCTCTGGAAAACTCATTTGCACCACTGCTGTGCCTTG
GAATGCTAGTTGGAGTAATAAATCTCTGGAACAGATTTGGAATCAC
ACGACCTGGATGGAGTGGGACAGAGAAATTAACAATTACACAAGC
TTAATACACTCCTTAATTGAAGAATCGCAAAACCAGCAAGAAAAGA
ATGAACAAGAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAA
TTGGTTTAACATAACAAATTGGCTGTGGTATATAAAATTATTCATAA
TGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGCTGTACTT
TCTATAGTGAATAGAGTTAGGCAGGGATATTCACCATTATCGTTTCA
GACCCACCTCCCAACCCCGAGGGGACCCGACAGGCCCGAAGGAAT
AGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTCGATT
AGTGAACGGATCTCGACGGTATCGCCTTTAAAAGAAAAGGGGGGA
TTGGGGGGTACAGTGCAGGGGAAAGAATAGTAGACATAATAGCAA
CAGACATACAAACTAAAGAACTACAAAAACAAATTACAAAAATTC
AAAATTTTCGGGTTTATTACAGGGACAGCAGAGATCCAGTTTATCG
ATAAGCTTGGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCG
CCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGA
CGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA
ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAA
GTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTA
AATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTACGGGACTT
TCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGG
TGATGCGGTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTGA
CTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGT
TTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACA
ACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGA
GGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGG
AGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGACTCT
AGCTAGAGGATCGCTAGCGCTACCGGACTCAGATCTCGAGCTCAAG
CTTCGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCTGGCCT

CGGCGGCCAAGCTTGGCAATCCGGTACTGTTGGTAAAGCCACCATG
GAAGATGCCAAAAACATTAAGAAGGGCCCAGCGCCATTCTACCCAC
TCGAAGACGGGACCGCCGGCGAGCAGCTGCACAAAGCCATGAAGC
GCTACGCCCTGGTGCCCGGCACCATCGCCTTTACCGACGCACATAT
CGAGGTGGACATTACCTACGCCGAGTACTTCGAGATGAGCGTTCGG
CTGGCAGAAGCTATGAAGCGCTATGGGCTGAATACAAACCATCGGA
TCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTGTTG
GGTGCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACATCT
ACAACGAGCGCGAGCTGCTGAACAGCATGGGCATCAGCCAGCCCA
CCGTCGTATTCGTGAGCAAGAAAGGGCTGCAAAAGATCCTCAACGT
GCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGATAGC
AAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTT
CCCATTTGCCACCCGGCTTCAACGAGTACGACTTCGTGCCCGAGAG
CTTCGACCGGGACAAAACCATCGCCCTGATCATGAACAGTAGTGGC
AGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGCACCGCTT
GTGTCCGATTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGAT
CATCCCCGACACCGCTATCCTCAGCGTGGTGCCATTTCACCACGGCT
TCGGCATGTTCACCACGCTGGGCTACTTGATCTGCGGCTTTCGGGTC
GTGCTCATGTACCGCTTCGAGGAGGAGCTATTCTTGCGCAGCTTGC
AAGACTATAAGATTCAATCTGCCCTGCTGGTGCCCACACTATTTAGC
TTCTTCGCTAAGAGCACTCTCATCGACAAGTACGACCTAAGCAACT
TGCACGAGATCGCCAGCGGCGGGGCGCCGCTCAGCAAGGAGGTAG
GTGAGGCCGTGGCCAAACGCTTCCACCTACCAGGCATCCGCCAGGG
CTACGGCCTGACAGAAACAACCAGCGCCATTCTGATCACCCCCGAA
GGGGACGACAAGCCTGGCGCAGTAGGCAAGGTGGTGCCCTTCTTCG
AGGCTAAGGTGGTGGACTTGGACACCGGTAAGACACTGGGTGTGA
ACCAGCGCGGCGAGCTGTGCGTCCGTGGCCCCATGATCATGAGCGG
CTACGTTAACAACCCCGAGGCTACAAACGCTCTCATCGACAAGGAC
GGCTGGCTGCACAGCGGCGACATCGCCTACTGGGACGAGGACGAG
CACTTCTTCATCGTGGACCGGCTGAAGAGCCTGATCAAATACAAGG
GCTACCAGGTAGCCCCAGCCGAACTGGAGAGCATCCTGCTGCAACA
CCCCAACATCTTCGACGCCGGGGTCGCCGGCCTGCCCGACGACGAT
GCCGGCGAGCTGCCCGCCGCAGTCGTCGTGCTGGAACACGGTAAAA
CCATGACCGAGAAGGAGATCGTGGACTATGTGGCCAGCCAGGTTAC
AACCGCCAAGAAGCTGCGCGGTGGTGTTGTGTTCGTGGACGAGGTG
CCTAAAGGACTGACCGGCAAGTTGGACGCCCGCAAGATCCGCGAG
ATTCTCATTAAGGCCAAGAAGGGCGGCAAGATCGCCGTGTAATAAT
TCTAGATAATTCTACCGGGTAGGGGAGGCGCTTTTCCCAAGGCAGT
CTGGAGCATGCGCTTTAGCAGCCCCGCTGGGCACTTGGCGCTACAC
AAGTGGCCTCTGGCCTCGCACACATTCCACATCCACCGGTAGGCGC
CAACCGGCTCCGTTCTTTGGTGGCCCCTTCGCGCCACCTTCTACTCC
TCCCCTAGTCAGGAAGTTCCCCCCCGCCCCGCAGCTCGCGTCGTGC
AGGACGTGACAAATGGAAGTAGCACGTCTCACTAGTCTCGTGCAGA
TGGACAGCACCGCTGAGCAATGGAAGCGGGTAGGCCTTTGGGGCA
GCGGCCAATAGCAGCTTTGCTCCTTCGCTTTCTGGGCTCAGAGGCTG
GGAAGGGGTGGGTCCGGGGGCGGGCTCAGGGGCGGGCTCAGGGGC
GGGGCGGGCGCCCGAAGGTCCTCCGGAGGCCCGGCATTCTGCACGC
TTCAAAAGCGCACGTCTGCCGCGCTGTTCTCCTCTTCCTCATCTCCG
GGCCTTTCGACCTGCAGCCCAAGCTTACCATGACCGAGTACAAGCC
CACGGTGCGCCTCGCCACCCGCGACGACGTCCCCAGGGCCGTACGC

ACCCTCGCCGCCGCGTTCGCCGACTACCCCGCCACGCGCCACACCG
TCGATCCGGACCGCCACATCGAGCGGGTCACCGAGCTGCAAGAACT
CTTCCTCACGCGCGTCGGGCTCGACATCGGCAAGGTGTGGGTCGCG
GACGACGGCGCCGCGGTGGCGGTCTGGACCACGCCGGAGAGCGTC
GAAGCGGGGGCGGTGTTCGCCGAGATCGGCCCGCGCATGGCCGAG
TTGAGCGGTTCCCGGCTGGCCGCGCAGCAACAGATGGAAGGCCTCC
TGGCGCCGCACCGGCCCAAGGAGCCCGCGTGGTTCCTGGCCACCGT
CGGCGTCTCGCCCGACCACCAGGGCAAGGGTCTGGGCAGCGCCGTC
GTGCTCCCCGGAGTGGAGGCGGCCGAGCGCGCCGGGGTGCCCGCCT
TCCTGGAGACCTCCGCGCCCCGCAACCTCCCCTTCTACGAGCGGCTC
GGCTTCACCGTCACCGCCGACGTCGAGGTGCCCGAAGGACCGCGCA
CCTGGTGCATGACCCGCAAGCCCGGTGCCTGACCGCGTCTGGAACA
ATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTT
AACTATGTTGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCC
TTTGTATCATGCTATTGCTTCCCGTATGGCTTTCATTTTCTCCTCCTT
GTATAAATCCTGGTTGCTGTCTCTTTATGAGGAGTTGTGGCCCGTTG
TCAGGCAACGTGGCGTGGTGTGCACTGTGTTTGCTGACGCAACCCC
CACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCCGGGACTT
TCGCTTTCCCCCTCCCTATTGCCACGGCGGAACTCATCGCCGCCTGC
CTTGCCCGCTGCTGGACAGGGGCTCGGCTGTTGGGCACTGACAATT
CCGTGGTGTTGTCGGGGAAGCTGACGTCCTTTCCATGGCTGCTCGCC
TGTGTTGCCACCTGGATTCTGCGCGGGACGTCCTTCTGCTACGTCCC
TTCGGCCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCTGCTGCCGG
CTCTGCGGCCTCTTCCGCGTCTTCGCCTTCGCCCTCAGACGAGTCGG
ATCTCCCTTTGGGCCGCCTCCCCGCCTGGAATTAATTCTGCAGTCGA
GACCTAGAAAAACATGGAGCAATCACAAGTAGCAATACAGCAGCT
ACCAATGCTGATTGTGCCTGGCTAGAAGCACAAGAGGAGGAGGAG
GTGGGTTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTT
ACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGAGGGG
ACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGAT
CTGTGGATCTACCACACACAAGGCTACTTCCCTGATTAGCAGAACT
ACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGATGGTG
CTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAA
TAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGG
ATGGATGACCCGGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGC
CGCCTAGCATTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACT
TCAAGAACTGCTGATATCGAGCTTGCTACAAGGGACTTTCCGCTGG
GGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCG
AGCCCTCAGATCCTGCATATAAGCAGCTGCTTTTTGCCTGTACTGGG
TCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACT
AGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTT
CAAGTAGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATC
CCTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGTAGTAGTT
CATGTCATCTTATTATTCAGTATTTATAACTTGCAAAGAAATGAATA
TCAGAGAGTGAGAGGCCTTGACATTGCTAGCGTTTTACCGTCGACC
TCTAGCTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTG
AAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGC
ATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACAT
TAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCG
TGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT

TGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGC
TCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGG
TAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACA
TGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCG
CGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCAC
AAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTA
TAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCC
TGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTT
CGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAG
TTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCC
CCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGA
GTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACT
GGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG
TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTAT
TTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGT
TGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTTTT
TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAG
AAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGA
AAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATC
TTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTA
AAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATC
AGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGT
TGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTA
CCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCAC
CGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGA
GCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTA
ATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTT
GCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGT
CGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGA
GTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCG
GTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTC
ATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGT
AAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAG
AATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACG
GGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATT
GGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGT
TGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCA
GCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAA
GGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTT
GAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAG
GGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAA
ATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACC
TGACGTCGACGGATCGGGAGATCAACTTGTTTATTGCAGCTTATAA
TGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCA
TTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTA
TCTTATCATGTCTGGATCAACTGGATAACTCAAGCTAACCAAAATC
ATCCCAAACTTCCCACCCCATACCCTATTACCACTGCCAATTACCTA
GTGGTTTCATTTACTCTAAACCTGTGATTCCTCTGAATTATTTTCATT
TTAAAGAAATTGTATTTGTTAAATATGTACTACAAACTTAGTAGTTT
TTAAAGAAATTGTATTTGTTAAATATGTACTACAAACTTAGTAGT

7.2 The CDK12pLVX-Luc plasmid sequence

Green(dark)=CMV promoter

Red=CDK12 insert

Turquoise=Luciferase ORF

Teal=sequencing primers

Pink=PGKprom

Green(light)=PuroR and WPRE

Blue= packaging signal, RREreg and cPPT elements

Purple=LTRs

TGGAAGGGCTAATTCACTCCCAAAGAAGACAAGATATCCTTGATCT
GTGGATCTACCACACACAAGGCTACTTCCCTGATTAGCAGAACTAC
ACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGATGGTGCT
ACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATA
AAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGGAT
GGATGACCCGGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCG
CCTAGCATTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTC
AAGAACTGCTGATATCGAGCTTGCTACAAGGGACTTTCCGCTGGGG
ACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGAG
CCCTCAGATCCTGCATATAAGCAGCTGCTTTTTGCCTGTACTGGGTC
TCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTA
GGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTC
AAGTAGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCC
CTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGTGGCGCCC
GAACATGGCGCCCGAACAGGGACTTGAAAGCGAAAGGGAAACCAG
AGGAGCTCTCTCGACGCAGGACTCGGCTTGCTGAAGCGCGCACGGC
AAGAGGCGAGGGGCGGCGACTGGTGAGTACGCCAAAAATTTTGAC
TAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTAT
TAAGCGGGGGAGAATTAGATCGCGATGGGAAAAAATTCGGTTAAG
GCCAGGGGGAAAGAAAAAATATAAATTAAAACATATAGTATGGGC
AAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAA
ACATCAGAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCC
TTCAGACAGGATCAGAAGAACTTAGATCATTATATAATACAGTAGC
AACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAGACACCAAG
GAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGACC
ACCGCACAGCAAGCGGCCGGCCGCTGATCTTCAGACCTGGAGGAG
GAGATATGAGGGACAATTGGAGAAGTGAATTATATAAATATAAAG
TAGTAAAAATTGAACCATTAGGAGTAGCACCCACCAAGGCAAAGA
GAAGAGTGGTGCAGAGAGAAAAAAGAGCAGTGGGAATAGGAGCTT
TGTTCCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGC
GTCAATGACGCTGACGGTACAGGCCAGACAATTATTGTCTGGTATA

GTGCAGCAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAACAGC
ATCTGTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGGCAAG
AATCCTGGCTGTGGAAAGATACCTAAAGGATCAACAGCTCCTGGGG
ATTTGGGGTTGCTCTGGAAAACTCATTTGCACCACTGCTGTGCCTTG
GAATGCTAGTTGGAGTAATAAATCTCTGGAACAGATTTGGAATCAC
ACGACCTGGATGGAGTGGGACAGAGAAATTAACAATTACACAAGC
TTAATACACTCCTTAATTGAAGAATCGCAAAACCAGCAAGAAAAGA
ATGAACAAGAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAA
TTGGTTTAACATAACAAATTGGCTGTGGTATATAAAATTATTCATAA
TGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGCTGTACTT
TCTATAGTGAATAGAGTTAGGCAGGGATATTCACCATTATCGTTTCA
GACCCACCTCCCAACCCCGAGGGGACCCGACAGGCCCGAAGGAAT
AGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTCGATT
AGTGAACGGATCTCGACGGTATCGCCTTTAAAAGAAAAGGGGGGA
TTGGGGGGTACAGTGCAGGGGAAAGAATAGTAGACATAATAGCAA
CAGACATACAAACTAAAGAACTACAAAAACAAATTACAAAAATTC
AAAATTTTCGGGTTTATTACAGGGACAGCAGAGATCCAGTTTATCG
ATAAGCTTGGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCG
CCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGA
CGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA
ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAA
GTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTA
AATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTACGGGACTT
TCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGG
TGATGCGGTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTGA
CTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGT
TTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACA
ACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGA
GGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGG
AGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGACTCT
AGCTAGAGGATCGCTAGCGCTACCGGACTCAGATCTCGAGACCATG
CCCAATTCAGAGAGACATGGGGGCAAGAAGGACGGGAGTGGAGGA
GCTTCTGGAACTTTGCAGCCGTCATCGGGAGGCGGCAGCTCTAACA
GCAGAGAGCGTCACCGCTTGGTATCGAAGCACAAGCGGCATAAGTC
CAAACACTCCAAAGACATGGGGTTGGTGACCCCCGAAGCAGCATCC
CTGGGCACAGTTATCAAACCTTTGGTGGAGTATGATGATATCAGCT
CTGATTCCGACACCTTCTCCGATGACATGGCCTTCAAACTAGACCGA
AGGGAGAACGACGAACGTCGTGGATCAGATCGGAGCGACCGCCTG
CACAAACATCGTCACCACCAGCACAGGCGTTCCCGGGACTTACTAA
AAGCTAAACAGACCGAAAAAGAAAAAAGCCAAGAAGTCTCCAGCA
AGTCGGGATCGATGAAGGACCGGATATCGGGAAGTTCAAAGCGTTC
GAATGAGGAGACTGATGACTATGGGAAGGCGCAGGTAGCCAAAAG
CAGCAGCAAGGAATCCAGGTCATCCAAGCTCCACAAGGAGAAGAC
CAGGAAAGAACGGGAGCTGAAGTCTGGGCACAAAGACCGGAGTAA
AAGTCATCGAAAAAGGGAAACACCCAAAAGTTACAAAACAGTGGA
CAGCCCAAAACGGAGATCCAGGAGCCCCCACAGGAAGTGGTCTGA
CAGCTCCAAACAAGATGATAGCCCCTCGGGAGCTTCTTATGGCCAA
GATTATGACCTTAGTCCCTCACGATCTCATACCTCGAGCAATTATGA
CTCCTACAAGAAAAGTCCTGGAAGTACCTCGAGAAGGCAGTCGGTC
AGTCCCCCTTACAAGGAGCCTTCGGCCTACCAGTCCAGCACCCGGT

CACCGAGCCCCTACAGTAGGCGACAGAGATCTGTCAGTCCCTATAG
CAGGAGACGGTCGTCCAGCTACGAAAGAAGTGGCTCTTACAGCGG
GCGATCGCCCAGTCCCTATGGTCGAAGGCGGTCCAGCAGCCCTTTC
CTGAGCAAGCGGTCTCTGAGTCGGAGTCCACTCCCCAGTAGGAAAT
CCATGAAGTCCAGAAGTAGAAGTCCTGCATATTCAAGACATTCATC
TTCTCATAGTAAAAAGAAGAGATCCAGTTCACGCAGTCGTCATTCC
AGTATCTCACCTGTCAGGCTTCCACTTAATTCCAGTCTGGGAGCTGA
ACTCAGTAGGAAAAAGAAGGAAAGAGCAGCTGCTGCTGCTGCAGC
AAAGATGGATGGAAAGGAGTCCAAGGGTTCACCTGTATTTTTGCCT
AGAAAAGAGAACAGTTCAGTAGAGGCTAAGGATTCAGGTTTGGAGT
CTAAAAAGTTACCCAGAAGTGTAAAATTGGAAAAATCTGCCCCAGA
TACTGAACTGGTGAATGTAACACATCTAAACACAGAGGTAAAAAAT
TCTTCAGATACAGGGAAAGTAAAGTTGGATGAGAACTCCGAGAAGC
ATCTTGTTAAAGATTTGAAAGCACAGGGAACAAGAGACTCTAAACC
CATAGCACTGAAAGAGGAGATTGTTACTCCAAAGGAGACAGAAAC
ATCAGAAAAGGAGACCCCTCCACCTCTTCCCACAATTGCTTCTCCCC
CACCCCCTCTACCAACTACTACCCCTCCACCTCAGACACCCCCTTTG
CCACCTTTGCCTCCAATACCAGCTCTTCCACAGCAACCACCTCTGCC
TCCTTCTCAGCCAGCATTTAGTCAGGTTCCTGCTTCCAGTACTTCAA
CTTTGCCCCCTTCTACTCACTCAAAGACATCTGCTGTGTCCTCTCAG
GCAAATTCTCAGCCCCCTGTACAGGTTTCTGTGAAGACTCAAGTATC
TGTAACAGCTGCTATTCCACACCTGAAAACTTCAACGTTGCCTCCTT
TGCCCCTCCCACCCTTATTACCTGGAGATGATGACATGGATAGTCCA
AAAGAAACTCTTCCTTCAAAACCTGTGAAGAAAGAGAAGGAACAG
AGGACACGTCACTTACTCACAGACCTTCCTCTCCCTCCAGAGCTCCC
TGGTGGAGATCTGTCTCCCCCAGACTCTCCAGAACCAAAGGCAATC
ACACCACCTCAGCAACCATATAAAAAGAGACCAAAAATTTGTTGTC
CTCGTTATGGAGAAAGAAGACAAACAGAAAGCGACTGGGGGAAAC
GCTGTGTGGACAAGTTTGACATTATTGGGATTATTGGAGAAGGAAC
CTATGGCCAAGTATATAAAGCCAAGGACAAAGACACAGGAGAACT
AGTGGCTCTGAAGAAGGTGAGACTAGACAATGAGAAAGAGGGCTT
CCCAATCACAGCCATTCGTGAAATCAAAATCCTTCGTCAGTTAATCC
ACCGAAGTGTTGTTAACATGAAGGAAATTGTCACAGATAAACAAGA
TGCACTGGATTTCAAGAAGGACAAAGGTGCCTTTTACCTTGTATTTG
AGTATATGGACCATGACTTAATGGGACTGCTAGAATCTGGTTTGGT
GCACTTTTCTGAGGACCATATCAAGTCGTTCATGAAACAGCTAATG
GAAGGATTGGAATACTGTCACAAAAAGAATTTCCTGCATCGGGATA
TTAAGTGTTCTAACATTTTGCTGAATAACAGTGGGCAAATCAAACT
AGCAGATTTTGGACTTGCTCGGCTCTATAACTCTGAAGAGAGTCGC
CCTTACACAAACAAAGTCATTACTTTGTGGTACCGACCTCCAGAAC
TACTGCTAGGAGAGGAACGTTACACACCAGCCATAGATGTTTGGAG
CTGTGGATGTATTCTTGGGGAACTATTCACAAAGAAGCCTATTTTTC
AAGCCAATCTGGAACTGGCTCAGCTAGAACTGATCAGCCGACTTTG
TGGTAGCCCTTGTCCAGCTGTGTGGCCTGATGTTATCAAACTGCCCT
ACTTCAACACCATGAAACCGAAGAAGCAATATCGAAGGCGTCTACG
AGAAGAATTCTCTTTCATTCCTTCTGCAGCACTTGATTTATTGGACC
ACATGCTGACACTAGATCCTAGTAAGCGGTGCACAGCTGAACAGAC
CCTACAGAGCGACTTCCTTAAAGATGTCGAACTCAGCAAAATGGCT
CCTCCAGACCTCCCCCACTGGCAGGATTGCCATGAGTTGTGGAGTA
AGAAACGGCGACGTCAGCGACAAAGTGGTGTTGTAGTCGAAGAGC

CACCTCCATCCAAAACTTCTCGAAAAGAAACTACCTCAGGGACAAG
TACTGAGCCTGTGAAGAACAGCAGCCCAGCACCACCTCAGCCTGCT
CCTGGCAAGGTGGAGTCTGGGGCTGGGGATGCAATAGGCCTTGCTG
ACATCACACAACAGCTGAATCAAAGTGAATTGGCAGTGTTATTAAA
CCTGCTGCAGAGCCAAACCGACCTGAGCATCCCTCAAATGGCACAG
CTGCTTAACATCCACTCCAACCCAGAGATGCAGCAGCAGCTGGAAG
CCCTGAACCAATCCATCAGTGCCCTGACGGAAGCTACTTCCCAGCA
GCAGGACTCAGAGACCATGGCCCCAGAGGAGTCTTTGAAGGAAGC
ACCCTCTGCCCCAGTGATCCTGCCTTCAGCAGAACAGACGACCCTTG
AAGCTTCAAGCACACCAGCTGACATGCAGAATATATTGGCAGTTCT
CTTGAGTCAGCTGATGAAAACCCAAGAGCCAGCAGGCAGTCTGGAG
GAAAACAACAGTGACAAGAACAGTGGGCCACAGGGGCCCCGAAGA
ACTCCCACAATGCCACAGGAGGAGGCAGCAGCATGTCCTCCTCACA
TTCTTCCACCAGAGAAGAGGCCCCCTGAGCCCCCCGGACCTCCACC
GCCGCCACCTCCACCCCCTCTGGTTGAAGGCGATCTTTCCAGCGCCC
CCCAGGAGTTGAACCCAGCCGTGACAGCCGCCTTGCTGCAACTTTT
ATCCCAGCCTGAAGCAGAGCCTCCTGGCCACCTGCCACATGAGCAC
CAGGCCTTGAGACCAATGGAGTACTCCACCCGACCCCGTCCAAACA
GGACTTATGGAAACACTGATGGGCCTGAAACAGGGTTCAGTGCCAT
TGACACTGATGAACGAAACTCTGGTCCAGCCTTGACAGAATCCTTG
GTCCAGACCCTGGTGAAGAACAGGACCTTCTCAGGCTCTCTGAGCC
ACCTTGGGGAGTCCAGCAGTTACCAGGGCACAGGGTCAGTGCAGTT
TCCAGGGGACCAGGACCTCCGTTTTGCCAGGGTCCCCTTAGCGTTA
CACCCGGTGGTCGGGCAACCATTCCTGAAGGCTGAGGGAAGCAGCA
ATTCTGTGGTACATGCAGAGACCAAATTGCAAAACTATGGGGAGCT
GGGGCCAGGAACCACTGGGGCCAGCAGCTCAGGAGCAGGCCTTCA
CTGGGGGGGCCCAACTCAGTCTTCTGCTTATGGAAAACTCTATCGGG
GGCCTACAAGAGTCCCACCAAGAGGGGGAAGAGGGAGAGGAGTTC
CTTACGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCTGGCC
TCGGCGGCCAAGCTTGGCAATCCGGTACTGTTGGTAAAGCCACCAT
GGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGCCATTCTACCC
ACTCGAAGACGGGACCGCCGGCGAGCAGCTGCACAAAGCCATGAA
GCGCTACGCCCTGGTGCCCGGCACCATCGCCTTTACCGACGCACAT
ATCGAGGTGGACATTACCTACGCCGAGTACTTCGAGATGAGCGTTC
GGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAATACAAACCATCG
GATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTG
TTGGGTGCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACAT
CTACAACGAGCGCGAGCTGCTGAACAGCATGGGCATCAGCCAGCCC
ACCGTCGTATTCGTGAGCAAGAAAGGGCTGCAAAAGATCCTCAACG
TGCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGATAG
CAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACT
TCCCATTTGCCACCCGGCTTCAACGAGTACGACTTCGTGCCCGAGA
GCTTCGACCGGGACAAAACCATCGCCCTGATCATGAACAGTAGTGG
CAGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGCACCGCT
TGTGTCCGATTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGAT
CATCCCCGACACCGCTATCCTCAGCGTGGTGCCATTTCACCACGGCT
TCGGCATGTTCACCACGCTGGGCTACTTGATCTGCGGCTTTCGGGTC
GTGCTCATGTACCGCTTCGAGGAGGAGCTATTCTTGCGCAGCTTGC
AAGACTATAAGATTCAATCTGCCCTGCTGGTGCCCACACTATTTAGC
TTCTTCGCTAAGAGCACTCTCATCGACAAGTACGACCTAAGCAACT

TGCACGAGATCGCCAGCGGCGGGGCGCCGCTCAGCAAGGAGGTAG
GTGAGGCCGTGGCCAAACGCTTCCACCTACCAGGCATCCGCCAGGG
CTACGGCCTGACAGAAACAACCAGCGCCATTCTGATCACCCCCGAA
GGGGACGACAAGCCTGGCGCAGTAGGCAAGGTGGTGCCCTTCTTCG
AGGCTAAGGTGGTGGACTTGGACACCGGTAAGACACTGGGTGTGA
ACCAGCGCGGCGAGCTGTGCGTCCGTGGCCCCATGATCATGAGCGG
CTACGTTAACAACCCCGAGGCTACAAACGCTCTCATCGACAAGGAC
GGCTGGCTGCACAGCGGCGACATCGCCTACTGGGACGAGGACGAG
CACTTCTTCATCGTGGACCGGCTGAAGAGCCTGATCAAATACAAGG
GCTACCAGGTAGCCCCAGCCGAACTGGAGAGCATCCTGCTGCAACA
CCCCAACATCTTCGACGCCGGGGTCGCCGGCCTGCCCGACGACGAT
GCCGGCGAGCTGCCCGCCGCAGTCGTCGTGCTGGAACACGGTAAAA
CCATGACCGAGAAGGAGATCGTGGACTATGTGGCCAGCCAGGTTAC
AACCGCCAAGAAGCTGCGCGGTGGTGTTGTGTTCGTGGACGAGGTG
CCTAAAGGACTGACCGGCAAGTTGGACGCCCGCAAGATCCGCGAG
ATTCTCATTAAGGCCAAGAAGGGCGGCAAGATCGCCGTGTAATAAT
TCTAGATAATTCTACCGGGTAGGGGAGGCGCTTTTCCCAAGGCAGT
CTGGAGCATGCGCTTTAGCAGCCCCGCTGGGCACTTGGCGCTACAC
AAGTGGCCTCTGGCCTCGCACACATTCCACATCCACCGGTAGGCGC
CAACCGGCTCCGTTCTTTGGTGGCCCCTTCGCGCCACCTTCTACTCC
TCCCCTAGTCAGGAAGTTCCCCCCCGCCCCGCAGCTCGCGTCGTGC
AGGACGTGACAAATGGAAGTAGCACGTCTCACTAGTCTCGTGCAGA
TGGACAGCACCGCTGAGCAATGGAAGCGGGTAGGCCTTTGGGGCA
GCGGCCAATAGCAGCTTTGCTCCTTCGCTTTCTGGGCTCAGAGGCTG
GGAAGGGGTGGGTCCGGGGGCGGGCTCAGGGGCGGGCTCAGGGGC
GGGGCGGGCGCCCGAAGGTCCTCCGGAGGCCCGGCATTCTGCACGC
TTCAAAAGCGCACGTCTGCCGCGCTGTTCTCCTCTTCCTCATCTCCG
GGCCTTTCGACCTGCAGCCCAAGCTTACCATGACCGAGTACAAGCC
CACGGTGCGCCTCGCCACCCGCGACGACGTCCCCAGGGCCGTACGC
ACCCTCGCCGCCGCGTTCGCCGACTACCCCGCCACGCGCCACACCG
TCGATCCGGACCGCCACATCGAGCGGGTCACCGAGCTGCAAGAACT
CTTCCTCACGCGCGTCGGGCTCGACATCGGCAAGGTGTGGGTCGCG
GACGACGGCGCCGCGGTGGCGGTCTGGACCACGCCGGAGAGCGTC
GAAGCGGGGGCGGTGTTCGCCGAGATCGGCCCGCGCATGGCCGAG
TTGAGCGGTTCCCGGCTGGCCGCGCAGCAACAGATGGAAGGCCTCC
TGGCGCCGCACCGGCCCAAGGAGCCCGCGTGGTTCCTGGCCACCGT
CGGCGTCTCGCCCGACCACCAGGGCAAGGGTCTGGGCAGCGCCGTC
GTGCTCCCCGGAGTGGAGGCGGCCGAGCGCGCCGGGGTGCCCGCCT
TCCTGGAGACCTCCGCGCCCCGCAACCTCCCCTTCTACGAGCGGCTC
GGCTTCACCGTCACCGCCGACGTCGAGGTGCCCGAAGGACCGCGCA
CCTGGTGCATGACCCGCAAGCCCGGTGCCTGACCGCGTCTGGAACA
ATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTT
AACTATGTTGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCC
TTTGTATCATGCTATTGCTTCCCGTATGGCTTTCATTTTCTCCTCCTT
GTATAAATCCTGGTTGCTGTCTCTTTATGAGGAGTTGTGGCCCGTTG
TCAGGCAACGTGGCGTGGTGTGCACTGTGTTTGCTGACGCAACCCC
CACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCCGGGACTT
TCGCTTTCCCCCTCCCTATTGCCACGGCGGAACTCATCGCCGCCTGC
CTTGCCCGCTGCTGGACAGGGGCTCGGCTGTTGGGCACTGACAATT
CCGTGGTGTTGTCGGGGAAGCTGACGTCCTTTCCATGGCTGCTCGCC

TGTGTTGCCACCTGGATTCTGCGCGGGACGTCCTTCTGCTACGTCCC
TTCGGCCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCTGCTGCCGG
CTCTGCGGCCTCTTCCGCGTCTTCGCCTTCGCCCTCAGACGAGTCGG
ATCTCCCTTTGGGCCGCCTCCCCGCCTGGAATTAATTCTGCAGTCGA
GACCTAGAAAAACATGGAGCAATCACAAGTAGCAATACAGCAGCT
ACCAATGCTGATTGTGCCTGGCTAGAAGCACAAGAGGAGGAGGAG
GTGGGTTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTT
ACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGAGGGG
ACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGAT
CTGTGGATCTACCACACACAAGGCTACTTCCCTGATTAGCAGAACT
ACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGATGGTG
CTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAA
TAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGG
ATGGATGACCCGGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGC
CGCCTAGCATTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACT
TCAAGAACTGCTGATATCGAGCTTGCTACAAGGGACTTTCCGCTGG
GGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCG
AGCCCTCAGATCCTGCATATAAGCAGCTGCTTTTTGCCTGTACTGGG
TCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACT
AGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTT
CAAGTAGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATC
CCTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGTAGTAGTT
CATGTCATCTTATTATTCAGTATTTATAACTTGCAAAGAAATGAATA
TCAGAGAGTGAGAGGCCTTGACATTGCTAGCGTTTTACCGTCGACC
TCTAGCTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTG
AAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGC
ATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACAT
TAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCG
TGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT
TGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGC
TCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGG
TAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACA
TGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCG
CGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCAC
AAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTA
TAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCC
TGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTT
CGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAG
TTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCC
CCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGA
GTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACT
GGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG
TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTAT
TTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGT
TGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTTTT
TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAG
AAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGA
AAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATC
TTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTA
AAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATC

AGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGT
TGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTA
CCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCAC
CGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGA
GCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTA
ATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTT
GCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGT
CGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGA
GTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCG
GTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTC
ATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGT
AAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAG
AATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACG
GGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATT
GGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGT
TGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCA
GCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAA
GGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTT
GAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAG
GGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAA
ATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACC
TGACGTCGACGGATCGGGAGATCAACTTGTTTATTGCAGCTTATAA
TGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCA
TTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTA
TCTTATCATGTCTGGATCAACTGGATAACTCAAGCTAACCAAAATC
ATCCCAAACTTCCCACCCCATACCCTATTACCACTGCCAATTACCTA
GTGGTTTCATTTACTCTAAACCTGTGATTCCTCTGAATTATTTTCATT
TTAAAGAAATTGTATTTGTTAAATATGTACTACAAACTTAGTAGTTT
TTAAAGAAATTGTATTTGTTAAATATGTACTACAAACTTAGTAGT

7.3 Location of the oligonucleotides (CDK experiments) relative to the sequence of hybrid protein

Green=CMV

Red=CDK12 insert

Blue=firefly luciferase ORF

Yellow highlight=CDK12 306 ASO target

Red highlight=Luc150 ASO target

AAGCTTGGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCC
TGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACG

TATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATG
GGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTG
TATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAAT
GGCCCGCCTGGCATTATGCCCAGTACATGACCTTACGGGACTTTCCT
ACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGAT
GCGGTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTGACTCA
CGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTT
TTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCC
GCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCT
ATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACG
CCATCCACGCTGTTTTGACCTCCATAGAAGACACCGACTCTAGCTA
GAGGATCGCTAGCGCTACCGGACTCAGATCTCGAGACCATGCCCAA
TTCAGAGAGACATGGGGGCAAGAAGGACGGGAGTGGAGGAGCTTC
TGGAACTTTGCAGCCGTCATCGGGAGGCGGCAGCTCTAACAGCAGA
GAGCGTCACCGCTTGGTATCGAAGCACAAGCGGCATAAGTCCAAAC
ACTCCAAAGACATGGGGTTGGTGACCCCCGAAGCAGCATCCCTGGG
CACAGTTATCAAACCTTTGGTGGAGTATGATGATATCAGCTCTGATT
CCGACACCTTCTCCGATGACATGGCCTTCAAACTAGACCGAAGGGA
GAACGACGAACGTCGTGGATCAGATCGGAGCGACCGCCTGCACAA
ACATCGTCACCACCAGCACAGGCGTTCCCGGGACTTACTAAAAGCT
AAACAGACCGAAAAAGAAAAAAGCCAAGAAGTCTCCAGCAAGTCG
GGATCGATGAAGGACCGGATATCGGGAAGTTCAAAGCGTTCGAAT
GAGGAGACTGATGACTATGGGAAGGCGCAGGTAGCCAAAAGCAGC
AGCAAGGAATCCAGGTCATCCAAGCTCCACAAGGAGAAGACCAGG
AAAGAACGGGAGCTGAAGTCTGGGCACAAAGACCGGAGTAAAAGT
CATCGAAAAAGGGAAACACCCAAAAGTTACAAAACAGTGGACAGC
CCAAAACGGAGATCCAGGAGCCCCCACAGGAAGTGGTCTGACAGC
TCCAAACAAGATGATAGCCCCTCGGGAGCTTCTTATGGCCAAGATT
ATGACCTTAGTCCCTCACGATCTCATACCTCGAGCAATTATGACTCC
TACAAGAAAAGTCCTGGAAGTACCTCGAGAAGGCAGTCGGTCAGTC
CCCCTTACAAGGAGCCTTCGGCCTACCAGTCCAGCACCCGGTCACC
GAGCCCCTACAGTAGGCGACAGAGATCTGTCAGTCCCTATAGCAGG
AGACGGTCGTCCAGCTACGAAAGAAGTGGCTCTTACAGCGGGCGAT
CGCCCAGTCCCTATGGTCGAAGGCGGTCCAGCAGCCCTTTCCTGAG
CAAGCGGTCTCTGAGTCGGAGTCCACTCCCCAGTAGGAAATCCATG
AAGTCCAGAAGTAGAAGTCCTGCATATTCAAGACATTCATCTTCTC
ATAGTAAAAAGAAGAGATCCAGTTCACGCAGTCGTCATTCCAGTAT
CTCACCTGTCAGGCTTCCACTTAATTCCAGTCTGGGAGCTGAACTCA
GTAGGAAAAAGAAGGAAAGAGCAGCTGCTGCTGCTGCAGCAAAGA
TGGATGGAAAGGAGTCCAAGGGTTCACCTGTATTTTTGCCTAGAAA
AGAGAACAGTTCAGTAGAGGCTAAGGATTCAGGTTTGGAGTCTAAA
AAGTTACCCAGAAGTGTAAAATTGGAAAAATCTGCCCCAGATACTG
AACTGGTGAATGTAACACATCTAAACACAGAGGTAAAAAATTCTTC
AGATACAGGGAAAGTAAAGTTGGATGAGAACTCCGAGAAGCATCT
TGTTAAAGATTTGAAAGCACAGGGAACAAGAGACTCTAAACCCATA
GCACTGAAAGAGGAGATTGTTACTCCAAAGGAGACAGAAACATCA
GAAAAGGAGACCCCTCCACCTCTTCCCACAATTGCTTCTCCCCCACC
CCCTCTACCAACTACTACCCCTCCACCTCAGACACCCCCTTTGCCAC
CTTTGCCTCCAATACCAGCTCTTCCACAGCAACCACCTCTGCCTCCT
TCTCAGCCAGCATTTAGTCAGGTTCCTGCTTCCAGTACTTCAACTTT

GCCCCCTTCTACTCACTCAAAGACATCTGCTGTGTCCTCTCAGGCAA
ATTCTCAGCCCCCTGTACAGGTTTCTGTGAAGACTCAAGTATCTGTA
ACAGCTGCTATTCCACACCTGAAAACTTCAACGTTGCCTCCTTTGCC
CCTCCCACCCTTATTACCTGGAGATGATGACATGGATAGTCCAAAA
GAAACTCTTCCTTCAAAACCTGTGAAGAAAGAGAAGGAACAGAGG
ACACGTCACTTACTCACAGACCTTCCTCTCCCTCCAGAGCTCCCTGG
TGGAGATCTGTCTCCCCCAGACTCTCCAGAACCAAAGGCAATCACA
CCACCTCAGCAACCATATAAAAAGAGACCAAAAATTTGTTGTCCTC
GTTATGGAGAAAGAAGACAAACAGAAAGCGACTGGGGGAAACGCT
GTGTGGACAAGTTTGACATTATTGGGATTATTGGAGAAGGAACCTA
TGGCCAAGTATATAAAGCCAAGGACAAAGACACAGGAGAACTAGT
GGCTCTGAAGAAGGTGAGACTAGACAATGAGAAAGAGGGCTTCCC
AATCACAGCCATTCGTGAAATCAAAATCCTTCGTCAGTTAATCCAC
CGAAGTGTTGTTAACATGAAGGAAATTGTCACAGATAAACAAGATG
CACTGGATTTCAAGAAGGACAAAGGTGCCTTTTACCTTGTATTTGA
GTATATGGACCATGACTTAATGGGACTGCTAGAATCTGGTTTGGTG
CACTTTTCTGAGGACCATATCAAGTCGTTCATGAAACAGCTAATGG
AAGGATTGGAATACTGTCACAAAAAGAATTTCCTGCATCGGGATAT
TAAGTGTTCTAACATTTTGCTGAATAACAGTGGGCAAATCAAACTA
GCAGATTTTGGACTTGCTCGGCTCTATAACTCTGAAGAGAGTCGCC
CTTACACAAACAAAGTCATTACTTTGTGGTACCGACCTCCAGAACT
ACTGCTAGGAGAGGAACGTTACACACCAGCCATAGATGTTTGGAGC
TGTGGATGTATTCTTGGGGAACTATTCACAAAGAAGCCTATTTTTCA
AGCCAATCTGGAACTGGCTCAGCTAGAACTGATCAGCCGACTTTGT
GGTAGCCCTTGTCCAGCTGTGTGGCCTGATGTTATCAAACTGCCCTA
CTTCAACACCATGAAACCGAAGAAGCAATATCGAAGGCGTCTACGA
GAAGAATTCTCTTTCATTCCTTCTGCAGCACTTGATTTATTGGACCA
CATGCTGACACTAGATCCTAGTAAGCGGTGCACAGCTGAACAGACC
CTACAGAGCGACTTCCTTAAAGATGTCGAACTCAGCAAAATGGCTC
CTCCAGACCTCCCCCACTGGCAGGATTGCCATGAGTTGTGGAGTAA
GAAACGGCGACGTCAGCGACAAAGTGGTGTTGTAGTCGAAGAGCC
ACCTCCATCCAAAACTTCTCGAAAAGAAACTACCTCAGGGACAAGT
ACTGAGCCTGTGAAGAACAGCAGCCCAGCACCACCTCAGCCTGCTC
CTGGCAAGGTGGAGTCTGGGGCTGGGGATGCAATAGGCCTTGCTGA
CATCACACAACAGCTGAATCAAAGTGAATTGGCAGTGTTATTAAAC
CTGCTGCAGAGCCAAACCGACCTGAGCATCCCTCAAATGGCACAGC
TGCTTAACATCCACTCCAACCCAGAGATGCAGCAGCAGCTGGAAGC
CCTGAACCAATCCATCAGTGCCCTGACGGAAGCTACTTCCCAGCAG
CAGGACTCAGAGACCATGGCCCCAGAGGAGTCTTTGAAGGAAGCA
CCCTCTGCCCCAGTGATCCTGCCTTCAGCAGAACAGACGACCCTTG
AAGCTTCAAGCACACCAGCTGACATGCAGAATATATTGGCAGTTCT
CTTGAGTCAGCTGATGAAAACCCAAGAGCCAGCAGGCAGTCTGGA
GGAAAACAACAGTGACAAGAACAGTGGGCCACAGGGGCCCCGAAG
AACTCCCACAATGCCACAGGAGGAGGCAGCAGCATGTCCTCCTCAC
ATTCTTCCACCAGAGAAGAGGCCCCCTGAGCCCCCCGGACCTCCAC
CGCCGCCACCTCCACCCCCTCTGGTTGAAGGCGATCTTTCCAGCGCC
CCCCAGGAGTTGAACCCAGCCGTGACAGCCGCCTTGCTGCAACTTT
TATCCCAGCCTGAAGCAGAGCCTCCTGGCCACCTGCCACATGAGCA
CCAGGCCTTGAGACCAATGGAGTACTCCACCCGACCCCGTCCAAAC
AGGACTTATGGAAACACTGATGGGCCTGAAACAGGGTTCAGTGCCA

TTGACACTGATGAACGAAACTCTGGTCCAGCCTTGACAGAATCCTT
GGTCCAGACCCTGGTGAAGAACAGGACCTTCTCAGGCTCTCTGAGC
CACCTTGGGGAGTCCAGCAGTTACCAGGGCACAGGGTCAGTGCAGT
TTCCAGGGGACCAGGACCTCCGTTTTGCCAGGGTCCCCTTAGCGTTA
CACCCGGTGGTCGGGCAACCATTCCTGAAGGCTGAGGGAAGCAGC
AATTCTGTGGTACATGCAGAGACCAAATTGCAAAACTATGGGGAGC
TGGGGCCAGGAACCACTGGGGCCAGCAGCTCAGGAGCAGGCCTTC
ACTGGGGGGGCCCAACTCAGTCTTCTGCTTATGGAAAACTCTATCG
GGGGCCTACAAGAGTCCCACCAAGAGGGGGAAGAGGGAGAGGAGT
TCCTTACGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCTGG
CCTCGGCGGCCAAGCTTGGCAATCCGGTACTGTTGGTAAAGCCACC
ATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGCCATTCTACC
CACTCGAAGACGGGACCGCCGGCGAGCAGCTGCACAAAGCCATGA
AGCGCTACGCCCTGGTGCCCGGCACCATCGCCTTTACCGACGCACA
TATCGAGGTGGACATTACCTACGCCGAGTACTTCGAGATGAGCGTT
CGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAATACAAACCATC
GGATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGT
GTTGGGTGCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGAC
ATCTACAACGAGCGCGAGCTGCTGAACAGCATGGGCATCAGCCAGC
CCACCGTCGTATTCGTGAGCAAGAAAGGGCTGCAAAAGATCCTCAA
CGTGCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGAT
AGCAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGA
CTTCCCATTTGCCACCCGGCTTCAACGAGTACGACTTCGTGCCCGAG
AGCTTCGACCGGGACAAAACCATCGCCCTGATCATGAACAGTAGTG
GCAGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGCACCGC
TTGTGTCCGATTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGA
TCATCCCCGACACCGCTATCCTCAGCGTGGTGCCATTTCACCACGGC
TTCGGCATGTTCACCACGCTGGGCTACTTGATCTGCGGCTTTCGGGT
CGTGCTCATGTACCGCTTCGAGGAGGAGCTATTCTTGCGCAGCTTGC
AAGACTATAAGATTCAATCTGCCCTGCTGGTGCCCACACTATTTAGC
TTCTTCGCTAAGAGCACTCTCATCGACAAGTACGACCTAAGCAACT
TGCACGAGATCGCCAGCGGCGGGGCGCCGCTCAGCAAGGAGGTAG
GTGAGGCCGTGGCCAAACGCTTCCACCTACCAGGCATCCGCCAGGG
CTACGGCCTGACAGAAACAACCAGCGCCATTCTGATCACCCCCGAA
GGGGACGACAAGCCTGGCGCAGTAGGCAAGGTGGTGCCCTTCTTCG
AGGCTAAGGTGGTGGACTTGGACACCGGTAAGACACTGGGTGTGA
ACCAGCGCGGCGAGCTGTGCGTCCGTGGCCCCATGATCATGAGCGG
CTACGTTAACAACCCCGAGGCTACAAACGCTCTCATCGACAAGGAC
GGCTGGCTGCACAGCGGCGACATCGCCTACTGGGACGAGGACGAG
CACTTCTTCATCGTGGACCGGCTGAAGAGCCTGATCAAATACAAGG
GCTACCAGGTAGCCCCAGCCGAACTGGAGAGCATCCTGCTGCAACA
CCCCAACATCTTCGACGCCGGGGTCGCCGGCCTGCCCGACGACGAT
GCCGGCGAGCTGCCCGCCGCAGTCGTCGTGCTGGAACACGGTAAAA
CCATGACCGAGAAGGAGATCGTGGACTATGTGGCCAGCCAGGTTAC
AACCGCCAAGAAGCTGCGCGGTGGTGTTGTGTTCGTGGACGAGGTG
CCTAAAGGACTGACCGGCAAGTTGGACGCCCGCAAGATCCGCGAG
ATTCTCATTAAGGCCAAGAAGGGCGGCAAGATCGCCGTGTAATAAT
TCTAGAT

7.4 Western blots

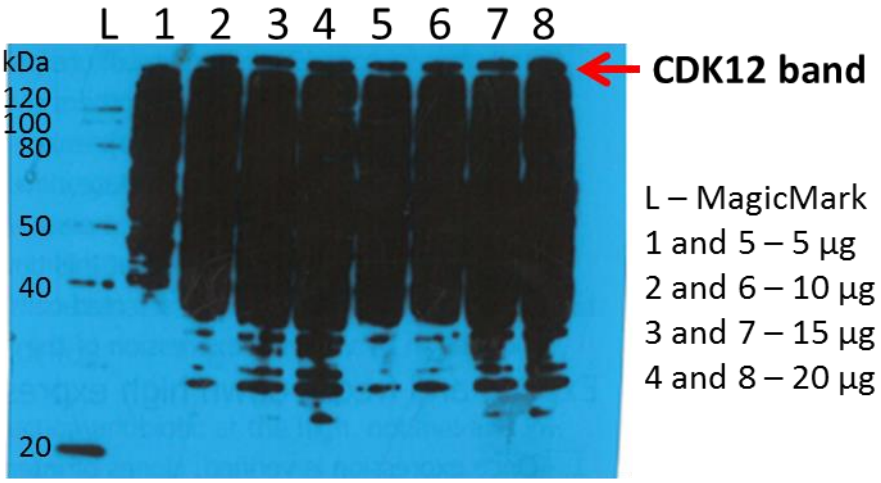


Figure 7.1 CDK12 western blot for MDA-MB fluc cells. Lanes represent different amounts of protein loaded.

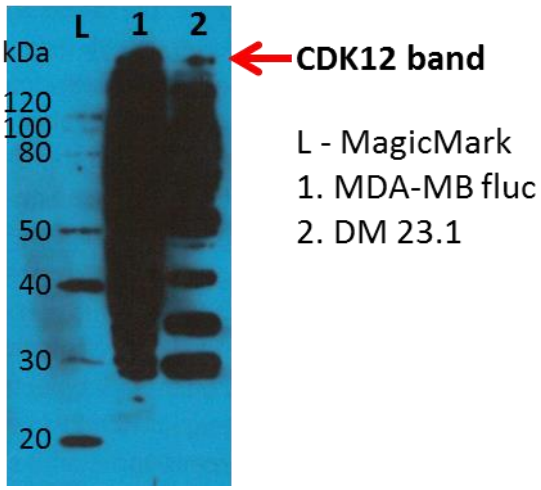


Figure 7.2 Blot showing an upper band corresponding to CDK12. Lysate from MDA-MB fluc cells was run together with muscle DM1 patient biopsy sample (DM 23.1) to confirm presence of the CDK12 band.

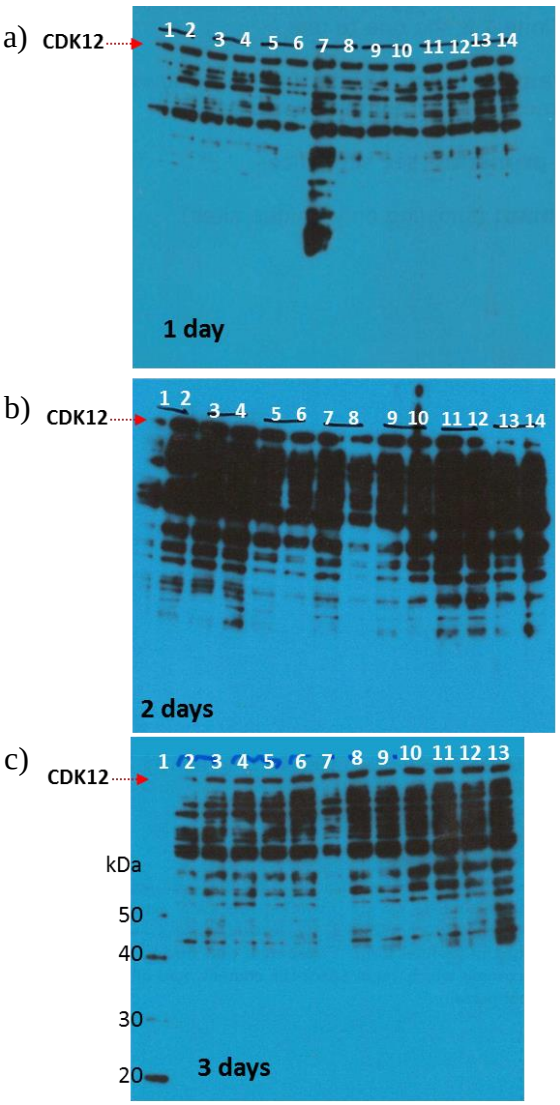


Figure 7.3 Western blots for attempts of CDK12 KD in MDA-MB fluc cells. 1 (a), 2 (b) and 3 (c) day treatments were carried out to see if CDK12 downregulation can be achieved. No inhibition of target was observed. The fainter bands in blot c (2 days) are result of incomplete transfer of the protein.

8 PIP reflective statement

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

PIP Reflective Statement:

My placement took place at Prosensa in Leiden, the Netherlands. Prosensa is a biotechnology company focused on the discovery, development and commercialisation of RNA-modulating therapeutics. The company targets genetic disorders with a large unmet medical need and with a focus on neuromuscular and neurodegenerative disorders, such as Duchenne Muscular Dystrophy, Myotonic Dystrophy (DM) and Huntington's disease.

My 3-month placement was divided into two parts, to provide me with a broader experience, both inside and outside the laboratory. The first part was a research project where I worked in the lab to analyse DM1 samples. In the second part I focused on learning and participating in Prosensa's internal processes, aiming to develop company's DM1 portfolio.

My first duty was to perform experiments using ddPCR to quantify splicing variants from the RNA samples extracted from mice. I also had to prepare muscle sections from tissues isolated from HSA^{LR} mice. To summarise my work I wrote a report and gave a presentation for my department. For the commercial part of placement I was responsible for preparing a flyer, targeting clinicians, with an overview of Prosensa's current DM1 program. Another responsibility was to prepare a comprehensive report on Prosensa's competitors in a DM1 field.

During my internship I developed many skills including new laboratory techniques and reporting in a format suitable for publication. I experienced new cultures and improved my communication methods. I showed that I have an ability to adapt to different environments. Undertaking a placement abroad raised my self-confidence. I demonstrated that I am able to work as a part of team, in both academia and industry. I developed strong research skills and an ability to analyse information and interpret data. Moreover, due to the relatively short duration of the project I proved that I have good time management skills and that I am capable of meeting deadlines.

In my PhD project I worked on developing antisense oligonucleotides. At Prosensa I could see a practical application of my work in the private sector. I observed several issues that may be faced by a company while trying to release their antisense product into the market. Overall it was a very valuable experience.