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NUCLEIC ACID BASED THERAPEUTIC APPROACHES

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Till min mor, far och syster

”Äntligen!”
—Gert Fylking

ABSTRACT

NUCLEIC ACID THERAPY comprises several approaches based on nucleic acid as the active therapeutic component to treat human disease. Antisense and short interfering RNA (siRNA) are two gene-silencing techniques in this category. Based on nucleic acid sequence recognition both techniques inhibit gene expression. This is highly attractive whenever there is deleterious expression of genetic material, as for example in viral diseases or cancer. In many of these diseases effective treatments are limited and/or toxic; therefore there is a need for new alternative therapeutics. We aimed to improve these gene-silencing techniques to facilitate future therapeutic application. We have made efforts in facilitating the use of viral vectors for nucleic acid delivery, but in particular, we have used the synthetic nucleotide analogue locked nucleic acid (LNA) in both antisense oligonucleotides and siRNA. As therapeutic targets we inhibited the human pathogens human immunodeficiency virus type 1 (HIV-1) and severe acute respiratory syndrome-associated corona virus (SARS-CoV) in cell culture. The results demonstrate that LNA is advantageous in both antisense and siRNA techniques.

Starting with delivery, as one major obstacle for nucleic acid based therapeutics, we aimed to facilitate the use of recombinant adeno-accosiated virus (AAV). In certain applications viruses can serve as suitable delivery vehicles for gene-silencing techniques. AAV has favourable properties for use as delivery vehicle, however there has been limited use of AAV due to production difficulties. We developed an alternative method for production that utilized baculoviruses for transfer of needed helper genes. We showed expression of the helper genes from the baculovirus and production of infec-

tious recombinant AAV. The protocol may lead to improved AAV production and facilitate increased use of recombinant AAV.

Continuing with the development of synthetic antisense oligonucleotides, we targeted the conserved HIV-1 dimerization initiation site with LNA modified antisense oligonucleotides, LNA/DNA mix-mers. We showed improved inhibition of dimerization by using LNA/DNA mix-mers and activation of RNase H by LNA/DNA mix-mers containing at least six consecutive DNA bases. We subsequently demonstrated inhibition of HIV-1 replication. LNA improves the antisense oligonuleotides and can function on a therapeutic target.

Using the more recently discovered gene-silencing technique siRNA, we targeted the SARS-CoV RNA dependent RNA polymerase. The siRNA inhibited SARS-CoV replication both when delivered pre and post infection. This study shows an example of how genetic information on an emerging pathogen can rapidly be converted to an antiviral tool by the means of siRNA.

Finally, we combined LNA with siRNA to a compound we term siLNA. We showed siLNA compatibility with the siRNA machinery in association with higher nuclease resistance and enhanced strand bias. siLNA provides a possibility to reduce undesired, off-target effects of siRNA. We also applied siLNA to inhibit SARS-CoV and showed improved efficiency over unmodified siRNA on certain target sites. LNA brings many favourable features to siRNA beneficial to future therapeutic use.

Taken together, these studies improve certain technical aspects of gene-silencing techniques and show potential applications of nucleic acid based therapeutics.

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which are referred to in the text by their roman numerals:

I. Sollerbrant K[†], **Elmén J**[†], Wahlestedt C, Acker J, Leblois-Prehaud H, Latta-Mahieu M and Perricaudet M. (2001) A novel method using baculovirus-mediated gene transfer for production of recombinant adeno-associated virus vectors. *Journal of General Virology*, **82**, 2051–2060.
[†]contributed equally

II. **Elmén J**, Zhang H-Y, Zuber B, Ljungberg K, Wahren B, Wahlestedt C and Liang Z (2004) Locked nucleic acid containing antisense oligonucleotides enhance inhibition of HIV-1 genome dimerization and inhibit virus replication. *FEBS Letters*, **578**, 285–290.

III. **Elmén J**, Wahlestedt C, Brytting M, Wahren B and Ljungberg K. (2004) SARS virus inhibited by siRNA. *Preclinica*, **2**, 135–142 (accessible on: www.preclinica.com).

IV. **Elmén J**, Thonberg H, Ljungberg K, Frieden M, Westergaard M, Xu Y, Wahren B, Liang Z, Ørum H, Koch T and Wahlestedt C. (2005) Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Research*, **33**, 439–447.

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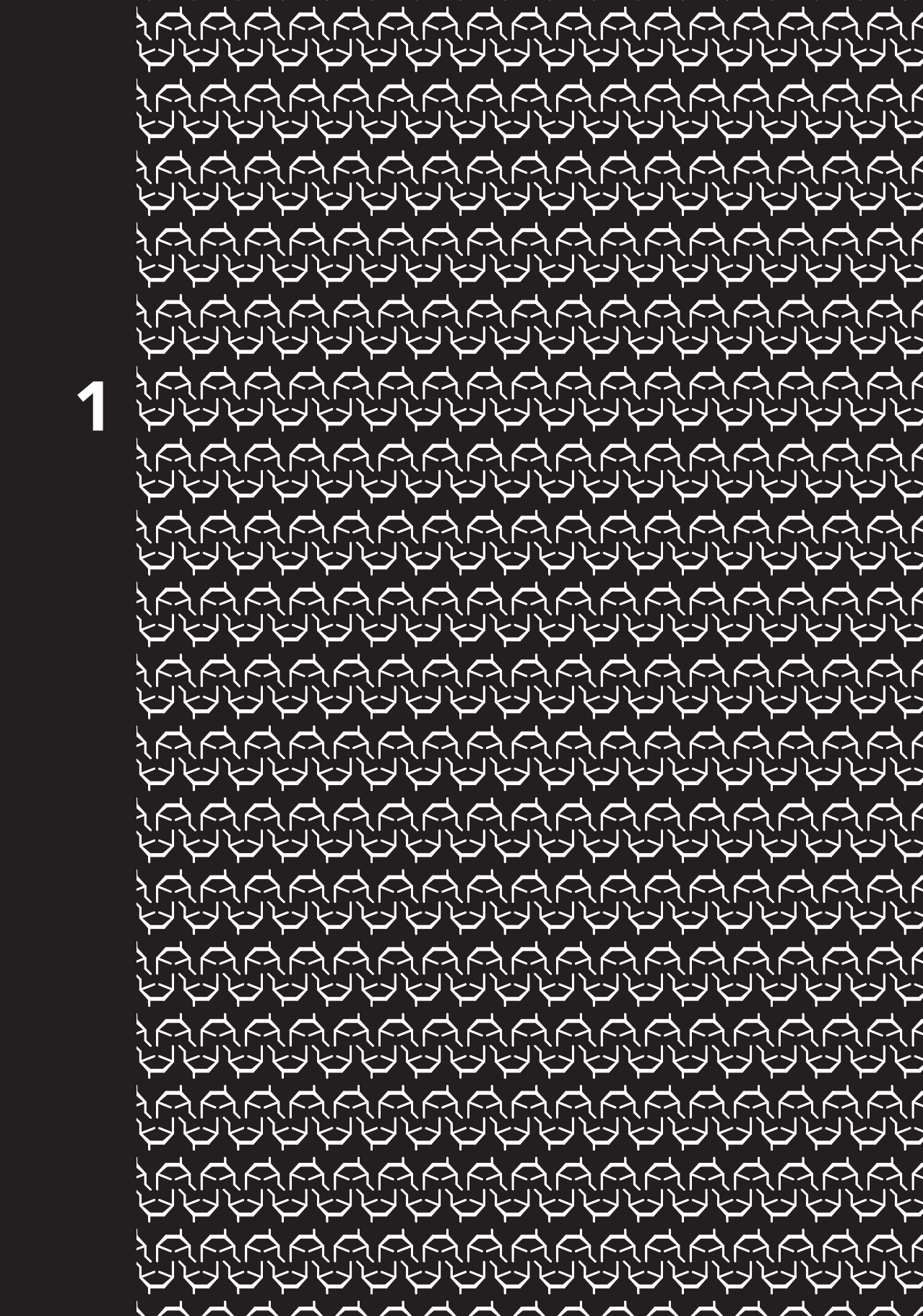
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INTRODUCTION

NUCLEIC ACID BASED therapy comprises several approaches based on nucleic acid as the active therapeutic component to treat human disease. A number of techniques can be classified in this category, for example: classical gene therapy, DNA vaccines, ribozymes, antisense and short interfering RNA (siRNA). Antisense, ribozymes and siRNA aim to inhibit gene expression and are also commonly called gene-silencing techniques. This thesis focuses on several technical aspects of antisense and siRNA technology. More specifically it describes how the synthetic nucleotide analogue locked nucleic acid (LNA) can be used to improve these techniques. The therapeutic action is to shut down a deleteriously expressed gene, for example in cancer or infectious diseases from viruses like HIV or SARS-CoV, to prevent the virus from replicating. The work also describes another aspect of viruses, as delivery vehicle for nucleic acids. Here the aim was to improve the production and facilitate the use of adeno-associated viral vectors. The therapeutic aspect of this work was extended to the application of gene-silencing techniques on the human pathogens HIV-1 and SARS-CoV in cell cultures. The obvious and necessary continuation of the work presented here is to progress to animal disease models, as well as improve delivery, as one of the main concerns. Pharmacokinetic and safety evaluations are also obligatory to proceed towards the clinic. Although several advances have been made in the development of nucleic acid based therapy, the clinical use is still limited. The technical approaches presented have the potential to advance the therapeutic application.

Nucleic acid; a brief historical overview

NUCLEIC ACID IS the carrier of the genetic hereditable material of all living matters. This was not known when nucleic acid was first isolated although the idea of hereditable element had been presented. In 1869 Friedrich Miescher was the first to isolate nucleic acid. He published his article "Über die chemische Zusammensetzung der Eiterzellen" (On the chemical composition of the pus cells) in 1871 (1). The extraction was made from pus, rich in white blood cells, which he obtained from bandages from the Hospital in Tübingen. He noted that this substance was acidic in nature and rich in phosphate compared to "Eiweiss" (protein) commonly part of the cell protoplasm. He called this substance nuclein. This was only a few years after Mendel presented his "Versuche über Pflanzen-Hybriden" (Experiments in Plant Hybrids) in 1865, published 1866 (2), where he discussed moving hereditable elements. These elements would later be known as genes. This is considered the beginning of modern genetics. Much later the two observations would merge, the nuclein would turn out to be the carrier of traits, the genetic material.

By the turn of the century nuclein was known as nucleic acid. It had been shown that the nuclein isolated by Miescher had contained a protein portion. After removing the protein portion the remaining portion was termed nucleic acid. One of the scientists that had a continued interest in nucleic acid was Albrecht Kossel, who continued the biochemical studies. His work on cell chemistry including nucleic acid was awarded the Nobel Prize in 1910. His Nobel lecture summarized the knowledge of nucleic acid at the time. The work of Miechers and others was recognized. He explained that nucleic acid contains nitrogen-rich bodies: pyrimidines; thymine and cytosine, as well as purines; adenine and guanine, which now are known as the bases in the nucleic acid and present the genetic code *(figure 1)*. Furthermore, Kossel noted that nucleic acid also contains a part that has the characteristics of a carbohydrate, a sugar, and another part that was phosphoric acid. The exact nature of the sugar was not known,

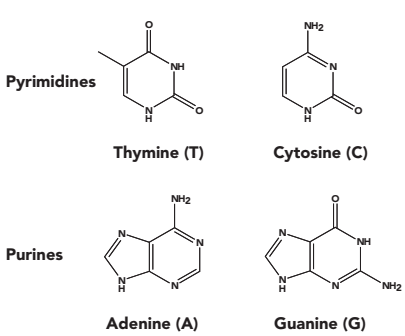


Figure 1. *Nucleic acid bases. Pyrimidines: Thymine (T), Cytosine (C), and Purines: Adenine (A) and Guanine (G).*

but it seemed to consist of a six carbons; however, in different studies the sugar isolated had five carbons. The connections between the different components were also not clear. However, to each of the four nitrogen-rich bodies there was one sugar and one phosphoric acid. Nevertheless, when nucleic acid was broken down carefully a larger component could be found; the sugar connected to one of either bases. Hence, these parts were most likely linked together *(figure 2)* (nucleoside: the sugar with a base connected, nucleotide: the same but phosphorylated).

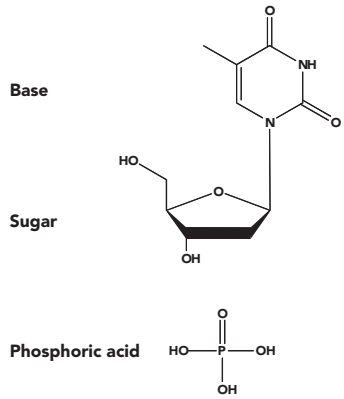
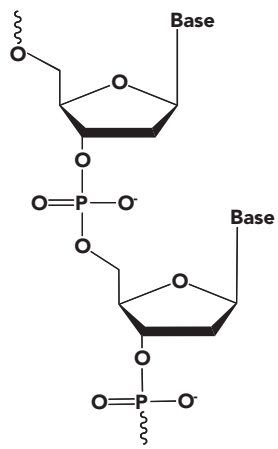


Illustration of a nucleoside (here: 2'-deoxythymidine); a base (thymine) connected to a sugar (2'-deoxyribose). Shown is also phosphoric acid. A nucleotide is a nucleoside with a phosphate group, the monomer unit in nucleic acid.

Figure 2.

The connection between the building blocks and the nature of the sugar came to be resolved. Nucleic acid was a linear polymer chain of the sugar deoxyribose connected by phosphodiester bonds and one of the four different bases; adenine, thymine, guanine, cytosine connected to each deoxyribose *(figure 3)*. Two types of nucleic acid were also recognized, deoxyribonucleic acid and ribonucleic acid (DNA and RNA). These nucleic acids differed in sugar and base composition. DNA had a deoxyribose sugar back-bone and RNA ribose *(figure 4)*. Deoxyribose lacks a hydroxyl group on the second carbon (C2') compared to the ribose. In addition, RNA used the pyrimidine uracil instead of thymine, whereas the other three bases were the same for both DNA and RNA. DNA was mainly found in the nucleus of the cell whereas RNA was found in the cytosol, the extra nuclear solution of the cell. The substance nuclein initially isolated by Miecher was of the first type, DNA.



Phophodiester bonds covalently link the sugars in the nucleic acid chain.

Figure 3.

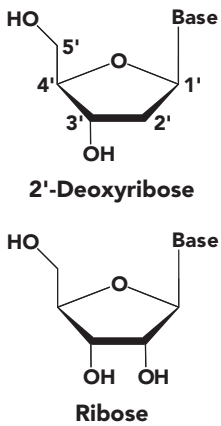


Figure 4. *2'-deoxyribose and ribose and their bond to the bases. These represent the sugars in the backbone of the two types of nucleic acid: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Also shown is the numbering of the carbons.*

The chemical components of nucleic acid were more or less known but it continued to be a molecule without function. The work leading an understanding of the function of DNA had significant help from a finding published by Griffith in 1928. He showed in his publication "The significance of pneumococcal types" how non-virulent pneumococcus (rough, R) can be transformed into virulent pnuemococcus (smooth, S) by a substance present in the heat killed virulent pneumococcus (3). This was called the transforming principle. The transformation occurred when both were injected into mice, in a living organism *in vivo*. This transformation principle was clearly due to a chemical substance changing the R phenotype to the S phenotype. Griffith hypothesized that the substance in the heat killed S form was a protein that was missing in the R form, and this substance, maybe an enzyme, was necessary to produce the missing carbohydrate in the R form. This finding indicated that a chemical component could be transferred and could transform (provide the missing information) to the non-virulent phenotype allowing it to thereby regain its virulence.

THE ROLE OF nucleic acid as a carrier of genetic information was unequivocally unravelled in 1944. Griffith's transformation system was further refined by Avery, MacLeod and McCarty to work *in vitro*, in a test tube. They presented a breakthrough paper 1944 in which they concluded: "The evidence presented supports the belief that nucleic acid of the desoxyribose type is the fundamental unit of the transforming principle of Pneumococcus Type III" (4). They provided compelling evidence that nucleic acid is the genetic material. Prior to this the chemical component which would contain our genetic information was generally thought to be protein due to its higher complexity; composed of about twenty building blocks, amino acids, compared to only four different bases in nucleic acid.

In this work they carefully purified nucleic acid from large bacterial batches. The purified substance was then rigorously tested for purity and content before it was used in transformation experiments. The purified nucleic acid could transform the R phenothype into S. They also used enzyme preparations that had the activity of degrading deoxyribonucleic acid, "depolymerase for desoxyribonucleate", and saw that it inactivated the transforming principle. This was one of the arguments for deoxyribonucleic acid being the active substance; as well as assessment of the purity and using several analytical methods confirming that the purified substance was deoxyribonucleic acid. Avery, MacLeod and McCarty showed a clear link between genes, hereditable elements, and nucleic acid. With this a function for nucleic acid was demostrated; it is the carrier of the genetic material.

The notion that nucleic acid is the genetic material was further established in another experiment, in 1952 by Hersey and Chase (5). They added different radioactive labels to the proteins (^{35}S, radioactive sulfur) and deoxyribonucleic acid (^{32}P, radioactive phosphor) of bacteriophage and thereafter infected unlabelled bacteria with these phage preparations. They saw that the major portion transferred from the phage to the bacteria was ^{32}P labelled deoxyribonucleic acid. Although it was known that the phage infect the bacteria and new phage were produced within the bacteria almost all phage DNA was found in the cell whereas at least 80 % of the ^{35}S containing protein remained at the cell surface. It was also shown that newly produced phage contained labelled DNA but little or nothing of the labelled protein. DNA was responsible, provided the information, to produce new phage.

At the same time the composition of nucleic acid was being continuously explored. Chargaff and co-workers saw that the composition of the bases in nucleic acid was different in different species (6–9). They found that there were different amounts of the bases (A, T, G, and C) in different organisms. However, adenine (A) and thymine (T) always were present in equal ratios (1:1). This was also true for guanine (G) and cytosine (C). Chargaff referred to the organisms to be of "AT type" or "GC type" depending on which pair was over-represented. In 1950 when Chargaff et al. investigated the composition of nucleic acid from human sperm, the ratio was "noteworthy, though possibly no more than accidental" (6). One year later Chargaff concluded that this ratio was a regularity in DNA after investigations of several species (7).

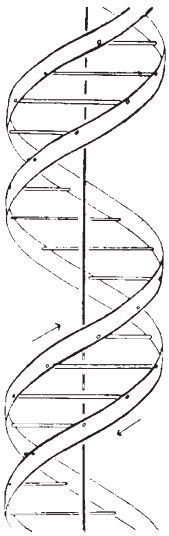


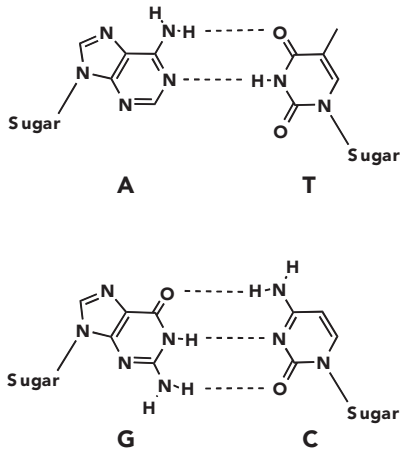
Figure 5. *The famous representation of DNA illustrated by Odile Crick (Watson, J.D. and Crick, F.H. (1953) Nature, 171, 737–738.). The ribbons depict the backbone, the sugar-phosphodiester chain and the horizontal bars the base-pairs (A-T or G-C) holding the two chains together. Reproduced with the permission from Nature and Odile Crick.*

THE STRUCTURE IS one of the most celebrated proposals concerning nucleic acid. In 1953 Watson and Crick suggested a structure of deoxyribonucleic acid, the double helix and the specific pairing of the bases (10), partly aided by the data from Chargaff. In their paper they proposed that two strands of deoxyribonucleic acid were joined together in a double spiral *(figure 5)*. The phosphate-sugar-backbone was presented outwards (the ribbons in *figure 5*) and the bases projected inwards (the bars in figure). The forces holding the two strands together were proposed to be hydrogen bonds that could form specifically between the bases A-T and G-C, respectively *(figure 6)*. As a result of this constraint Watson and Crick stated; "It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material". One strands base composition/order would determine the composition of the other strand in this structure. Therefore, one strand could serve as a template for replication and the new strand would inherit the information from the parental strand. The two strands were complementary to each other and run in anti-parallel direction. This work resolving the structure of DNA was awarded the Nobel Prize in 1962 and was shared between Watson, Crick and Wilkins. Wilkins contributed with x-ray crystallographic measurements proving the proposed structure by Watson and Crick.

The suggested copying mechanism of DNA in the Watson and Crick paper was soon proven. Evidence for the possibility to inherit the information from one strand to another was provided by the discovery of a DNA polymerase and that the mechanisms of DNA replication. Kornberg and colleagues played a role in elucidating the mechanism of DNA replication (11–13) (Kornberg along with Ochoa were rewarded with the Nobel Prize in 1959 "for their discovery of the mechanisms in the biological synthesis of ribonucleic acid and deoxyribonucleic acid"). Another finding by Meselson and Stahl also provided evidence for Watson and Crick's theories. They showed evidence for semi-conservative replication of DNA; that the two strands sepa-

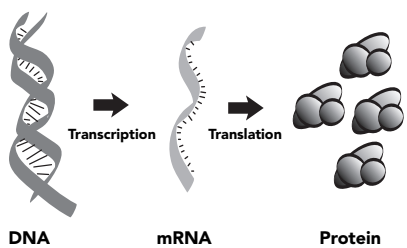
rate and both serve as templates for new double strands (14). A self-copying mechanism of DNA was evident, which was a prerequisite for inheritance.

Crick was also interested in the flow of genetic information and how it was transferred. He hypothesized that the base sequence of nucleic acid would determine the amino acid sequence of proteins. He further added constraint to this hypothesis that "once 'information' passed onto protein it cannot get out again". He called this the Central dogma and it was presented in 1958 *(figure 7)*(15). He continued to discuss that the RNA in the cytoplasm must be in control of the DNA in the nucleus. The base-pairing was also applicable to copying the DNA genetic information onto an RNA template. At this time evidence of a short-lived RNA species was accumulating and in 1961 Jacob and Monod presented arguments that this RNA species, which they designated messenger RNA (mRNA), would be the intermediate between DNA and protein (16)(Jacob and Monod along with Lwoff received the Nobel Prize 1965). At the time a gene was designated as a part of DNA that is translated into a specific protein, and RNA was the messenger in between.



The pairing of the bases suggested by Watson and Crick. The two chains in DNA are held together by hydrogen bonds (dashed lines) possible between the bases adenine and thymine as well as between guanine and cytosine.

Figure 6.



The Central dogma presented by Crick 1958, illustrates the flow of genetic information from DNA to messenger RNA (mRNA) to protein. The copying of the genetic code from DNA to mRNA is called transcription. The transfer of the information from mRNA to a protein product is called translation.

Figure 7.

Both forms of nucleic acid, deoxyribonucleic acid and ribonucleic acid, now had their respective functions identified. DNA contained genetic information and was capable of replication, and RNA carried the message to provide the information for production of proteins. The process of copying the information from DNA to RNA was called transcription and interpreting the

genetic information from RNA to produce proteins was called translation. Since the genetic information seemed to be contained in the sequence of the nucleic acid bases, the next major question was how this sequence would be translated to proteins. What was the code?

THE GENETIC CODE obtained its first part of the translation in 1961. Nirenberg and Matthaei described in different experiments how radio-labelled amino acids were incorporated into proteins in a cell free protein synthesis translation system (17,18). In their next step they added synthetic strands of RNA and observed that this stimulated the production of proteins. Strikingly, when a synthetic RNA strand containing only uridylic acid, polyuridylic acid (polyU) was added, the protein polyphenylalanine, a polymer chain of the amino acid phenylalanine, was produced. Clearly polyU was coding for polyphenylalanine. This was also evidence for the messenger RNA theory. At this stage it was unknown how many U's encoded one phenylalanine or if code was consecutive or overlapping. In this paper Nirenberg and Matthaei also showed that when the polyuridylic acid was combined with polyadenylic acid there was no protein synthesis. They concluded that this was probably due to duplex and/or triplex formation and that the template (messenger) RNA had to be single stranded. Indeed they had not only initiated solving the genetic code, but also shown the principle for translational block by hybridizing a complementary strand to the messenger RNA; the essence of the antisense technique described later in this thesis. A therapeutic application of this finding was however not a part of the discussion in their paper.

Crick also had an interest in breaking the genetic code and discussed this in his 1962 Nobel lecture. He assumed that the genetic code would consist of bases in triplets, which he termed codons. This was the smallest number of bases that would give enough combinations to make up for the twenty different amino acids in proteins. He also postulated that there would be no overlap of the codons; they would be aligned one after the other. Watson described another part of the puzzle in his Nobel lecture the same year. This was how they had struggled to obtain the structure of messenger RNAs and to identify the three dimensional structure which would be unique to each of the 20 amino acids. Crick had a radical proposal around this problem; that there would be an adaptor molecule between the messenger RNA and amino acid in the translation of the genetic code to proteins. The adaptor would recognize the codon and bring in the correct amino acid. The postu-

lated adaptor molecule, termed transfer RNA (tRNA), was first identified by Zamecnik and Hoagland. However, Holley managed to purify tRNA and show that it was indeed responsible for the translation of the code into proteins. He also determined the structure of tRNA. The genetic code was solved soon after the discovery that polyU coded for the polyphenylalanine along with advances in oligonucleotide (short nucleic acid strands) synthesis. Nirenberg, Holley and Khorana shared the Nobel Prize in 1968, "for their interpretation of the genetic code and its function in protein synthesis". The chemical composition, structure, function and the code contained in nucleic acid was now known. The scene was then set for many discoveries and a variety of new techniques concerning nucleic acid.

THE DISCOVERY OF nucleic acid has since lead to many new advances and discoveries. One method to be mentioned is DNA sequencing, reading the code, for which the Nobel Prize awarded Berg, Gilbert, and Sanger in 1980. Today, we know the whole sequence of the human genome, 3 billion bases (19,20), along with many other genomes. These bases in the human genome represent about 20,000–25,000 protein coding genes (21). Additionally, the sequence information of a gene instantly makes possible the use of gene-silencing techniques, for functional studies of the gene or therapeutic purposes. The basic concepts behind the two gene-silencing techniques, antisense and siRNA are discussed in the next section of this thesis.

Note

Additional references for this overview (22–31), as well as the official home page of the Nobel foundation: nobelprize.org.

Nucleic acid based therapeutic approaches; gene-silencing techniques

GENE-SILENCING CAN BE achieved in different ways. This section will describe two approaches, antisense oligonucleotides and short interfering RNA (siRNA). These two techniques aim to inhibit gene expression but rely on different biological mechanisms. Both are based on nucleic acid and mainly act at the mRNA level. In context of the central dogma both bind to the mRNA and either degrade this or inhibit the translation *(figure*

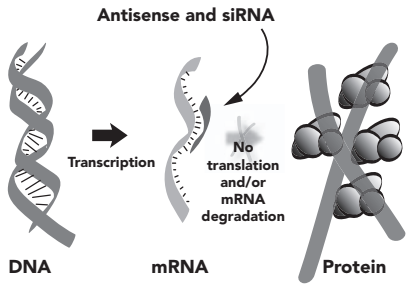


Figure 8.

General model for antisense and siRNA gene-silencing. Both antisense and siRNA rely on sequence recognition of the genetic message at the mRNA level and cleave the mRNA or block translation. This results in reduced or abolished protein production.

8). In both cases, there will be less or no protein produced. The target recognition occurs through the base-pairing described by Watson and Crick. As mentioned, the sequence of the entire human genome is known, as well as many other genomes, from viruses for example. All this sequence information provides a means to design a gene-silencing molecule to the gene of our choice. Emerging viruses like SARS-CoV or new strains of influenza can rapidly be sequenced and an inhibitory molecule made. This is an appealing idea, the simple conversion of nucleic acid sequence information of a deleterious expressed gene into an inhibitory molecule. In addition to mRNA degradation and inhibition of translation, other processes can be inhibited. Protein interactions with RNA can be blocked as well as degradation of viral genomic RNA, but there can also be inhibitory interactions with genomic DNA. The following description mainly implies mRNA degradation and inhibition of translation by synthetic antisense oligonucleotides and siRNA.

Antisense oligonucleotides

ANTISENSE OLIGONUCLEOTIDES ARE short stretches (usually 15–20 nucleotides long) of DNA or nucleotide analogues complementary to their target mRNA. They bind to their target sequence and inhibit the function of the mRNA by two mechanisms *(figure 9)*. First, inhibition of gene expression can be achieved through steric hindrance by the antisense oligonucleotides bound to the target mRNA. This blocks the ribosome access or processivity and hence inhibits translation. Second, antisense oligonucleotides can promote degradation of mRNA by activating the enzyme RNase H. RNase H is a ubiquitous cellular enzyme that cleaves the RNA strand in a DNA/RNA hybrid. Cleavage of the mRNA will lead to a truncated message but also to rapid degradation of the mRNA via the exposed unprotected RNA ends (32–35).

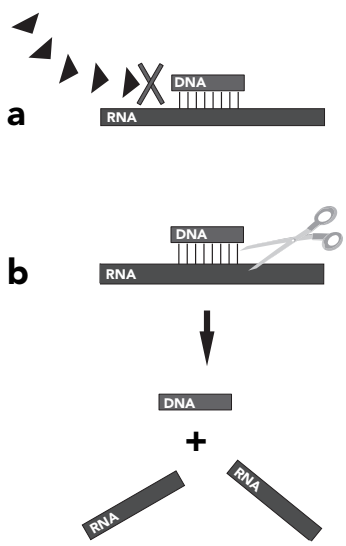


Figure 9. *Antisense oligonucleotides bind to the target RNA and either block the translation or other interactions with protein but can also promote cleavage of the RNA by the enzyme RNase H. RNase H recognize a DNA/RNA hybrid and cleaves the RNA in this hybrid.*

The first inhibition of translation by hybridization, binding of a complementary nucleic acid strand to the mRNA, was shown in 1961 when Nirenberg and Matthaei saw that when a polyU hybridized to a polyA would not be translated into protein whereas polyU alone would. Single-strandedness was necessary for translation. This was a demonstration of translational blockade. However, no potential therapeutic applications were discussed (18). The theory of translation blockade, by hybridizing a complementary nucleic acid strand to the mRNA was developed into an application to identify genes in 1977 by Paterson *et al.* (36). DNA fragments of interest were hybridized to total RNA extracts from cells. This mix was translated into proteins *in vitro*. The untranslated, missing proteins could then be mapped to the DNA fragment added. They called this hybrid-arrested translation.

A year later, in 1978 Zamecnik and Stephenson suggested a therapeutic application. This appeared in two consecutive papers (37,38). They described how a 13 bases long DNA oligonucleotide complementary to Rous sarcoma virus (RSV) inhibited both protein translation *in vitro*, as well as virus replication. This is considered the antisense proof of principle. They also suggested different mechanisms to be responsible for the inhibition such as blockage of transcription, translation, genome circularization and integration. RNase H was not discussed as a possible mechanism for the inhibition in this paper even though RNase H was known at this time.

RNase H cleavage of the target RNA, one of the mechanisms today considered an important part of the antisense effect, was also discovered in Tübingen exactly 100 years after Miechers first isolation of nucleic acid. In 1969 Stein and Hausen isolated an enzyme from calf thymus that specifically degraded the RNA in an RNA-DNA hybrid (39). They stated: "The biological function of the hybrid-degrading enzyme is obscure". Today the enzymes in this family are considered to play a role in DNA replication and transcription and are found in many organisms (40-42). Chargaff and co-workers, who found the one to one ratio between the bases A-T and G-C in the early 50's, also published in the RNase H area, reporting that DNA oligomers as short as tetranucleotides (four bases long oligonucleotides) could mediate the degradation of the target RNA (43).

The antisense theory is simple, but its application is more complex. Issues in the field include selection of good target sites, delivery, affinity and stability of the oligonucleotide and avoiding unwanted side effects. Target selection is mentioned here and the other issues will be partly covered in

the remaining sections. Target selection has been largely dependent on trial and error. However, the low efficiency of this empirical selection has lead to the development of more rational selection processes. These methods were developed to facilitate target selection especially when large sets of genes were targeted in functional genomics applications. Different assay scenarios deployed for *in vitro* screening include RNaseH mapping (44,45), gel shift (46), oligonucleotide array (47) and random RT priming (48), as well as computational predictions (49,50). However, secondary structures can be hard to predict by computer algorithms. An example of an *in vitro* screening method is for example mRNA accessible site tagging MAST (51). A random library of oligonucleotides is hybridized to the mRNA of choice. Unbound oligonucleotides are washed away and the remaining are cloned and sequenced. In this way oligonucleotides bound to the mRNA despite its secondary structures can be identified.

Short interfering RNA (siRNA)

SHORT INTERFERING RNA (siRNA) is typically a 21 nucleotides long double stranded stretch of RNA. The two strands in the siRNA usually have 19 complementary nucleotides and two overhanging nucleotides at each 3'-end. In the siRNA, one strand is complementary to the target RNA and guidies the siRNA to the target. It is referred to as the antisense or guiding strand. The other strand is referred to as the sense strand. The guiding siRNA strand is associated with a protein complex that will cleave the target RNA once the complementary target is found.

siRNA is a relatively new tool compared to the antisense technology and have had a rapid expansion and found many applications since it was shown to be a potent gene-silencing tool in mammalian cells in 2001. It has also drawn interest to the field of small non-coding RNAs. siRNA sprung from the discovery of RNA interference (RNAi). This discovery was made in 1998 by Fire *et al.* in the nematode *Ceanorhabditis elegans (C. elegans)* (52). To their surprise long double stranded RNA (dsRNA) was much more potent in inhibiting gene expression and creating phenotypes than the antisense or sense RNA alone. The effect could spread to different cells and last for generations in the worm. RNAi rapidly became a functional genomics tool in *C. elegans* (53,54), also because the ease of administration. The worms could simply be soaked in a dsRNA solution or fed bacteria that expressed the dsRNA of interest.

This stunning tool immediately raised hopes for the same function in vertebrates and in particular mammals. The first studies showing RNAi in vertebrate cells were performed in zebra fish embryos and mouse oocytes (55,56). However, at the time no publications were presented that could repeat this in other mammalian cells. Long stretches of double stranded RNA induce non-specific effects in mammalian cells. For instance, there is a general inhibition of translation by dsRNA, mediated by protein kinase R (PKR) and also non-specific RNA degradation by RNase L (57–59). It was argued that these mechanisms were not fully developed in the mouse embryonic system and that was why long dsRNA worked and provided an RNAi effect.

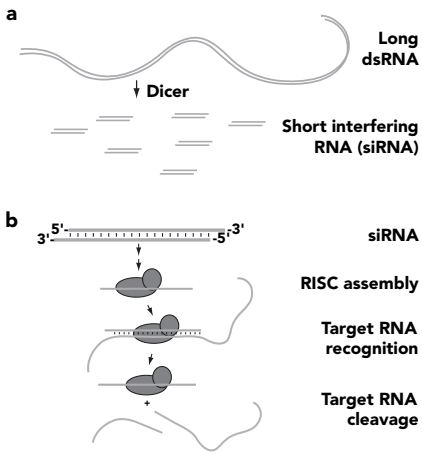


Figure 10. *RNA interference (RNAi) and short interfering RNA (siRNA). Gene-silencing induced by long double stranded RNA (dsRNA) in Drosophila and C. elegans called RNAi is mediated through siRNA. (a)The long dsRNAs are cleaved by an enzyme called Dicer to the active siRNAs. (b) The two short RNA strands in the siRNA are separated and one of them is incorporated into the RNA induced silencing complex (RISC). Loaded RISC search for the target RNA and cleave upon sequence recognition.*

It was shown that the long dsRNA inducing RNAi was degraded to short RNA double strands (21-23 nt). These short interfering RNAs (siRNAs) were actually responsible for the interference, the gene-silencing (60,61). An enzyme called Dicer was responsible for the processing of long dsRNA to siRNA *(figure 10)*. This led to the breakthrough for the siRNA technology in mammalian cells in 2001. Elbashir *et al.* unambiguously showed that siRNA mediated a potent and specific gene-silencing in a variety of mammalian cell lines and escaped the adverse, non-specific effects associated with long dsRNA (62). This discovery was a start for immense use and development of applications for siRNA.

Antisense oligonuleotides and siRNA work through different pathways. However, in both cases target recognition still occurs through Watson-Crick base-pair recognition. The siRNA double-strand is unwound and one of the single strands is incorporated into a protein complex called the RNA-induced silencing complex (RISC). The complex finds the complementary mRNA target guided by the siRNA strand and then cleaves this target

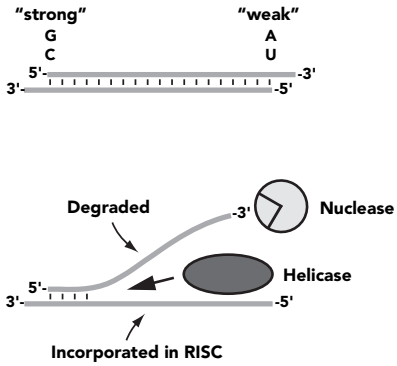


Figure 11. *An siRNA has two strands, one antisense, guiding strand complementary to the target RNA sequence and one accompanying sense strand. A helicase unwinds the two siRNA strands starting at the least stable 5'-end. The strand with the least stable 5'-end is subsequently incorporated into RISC and will mediate the sequence recognition. The released strand is most likely immediately degraded.*

(figure 10). The proteins in the siRNA pathway are evolutionarily conserved and are found in plants, invertebrates and mammals alike. Extensive reviews on RNAi and siRNA mechanisms have been written (63–71).

Selecting target sequences using siRNA has been fairly simple compared to antisense. Almost random target selection displayed greater success than using the same approach for selection of antisense targets. Initially simple design rules were presented by Tuschl that were based on the degradation products generated from long dsRNA to siRNA by Dicer. These products were roughly 21–23 nt long with 3' UU overhangs. However, more rational selection procedures have been presented. One important feature here is that strand bias has been described (72,73). One strand of the two in the siRNA is preferentially incorporated into RISC. The strand with the 5' end that has the weaker base-pairing, lower binding energy, is incorporated into RISC *(figure 11)* and subsequently the strand that will guide to the target. Computer algorithms considering these energy rules, as well as other criteria return siRNA sequences with high success rates (74,75).

Synthetic nucleotide analogues

IT WAS EARLY realized that oligonucleotides needed increased resistance to nucleases. The natural nucleic acids are not persistent enough and would be mostly degraded before they exert their effect. Hence, they were not suitable for therapeutic use. A solution to this problem was the development of synthetic nucleotide analogues that would be highly resistant to nuclease attacks. It was also recognized that enhanced binding to the target sequence was a desirable feature, which also could be modulated by different chemical modifications to the natural nucleic acids.

The phosphorothioate chemistry is one of the early modifications of nucleic acids. It has been one of the most popular modifications for anti-

sense applications. The phosphorothioate modified oligonucleotides contains a sulphur atom instead of oxygen in a non-bridging position of the phosphodiester bond *(figure 12)*. Phosphorothioates are more stable than DNA, but suffer from toxic side effects as well as decreased binding to the target (76–78). Quite simple modifications can also be protective towards nucleases, like changing the direction of the last nucleotide from 5'–3' to 3'–5', creating a 3'–3' link (3'-inverted)*(figure 12)*. As illustrated in *figure 13*, unmodified DNA oligonucleotides are rapidly degraded, whereas a 3' inverted or phosphorothioate modified oligonucleotides have decreased degradation rates. Exonuclease attack at the 3' end is a major path for degradation of oligonucleotides (79).

Later modifications are situated at the 2' carbon, such as 2'-O-methyl (2'OMe) and 2'-O-metoxyethyl (2'MOE) ribonucleic acid *(figure 14)*. These modifications have less toxicity associated with them compared to the phosphorothioate chemistry and have seen increased use. They also have a slight increase in affinity to the target (77,80). Even more recent nucleotide analogues, with very high affinity, are for example LNA or peptide nucleic acid (PNA) with extensively modified or with a fully replaced backbone *(figure 14)*. Synthesis of novel DNA analogues with interesting properties has

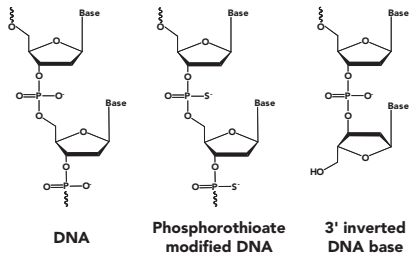


Figure 12. *Chemical modifications of DNA; phosphorothioate modified DNA, in which one of the non-bridging oxygen (O) in the phosphodiester bond is exchanged to sulphur (S). Another simple modification to improve nuclease resistance is to invert the last DNA base with a 3'-3' link, exposing the 5'-end.*

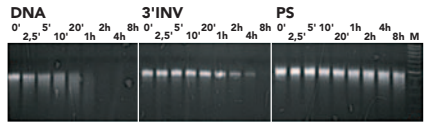


Figure 13. *Stability of DNA, DNA with a 3' inverted base (3'INV) and phosphorothioate (PS) oligonucleotides in rat serum. Marker (M) bright band 20 nt.*

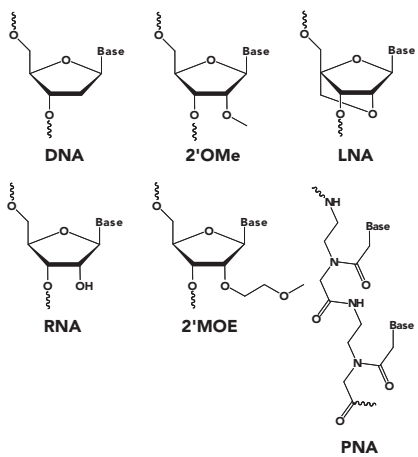


Figure 14. *Nucleotide analogue examples compared to DNA and RNA. Modification of the 2' carbon: 2'-O-methyl ribonucleic acid (2'OMe) or 2'-O-methoxyethyl ribonucleic acid (2'MOE). Heavier backbone modifications or completely replaced backbone: locked nucleic acid (LNA) or peptide nucleic acid (PNA).*

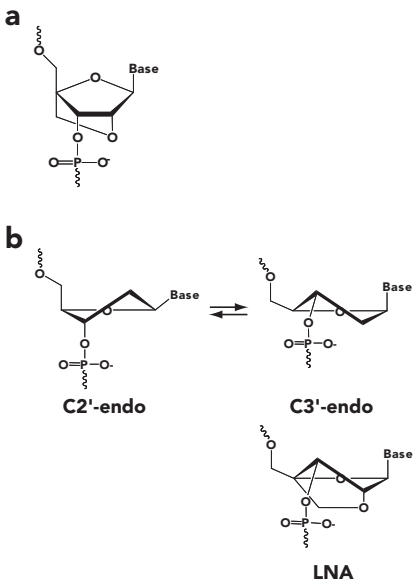


Figure 15. *Locked nucleic acid (LNA). (a) Chemical structure of LNA. (b) Structures and conformations of DNA and LNA. DNA can adopt two sugar conformations, C2'-endo and C3'-endo. C2'-endo is the preferred sugar conformation of DNA, supporting a B-type double strand. C3'-endo is the preferred sugar conformation of RNA, promoting an A-type double strand. LNA is locked in a C3'-endo conformation.*

continued and has increased greatly the last couple years. Phosphorothioates are still common in clinical trials but are being replaced with newer nucleotide analogues (81). LNA is one of the most promising, recently developed nucleotide analogues and will be described in the following section.

Locked nucleic acid (LNA)

LOCKED NUCLEIC ACID (LNA) was first described in 1998 (82–85). LNA shows minimal toxicity, enhanced binding to a the complementary sequence and is more stable to nuclease degradation than the traditional analogue, phosphorothioate (86,87). LNA alone can not activate RNase H, but when in a mix-mer with DNA the RNase H activation can be modulated as well as binding affinity and stability (86–90). One LNA nucleotide can increase the melting temperature (T_m) of an oligonucleotide by up to 10°C depending on oligonucleotide length and its base composition (91). The excellent binding property of LNA comes from the molecules lock in C3'-*endo* conformation *(figure 15)*, resulting in an A-type duplex like natural RNA and an increase in backbone organization. The ribose in DNA can flip between C2'-*endo* and C3'-*endo* where the C2'-*endo* is the preferred low energy state. C2'-*endo* leads to a B-type double strand. RNA on the other hand has a preference for the C3'-*endo* conformation which leads to the tighter A-type double strand. The C3'-*endo* lock in LNA is hence ideal to fit the natural RNA conformation. The A-type conformation is associated with more stable hybridization properties (77,82,91). LNA has been used for example in antisense inhibiting HIV Tat/TAR transactivation and tumor growth (92–94). Recent reports provide knowledge about design of LNA containing oligonucleotides (90,95).

Delivery of nucleic acids

NUCLEIC ACID DELIVERY to cells is usually divided into two categories: viral and non-viral. Unformulated or naked nucleic acid may also be used. Viral vectors express RNA from their genome using the cells own machinery, therefore expressed antisense RNA or short hairpin RNA (shRNA), which will be processed in the cell to siRNA (96,97), can be used in combination (98–100). Viral vectors are usually efficient in delivering and expressing their message. They can also be used for long term expression of the gene-silencing molecules, but add a level of complexity and risk.

Non-viral formulations have reduced complexity and risk, but are not always as efficient in delivering the nucleic acid. However, non-viral formulations can be used in combination with antisense oligonucleotides as well as synthesized siRNA. In cell culture, unformulated antisense oligonucleotides and siRNA are mainly not taken up. The use of non-viral formulations, like liposomes, can greatly enhance the uptake, but further improvements are needed for *in vivo* applications.

Viral vectors

ANTISENSE RNA AS well as shRNA can be expressed from different kinds of vectors. Gene-silencing over a longer period of time would require repeated administration of antisense oligonucleotides or synthetic siRNA, while a viral vector could be delivered once and in some cases provide a stable, long-term expression of the therapeutic molecule. Viral vectors tend to be more efficient in delivering genetic material compared to the non-viral delivery systems. They also provide better possibilities for targeted delivery and expression in different cells and tissues. However, viral vectors are labour intensive to produce and also interact with the host immune system possibly leading to adverse effects (101). However, one interesting virus for vector use is the adeno-associated virus (AAV).

Adeno-associated virus (AAV)

ADENO-ASSOCIATED VIRUS (AAV) is a single stranded DNA virus that belongs to the family *Parvoviridae* and genus dependovirus (102–104). It was first discovered as a satellite virus to adenovirus (Ad). AAV has several desirable features that make it suitable as a vector in gene therapy and similar applications. AAV is non-pathogenic, evokes a limited immune response, and is naturally replication-deficient due to the requirements of a

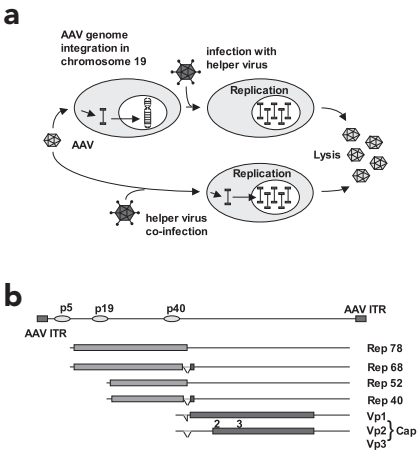


Figure 16. *Adeno-associated virus (AAV) life cycle and genome. (a) AAV infects cells and integrates in chromosome 19 without helper-virus or produces a lytic, reproductive, infection with helper virus. Integrated AAV is rescued by a subsequent helper-virus infection and lytic cycle begins. (b) The AAV genome and transcripts. The genome is 4,7 kb long and has two open reading frames from which seven proteins are expressed, four Rep proteins (Rep 78,68, 52, and 40) and three capsid proteins (Vp1-3).*

co-infecting helper virus (usually adenovirus) for lytic infection. The AAV genome incorporates site-specifically in chromosome 19 in the absence of helper virus. Without a helper infection, AAV remains silently incorporated in the host genome. A susequent helper virus infection can rescue the integrated AAV genome and promote replication *(figure 16 a)*. In addition AAV infects both dividing and non-dividing cells (105). AAV also infects a broad range of hosts and tissues, which is explained by the ubiquitously expressed receptors for AAV, heparan sulfate proteoglycan and co-receptors (106–108).

The AAV genome is a 4.7 kb long single stranded DNA and contains two open reading frames, rep and cap, and two inverted terminal repeats (ITRs) *(figure 16 b)*. The ITRs function as integration and packaging signals. Rep and cap define two open reading frames from which seven proteins are expressed (109). Three promoters control the expression; p5, p19, and p40. The p5 promoter controls the expression of Rep 78 and the splice version Rep 68. The Rep 78/68 proteins bind specific sites on the AAV genome, the promoters (p5, p19, p40) and the ITRs. Rep 78/68 have ATPase, helicase, and site-specific nickase activity when bound to ITR. Rep can rescue the integrated AAV genome and is needed for site-specific integration. The next promoter, p19, controls Rep 52 and its corresponding splice variant Rep 40. The Rep 52/40 proteins are thought to help in packaging of the genomic viral DNA into the virons. The last promoter, p40, directs expression of the capsid proteins (VP1, VP2, and VP3). VP1 and VP2/VP3 are translated from two differently spliced mRNAs. VP2 is initiated at an ACG codon whilst VP3 is initiated down stream at an AUG on the same mRNA. VP3 constitutes 80 % of the capsid proteins. It is likely that the low number of viral proteins is the reason why the host immune response is limited.

The functions not present in the AAV genome but required for AAV propagation are supplemented in *trans*, from infection with for example an adenovirus. Adenovirus genes that have been implicated in the process are; E1A, E1B, E4orf6, E2A, and VA I (110,111). E1A *trans*-activates the AAV promoters, E1B is thought to be involved in replication, E4orf6 deals with second strand synthesis and transcript transport from nucleus to cytoplasm, E2A stimulates transcription from the AAV promoters and is also involved in transport to the cytoplasm, and finally VA I facilitates the initiation of the AAV protein synthesis (111).

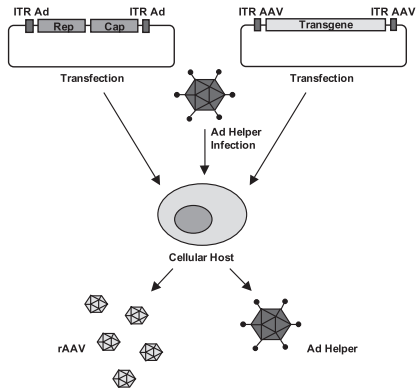


Figure 17. *Classical production protocol for recombinant AAV. Co-transfection of two plasmids containing helper functions and transgene, along with an adeno helper-virus infection.*

Cumbersome production procedure and low titers have earlier limited the use of recombinant AAV (rAAV). The classical method for producing rAAV is dependent on adenovirus infection and co-transfection of a two plasmids: one containing the transgene of interest flanked by the AAV packaging signals, ITRs, and the other the missing AAV functions, rep and cap on the other *(figure 17)*. Currently, the adenovirus infection step has been avoided by transferring the necessary andenovirus genes onto yet another plasmid. In this way adenovirus contamination of the final rAAV stock is avoided. This triple plasmid transfection protocol has become the current choice for production (111–113).

Non-viral vectors

UPTAKE OF OLIGONUCLEOTIDES in cell cultures normally has to be facilitated by transfection agents such as liposomes or polycations formulations. Without transfection agents the uptake in cell culture is poor or non-existent. Agents used for delivery of nucleic acids are for example polycations like polyethyleneimine along with liposomal vehicles. Delivery agents could also be used for targeting the oligonucleotides to specific cells and tissues (114–116).

Using commercially available transfection agents, we have observed that oligonucleotides are readily taken up by many adherent cell lines. In cell culture antisense oligonucleotides and siRNA show similar transfection

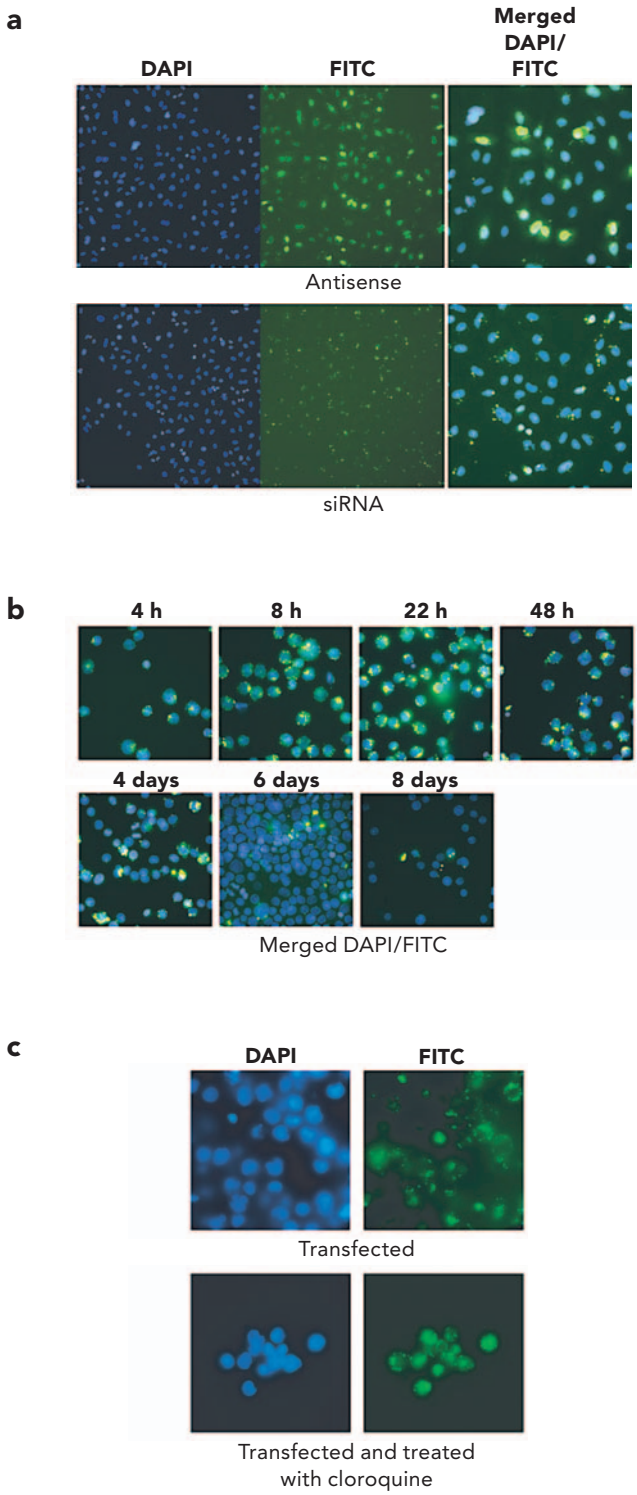


Figure 18. *Antisense oligonucleotide and siRNA transfections of adherent and suspension cell lines. (a) The human lung epithelial cell line A549 (adherent), show similar transfection efficiency of FITC labelled antisense oligonucleotides and siRNA (26 nM each, transfection agent Lipofectamine 2000, Invitrogen). The cellular distribution is different. Antisense oligonucleotide display nuclear localization whereas siRNA is localized in extranuclear compartments. (b) Uptake and duration of FITC-LNA oligonucleotides transfected with PEI-transferrin (Duofect, Qbiogene) into the human T-cell line Jurkat-Tat cells (suspension). Most cells contain oligonucleotides already after 4 hours. FITC intensity peaks at 22 hours. After 6 to 8 days only a few cells contain oligonucleotides. (c) Cells transfected (same as in (b), Jurkat-Tat cells, FITC-LNA oligonucleotide, PEI-transferrin) and treated with the endosomolytic agent chloroquine. The oligonucleotides show endosomal distribution without chloroquine, but there is a tendency for whole cell distribution with chloroquine.*

DAPI nuclear stain: blue (DAPI), FITC oligonucleotide label: green (FITC).

efficiencies. However, distribution in the cell can be different. Antisense oligonucleotides are strongly visibly associated with the nucleus, whilst siRNA can be found in a similar number of cells but in extranuclear compartments (dotty signals outside the nucleus, *figure 18 a*).

Suspension cell lines like Jurkat-Tat can be more difficult to transfect. Oligonucleotide delivery to T-cells is commonly achieved using electroporation, something that may not be feasible under physiological conditions in a therapeutic setting. An alternative can be to use receptor-mediated transfection, for instance transferrin bound to polyethyleneimine (PEI) (commercially available as Duofect, Quantum Appligene, IIkirch, France). T-cells express CD-71, which is the transferrin receptor and function as an iron transporter together with its ligand transferrin. The transferrin ligand can be bound to PEI, which have good DNA condensing and transfecting properties (117), and this complex has been shown to transfect the T-cell line Jurkat (118). The method relies on receptor-mediated endocytosis of the transferrin-PEI-nucleic acid complex. For example, Jurkat-Tat cells show good uptake of oligonucleotides by this method *(figure 18 b)*. However, oligonucleotides can be trapped in endosomal compartments. Here, endosomolytic agents such as chloroquine can enhance the release of oligonucleotides from the endosomes to the cytoplasm *(figure 18 c)*. Non-viral delivery has moved forward and there are many possibilities. However, *in vivo* result does not necessarily extrapolate from cell culture data.

Potential therapeutic applications; viral examples

VIRUSES ARE ATTRACTIVE targets for gene-silencing techniques, especially as efficient drug treatments are limited. Viruses hide in the host cells and use their cellular machinery to replicate. They transfer their genetic message and over-ride the natural message of the cell. Gene-silencing techniques can be used to specifically target the virus message. Several publications demonstrate that antisense and siRNA inhibit viruses (119–121). Two viruses having human impact are the human immunodeficiency virus (HIV) and severe acute respiratory syndrome-associated corona virus (SARS-CoV).

Human Immunodeficiency Virus (HIV)

HUMAN IMMUNODEFICIENCY VIRUS (HIV) has become an epidemic spread through-out the world. According to the UNAIDS report from December 2004, 40 million people are living with HIV and 3 million died of AIDS in

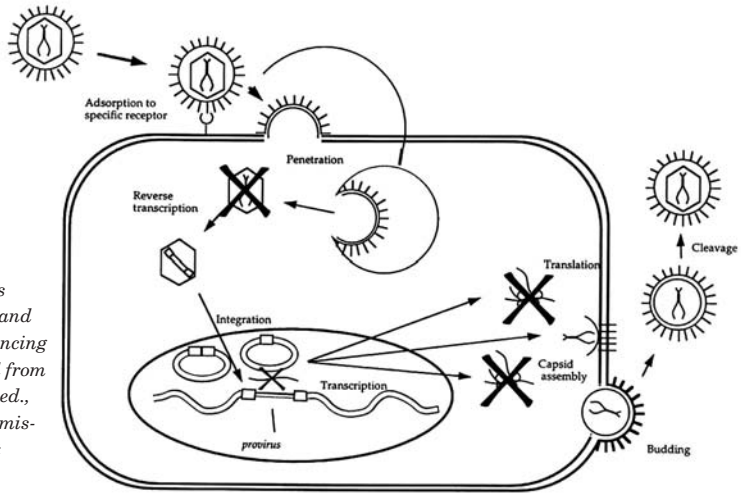


Figure 19. *HIV life cycle and possible RNA targets (both genomic RNA and mRNA) for gene-silencing techniques. Adapted from Fields Virology, 4th ed., reproduced with permission from Lippincott Williams & Wilkins.*

2004. The need for development of new drugs and vaccines is still high, due to virus resistance and side effects of current therapies. HIV is a difficult viral target due to its high variability (122). On the genomic level there are conserved regions that could serve as suitable targets for gene-silencing techniques. Moreover, gene-targeting tools could also rapidly be adapted to an altered target sequences.

HIV belongs to the family *Retroviridae* and genus lentivirus. HIV is an enveloped virus and has a genome of two positive single RNA strands in a dimer. The genome is 9.7 kb. The life cycle is shown in *figure 19* along with potential gene-silencing targets (102). HIV binds to the cell and transfers its genetic material. The RNA genome is reverse transcribed to DNA and integrated in the host genome. From the integrated copy, mRNA and new genomic RNA are expressed. Finally new particles are formed and released.

There are several possible points of action for gene-silencing techniques. Initially the incoming viral RNA genomes could be attacked for degradation and possibly the reverse transcription could be sterically hindered. Next step, expressed mRNA and genomic RNA could be targeted for degradation both in the nucleus and cytoplasm, as well as blocking the mRNA for translation. Finally, genome dimerization could be sterically hindered and thereby assembly of new viral particles inhibited *(figure 19)*. A cocktail of several antisense oligonucleotides and siRNA targeting different genomic regions but also different stages of the replication might function as a broad-spectrum HIV therapeutic with possible synergistic effects (123,124).

Severe acute respiratory syndrome-associated corona virus (SARS-CoV)

SEVERE ACUTE RESPIRATORY Syndrome (SARS) first appeared in the Guandong province in China in November 2002. The outbreak was contained in July 2003. Over 8,000 people were infected world-wide and almost 800 died (125). A previously unknown coronavirus was identified as the causative agent for the SARS epidemic (126–128). Identification of the SARS-CoV was followed by rapid sequencing of the viral genome from multiple isolates (129–131). The sequence information immediately made possible the development of potential SARS antivirals by nucleic acid based gene-silencing techniques such as siRNA (119,132–136).

SARS-CoV belongs to the family *Coronaviridae* but does not fit into any of the previously characterized groups. SARS-CoV is an enveloped virus with a 29 kb positive single stranded RNA genome. Upon infection the RNA genome is immediately translated and one of the first protein products is the RNA dependent RNA polymerase. As with HIV the RNA genome could be an immediate target both to cleave and to inhibit translation. Also the newly produced mRNAs can be targets. Because the virus is dependent on the RNA dependent RNA polymerase for production of new RNA genomes and mRNA, there is no DNA intermediate, the polymerase might be a suitable target. The polymerase region is also well conserved among corona viruses.

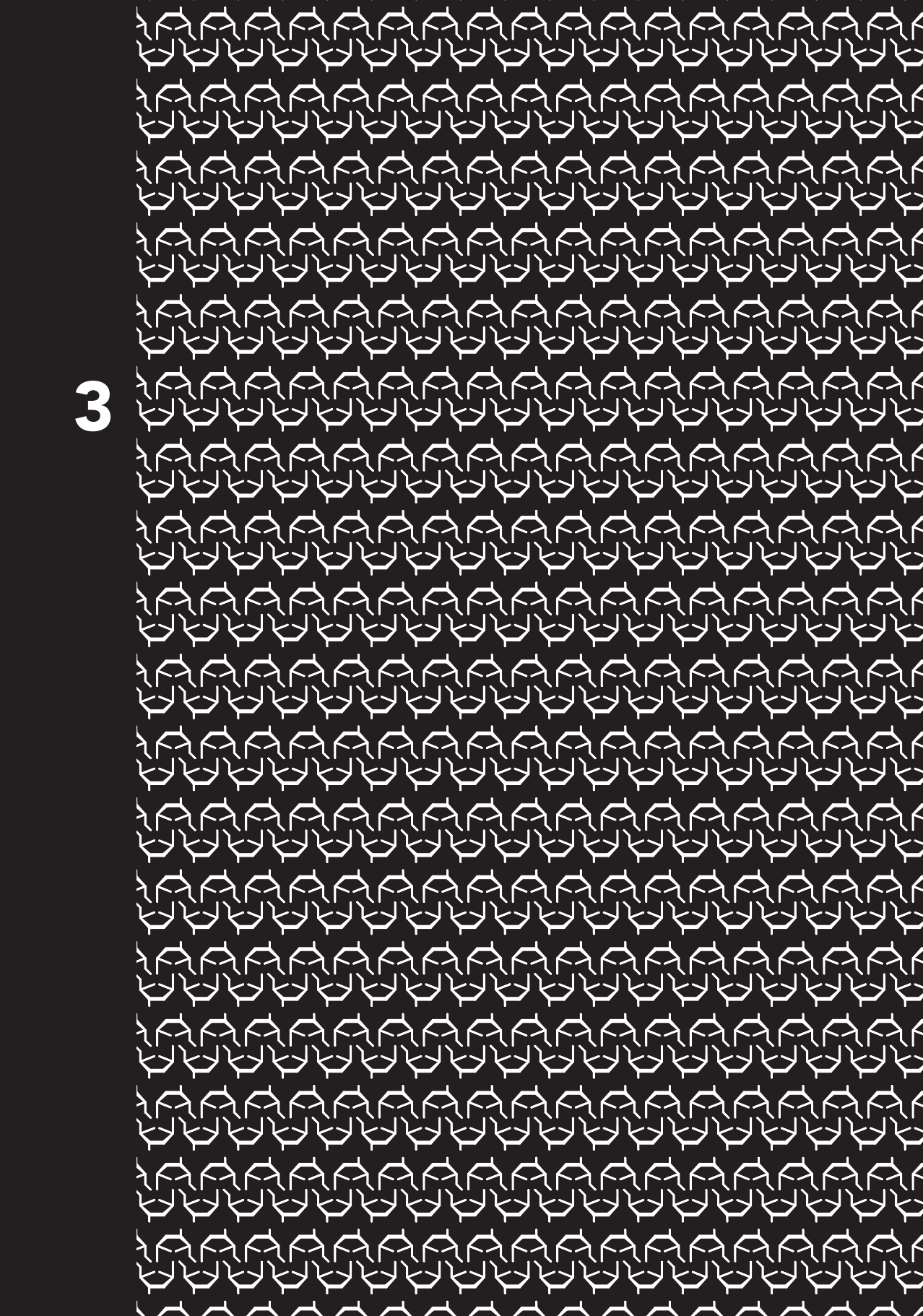
2

AIMS

THE AIM OF this thesis was to improve several technical aspects of the antisense and siRNA techniques to facilitate the use of gene-silencing techniques *in vivo*, including future therapeutic applications.

Specific aims in the different papers were:

I. To establish a reliable, efficient rAAV vector production method.
II. To further characterize the high affinity, nuclease resistant, synthetic nucleotide analogue LNA, and show its benefits in antisense inhibition on a therapeutic target like HIV.
III. To show that gene-silencing techniques such as siRNA can rapidly convert sequence information into possible virus fighting tool and inhibit an emerging virus such as SARS-CoV.
IV. To ask whether LNA components in siRNA can enhance siRNA properties.


3

RESULTS AND DISCUSSION

Paper I.

IN THE FIRST study, we investigated the possibility of using baculovirus as a tool to produce recombinant adeno-associated virus (rAAV). AAV has become increasingly popular as a vector for gene delivery, although its use is hampered by the lack of a simple and efficient vector production method. The baculovirus *Autographa californica* multiple nuclear polyhedrosis virus causes non-productive infection in mammalian cells. Therefore, recombinant baculoviruses have the capability to transfer and express heterologous genes in these cells if a mammalian promoter governs the gene of interest. We showed that co-infection of mammalian producer cells with three viruses – a baculovirus containing the reporter gene flanked by AAV ITRs, a baculovirus expressing the AAV *rep* gene and a helper adenovirus expressing the AAV *cap* gene – produced infectious replication competent rAAV particles. Initially, we verified that baculoviruses can infect the producer cell line (HEK293) and *trans*-complement functions that are necessary to produce rAAV. The newly formed rAAV particles could transduce cells and express the reporter gene, *lacZ*. This was not caused by vBac-LacZ contamination, which was clear from the fact that baculovirus were heat-sensitive and did not survive the heat treatment used to kill off the adenovirus helper in the crude rAAV preparation. We also observed synthesis of AAV genomic monomer and dimer form characteristic of AAV replication in the presence of the helper functions, confirming replication of rAAV.

The AAV vector and the Rep expression cassette were introduced into the producer cells by different baculoviruses, which enabled us to investigate the requirement of these components separately. Previous investigations

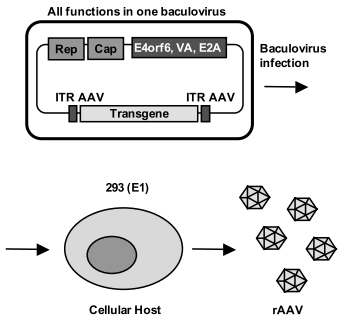


Figure 20. *Future recombinant AAV production-scenario. All needed functions inserted in one baculovirus, which eliminates the helper-virus and leaves one easy to use, scaleable, and reproducible infection step.*

have shown that unregulated overexpression of Rep inhibits rAAV production (137–139). Our results also clearly demonstrated that attenuated Rep synthesis significantly increased rAAV titers in our baculovirus-based production protocol. The best rAAV yields were achieved when Rep was expressed from the p5 promoter and when the original ATG start codon was mutated into an inefficient ACG codon. However, although this mutation resulted in higher rAAV yields compared to non-mutated *rep*, the increase was not as dramatic as described when the same mutation was used in the classical production protocol (137–139). The increase was not possible to achieve by simply lowering the multiplicity of infection of the Rep-expressing baculovirus.

The size of heterologous DNA that can be inserted into a baculovirus backbone is large (> 15 kb). It would therefore be possible to construct a baculovirus harbouring all helper functions necessary to produce rAAV, that is *rep*, *cap* and the adenovirus specific helper genes (vBac-all, including a transgene, *figure 20*). The adenovirus genes required for an AAV lytic life cycle have been determined. These are the early region 1 (E1) expressing several multifunctional proteins, the E2A transcription unit expressing the single-strand DNA binding protein (ssDBP), E4orf6 and VA1 (102). Since the producer cell line, HEK293 already expresses E1, the adenovirus genes that would have to be cloned into baculovirus can be limited to E4orf6, E2A and VA. With such a virus, rAAV would be produced simply by a one-step infection of the producer cells with vBac-all. Construction of a vBac-all virus will probably require a careful examination of the proper combination of promoters for all helper-genes to obtain the right conditions for optimal rAAV yield.

Compared to the classical production method, the need for adenovirus helper infection has been circumvented by the development of adenovirus mini-genomes expressing only the adenovirus genes needed for AAV production. Functional rAAV can be produced by expression of selected adenovirus genes only, in the absence of adenovirus helper infection (111,113). Xiao *et al.* described a system with adenovirus genes on one plasmid, *rep* and *cap* on

a second and the third containing the transgene (111). This triple-transfection procedure shows equal or higher virus titers compared to the one using an adenovirus helper infection.

The titers we obtained with the baculovirus-based production protocol were comparable or better than those obtained in small-scale preparations using the triple-transfection method in our hands. A further development of this technique may have an impact on future large-scale rAAV vector production. However the titers (or rAAV produced per cell) generated in this study were about two logs lower than what is standard today (112) and optimization is still crucial. The triple-transfection technique has become the standard method along with improved purification through chromatography. However, it is still not considered suitable for scale up and virus mediated transduction, as an alternative production method, is still of interest (140).

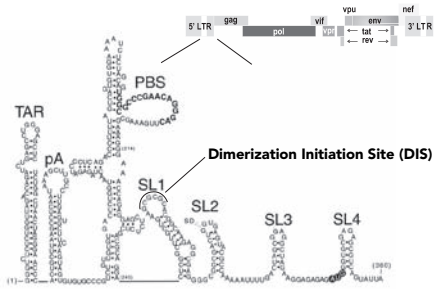


Figure 21. *The HIV genome and secondary structures in the long terminal repeat (LTR). The dimerization initiation site (DIS) in stem loop 1 (SL1) is marked. Adapted from; Joint United Nation Programme on HIV/AIDS (UNAIDS) and Fields Virology, 4th ed., reproduced with permission from Lippincott Williams & Wilkins.*

Paper II.

THE SECOND STUDY aimed to evaluate antisense design and enhance efficacy of LNA/DNA mix-mers and use them in a biological context. We also wanted to investigat the potential of the HIV-1 dimerization initiation site (DIS) *(figue 21)* as a suitable antisense target for the designed LNA/DNA mix-mers. Earlier studies have shown that RNA oligonucleotides are potent inhibitor of HIV-1 subtype A dimerization while DNA is not. Since LNA confer these properties to DNA and make an LNA/DNA mix-mer interfere equally well as RNA while being nuclease resistant. Our laboratory has previously showed that LNA containing oligonucleotides have increased the serum stability and has minimal toxicity *in vivo* (87). We showed here that addition of LNA bases to the oligonucleotide improve the interference of the HIV-1 genome dimerization. We also confirmed activation of RNase H by six consecutive DNA bases in a LNA/DNA mix-mer. Finally we inhibited the HIV-1 replication with the LNA/DNA mix-mers delivered through non-viral receptor mediated transfection of oligonucleotides to a T-cell line.

The results of the in vitro HIV genome dimerization confirmed the data

from Skripkin *et al.* (141), where a 9-mer RNA antisense oligonucleotide (asDIS 1, for oligonucleotide labels see table 1 paper II) targeting the DIS loop showed inhibition and the corresponding DNA oligonucleotide (asDIS 2) did not. The same inhibitory effect as with the RNA oligonucleotide was seen when LNA was introduced in the DNA oligonucleotide, three flanking DNA bases (one 5' and two 3') were exchanged (asDIS 3). Our aim to augment the effect of the DNA oligonucleotide with the enhanced binding capacity of LNA was indeed possible. Further increase of the LNA content did not increase the dimerization inhibitory effect (asDIS 4 and asDIS 5).

Both asDIS 2, with all nine bases DNA, and asDIS 3, with a 6 DNA nt window, activated RNase H. This is in-line with previous results (87,90). Kurreck *et al.* found that eight consecutive DNA bases in an LNA/DNA mixmer antisense oligonucleotide give full RNase H activation but six bases still significantly activated RNase H. Shen *et al.* (142) showed that as few as 4 consecutive DNA bases activate RNase H even with a mismatch. However, this was not in chimera with LNA.

The 9-mer oligonucleotides did not inhibit HIV-1 replication. This is most likely due to binding of the oligonucleotides to many other cellular mRNAs. 9-mers may not be useful *in vivo*, but could still be a tool to evaluate the RNA oligonucleotide interaction.

Continuing with the LNA/DNA 18-mers, asDIS 8 was effective at both inhibiting dimerization and activating RNase H. This oligonucleotide subsequently also inhibited the HIV-1 replication. Here, the oligonucleotide was longer and thereby gained affinity for the target. The uninterrupted DNA stretch was nine nucleotides long, which was sufficient for RNase H activation. The sense version sDIS 11 was a surprisingly good inhibitor of virus replication although it did not activate RNase H or showed strong inhibition of dimerization. The other two 18-mers, antisense asDIS 9 and sense sDIS 10 had little or no significant effect on virus production.

RNase H was activated by both antisense 18-mers but not by the corresponding sense oligonucleotides. However, some non-target specific degradation was seen. The 18-mer antisense oligonucleotides also showed good inhibition of *in vitro* dimerization whereas the sense oligonucleotides were less efficient. No correlation was found between the *in vitro* performance and inhibition of HIV replication, even though inhibition of dimerization seemed to be more important than RNase H activation.

The clinical isolate 2102 in this study replicated very slowly. It was dif-

ficult to amplify and gave low titers. The low titer may result in poor infection of the cell and be a reason for the high standard deviations in the RT levels. Nevertheless, we demonstrated inhibition of HIV-1 replication. LNA has been successfully used against HIV in inhibiting Tat dependent transactivation in reporter systems (92,93,143). However the current results represent the first study that shows inhibition of HIV-1 replication by LNA/DNA mix-mers targeting the dimerization initiation. In conclusion, LNA in antisense oligonucleotides enhance interaction with the target, activates RNase H in combination with DNA and can inhibit HIV replication.

Paper III.

DURING THE FIRST half of 2003 there was an outbreak of severe acute respiratory syndrome (SARS). This was a reminder of the deadly influenza pandemic (1918–1920), which killed at least 50 million (144). Luckily the SARS epidemic far from reach this number of deaths. It spread rapidly, over a few months, around the World, infected over 8,000 people and killed almost 800. At its peak in May 2003 the SARS causing virus (severe acute respiratory syndrome-associated coronavirus, SARS-CoV) had been identified and the first genetic sequence published. To demonstrate the potential of gene-silencing techniques we decided to employ siRNA in an attempt to rapidly convert the sequence information to a virus-combating tool. Two months later the first part of the study was completed showing the possibility to inhibit the virus. The outbreak was also almost over. This was by far not a therapeutic treatment, but was a demonstration how the sequence information, from a completely new virus, could be easily and rapidly converted into a virus inhibitory tool through siRNA.

In brief we showed that synthetic siRNA could specifically inhibit viral replication in cells infected with high doses of the SARS-CoV. We transfected Vero cells with synthetic siRNA oligonucleotides targeting the SARS-CoV RNA dependent RNA polymerase (Pol) before viral challenge. At 600 tissue culture infectious dose 50 % ($TCID_{50}$) of SARS-CoV infection, the most efficient siRNA reduced viral-induced cytotoxicity of Vero cells by up to 80 % compared to control treated cells. At 6,000 $TCID_{50}$, the cell death was reduced up to 70 %. Dose-response studies demonstrated that the protection by the siRNA was dose-dependent with an EC_{50} of 35 nM for the most efficient siRNA. We also showed that siRNA effectively prevented cell death in cells already infected with SARS-CoV.

We tested four siRNA sequences for the SARS-CoV RNA-dependent RNA polymerase (siSARS 1–4, see figure 1 paper III). siSARS 1 was the most prominent inhibitor of cell death at high viral challenge doses. siSARS 3 was an intermediate inhibitor of cytotoxicity, whereas siSARS 2 and siSARS 4 had no considerable effect. Only a few rules for siRNA design had been postulated at the time of the study, and the determinants for effective siRNA were not fully understood. The mechanisms underlying the differences in effectiveness and the siRNA-RISC association as well as target binding are under intensive studies. We can only speculate that the mRNA target sites for siSARS 2 and siSARS 4 are, for some reason, less accessible to RISC, perhaps due to formation of secondary structures or protein binding. Another explanation would be that the non-efficient siRNA are more prone to incorporate the sense strand into RISC. Incorporation of the sense strand would not guide RISC to the target mRNA, since it lacks complementarity. It has recently been shown that incorporation of the different siRNA strands into RISC is asymmetric, and that unwinding is preferably initiated at the weakest base-pairing 5'-end of the siRNA duplex (72,73). The strand containing the 5'-end from where the unwinding starts is subsequently incorporated into RISC. We therefore estimated the free energy values of the four 5' terminal base pairs using the nearest neighbor method as described in Khvorova *et al.* (72) at the different ends of the SARS-specific siRNA. The most efficient siRNA, siSARS 1, had the largest difference in binding energy between the two 5'-ends, with the weakest binding in the 5'-end of the antisense strand. Hence, the antisense strand would efficiently associate with RISC. siSARS 3 had a slightly smaller difference, still with the weakest binding in the 5'-end of the antisense strand. siSARS 2 and 4 showed virtually no difference in binding energy between the two ends. Less efficient incorporation of the active antisense strand into RISC could indeed explain the lower effect of siSARS 3 and absence of effect of siSARS 2 and 4. Once the mechanisms for RISC association have been fully understood, target sequence selection may become more accurate.

In the dose-response assay, increasing amounts of siRNA correlated with increased control of cytotoxicity. This is similar to what other investigators have reported for siRNA targeting influenza virus and hepatitis B virus genes (120,145). Our transfection efficiency data suggest that the reduced cytotoxicity at higher siRNA concentrations is not due to increased transfection efficiency. Within the effective range of siRNA, the transfection ef-

ficiency was equally high. At the lower end of the effective range, less siRNA was delivered to each cell and would account for the loss of effect. However, at concentrations below the effective range, we cannot exclude a decrease in transfection efficiency.

We also investigated the ability of the effective siRNA to inhibit the SARS-CoV induced cell death when administered after infection. Cells were transfected two hours after infection utilizing a high challenge dose to compensate for the short time between infection and subsequent transfection. Therapeutic siRNA treatment of cells after viral challenge was less effective compared to prophylactic treatment. This is in accordance with the finding by Ge *et al.* that influenza virus can be effectively inhibited pre and post infection of MDCK cells (145). Similarly, post challenge treatment was less effective than prophylactic treatment also in this report. There may be multiple reasons why post challenge treatment is less effective. For instance, it is possible that two hours of infection is sufficient for some viral replication. Thus, when siRNAs enter cells, the number of SARS-CoV transcripts may outnumber the siRNA-RISC complexes. The rate of mRNA degradation may no longer be sufficient to efficiently prevent viral replication, lysis and spread in all cells. Alternatively, expression of a putative SARS-CoV RNAi suppressor protein could interfere with the RNA silencing pathway. Indeed, various RNAi suppressor proteins have been described in plants (146). Moreover, the flock house virus (FHV) B2 protein has been shown to suppress the RNAi machinery in *Drosophila* host cells (147). Another possibility is that infected cells would be less prone to uptake of the siRNA-transfection agent complexes. The possibility to treat cells after infection gives hope that post-exposure treatment of infected patients would also be possible with siRNA.

SARS symptoms are initially related to virus replication and cytolysis, which can progress to the more severe condition, acute respiratory distress syndrome (148). The reduction of cytotoxicity reported here, might be extended to clinical relief of symptoms and disease progression. While these experiments were performed in cell culture, the lung is also accessible to antisense therapies. In fact, there is currently an antisense oligonucleotide compound targeting the asthmatic inflammatory pathway in clinical phase II trials (149). This study provides a potential new therapeutic approach to combat SARS and shows that siRNA can be an instrument to rapidly convert sequence information into infectious disease control.

Paper IV

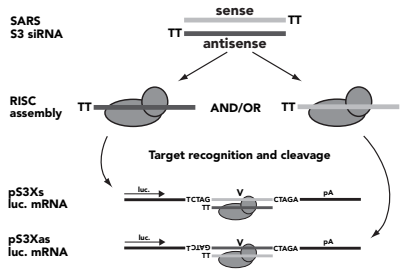
THE FOURTH AND final paper described the combination of LNA and siRNA, siLNA, and its application on SARS-CoV. For future therapeutic applications of siRNA, stability is one important issue, as it has been for antisense oligonucleotides. Nucleic acid analogues developed for antisense oligonucleotides could possibly also improve siRNA. Several of these analogues have been introduced into siRNA (150–154). Some more or less compatible with the gene-silencing. We aimed to investigate LNA modified siRNA, what we termed siLNA. We have systematically modified siRNA with the synthetic RNA-like high affinity nucleotide analogue LNA. LNA is known to enhance nuclease resistance and target binding in antisense oligonucleotides and has low toxicity.

We showed that incorporation of LNA substantially enhanced serum half-life of siRNA. Unmodified siRNA is degraded within six hours in both human and mouse serum. siRNA with LNA modified ends was still present after 48 hours. More extensively LNA modified siRNA was mostly intact even at 48 hours. We also provided evidence that LNA is compatible with the intracellular siRNA machinery. LNA was well tolerated at both the 3' and 5' end of the sense strand, as well as the 3' end of the antisense strand. Notably, LNA in the 5' end of the antisense strand reduced efficiency. Substantial internal modifications to the sense strand could also reduce the efficiency. However, this also contributed to the dramatic increase in nuclease resistance.

Single LNA exchanges along the siRNA antisense strand were tolerated in most positions, except in position 1, as stated above, and in positions 10, 12 and 14 from the antisense 5'-end, all of which reduced gene-scilencing efficiency. The reduced efficiency introduced by an LNA modification in 5'-end of the antisense strand could partly be rescued by a similar modification in the 5'-end of the sense strand. This could be explained by the RISC strand bias. RISC incorporates the strand that has the weakest energy in the 5' end and deselect the strand with high binding energy in the 5' end (72,73). LNA can enhance binding energy and this should influence strand selection. We aimed to improve the siRNAs we used to target SARS. Indeed the mediocre target SARS 3 (for labels see table 1 paper IV) became as efficient as the already efficent SARS 1 siRNA. SARS 2 and 4 did not benefit from the LNA treatement.

To investigate if this was due to different strands incorporated into RISC, we cloned both the sense and antisense SARS 3 target in the 3' UTR of

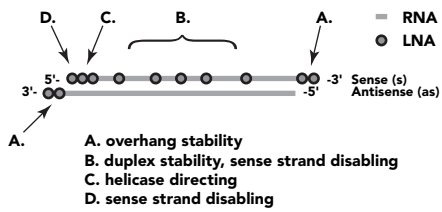
the luciferase gene. We then used the siRNA and siLNA and investigated the reporter expression. The siRNA and siLNA antisense strand targeted the sense clone and the siRNA and siLNA sense strand targeted the antisense clone *(figure 22)*. SARS 3 siLNA abolished the effect seen by the sense strand in the unmodified siRNA. LNA substitution could alter strand-bias by selectively increasing the affinity of the closing base-pair at the 5'-end of the siRNA sense strand.



Schematic of the two siRNA strands and their incorporation into RISC. Assessing sense or antisense strand activity of the siRNA by cloning the target in antisense or sense direction respectively. SARS 3 target cloned in sense (pS3Xs) or antisense (pS3Xas) direction behind firefly luciferase.

Figure 22.

We believe that off-target effects brought about by inappropriate loading of siRNA sense strands constitute a concern for the use of siRNAs as genomic tools and prospective drugs. This data provide evidence that LNA can be used to minimize such effects. In addition LNA may be incorporated into positions in the sense strand that, once loaded into the RISC, impair its ability to participate in target cleavage, like the activity-impairing positions (pos. 10,12 and 14). The ability to influence strand loading by LNA modification at the 5'-sense end also provides an opportunity to improve the potency of ineffective siRNAs by further enhancing antisense strand incorporation into RISC. Although our data demonstrate that such enhanced loading is not a general phenomenon, it provides an option to incorporate LNA in this way where the choice of target sequence is restrained.

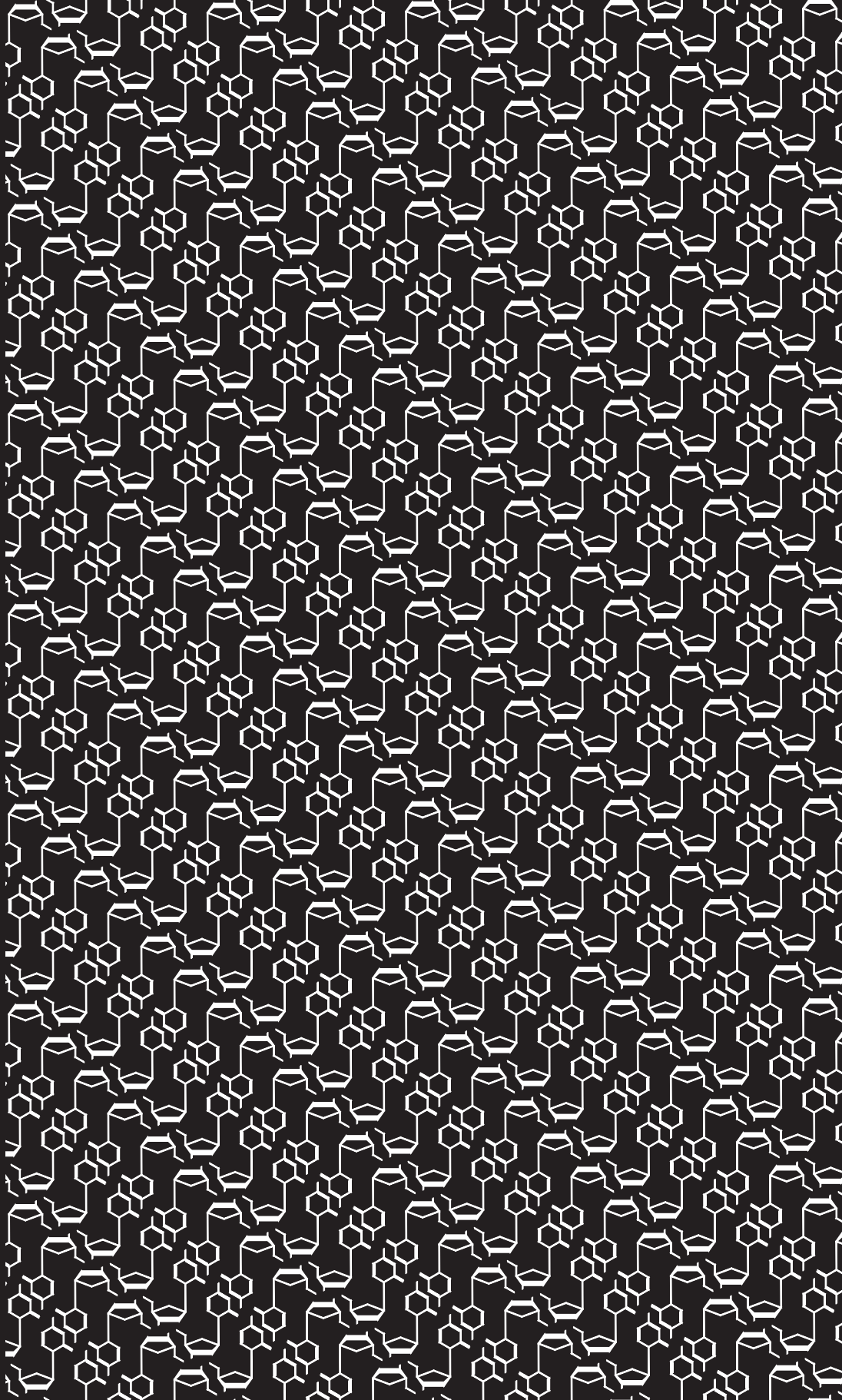


siLNA summary, LNA modifications of siRNA and its function depending on position.

Figure 23.

In conclusion, the RNA-like character of LNA combined with its increased nuclease resistance and affinity, enable us to construct hybrid RNA-LNA molecules with favourable properties over unmodified siRNA. We have summarized the LNA modifications, their position and added function to siRNA in *figure 23*. We anticipate that these new molecules will impact positively the use of RNAi technology in functional genomics and the broader perspective of translating the technology into a drug platform.

4



CONCLUDING REMARKS

THIS STUDY EXPLORED different technical approaches towards nucleic acid based therapeutics. In particular this thesis demonstrates that LNA can be beneficial to both antisense and siRNA methodology as well as its application on two relevant viral pathogens, HIV and SARS-CoV. In addition, another aspect of viruses was examined, how to facilitate the use of AAV as delivery vectors for nucleic acid based therapeutics. Clinical applications of the work presented would be most exiting, but would require extensive efforts. Only at the last step would we know if the approaches described in this thesis would have a clinical impact. This work was performed strictly *in vitro* and should be treated as such.

Specific conclusions from the papers are:

I. Baculoviruses can be used as a tool in the production of rAAV.

II. LNA/DNA mix-mer antisense oligonucleotides targeting the HIV-1 dimerization initiation site can inhibit the dimerization process and virus replication.

III. siRNA can inhibit the human pathogenic virus SARS-CoV.

IV. LNA can be used to modify siRNA and will bring along favourable properties, like nuclease resistance, possibility to improve certain targets and inactivate the non-targeting sense strand.

Antisense oligonucleotides have become a major tool for functional genomics. Antisense is also the only gene-silencing technique currently with a product on the market in clinical use (Vitravene, Isis Pharmaceuticals). There is more to be expect since there are several lead products in clinical

trials. However, there have been some disappointments and no other antisense product has yet reached the market (34,81,155). Difficulties in applying antisense oligonucleotides could partly be explained by the phosphorothioate chemistry and non-specific binding to proteins. However, uptake *in vivo* is facilitated by the phosphorothioate chemistry (156). Also there are side-effects due to the presence of hypomethylated CpG motifs, which are immunostimulatory (157). Specificity is another issue; will targets with similar gene sequences also be down-regulated? New nucleic acid analogues might circumvent some of the obstacles. It has been reported that LNA, has a low toxicity compared to phosphorothioates (87). In addition they seem to be less immunostimulatory through CpG motifs (158). The specificity problem remains, but can also be utilized in reducing several similar targets at the same time (159). The use of high affinity nucleic acid analogues might also reduce side effects by being effective at lower concentrations.

Short interfering RNA has despite its few years as a gene-silencing technique already resulted in a first clinical trial (Sirna therapeutics, www.sirna.com). A lot of the knowledge from the antisense field can be applied to siRNA development as and this surely has benefited siRNA development. The stability issue in antisense has lead to several nuclease resistant nucleotide analogues, which now can be applied to siRNA. However, a new technique also has new potential side effects to be considered. Off-target effects are being discussed. Similar immuno-stimulatory pathways as those induced by CpG motifs in antisense oligonucleotides have been noted (57,160-162). Other side effects are related to specificity, irrelevant cleavage and translational block of similar target sequences (163–165). Finally, there is a risk/possibility that siRNA can methylate DNA (166,167). When it comes to siRNA there is limited *in vivo* effects and delivery vehicles might be a necessity for clinical application. A part from viral mediated delivery of siRNA expressed as shRNA (98-100), conjugating the synthetic siRNA to molecules that facilitate uptake and enhance *in vivo* properties could be a solution (168). Local delivery to the eye is the choice of administration for first siRNA clinical trial and also a recent publication shows a possible therapeutic application via gel-based delivery (169). Local application of nucleic acid based therapeutics might be beneficial for the whole field. Small successes relying on local application might build trust in the techniques as well as slowly teaching us the dangers on a small scale.

For the future, in my opinion, the most interesting is chemically modified

siRNA for moving siRNA technology towards the clinic. This in combination with a potential therapeutic target like SARS-CoV or similar diseases infecting through the respiratory tract and aerosol delivery of siRNA is an appealing therapeutic scenario. Hopefully, local administration to the lung of siRNA could reduce the severity of such of diseases. Despite unclear side effects and delivery problems the potential of nucleic acid based therapy remains exciting. Several technical advances have been made. But also, careful examination of the biology and knowledge of the pathways involved are necessary for successful applications.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and Ana it is quite some time since we first got together. Even though things change: Gitt och Elisabeth, vi har varit med här ett tag tillsammans, hur ska jag klara ett liv utan er?

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Anna (and sorry Martin I left you out, I have tried to limit this to work people, but I dream of dirt), Shane, Emily, Marcela, Alistair (I'm picturing you and me at a crayfish party many years ago, maybe the first, I know I'm less party now a days but I hope I'm excused), Natalya, Agne thanks for all the good times. The old pub crew is also in my memory. CMB, Ludwig, MTC, SMI, I've met so many nice people all over the place even though you're not in print you are in my mind, thank you.

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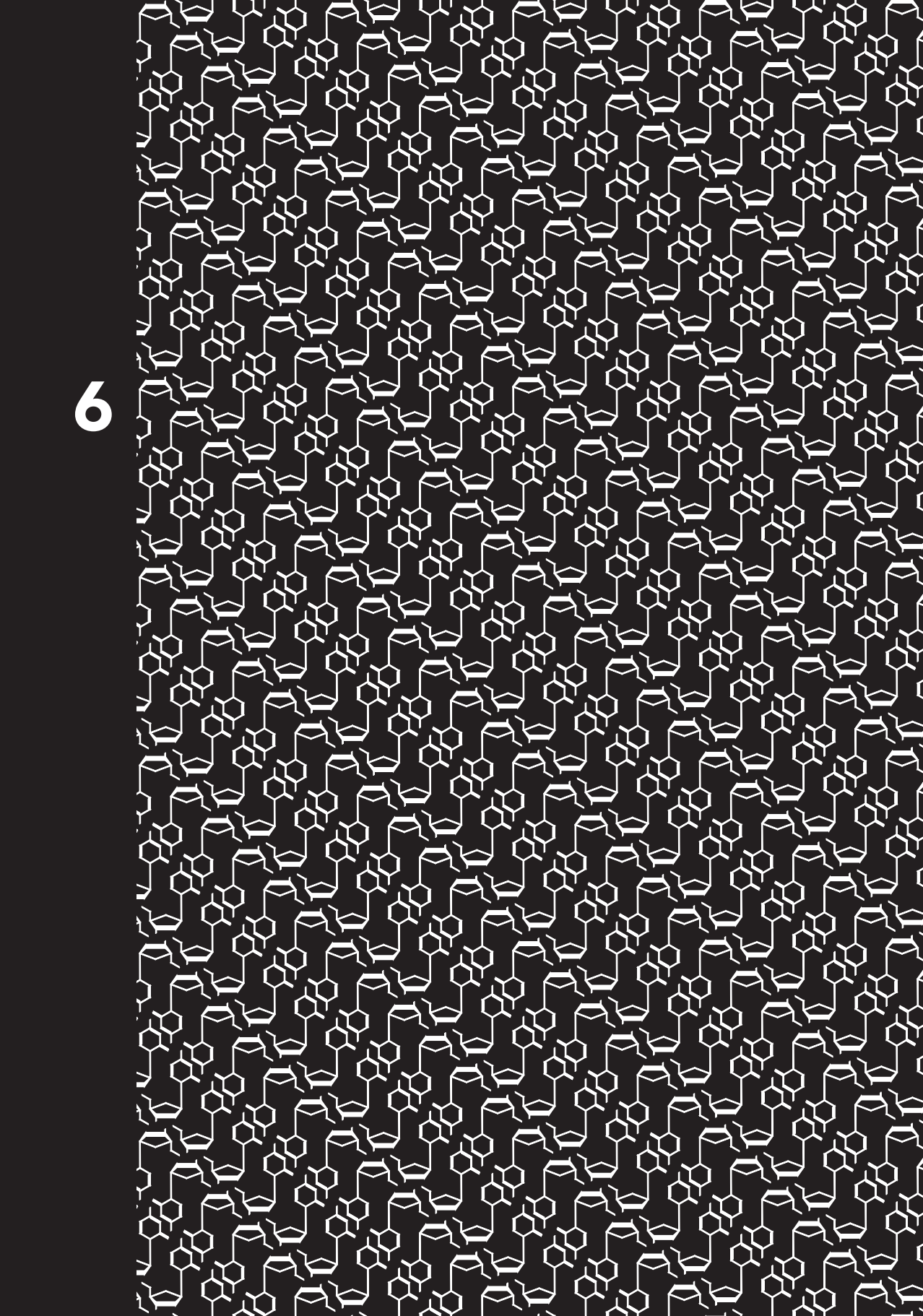
Tack till mamma, pappa och syrran (inte bara syrra utan även världen bästa rumskompis).

MY LITTLE ALBIGER! I am dreaming of France, dream with me!



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