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Zusammenfassung

Diese Arbeit beschäftigt sich mit der Evaluierung von chemisch modifizierten splice-switching Oligonukleotiden in einem in vitro Luciferase Assay.

Wenn der äußerst komplexe Vorgang des Spleißens fehlerhaft durchgeführt wird, ist das oft der Auslöser für genetische Erkrankungen wie etwa die Duchenne Muskeldystrophie und die Mittelmeeranämie. Splice-switching Oligonukleotide können defekte Spleißvorgänge beeinflussen und bieten daher eine praktikable Therapiemöglichkeit. Um die Effizienz der Oligonukleotidtherapie zu steigern, muss allerdings die Aufnahme in die Zellen verbessert werden, bestenfalls ohne die Verwendung der toxischen, kationischen Transfektionsreagenzien.

Die Oligonukleotide, die in dieser Arbeit verwendet wurden, sind chemisch modifiziert und tragen meist in 2'-Position einen Thiol- oder Aminohexyllinker. Die Linker wurden in weiterer Folge zur Konjugation mit PEG-Ketten oder Oligolysin-Peptiden verwendet, dadurch soll den Oligonukleotiden die Fähigkeit verliehen werden aus dem Endosom und in den Zellkern zu gelangen.

Im ersten Schritt wurde der Reporterassay in Bezug auf Zellzahl, Inkubationszeit sowie Auswahl und Konzentration der Vergleichssubstanzen optimiert. Die Testung auf spleißmodulierende Aktivität und zeitgleich auf Zellaufnahme ohne Transfektionsreagenz wurde mittels HeLa-Zellen ermittelt, die stabil mit dem Plasmid 705 transfiziert waren. Dieses Zellkulturmodell exprimiert ein inaktives Luciferaseenzym, welches erst durch Spleiß-Modulation aktiviert wird.

Die Testsubstanzen wurden jeweils in Triplets gemessen, gegen die unbehandelten Zellen standardisiert und über ein Bradford Assay normalisiert.

Die Ergebnisse zeigten Hochregulation der Luciferase in Kombination mit Transfektionsreagenz bei acht von acht Peptid-konjugierten Substanzen und vier von sieben PEG-konjugierten. Die Zellaufnahme (ohne Transfektionsreagenz) war bei drei Substanzen erfolgreich, diese waren alle mit dem Oligolysin C5K konjugiert. Die Zytotoxizitätstests zeigten keinen toxischen Effekt der Peptid-gekoppelten Substanzen.

Die Messung der Partikelgrößenverteilung zeigte eine Komplexbildung von Lipofectamin mit den Peptid-gekoppelten Testsubstanzen ähnlich der mit SSO.

Die CD-Spektren zeigen die Ausbildung von Duplexen, wobei die Peptide die Sekundärstruktur mehr verändern als die PEG-Ketten.

Zusammenfassend wurde die Zellaufnahme und die daraus folgende Hochregulation des Luciferasegens für die Substanzen bewiesen, die mit C5K konjugiert waren.

Abstract

This thesis is an evaluation of chemically modified splice-switching oligonucleotides in an in vitro luciferase assay.

If the highly complex process of splicing is defective, it is often the trigger of genetic diseases like the Duchenne muscular dystrophy (DMD) or β -Thalassaemia. Splice-switching oligonucleotides have the ability of altering defect splicing and are therefore a viable strategy in curing those diseases. To enhance the effectiveness of oligonucleotide therapy, it is necessary to improve cellular uptake of oligonucleotides without the use of cationic lipid preparations, which are often toxic.

The oligonucleotides in this thesis were synthesized with chemically modified nucleotides which carry a linker, like thiol- or aminohexyllinker mostly in 2'-position. These linkers were then used for the conjugation with PEG₈-chains or oligolysine-peptides to enable the oligonucleotides to escape endosomal trapping and cross into the nucleus.

In the first step the dual-luciferase reporter assay was optimized for the use with oligonucleotides concerning cell-count, incubation time and concentration and selection of control substances. The evaluation of splice-switching activity and simultaneously the evaluation of cellular uptake were done using HeLa cells, stably transfected with the pLuc705 plasmid. This reporter assay cell culture model produced an inactive Luciferase enzyme, which could only be activated by splice-switching oligonucleotides.

The substances were all measured in triplets, with and without the use of lipofectamine, data was then standardized over untreated cells and normalized using Bradford assay.

The results showed up-regulation of luciferase in combination with lipofectamine in eight out of eight oligolysine conjugated oligonucleotides but only in four out of seven with PEG-conjugation. Cellular uptake (without the use of lipofectamine) was observed in three substances: All of them were conjugated with the oligolysine-peptide C5K. During the cell viability assay, no toxic effect induced by the peptide-conjugated substances was detected.

The size distribution measurement using light scattering proved the formation of lipoplexes with peptide-coupled test substances and lipofectamine similar to those with the unmodified SSO.

The measurement of the CD-spectra proved the formation of duplexes. The secondary structure is influenced more heavily by the peptides than by the PEG-chains.

In total, cellular uptake and consequential up regulation of luciferase genes was proved for the substances conjugated with the oligolysine-peptide C5K.

“Because up to 50% of human disease-causing mutations affect splicing, the SSO approach is emerging as a promising alternative to gene therapy” (1)

1. Introduction

Since it was first discovered that RNA is more than just an intermediate between DNA and proteins, the process of transcription and splicing has become an interesting target. When it was discovered that numerous human diseases are based on mutations causing aberrant splicing, the interest rose even higher. (2, 3)

1.1. The basic principles of gene expression:

1.1.1. DNA transcription

Although all cells in the human body carry identical DNA, they are able to differentiate in multiple ways to create the wide variety that is the basis of our complex organism. The key to this diversity lies in gene expression and the ability to read the information in different ways.

Transcription of RNA is the first step of gene expression, it takes place in the nucleus (of eukaryotic cells) and produces the primary transcript: a complementary, antiparallel RNA strand.

Transcription ends in three main products: messengerRNA (mRNA), ribosomalRNA (rRNA) and transferRNA (tRNA) accompanied by other types such as snRNAs (small nuclear RNA), which is important for the splicing-process, and other RNAs with catalytic functions. RNA similar to DNA, is composed of adenosine, guanosine, cytidine and uridine as a replacement for thymidine.

Transcription starts at the Promotor TATA-box, where the RNA-Polymerase produces the primary transcript antiparallel to the desired DNA template. (4)

1.1.2. Splicing

The primary transcript called pre-mRNA is further processed by splicing. Most primary transcripts are four to six times longer than the translated protein, as they consist of introns and exons. Introns are the sections that are removed from the sequence before translation, exons make up the generated mRNA strand which will be translated into protein.

Splicing is executed by the spliceosome, a protein complex built out of snRNP (small nuclear ribonucleoprotein) units.

The spliceosome detects the splice sites of introns due to their specific consensus sequences, the GU nucleotide sequence in 5' and its AG at the 3' end splice site. The branchpoint sequence (BPS) consists mostly of polypyrimidines and a conserved adenosine.

In the first step of splicing the 2'OH of the branchpoint adenosine performs a nucleophilic attack

on the guanosine of the GU nucleotide sequence that marks the 5' splice site of the intron, this creates a cyclic lariat intermediate. In the second step the free 3'OH of the first exon performs a nucleophilic attack on the guanosine of the AG splice site of the intron, fusing the two exons and releasing the cyclic intron, which will be degraded quickly by nucleases. The processed mRNA strand can now be transported to the cytoplasm, where the translation of the mRNAs to proteins takes place. (4)

1.1.3.Alternative splicing

The human proteome is considerably more diverse than the human genome. To increase the variability of gene expression, splicing mechanisms can be altered: This process is called “alternative splicing”. Alternative splicing is extremely complex and still not comprehended in its entirety, an effort only higher eukaryotes make.

The basic concept of splicing is that various protein isoforms can be developed from one single pre-mRNA by discharging exons or including introns, sometimes even only partially. Since some exons code for a specific function in the protein, the functionality of proteins can be switched on and off by alternative splicing. Furthermore the functionality of proteins can be altered and even turned into opposites. (1, 4)

Alternative splicing can be achieved by: (Figure 1)

1. exon skipping
2. use of an alternative donor site
3. use of an alternative acceptor site
4. intron retention
5. use of an alternative exon

A major aspect in splicing regulation is the contest for splicing factors between splicing elements and the spliceosome, which leads to a selection of splicing sites. The spliceosome consist of different proteins and RNA joined together right before the splicing process. (2, 5)

The significance of alternative splicing is self-evident considering that evidence of it can be found in 35% of genes. (6)

Apart from enabling the diversity, alternative splicing also controls, the down regulation of gene expression by insertion of premature stop codons and consequent nonsense mediated decay of the transcript, if necessary. (1)

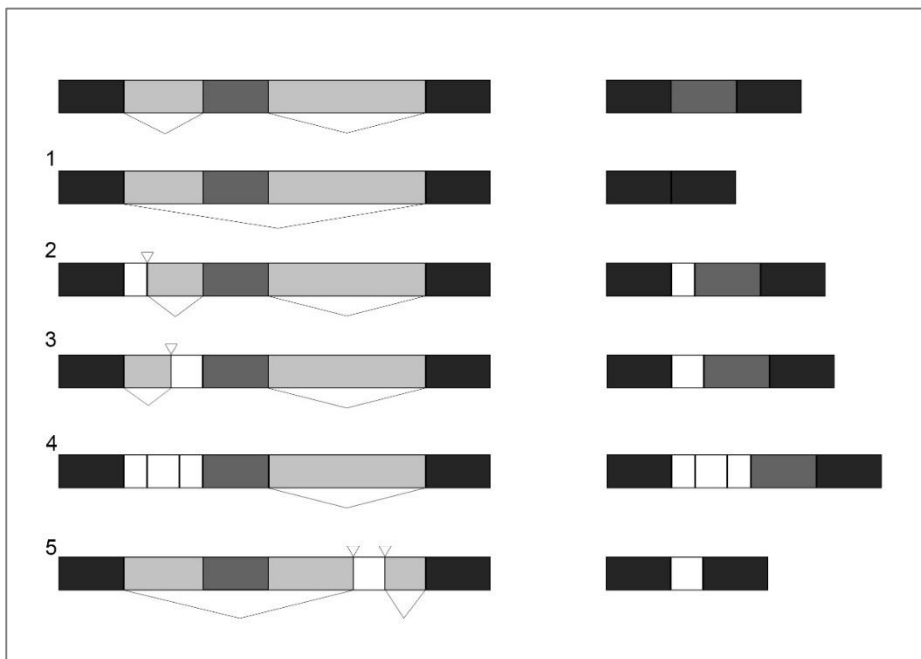


Figure 1 alternative splicing 1 - exon skipping, 2- use of an alternative donor site, 3- use of an alternative acceptor sit, 4 - intron retention, 5 - use of an alternative exon

1.1.4. Defect splicing

The process of splicing is highly complex and very susceptible to faults. One single remaining nucleotide can lead to a shift in the reading frame and following to defect RNA, which in most cases is immediately degraded by nonsense-mediated mRNA decay (NMD). Missing exons lead to dysfunctional proteins in the majority of cases. (4) Mutations in natural splice sites can lead to the activation of cryptic splice sites by the competing splice factors and skipping of the bordered exon. On the other hand, experiments showed that mutations can increase the affinity of a splice site or a branch point to their consensus sequence and lead to the inclusion of formerly ignored exons. (2)

1.1.4.1. Diseases caused by aberrant splicing

Although splicing grants access to a large amount of indispensable proteins, aberrant splicing can lead to serious diseases and malfunctions. It is estimated that up to 50% of all mutations leading to genetic diseases may be correlated with defect pre-mRNA splicing. (5, 7)

Thalassemia, also known as Mediterranean anemia, is one of the most profoundly investigated diseases caused by aberrant splicing. The dysfunctioning of the gene coding for the β -globin-protein is caused by over 100 mutations. Typical for this kind of mutation is that splice sites are

removed or cryptic splice sites are activated without shifting the reading frame. The mRNA can be translated into a protein but due to the intron included by the activated aberrant splice site a premature stop codon leads to the production of a truncated, non-functional β -globin. The consequence is a reduced synthesis of haemoglobin and therefore improper oxygen supply. (1, 4)

The Duchenne muscular dystrophy (DMD) is a progressive, severely disabling genetic disease of the skeletal muscle caused by genetic mutations, which result in premature termination of translation and hence in depletion of functional dystrophin protein. Due to the x-chromosomal heredity transmission, it affects one in 3500 new-born boys, which suffer from loss of muscle strength. The rapid degeneration of the skeletal and cardiac muscles often cause death by respiratory or cardiac failure before the age of 30. (1, 8, 9, 10) The dystrophin protein in the muscle connects the cytoskeleton with the sarcolemma, a loss of function often leads to inflammation and muscle degeneration. (11)

Furthermore, splice-modulation induced by oligonucleotides could lead to viable strategy in the treatment of primary immunodeficiency diseases (PIDs). This group of genetic diseases affects the development of the immune system and is often caused by mutations. (7)

1.2. Targets and structures

Since RNA and DNA have evolved as major targets for curing diseases, a large count of therapeutic agents have been developed. With their ability to recruit mRNA or pre-mRNA via Watson-Crick base pairing, oligonucleotides offer high selectivity and precision in the manipulation of gene expression. (11)

The classic **antisense oligonucleotides** have a relatively short (≥ 16 bases) sequence. After introduction into cells they are able to enter the nucleus and hybridize with their complementary pre-mRNA. (5) This leads to the recruiting of RNase H which is able to cleave the RNA-complex, other enzymes then continue the degradation. (11) RNase H activation is dependent on the chemical structure of the antisense oligonucleotide, and a stretch of at least seven 2'-desoxy nucleotides is necessary for mRNA degradation.

With their ability to restore the expression of genes inactivated by numerous mutations, while simultaneously being able to down regulate gene expression, antisense oligonucleotides have a wide field of application. (2)

Furthermore, they have emerged as important sequence specific research tools in various fields,

since they are able to address issues concerning the basic rules of pre-mRNA splicing and splice site selection. (2)

Another mechanism for post-transcriptional gene silencing is RNA interference (RNAi), which can be induced by small interfering RNAs (**siRNAs**) and micro RNAs (**miRNAs**). RNAi can be used as therapeutic target for diseases like cancer and HIV. (12) Gene silencing is a natural process used by all eukaryotic cells, in which siRNA targets mRNA to cleave a specific sequence. For therapeutic use the siRNA can be introduced into the cells, where it is incorporated by the RISC (the RNA-induced silencing complex) to destroy specific mRNA strands. MicroRNA on the other hand is a single stranded RNA molecule, which is catalytic and used for regulating gene expression. (9)

Splice switching antisense oligonucleotides (**SSOs**) have the ability to affect pre-mRNA splicing by complimentary binding to the transcript at the splice junction. The access for the spliceosome and other splicing factors is now blocked. This, however, does not result in a termination, but the splicing machinery will be redirected to another splice site, where it may include or exclude particular exons, or include parts of introns. (5, 11) The SSO approach, first introduced in 1993, is a promising path to cure diseases like the Duchenne muscular dystrophy (DMD). Apart from their activity in in-vitro assays, where they repaired aberrant splicing or activated new splice sites, SSOs also showed potential in animal disease models and in various clinical trials: two distinct oligonucleotide agents showed promising outcomes.

Their ability to put new splice variants to account or favour one variant over the other is equally valuable. (1)

1.3. Obstacles

For the effective therapy of gene expression the drugs need to enter the cells or tissues through the hydrophobic plasma membrane. (7)

The first clinical trials using oligonucleotides had disappointing results, this was due to the fact that oligonucleotides have many properties with bad influence on their pharmacokinetic and pharmacodynamics. (9) The common oligonucleotides have a relatively high molecular weight and are mostly negative charged molecules, therefore crossing cellular membranes is not easily achieved. (11)

Furthermore, RNA is not able to reach the target cells on its own and needs an efficient delivery system. (1)

Although in theory oligonucleotides should work very specific by binding only complementary strands, there are several routes for off-target effects. Incomplete binding to similar sequences can

induce downstream effects. Moreover oligonucleotides depending of their sequence and chemical structure often activate the expression of cytokines by interaction with Toll-like Receptors and other factors of the immune system. (8) Concerning the use of splice switching oligonucleotides the primary problem is the RNase H, which degrades loose RNA efficiently. (1, 10)

1.4. Chemical modifications

Cells have a vigilant defence system against unfamiliar or extrinsic DNA and RNA, this complicates the in-vivo use of RNA. In serum and in cells ribonucleases work efficiently to annihilate the intruders. To counter this defence system a wide variety of chemical modifications has been developed. The chemical modifications can affect all units of the nucleotides: backbone, sugar, base and terminal modifications. (12)

These modifications not only induce a longer in-vivo half-life by lowering vulnerability to degradation, but can also influence the pharmacokinetic and pharmacodynamics parameters, and immunogenicity, and can increase or decrease target affinity and even specificity . (3, 12, 13)

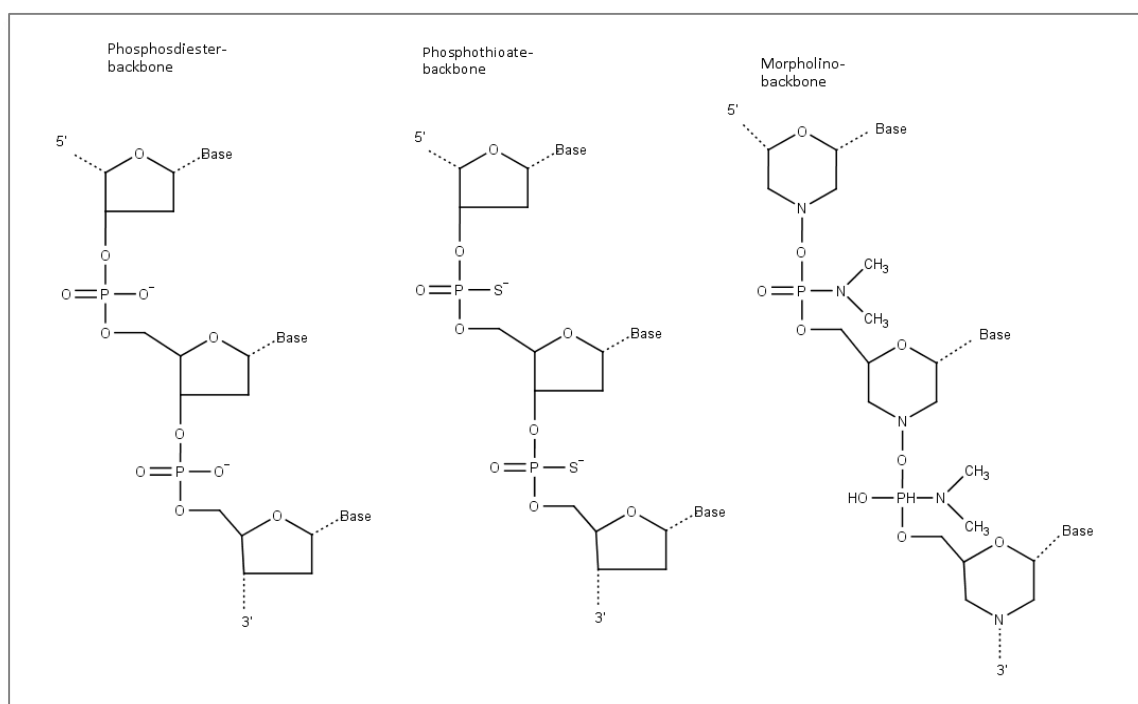


Figure 2 backbone modifications

1.4.1.Backbone modifications

Some of the first chemical modifications for oligonucleotides were modifications of the backbone, where a nonbridging oxygen atom is replaced by a methyl group forming a **methylphosphonate**. The loss of the oxygen of course affects the solubility, since the molecule loses its charge. Furthermore, this can affect cellular uptake and the RNA-DNA interaction, methylphosphonate oligonucleotides therefore do not activate RNase H. Another backbone modification is represented by **phosphorothioates (PS)**, the most profoundly researched chemical modification. (10) Being one of the earliest ON modifications, PS backbone modifications are still not outdated but are frequently used because of their striking advantages. In this modification the non-bridging oxygen of the phosphate-group is exchanged with a sulphur atom, which makes it cheap and easy to use. Furthermore, it increases the resistance against nucleases, is able to trigger RNase H-mediated cleavage for the use as antisense drugs and can improve the affinity to plasma proteins, which lessens the renal clearance. (3) One potential disadvantage remains: the PS oligonucleotides often induce sequence independent off-target effects. These highly specific drugs can lead to highly specific side effects. (14) They also tend to be “sticky” which can lead to non-specific protein binding. (11) Especially heparin-binding growth factors like platelet-derived growth factor, laminin, fibronectin and many more are affected. (10)

There are also chemical modifications that go as far as replacing the phosphate-ribose backbone. The **PNAs** (peptide nucleic acids) are based on nucleic acid analogues with a polyamide backbone, which results in an uncharged and flexible molecule. These oligonucleotides are suitable to form DNA or RNA duplexes or triplexes.

The fourth approach described here are the **phosphorodiamidate morpholinos**, where the ribose is replaced by a morpholino ring and the phosphodiester bond is replaced by an uncharged phosphoroamidate linkage. However these uncharged molecules do not form complexes with the most common, cationic delivery reagents. (10)

1.4.2.Sugar modifications

Ribose, the sugar in the nucleotide, can be altered at the 2'-position. The hydrogen can be replaced, in case of **2'-OMe** it is replaced by a methylene group. Oligonucleotides with this modification form heteroduplexes with high melting points. (10)

Other sugar modifications are **2'-fluoro**, where the hydroxygroup is replaced by a fluoride, and **2'-MOE**, where the hydrogen is replaced by a methoxyethylene group. Both modifications form thermally stable duplexes. (12)

A very special modification is the synthesis with locked nucleic acids short **LNA**. In this modification

the 2' and the 4' position of the ribose ring are linked using a methylene group, forming a furanose ring “locked” in the 3'-endo conformation, which makes it conformationally analogous to the natural RNA-monomer. This modification produces very rigid oligonucleotides, which result in stronger hybridization with the target strand. (12)

Another very interesting approach is the substitution of the oxygen atom of the ribose ring with a sulphur atom, which can lead to nuclease stability and improved target binding. (12)

1.4.3. Other modifications

Of course the list of possible modifications is sheer endless. A great summary of the base modifications can be found in: (12)

1.5. Cellular uptake and delivery systems

On basis of the chemical and physical properties of oligonucleotides it is clear, that they cannot permeate through biological membranes, therefore their cellular uptake depends on endocytosis. There are different mechanisms for leaving the endosome, but most of the oligonucleotide that accumulates in cells stays trapped in endomembrane compartments. Only a small amount can “leak” into the cytosol and from there diffuse into the nucleus. (11)

There are different ways to get the oligonucleotides into the nucleus.

For instance, the target organ can play a big role, since topical delivery is easier in the eye, lung, skin, mucus membranes and local tumors. (9)

For the use in cell culture cellular uptake for oligonucleotides with a negative charge is often enhanced by cationic lipid preparations (Lipofectamine and others), which remains to be the most reliable strategy in-vitro. The in-vivo use however is limited to local administration. (5)

SSO delivery can be significantly enhanced by the use of cell-penetrating peptides, with lower risk of increasing toxicity than with lipid preparations. (1) These peptides can be designed to specifically bind to receptors, or to provoke escape from intracellular vesicular compartments. (11)

Other interesting approaches are being investigated such as microinjection, electroporation, liposomes and viral vectors. (7)

1.6. Conjugation of oligonucleotides

The conjugation of oligonucleotides is mostly done to improve cellular uptake or in-vivo parameters like biodistribution. However, the influence of the linker on biological activity is still being discussed, as well as the question if the linker used for conjugation has to be bioreversible

or not. (11)

There are numerous possible molecules, which can be conjugated with oligonucleotides like antibodies, saccharides, hormones, proteins, toxins, enzymes, growth factors or vitamins, these conjugates not only improve the properties but can give the oligonucleotides completely new functionality. (9)

The most common conjugation partners of oligonucleotides are **peptides**, which can be added using a variety of linkages such as amide, thioether, thiolmaleimide, ester and disulfide.

Concerning **lipids** especially cholesterol has proved to be useful, as it promotes the interaction with albumin and other serum-lipoproteins.

The third possibility is the conjugation with **carbohydrates**, which mainly promotes targeting of lectin-like proteins active in immune-cells or carbohydrate-clearing receptors.

Finally, oligonucleotides have been conjugated with **nucleic acids** forming aptamers to specifically target cell surface receptors. (11)

1.6.1. Cell penetrating peptides

Cell penetrating peptides (CPPs) are a group of delivery vectors used for the enhancement of entry of a variety of molecules used in gene modulation both in vitro and in vivo. CPPs also known as “Trojan peptides” are usually highly cationic peptides, that are used in oligonucleotide conjugation for their ability of transporting attached cargo across cell membranes. (7) Older hypotheses suggested that CPPs were able to directly cross plasma membranes, but now it is suspected that they are dependent on escaping from endosomes into the cytosol. It is, of course predictable, that the size and charge of the cargo influences the effectiveness of cytosolic delivery. (11)

1.7. Clinical use

Oligonucleotides are administered by injection into the blood compartment from where they spread quickly into various tissues. In a second phase the oligonucleotides are redistributed from the tissues and because of their low molecular mass are excreted in the urine. Phosphorothioates are an exception because of their affinity to plasma proteins. (11)

In the majority of clinical studies involving human testing the oligonucleotides are administered without any carrier or delivery system, few trials involve lipid-based delivery systems. Since the development of clinical trials takes a lot of time, many oligonucleotides, that are being tested now, are older substances with simple chemical modifications. (11)

In Duchenne muscular dystrophy the researcher's goal is to create a functional dystrophin transcript, that is able to generate a partially functional dystrophin protein. This was achieved by shifting the reading frame of the transcript with the help of an SSO, that blocked the pre-mRNA splicing proteins and thus excluded the exons (exon 51) containing the mutations. (1, 8) The results in cell culture were so convincing that the exon skipping via SSO is now being tested in animal models and clinical trials.

In β -Thalassemia the correction of the β -globin pre-m-RNA splicing was achieved in erythroid progenitor cell by treating them with PMOs targeted to the aberrant splice sites, the production of a fully functioning HbA was observed. These results are very promising and suggest that PMOs are a realizable in-vivo model for β -thalassemia therapy. (11)

1.7.1.Eteplirsen and Drisapersen in DMD

There are two hopeful candidates for the treatment of Duchenne muscular dystrophy tested in clinical trials and discussed for registration by the FDA. The drugs are eteplirsen (Sarepta Therapeutics Inc) and drisapersen (Prosensa Holding).

Eteplirsen is a phosphorodiamidate based oligonucleotide, drisapersen is a 2'-O-methyl-phosphorthioate. Both drugs are designed to induce the skipping of the exon 51 of the human dystrophin pre-mRNA. The goal is to create a truncated, but partially functional dystrophin. The oligonucleotides are administered by subcutaneous injection, which mostly lead to local inflammatory reactions. (15, 16) In January 2016 the FDA (U.S. Food and Drug Administration) rejected the approval of Kyndrisa (drisapersen, BioMarin pharmaceutical Inc), due to the lack of substantial evidence of the effectiveness. (17) A decision about approval for eteplirsen is expected in February 2016. (17)

1.8. The substances

Several chemical modifications were recently developed for enhancing cellular uptake in absence of transfection agents. They include mainly 2'-modifications, either oligolysine or short polyethylene glycol chains. The sequence used for the substances is the sequence of the splice switching oligonucleotide (GSK SSO) used for testing in luciferase reporter assays.

1.8.1. Ethylene glycol chains linked to 2'-amphiphilic amino linkers - the AL-series

The substances of the AL-series have 2'-modifications attached to a short amphiphilic amino-linker. This linker was then used to add a PEG chain with eight ethylene units. (Figure 3) (18)

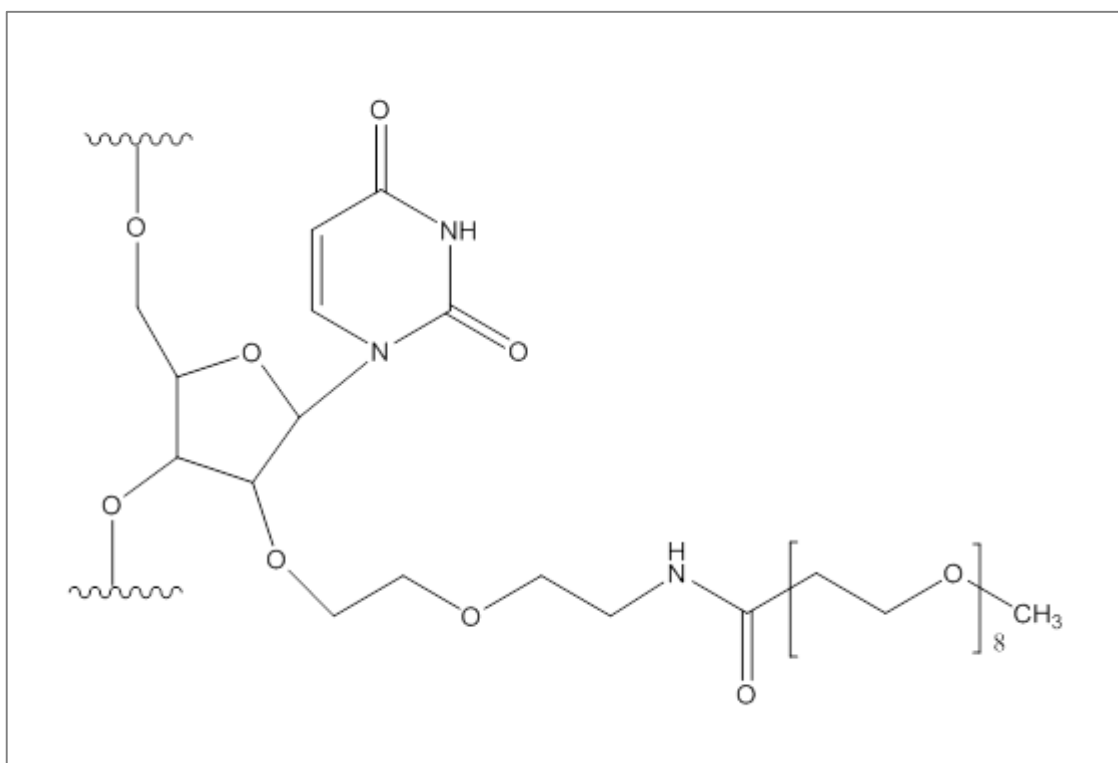


Figure 3 modified uridine

Name	Modifications	Sequence
AL-1	1 mod	CCU*-CUU-ACC-UCA-GUU-ACA
AL-2	2 mod	CCU*-CUU*-ACC-UCA-GUU-ACA

Table 1 oligonucleotides with lipophilic amino linker, mod = modifications, U* = modified uridine

The oligonucleotides of the AL-series are all based on phosphoramidite synthesis. The sequence was varied with the modified nucleotides. The substance AL-1 has one modification, for AL-2 two uridines were exchanged with the modified uridines. (Table 1)

1.8.2. Ethylene glycol chains attached at 2'-O-amino linkers - the BP-series

The adenosine used in the synthesis of the BP-substances was modified in 2'-position, where an aminohexyl linker replaced the hydrogen. (Figure 4) (19)

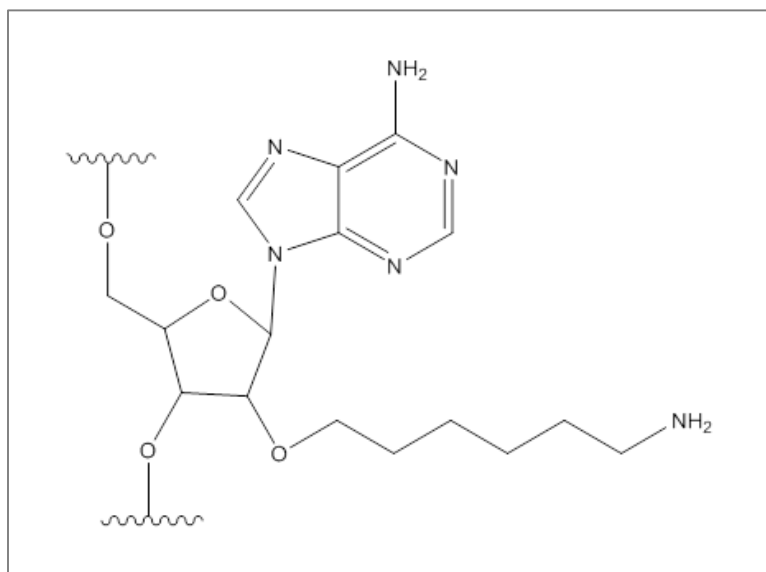


Figure 4 modified adenosine

Name	Modifications	coupled	Sequence
BP-1	1 mod		CCU-CUU-ACC-UCA-GUU-AC3'A*
BP-2	2 mod		CCU-CUU-ACC-UC2'A*-GUU-AC3'A*
BP-3	2 mod	PEG ₈	CCU-CUU-ACC-UC2'A*-GUU-AC3'A*
BP-4	3mod	PEG ₈	CCU-CUU-2'A*CC-UC2'A*-GUU-AC3'A*
BP-5	4 mod		CCU-CUU-2'A*CC-UC2'A*-GUU-2'A*C3'A*

Table 2 Oligonucleotides with ethylene glycol modifications, mod = modifications, 2'A* = modified adenosine,

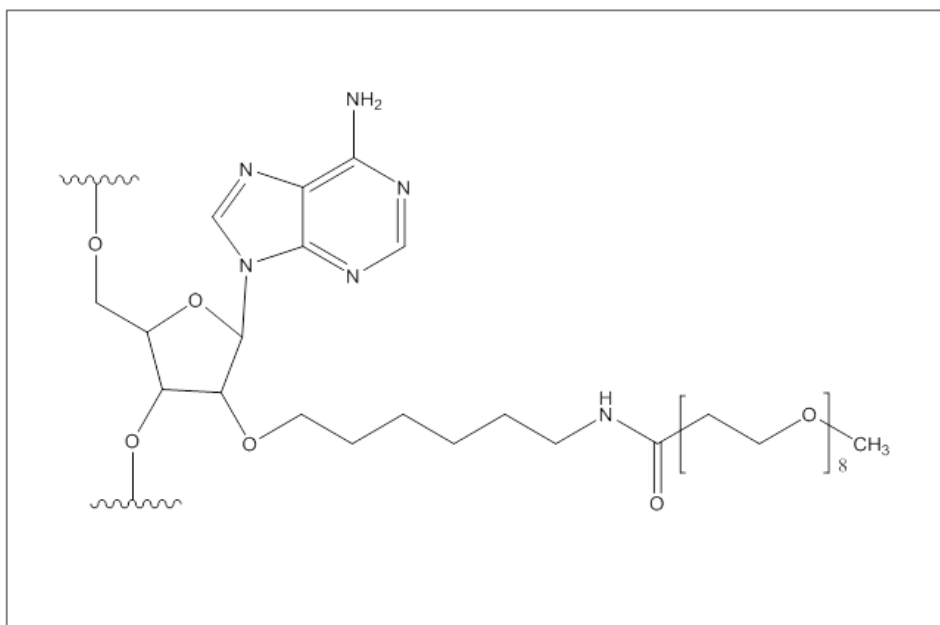


Figure 5 modified adenosine with PEG₈

These aminohexyl-linkers were then used for the coupling with PEG for substances BP-3 and BP-4. (**Table 2**) The PEGylation was used to improve solubility in aqueous and lipophilic environments and induce a certain degree of cellular membrane permeation.

1.8.1. Disulfide-linked Oligolysine-Oligonucleotide Conjugates – the FP-series

Oligonucleotides modified with 2'-O-thioethylether-uridines were coupled to short lysine peptides, generating a labile disulfide linkage between peptide and nucleic acid. (20) (Figure 6)

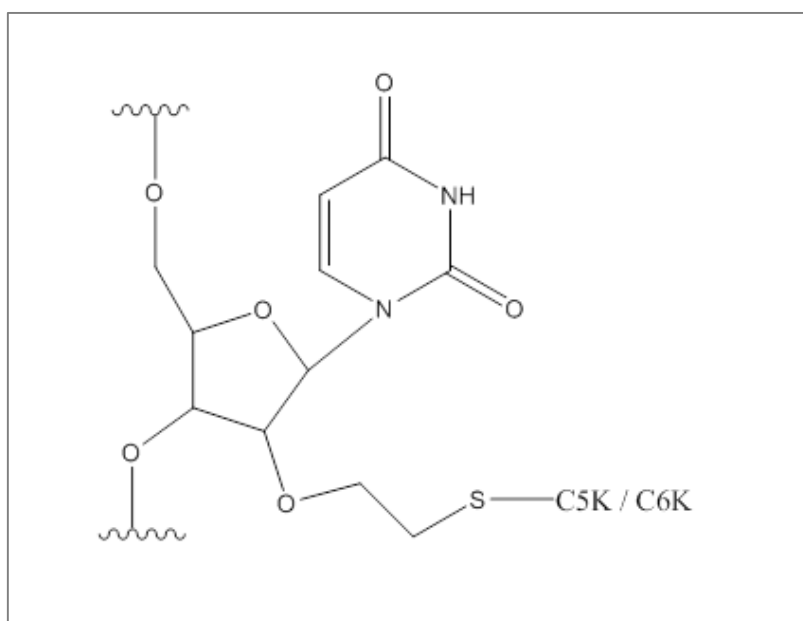


Figure 6 modified uridine

name	modifications	coupled	Sequence
FP-1	1 mod	C5K	CCT-CTT-ACC-TCA-GT*U*-ACA
FP-2	2 mod	C5K	CC*U*-CTT-ACC-TCA-GT*U*-ACA
FP-3	2 mod	C5K	CCT-CTT-ACC-*U*CA-GT*U*-ACA
FP-4	3 mod	C5K	CC*U*-CTT-ACC-*U*CA-GT*U*-ACA
FP-5	1 mod	C6K	CCT-CTT-ACC-TCA-GT*U*-ACA
FP-6	2 mod	C6K	CCT-CTT-ACC-*U*CA-GT*U*-ACA

Table 3 Oligonucleotide conjugates with penta- and hexalysine peptides, mod = number of modifications, *U* = modified uridine

The substances of the FP-series carry the modified uridines in different positions and different numbers. Some oligonucleotides were coupled with the C5K-peptide and some with the C6K-peptide. (Table 3)

1.8.2. Disulfide-linked oligolysine-oligonucleotide conjugates – the SK-series

The substances of the SK-series contained two different modifications, a modified uridine nucleotide was incorporated at the 3'-end, for terminal attachment of oligolysine peptides. A modified cytidine with a thiol linker added to the 2'-position of the nucleobase was used to couple the synthesized oligonucleotides with the C5K Peptides at the 5'-end. (21)

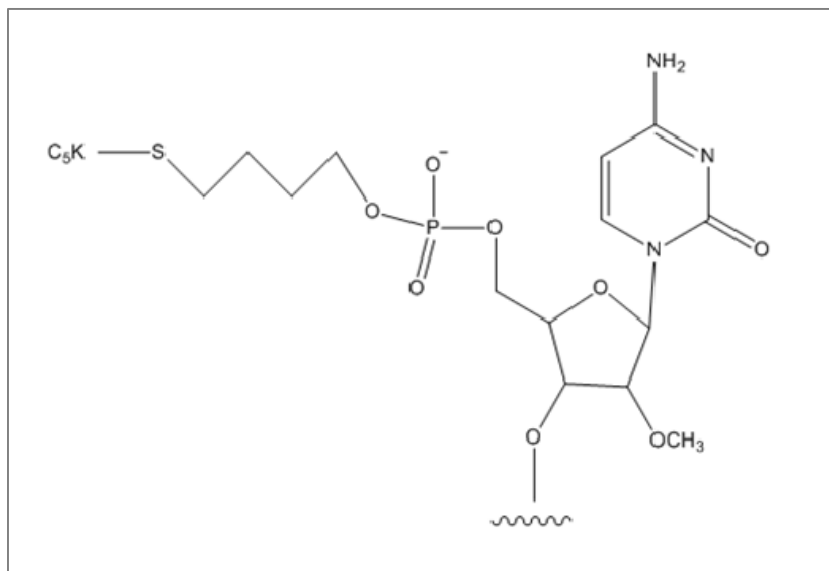


Figure 7 modified cytidine

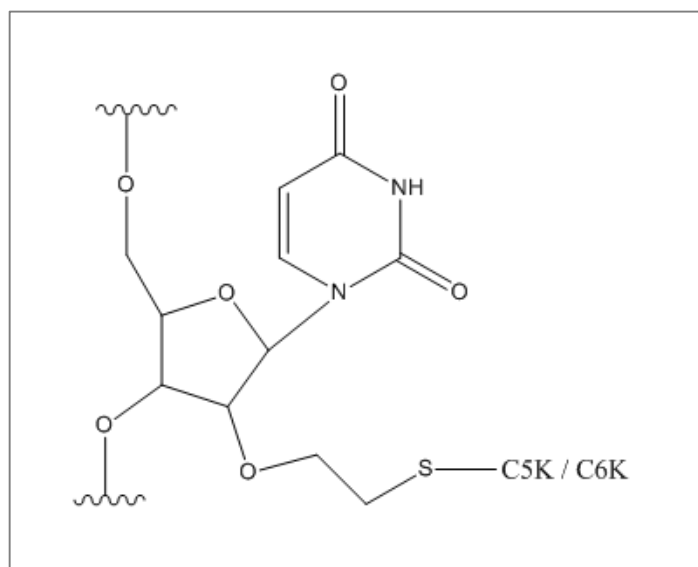


Figure 8 modified uridine

Name	Modifications	coupled	Sequence
SK-1	2 mod	C5K	C*CU-CUU-ACC-UCA-GUU-ACAU*
SK-2	1 mod	C5K	CCU-CUU-ACC-UCA-GUU-ACAU*

Table 4 Oligonucleotide conjugates with penta- and hexalysine peptides, mod = number of modifications, *U* = modified uridine

The SK-1 oligonucleotide has two modified nucleotides, the SK-2 oligonucleotide has only the 5'-modification, but both substances were coupled with the C5K peptide. (**Table 4**)

1.8.3. The C5K and C6K peptide

The peptides C5K and C6K are used for coupling with oligonucleotides via thiol-linker. The goal is to increase cellular uptake of the oligonucleotides to the nucleus. The peptides consist of 5 and 6 lysines, which give the peptides a highly cationic characteristic. The positive charges can then be used to partially neutralize the negative charges of the oligonucleotide backbone. (22)

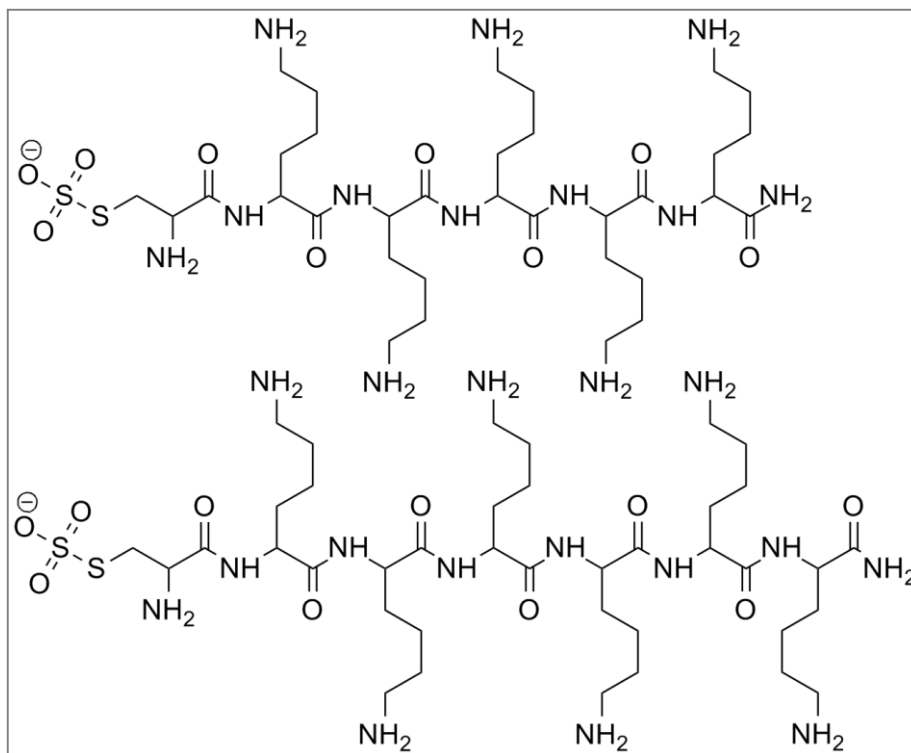


Figure 9. C5K-peptide and the C6K-peptides used for coupling to thiol modified oligonucleotides

2. Aim

The goal was to evaluate the previously described chemically modified splice-switching oligonucleotides, on their ability to regulate the gene expression of luciferase in HeLa pLuc705 cells.

The first part of the process established and optimized the Dual-Luciferase reporter assay in matters of cell count, incubation time, concentration of test substances and control substances, as well as the selection of the best negative and positive control substances for the use of oligonucleotide testing.

The second part was the individual testing of the in-vitro activity of the substances carrying different chemical modifications and conjugations.

The evaluation of the up regulation lead to conclusions about the ability to induce lipofectamine-mediated splice switching. Of equal interest was the ability of the cationic peptides to affect cellular uptake, which was tested by applying the oligonucleotides without any transfection enhancing agents.

Those substances, which showed the ability to up regulate the expression of luciferase, were tested for potential unspecific toxic-effects in a cell-viability assay in wildtype HeLa cells.

Since it was suspected that the cationic peptides possibly influence the complexation properties with lipofectamine, particle sizes of cationic lipoplexes were measured with dynamic light scattering in a Malver Zetasizer.

The last step was the evaluation of eventual changes in the secondary structure and the potential to form stable duplexes with the complimentary DNA strand. This was done by recording Circular Dichroism spectra and determing duplex denaturation temperatures.

3. Material and Methods

3.1. Cell culture

The culture media used was DMEM (Dulbecco's Modified Eagle Medium) (Thermo Fisher Scientific, Waltham, MA, USA) with GlutaMAX™ (Gibco, Thermo Fisher Scientific) which was supplemented with 10% FBS (fetal bovine serum) (Gibco). The cells were cultured under standard conditions at 37°C in a 5% CO₂ humidified atmosphere using an incubator.

The cell lines used were HeLa (human cervix cancer cells) and HeLa pLuc (with stabilised Luciferase genes). TripLE (Gibco) was used to detach the cells before resuspending them in preheated culture media. A Neubauer chamber was used to count the cells.

The HeLa pLuc were treated with DMEM + GlutaMAX™+10% FBS +200µg/µl Hygromycin every 5th passage.

3.2. Dual-Luciferase Reporter Assay

The Luciferase assay protocol and kit from Promega (Promega Corporation, Madison, WI, USA) was used according to the manufacturer's instructions.

Samples were mixed with Lipofectamine RNAiMAX Reagent (Invitrogen, Thermo Fisher Scientific): 0.3 µl lipofectamine were added per pmol SSO (splice switch oligonucleotide). The lipofectamine was mixed with at least the same volume of Opti-MEM (Gibco) before use and incubated for 5 minutes at room temperature, so the lipoplexes can develop their structure.

A reverse transfection was done: the samples were added to a 96-well plate according to the plate plan.

10.000 cells per well were seeded in DMEM (+10% FBS).

The plates were incubated for 48 hours, cultured under standard conditions at 37°C in a 5% CO₂ humidified atmosphere.

After 48 hours the growth of the cells was reviewed.

The culture media was removed, the cells were washed carefully with 100 µl PBS (Thermo Fisher) and treated with 20 µl PLB (passive lysis buffer) (Promega Corporation). The plate was shaken for 30 minutes.

After complete separation of the cells, small volumes of each sample were separated for Bradford assay (2 µl).

50 µl LAR II (Luciferase Assay Reagent II) (Promega Corporation) were injected with Tecan infinite M200Pro (Tecan Group Ltd., Männedorf, Switzerland) and the luminescence was measured immediately.

For normalization of the transfection efficiency and cell growth a Bradford assay was performed.

3.3. Bradford Assay

The Bradford assay was done by manufacturer's instructions and used to normalize the results of the Luciferase assay.

All the wells of a clear 96 well plate were filled with different amounts of H₂O dd (double distilled), so they could be topped up with the samples to 20 µl. Two µl of the wells containing the lysed cells (from the luciferase assay) were transferred to the new plate and added to the water. For the calibration curve BSA 1 mg/ml (bovine serum albumin) was pipetted into the water and treated exactly like the samples.

One hundred and eighty µl Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were added to each well. The absorbance values were measured at 595 nm with Tecan scan.

3.4. Cell viability Assay

9x10³ HeLa cells were seeded in 100 µl DMEM (+10% FBS) into a clear 96-well plate. The cells were cultured under standard conditions at 37°C in a 5% CO₂ humidified atmosphere.

Preparation of stocks:

Preparation of 10 M sterile hHBSS Buffer: To 495 ml Hank's balanced salt solution (Sigma-Aldrich Corp.

St. Louis, MO, USA) 5 ml HEPES buffer solution (Thermo Fisher Scientific) 1 M was added. Under sterile conditions NaOH (sterile filtered) was added to adjust the pH to 7.5.

Preparation of MTS Stock solution: 21 mg MTS reagent powder (Promega Corporation) were dissolved in 10 ml HEPES buffer under light protection. The pH was adjusted to 6.5. After sterile filtration, the MTS was divided into 1.3 ml aliquots and stored at -20°C under light protection.

Preparation of PMS stock solution: 1 mg PMS (Sigma-Aldrich Corp.) was dissolved in 1 ml HBSS and sterile filtrated. The PMS was divided into 0.1 aliquots and stored under light protection at -20°C. After 24 hours the culture media was removed and the test substances were pipetted in triplets to the wells according to the plate plan.

After 60 minutes of incubation the substances were removed and the cells were washed with 37°C hHBSS two times with 200 µl.

1200 µl MTS and 24 µl PMS were diluted in 8,776 ml hHBSS 37°C. The hHBSS was removed from the wells and 100 µl of MTS/PMS mixture were added.

The absorbance was measured with Tecan Scan every 30 minutes until the intensity of the signal was steady.

3.5. Oligonucleotide synthesis

All the modified oligonucleotide test substances were prepared in earlier works. (4, 5, 6, 7)

The test substances consist of oligonucleotides which were synthesized via a PolyGen 10 column DNA/RNA-synthesizer (Polygen GmbH, Langen, Germany). The synthesis was fully automatised using the phosphoramidite method in 3' to 5' direction. The first nucleotide, an adenosine, was pre-attached to the controlled pore glass (CPG), that was used as solid phase. With every cycle of the synthesis one nucleotide was added. The object was to replace some of the nucleotides in the sequence with the modified nucleotides to create the new, modified oligonucleotide.

After synthesis the solid phase was transferred into a glass vial and incubated over night with 1.5 ml ammonium hydroxide solution (25-30%) at 55°C. Subsequently, the vial was cooled to room temperature and the excessive NH₃ was removed by blowing a thin stream of air on the surface for 15 minutes or until the characteristic smell was no longer detected. The supernatant was then transferred into a sterile tube with extreme care not to collect the solid phase. The support was rinsed twice with ammonium acetate. The collected liquid was then dried under vacuum using a Speed-Vac (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The dried oligonucleotides were dissolved in 2 µL ammonium acetate-buffer (3 M) and precipitated by addition of 200 µL isopropanol -20°C. After incubating for 20 minutes at -20°C the samples were centrifuged for 15 minutes at 14 000 rpm. The supernatant was decanted and the pellet was washed twice with -20°C 75% ethanol. The supernatant was carefully decanted once more and the dried pellet was dissolved in 50 µL RNase free water for absorption measurement using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

Purification of the oligonucleotides was done by sephadex 50 (GE Healthcare UK Limited, Buckinghamshire, England) column. The samples were loaded and the resin was washed thoroughly with ammonium acetate-solution (0.1 mM). The fractions containing oligonucleotides were detected by Nanodrop-measurement. Those were united, dried with the SpeedVac and stored at -20°C.

3.5.1.PV-3 and PV-4

Two substances were synthesized to be used for standardization. The goal was to compare the substances of the AL-, BP-, FP- and SK-series with standards produced and purified under similar conditions.

Name	Sequence
GSK SSO	CCU-CUU-ACC-UCA-GUU-ACA
GSK NEG	CCU-GUU-AUA-CCA-CTT-ACA

Table 5 Standard substances, SSO = splice-switching oligonucleotide, NEG = negative control

The standard substances used for testing were splice-switching oligonucleotides with the sequences shown in Table 5, from gsk group (Philadelphia, Pennsylvania, U.S.), which was a 2'-O-Me modified Phosphothioate.

Name	Sequence
PV-3	CCU-CUU-ACC-UCA-GUU-ACA
PV-4	CCU-GUU-AUA-CCA-CTT-ACA

Table 6 oligonucleotides for standardization

The substance PV-3 has the same sequence as the GSK SSO and can therefore be used as a positive control. The substance PV-4 has the same sequence as the negative control (NEG) and is used accordingly.

3.6. Particle size distribution analysis

To confirm that there was no significant difference in particle size between the standard SSO preparation and the preparation with the modified SSO a light scattering measurement was done, using Malvern Zetasizer (Malvern Instruments Ltd, Worcestershire, UK).

Although all the cell culture assays were done with Optimem HEPES Buffer was used for the size distribution measurement the particle count of Optimem itself was a too high.

The standard SSO and the Substance FP-2 were both measured in a 200 nM concentration, prepared with lipofectamine in the same way as it was done in cell-culture assay.

3.7. Circular dichroism spectropolarimeter

To confirm that the hybridization of the modified oligonucleotides with its target mRNA was still possible, a melting curve was measured with variable temperature.

First a spectrum was recorded with Jasco's J-810 L Circular Dichroism (CD) Spectropolarimeter (Jasco Analytical Instruments, Easton, MD, U.S.). The peak wave length was then used for the variable temperature program of Neslab Thermoflex (Thermo Fisher Scientific, Waltham, MA, USA).

The substances were used in a 1 nM concentration with CD Tris buffer 0.01 M pH 7.

4. Results

4.1. Optimization of methods

The first goal was to optimize the conditions of the luciferase assay, that would be used for the testing of the oligonucleotides. Parameters were cell count, incubation time, amount of test substance and thus amount of lipofectamine. A reverse transfection was done, the samples were pipetted into white 96 well plates, and afterwards cells were seeded. The samples consisted of ASO 3'amine = SSO with or without lipofectamine RNAiMAX reagent (Invitrogen) in different concentrations.

4.1.1. Luciferase assay: influence of incubation time and amount of lipofectamine

A reverse transfection was done, samples (10 µL) were added to three white 96-well plates and 30.000 cells (in 90 µL) were seeded in DMEM (+ 10% FBS). The samples contained five different concentrations of SSO with lipofectamine and three different concentrations of SSO without lipofectamine. The plates were then incubated for 24, 48 and 72 hours. Luminescence was measured with Promega Dual-Luciferase Reporter Assay.

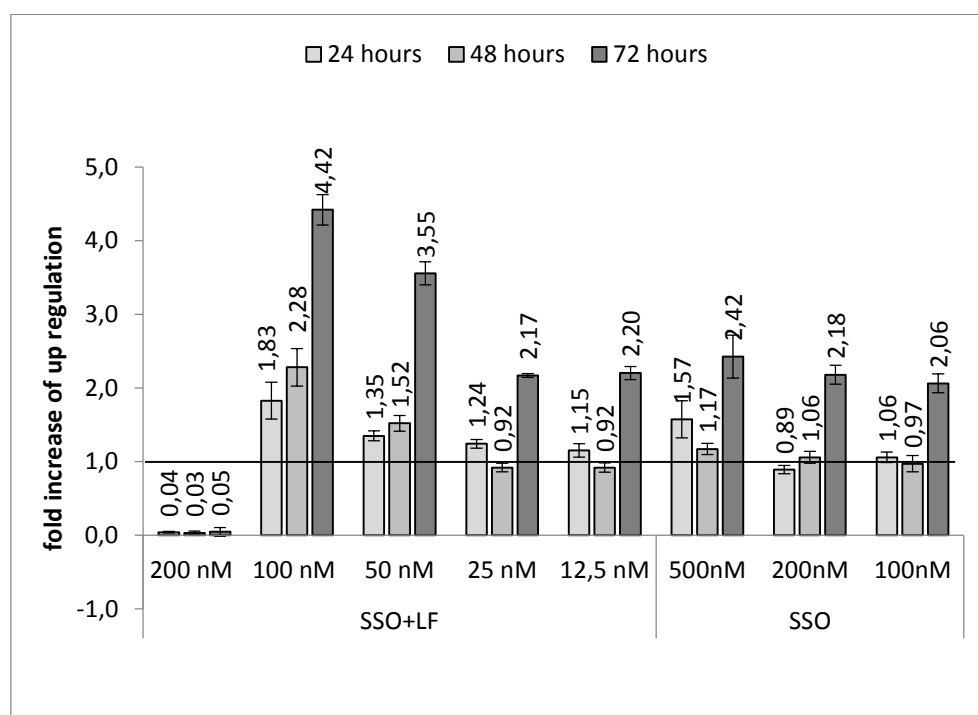


Figure 10: Dual-Luciferase reporter assay, Luminescence up regulation, *significant, **very significant, SSO = ASO 3'amine, LF = Lipofectamine

After the measurement of the luminescence and the visual analysis of the cells, it was clear that the assay works best with an incubation time of 48 hours. The up regulation is not higher after 72 hours, but the standard deviation is, therefore after 48 hours more of the data is significant (P-value <0,05) than after 72 hours.

4.1.2. Luciferase assay: influence of cell count and amount of Lipofectamine

A reverse transfection was done, samples (10 μ L) were added to three white 96-well plates and 30,000 cells (in 90 μ L) were seeded on the left half of the plate, 15,000 (in 90 μ L) were seeded on the right half of the plate in DMEM (+ 10% FBS). The samples contained five different concentrations of SSO with lipofectamine. The plates were incubated for 48 hours. Luminescence was then measured with Promega Dual-Luciferase Reporter Assay.

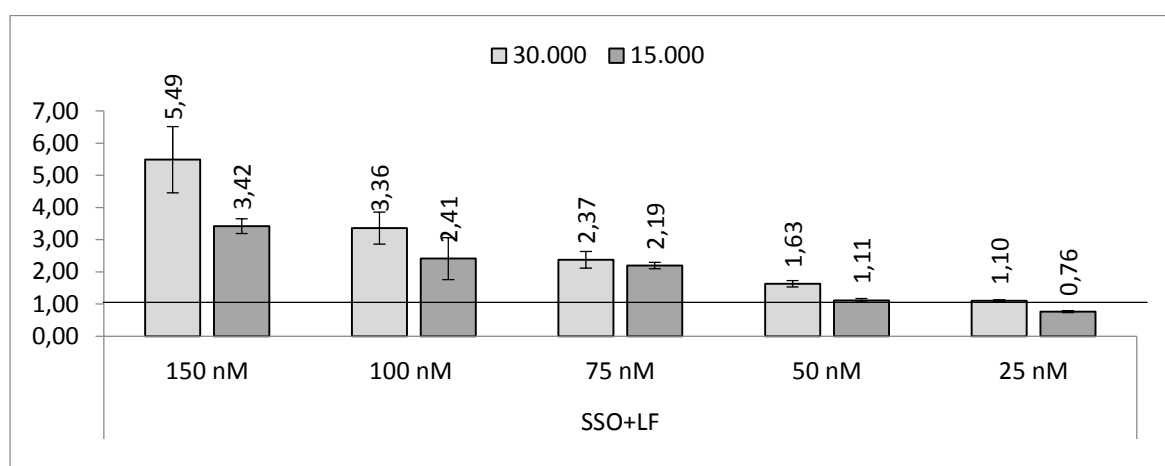


Figure 11 Dual-Luciferase reporter assay, SSO = ASO 3'amine, LF = Lipofectamine

The assay worked well with both cell counts. Although the 30,000 cells lead to higher luminescence signals, the variations were also higher. Furthermore, the visual examination suggested to use the lower cell count.

4.1.3. Luciferase assay: influence of cell count and amount of Lipofectamine

A reverse transfection was done, samples (10 μ L) were added to three white 96-well plates and 5,000 cells (in 90 μ L) were seeded on the left half of the plate, 15,000 (in 90 μ L) were seeded on the right half of the plate in DMEM (+ 10% FBS). The samples contained four different concentrations of SSO with lipofectamine. The plates were incubated for 48 hours. Luminescence was then measured with Promega Dual-Luciferase Reporter Assay.

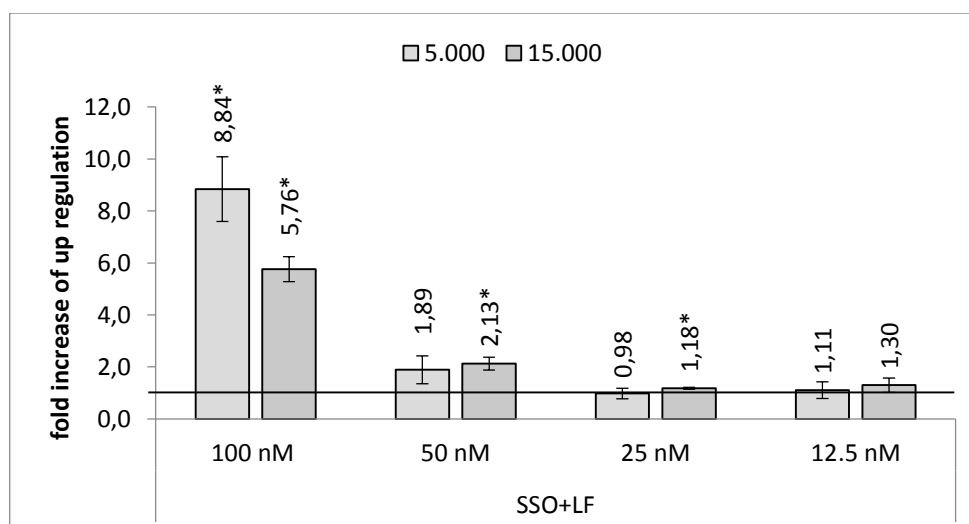


Figure 12 Dual-Luciferase reporter assay, normalized via Bradford, *significant, **very significant, SSO = ASO 3'amine, LF = Lipofectamine

Concerning the luminescence signals the difference between 5,000 and 15,000 cells is not significant. Although the standard deviation is lower with 15,000 cells.

Henceforth 10,000 cells will be seeded.

4.2. Gene upregulation via modified SSOs by Luciferase assay

The test substances were evaluated for their ability to correct splicing, which was detected by up-regulation of luciferase expression.

4.2.1. Disulfide-linked Oligolysine-Oligonucleotide Conjugates

Oligonucleotides modified with a 2'-O-thioethylether were coupled to short lysine peptides, generating a labile disulfide linkage between peptide and nucleic acid. (10)

4.2.1.1. FP-1

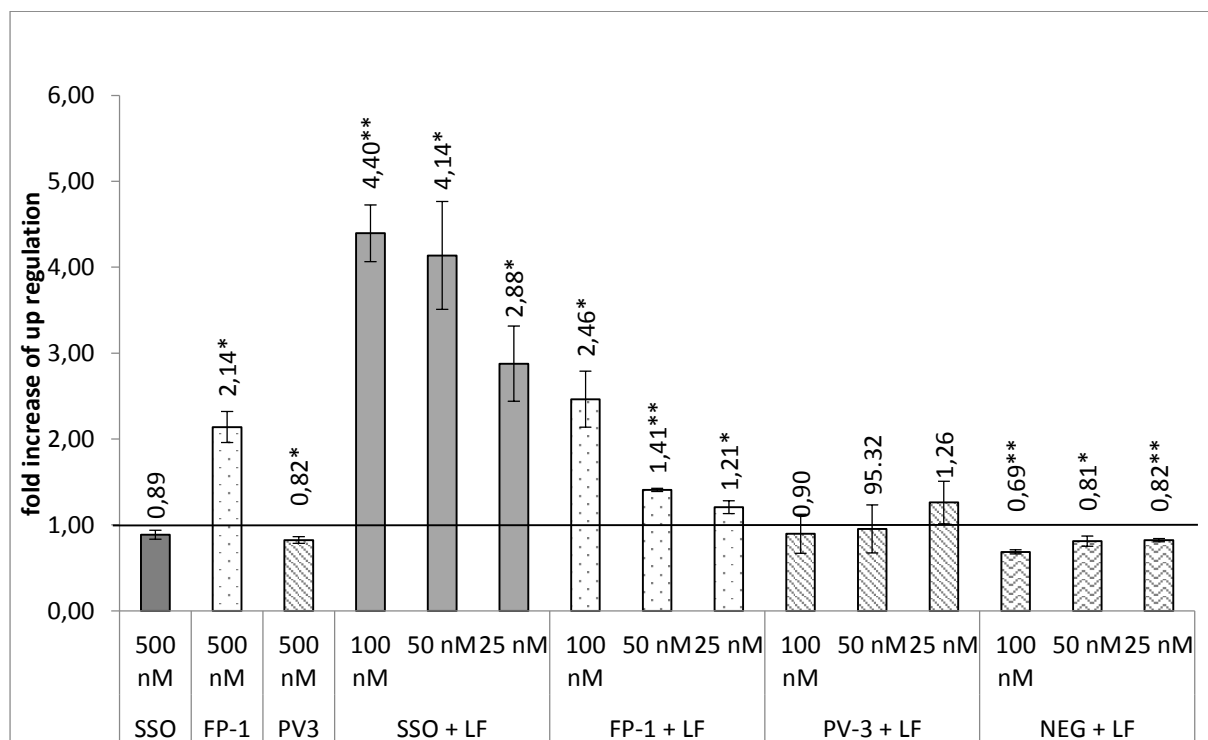


Figure 13 Dual-Luciferase reporter assay, normalized via Bradford, *significant, **very significant, SSO = ASO 3'amine, LF = Lipofectamine, Sequence: CCT-CTT-ACC-TCA-GT*U*-ACA

4.2.1.2. FP-2

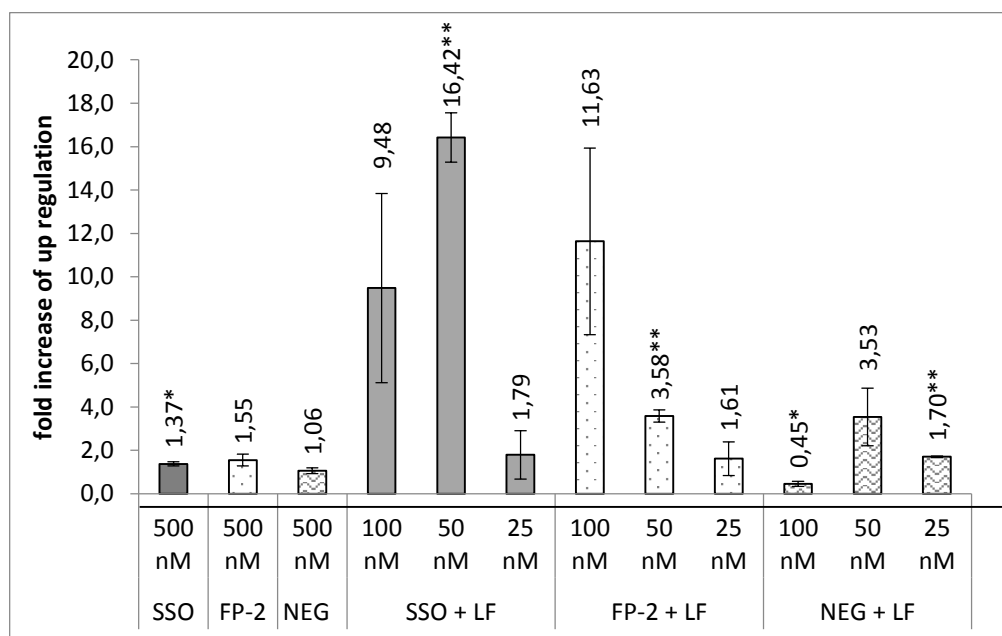


Figure 14 Dual-Luciferase reporter assay, normalized via Bradford, *significant, **very significant, SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CC*U*-CTT-ACC-TCA-GT*U*-ACA

4.2.1.3. FP-3

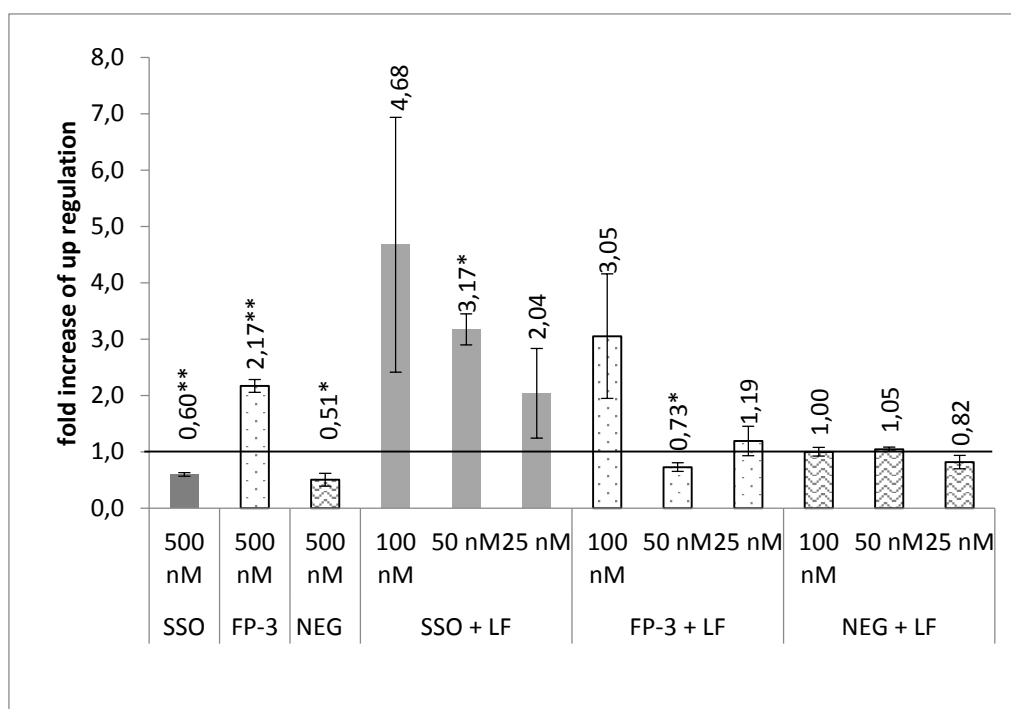


Figure 15 Dual-Luciferase reporter assay, normalized via Bradford, *significant, **very significant, SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CCT-CTT-ACC-*U*CA-GT*U*-ACA

4.2.1.4. FP-4

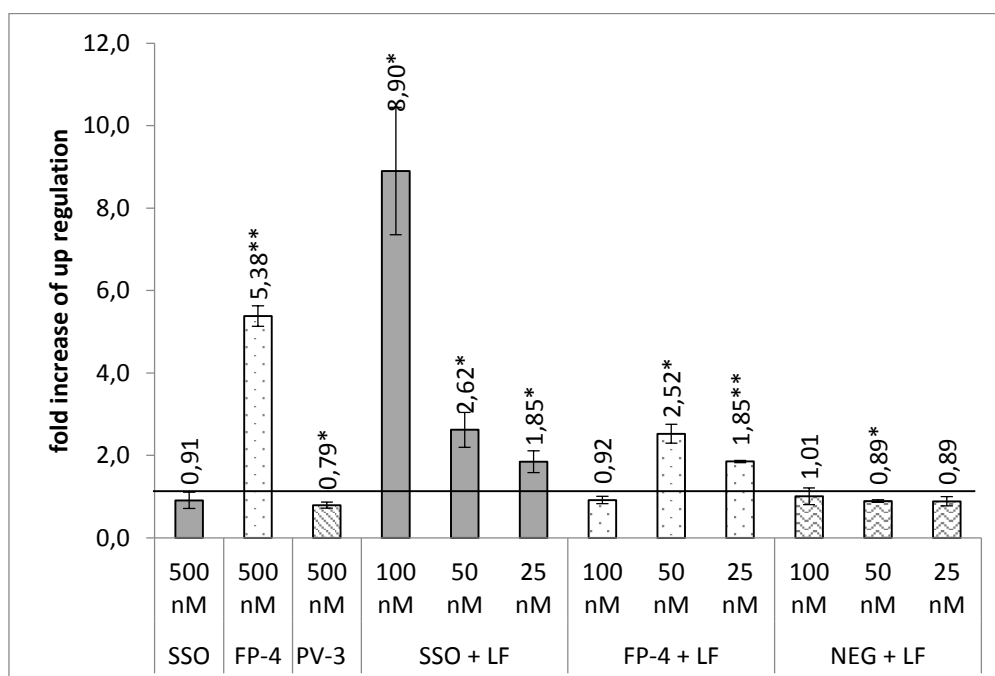


Figure 16 Dual-Luciferase reporter assay, normalized via Bradford, *significant (P<0.05), **very significant (P<0.01), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence CC*U*-CTT-ACC-*U*CA-GT*U*-ACA

4.1.1.1. FP-5

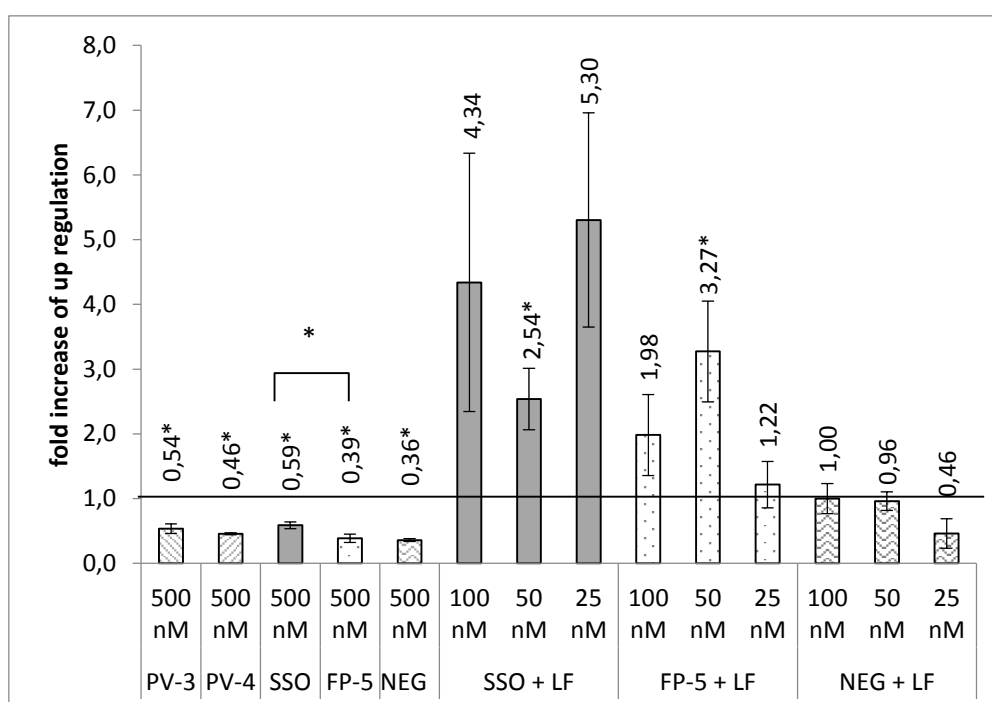


Figure 17 Dual-Luciferase reporter assay, normalized via Bradford, *significant ($P < 0.05$), **very significant ($P < 0.01$), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CCT-CTT-ACC-TCA-GT*U*-ACA

4.1.1.2. FP-6

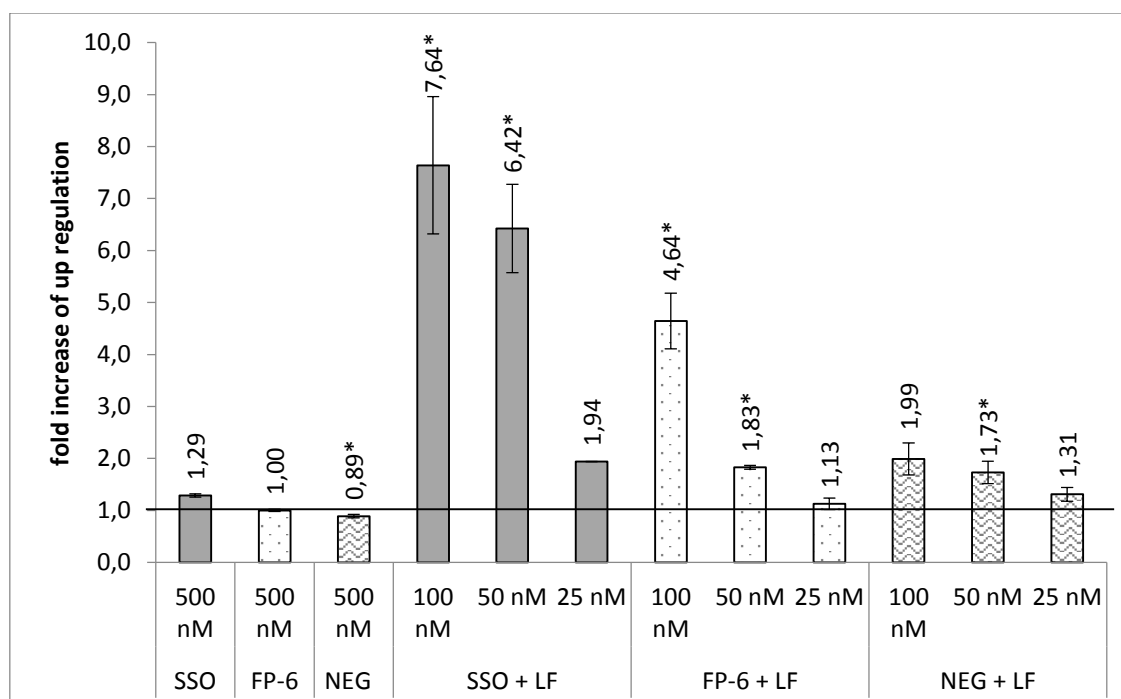


Figure 18 Dual-Luciferase reporter assay, normalized via Bradford, *significant ($P < 0.05$), **very significant ($P < 0.01$), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CCT-CTT-ACC-*U*CA-GT*U*-ACA

4.1.2. Summary of the FP-substances

Evaluating the luciferase reporter assays it is obvious, that the substances of the FP-series were able to induce up regulation of luciferase genes when combined with the cationic lipofectamine. In all six assays the standard SSO did induce higher up regulation than the test-substances. However all six of the FP substances were able to induce a significant up regulation in at least one of the three concentrations.

Three of the six tested substances showed significant up regulation of the luciferase genes without the use of lipofectamine when compared to the standard SSO. FP-1 showed significant twofold up regulation, FP-3 very significant two fold upregulation and substance FP-4 very significant 5-fold up regulation. FP-5 and FP-6 showed no up regulation at all, whereas FP-2 showed up regulation which was not significant.

The substance PV-3, which was introduced as a standard, did not produce significant results and was therefore excluded from further evaluations.

4.1.1. Disulfide-linked Oligolysine-Oligonucleotide Conjugates – the SK-series

A modified uridine nucleotide was incorporated at the 3'-end for terminal attachment of oligolysine peptides, and a thiolinker was attached at the 5'-end.

4.1.1.1. SK-1

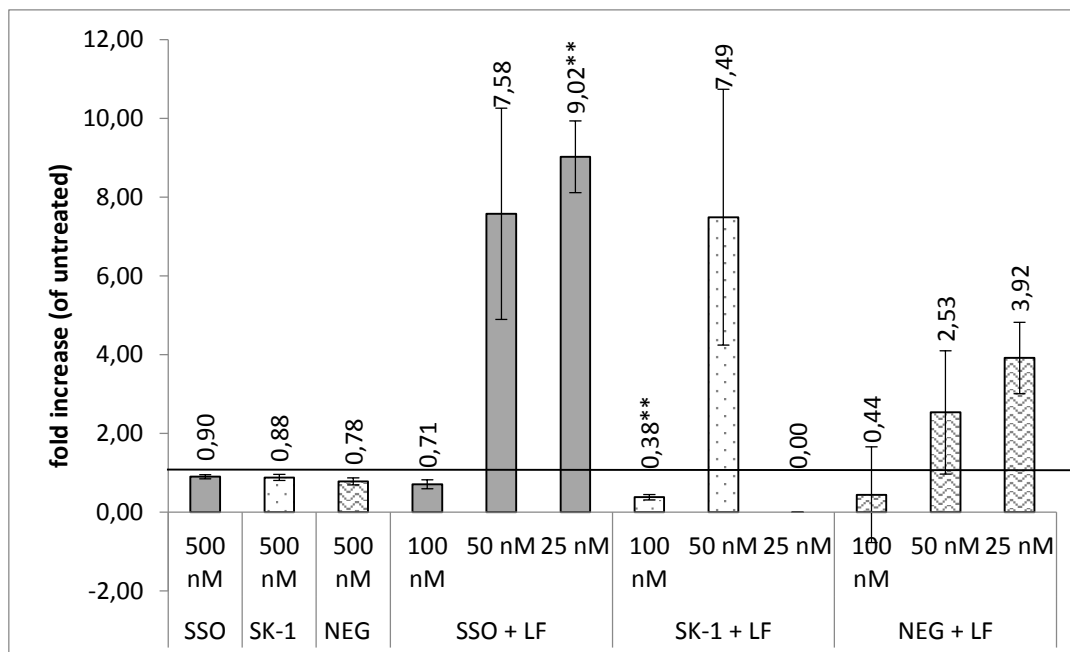


Figure 19 Dual-Luciferase reporter assay, *significant ($P < 0.05$), **very significant ($P < 0.01$), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: C*CU-CUU-ACC-UCA-GUU-ACAU*

4.1.1.2. SK-2

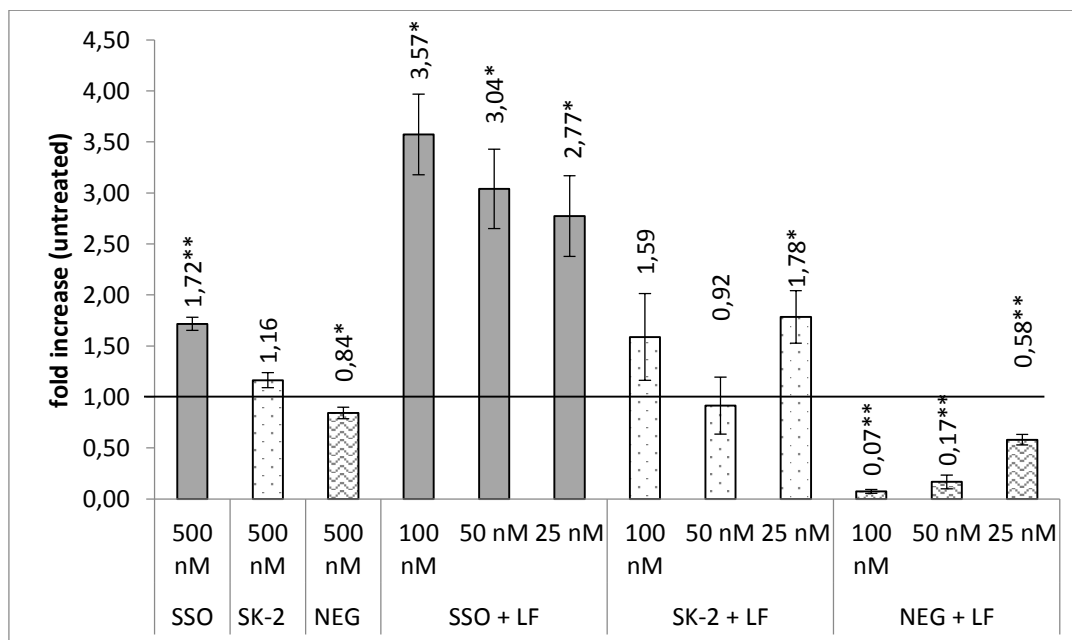


Figure 20 Dual-Luciferase reporter assay, *significant ($P < 0.05$), **very significant ($P < 0.01$), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CCU-CUU-ACC-UCA-GUU-ACAU*

4.1.2. Summary of the SK substances

Both Substances of the SK-series were not able to induce an up regulation without the use of lipofectamine. They did however induce a significant up regulation in combination with lipofectamine.

4.1.3. Ethylene glycol chains attached at 2'-O-amino linkers – the BP-series

Short ethylene glycol chains were attached at terminal and internal nucleotide sites via the 2'-position. (11)

The Substances of the BP-Group were tested with and without the use of lipofectamine using the Luciferase reporter assay. The graphics below display the results.

4.1.2.1. BP-1

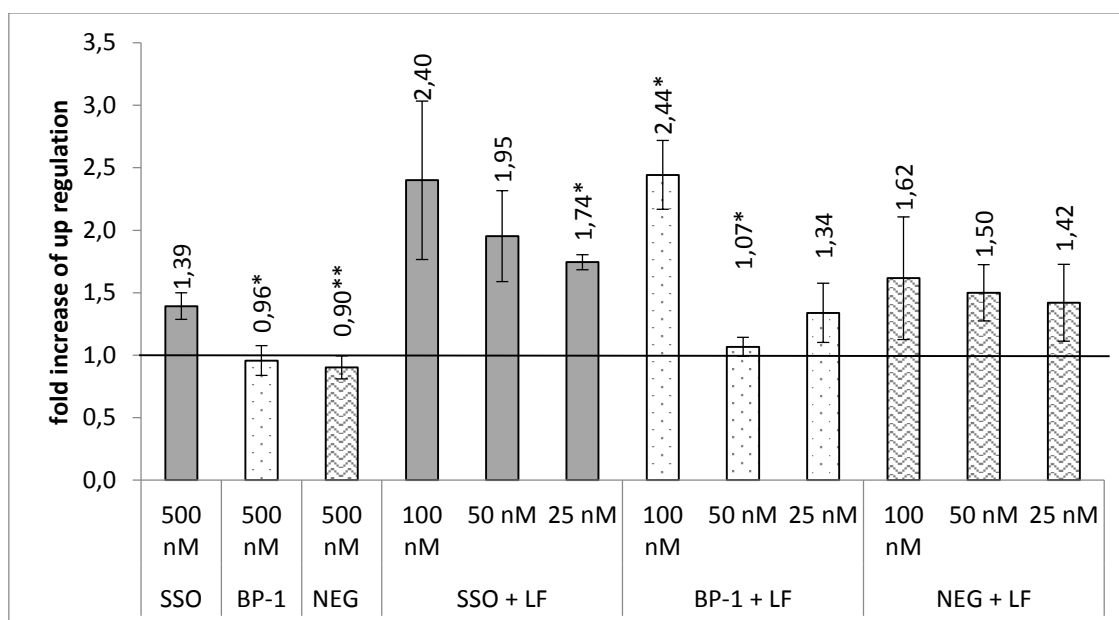


Figure 21 Dual-Luciferase reporter assay, normalized via Bradford, *significant ($P < 0.05$), **very significant ($P < 0.01$), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CCU-CUU-ACC-UCA-GUU-AC3'A*

4.1.3.1. BP-2

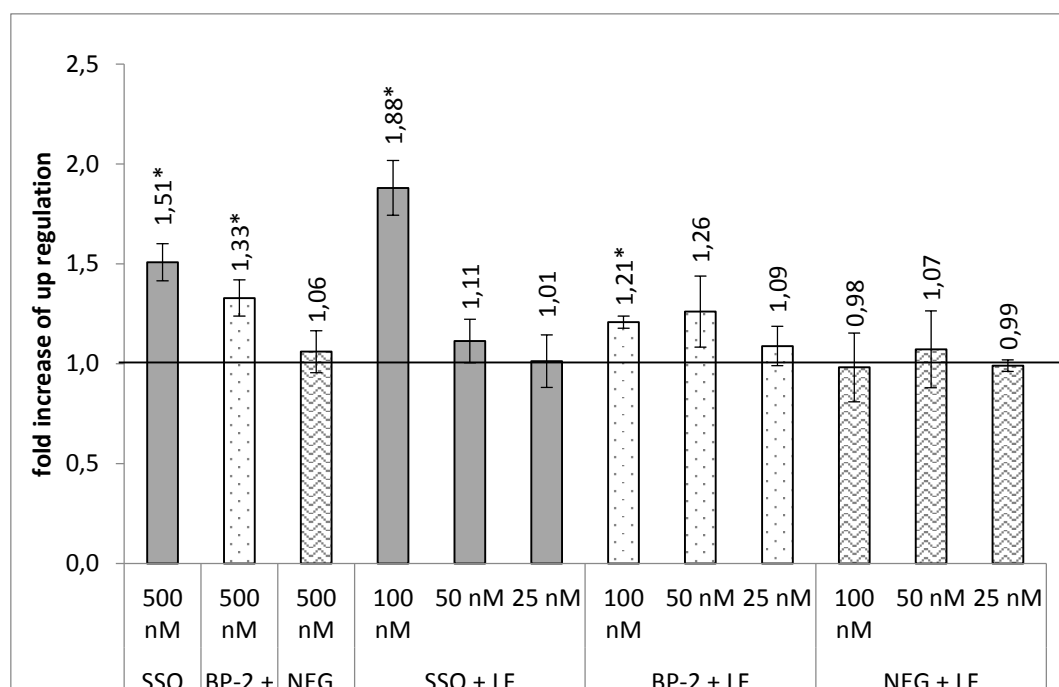


Figure 22 Dual-Luciferase reporter assay, normalized via Bradford, *significant ($P < 0.05$), **very significant ($P < 0.01$), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CCU-CUU-ACC-UC2'A*-GUU-AC3'A*

4.1.2.2. BP-3

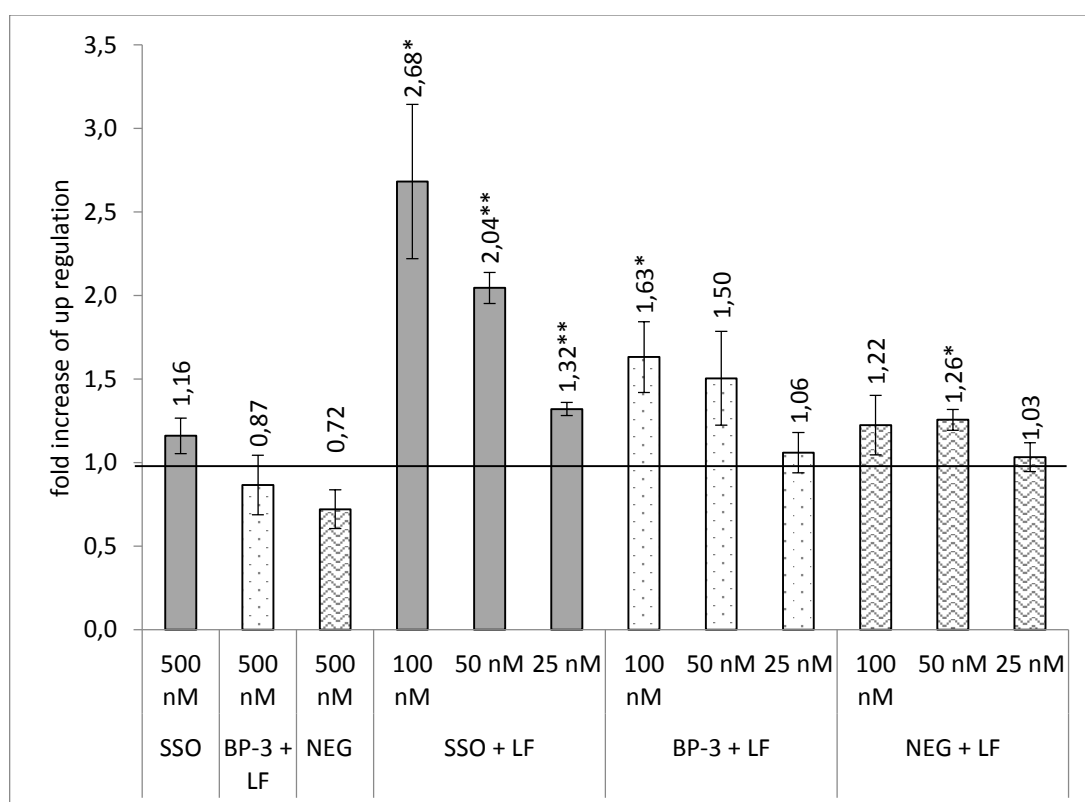


Figure 23 Dual-Luciferase reporter assay, normalized via Bradford, *significant ($P < 0.05$), **very significant ($P < 0.01$), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CCU-CUU-ACC-UC2'A*-GUU-AC3'A*

4.1.2.3. BP-4

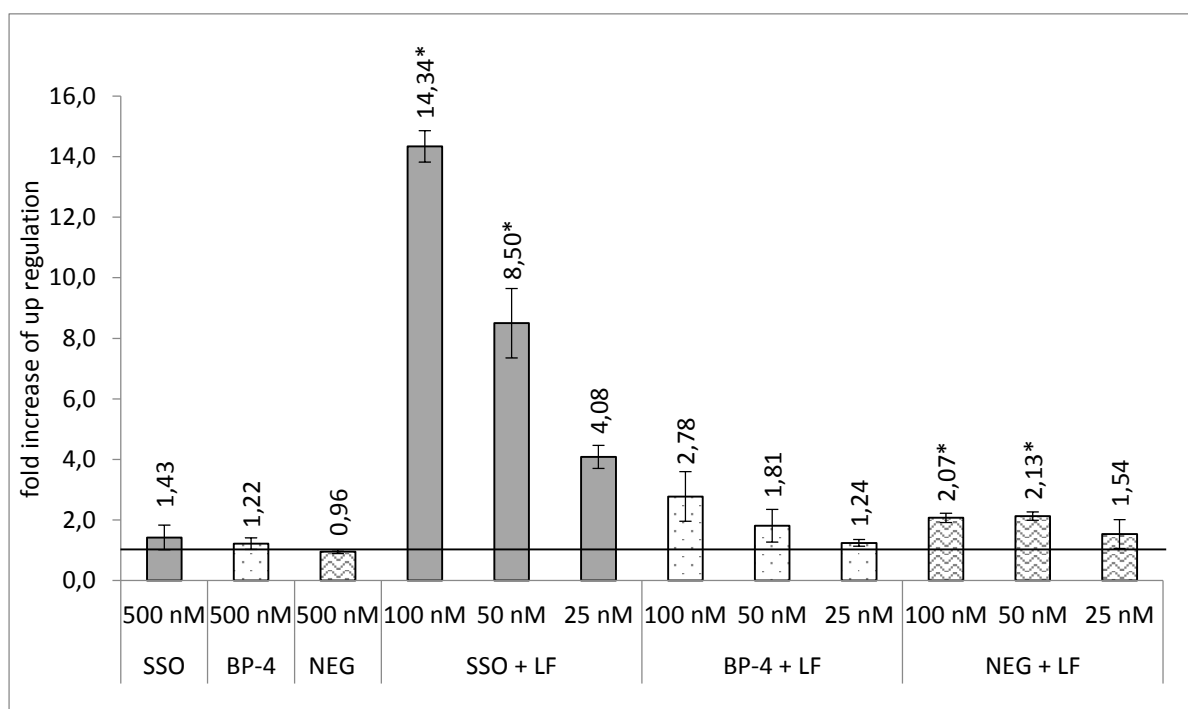


Figure 24 Dual-Luciferase reporter assay, normalized via Bradford, *significant ($P < 0.05$), **very significant ($P < 0.01$), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CCU-CUU-2'A*CC-UC2'A*-GUU-AC3'A*

4.1.2.4. BP-5

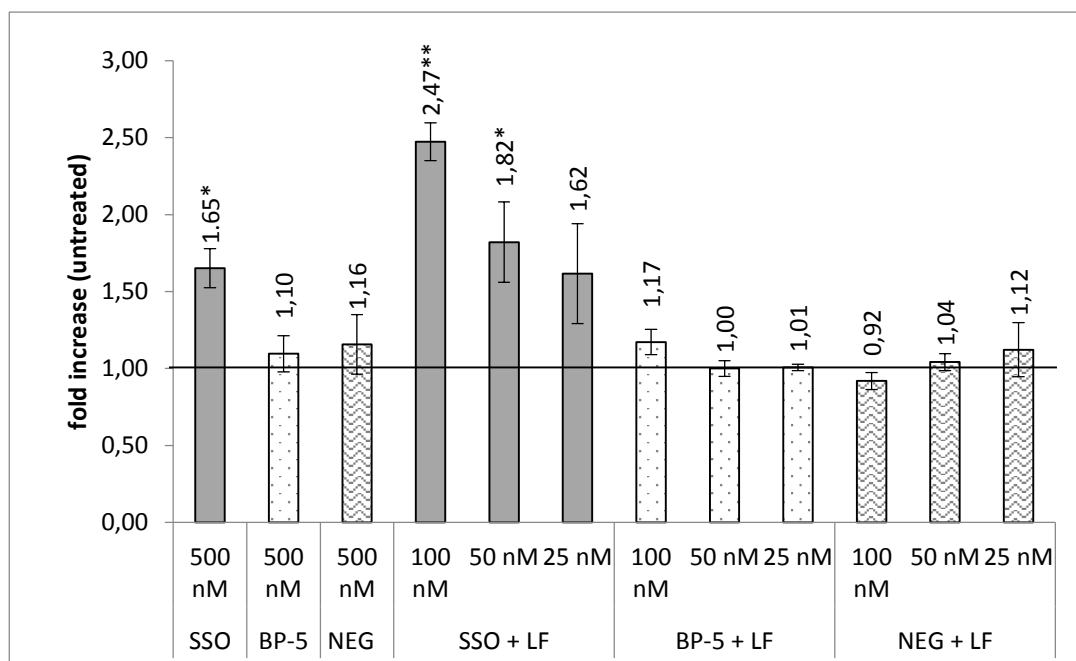


Figure 25 Dual-Luciferase reporter assay, normalized via Bradford, *significant ($P < 0.05$), **very significant ($P < 0.01$), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CCU-CUU-2'A*CC-UC2'A*-GUU-2'A*C3'A*

4.1.3. Summary of the BP-series:

The test-substances of the BP-series were tested with lipofectamine, three of them were able to induce a significant up regulation of luciferase genes (BP-1, BP-2 and BP-3), but only one was able to induce an up regulation higher than the one induced by the standard SSO.

Without the use of lipofectamine only one of the substances (BP-2) was able to induce a significant up regulation, although it was not higher than the one induced by the standard SSO, the other four substances did not induce any up regulation.

4.1.4. Ethylene glycol chains linked to 2'-amphiphilic amino linkers - the AL-series

Oligonucleotides with a less lipophilic amino linker were used for attachment of short ethylene glycol chains. (7)

4.1.4.1. AL-1

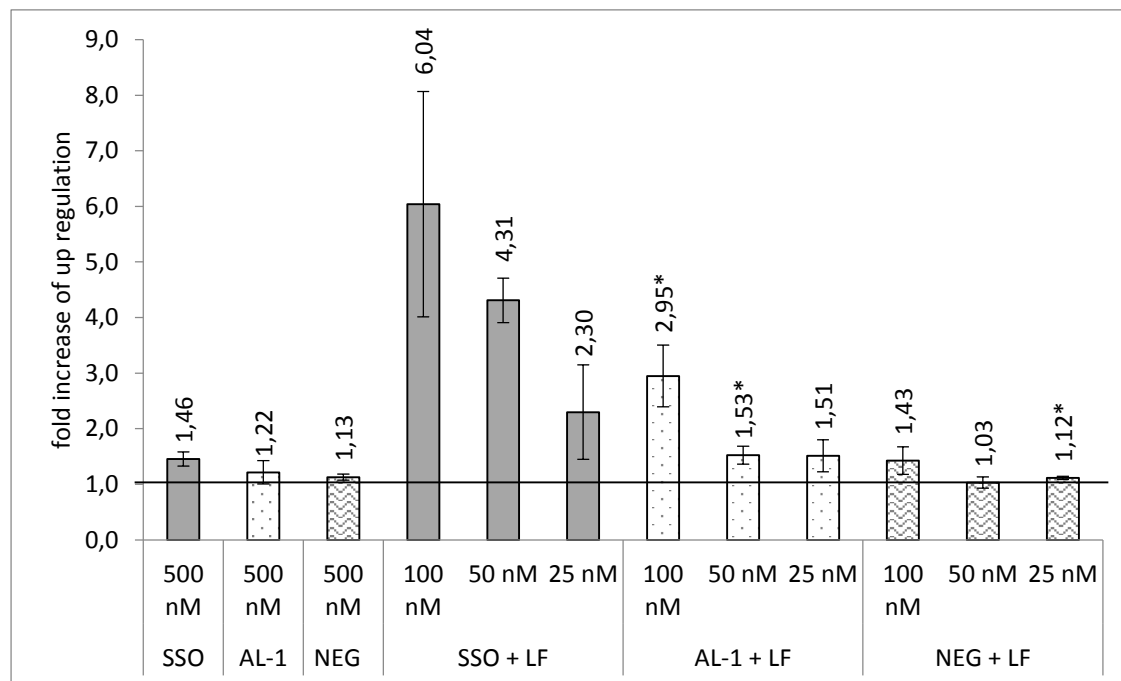


Figure 26 Dual-Luciferase reporter assay, normalized via Bradford, *significant ($P < 0.05$), **very significant ($P < 0.01$), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CCU*-CUU-ACC-UCA-GUU-ACA

4.1.4.2. AL-2.1

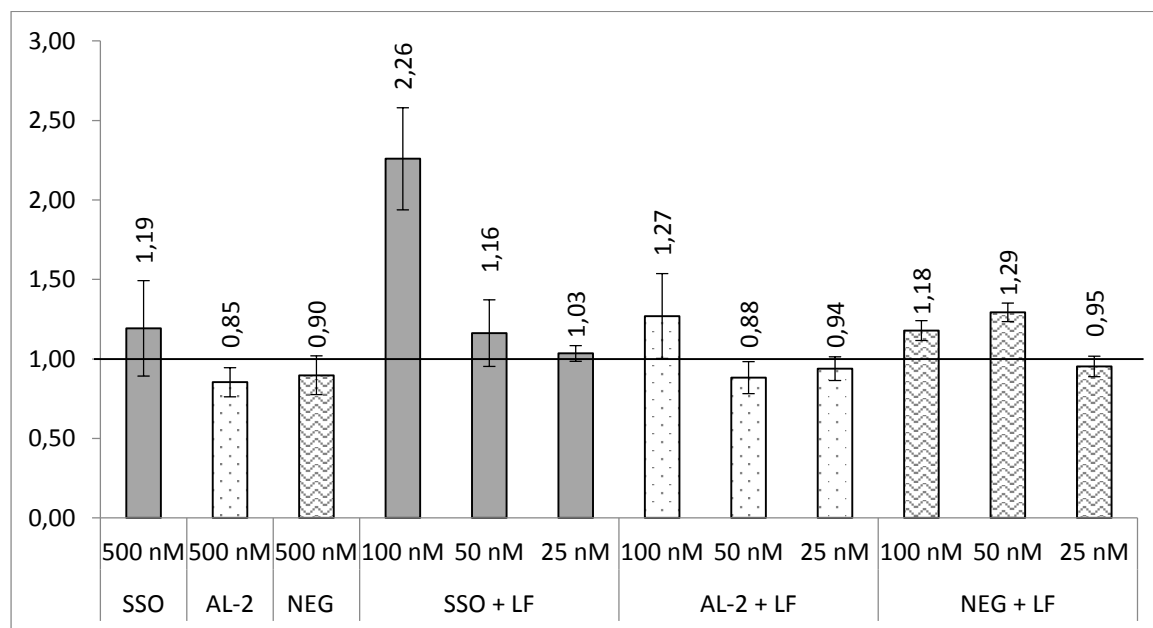


Figure 27 Dual-Luciferase reporter assay, normalized via Bradford, *significant ($P < 0.05$), **very significant ($P < 0.01$), SSO = ASO 3'amine, LF = Lipofectamine, NEG = GSK negative control, Sequence: CCU*-CUU*-ACC-UC2'A*-GUU-ACA

4.1.4.3. Summary of the AL-Substances

The substances AL-1 and AL-2 were not able to induce up regulation of luciferase genes in HeLa pLuc 705 cells in a 500 nM concentration. However in a 100 nM concentration in the combination with lipofectamine AL-1 did induce a significant up regulation ($P=0.03$), the up regulation of AL-2 was not significant.

4.2. Cell viability after treatment with modified SSO by MTS/PMS assay

The MTS/PMS assay was used to measure the viability of HeLa cells. It is based on measuring absorbance levels. In living cells mitochondrial reductase enzymes are active, which can reduce MTS to a coloured formazan. The colour can be determined by absorbance and then related to the number of living cells.

4.2.1.FP-1

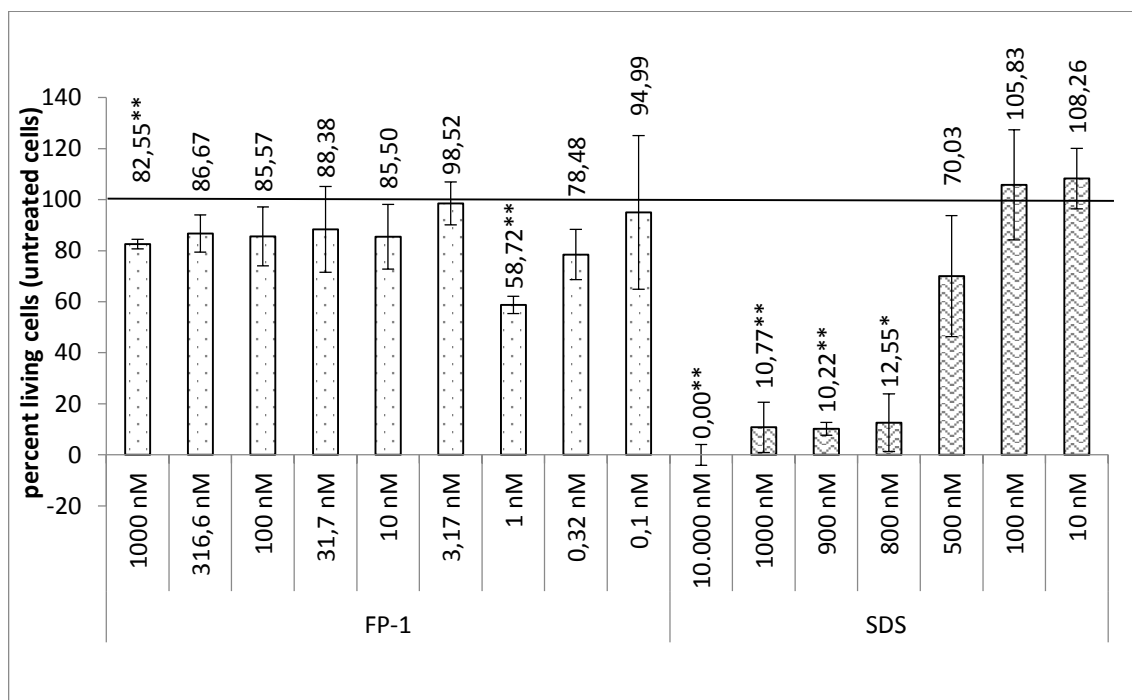


Figure 28 MTS/PMS cell viability assay, *significant ($P<0.05$), **very significant ($P<0.01$),

4.2.2.FP-2

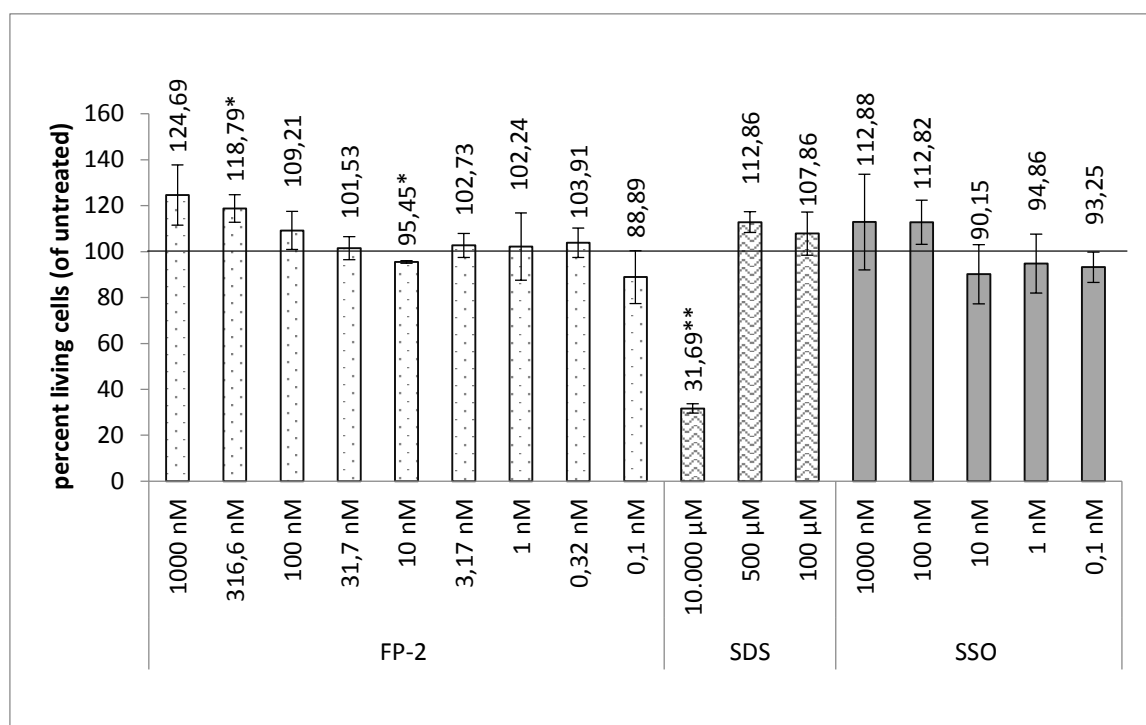


Figure 29 MTS/PMS cell viability assay, *significant ($P < 0.05$), **very significant ($P < 0.01$)

4.2.3.FP-3

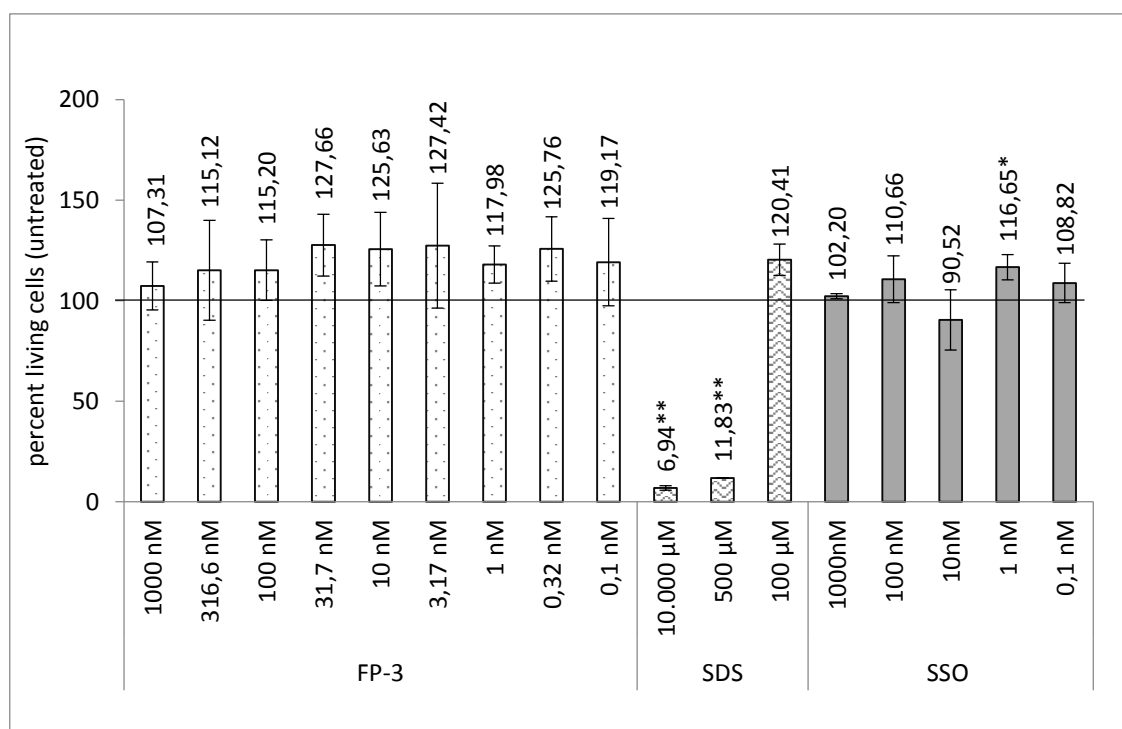


Figure 30 MTS/PMS cell viability assay, *significant ($P < 0.05$), **very significant ($P < 0.01$)

4.2.4.FP-4

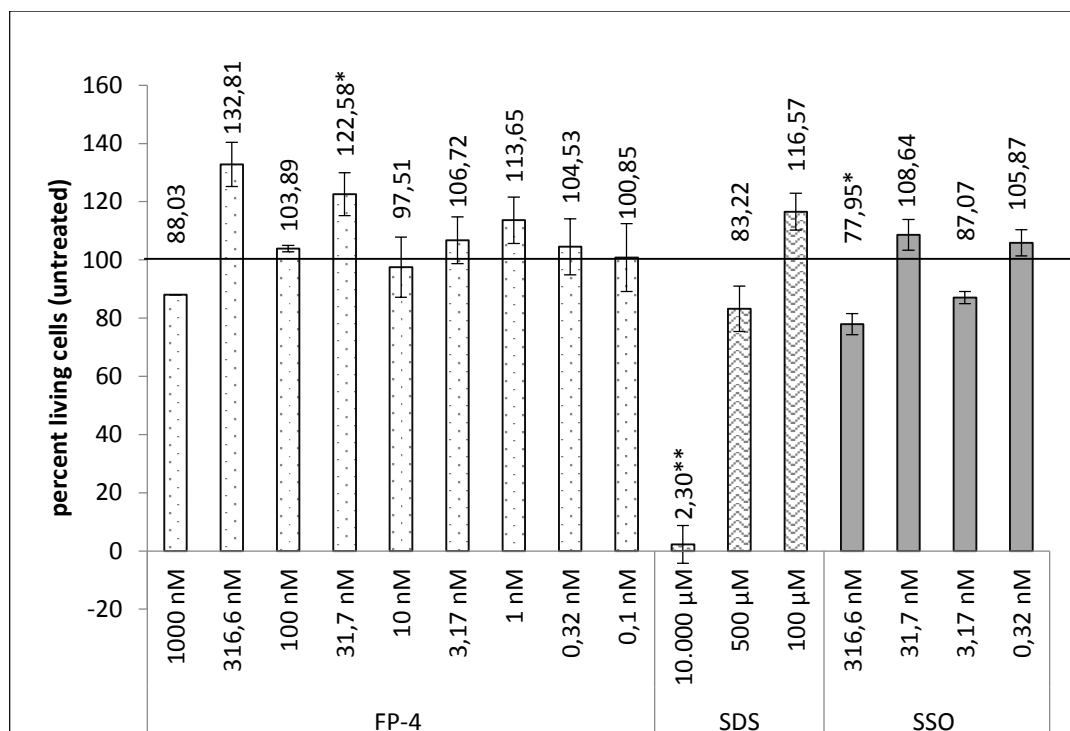


Figure 31 MTS/PMS cell viability assay, *significant ($P<0.05$), **very significant ($P<0.01$)

4.2.5.FP-5

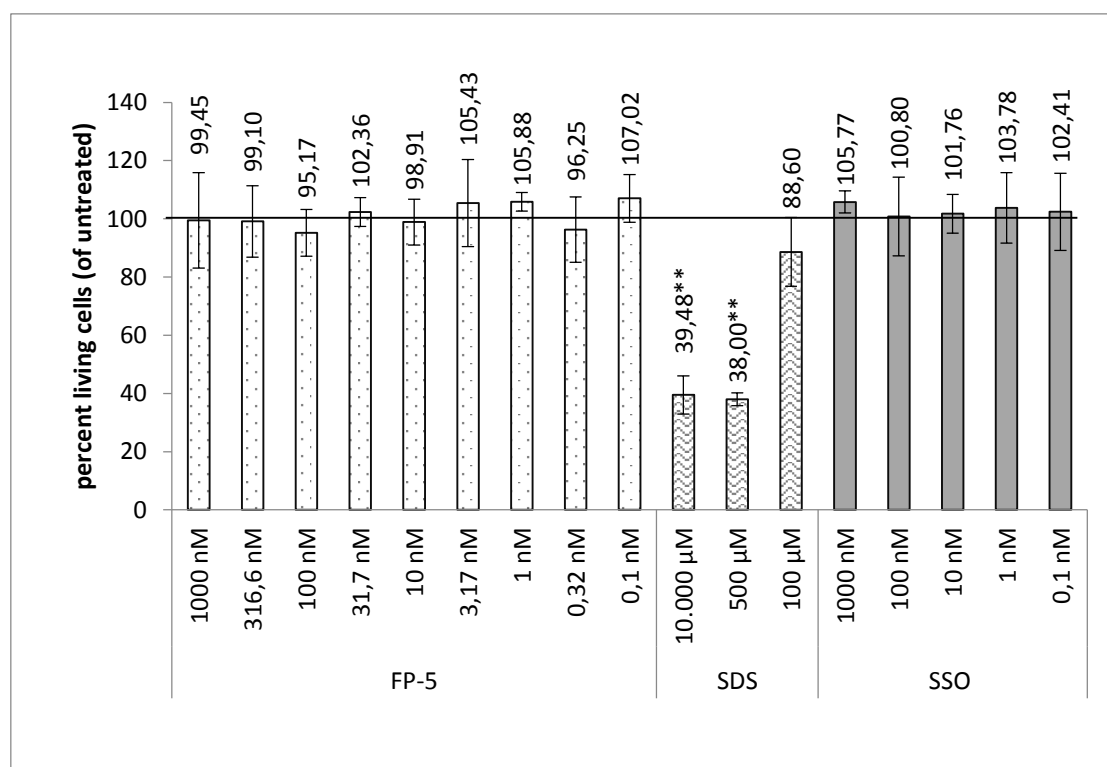


Figure 32 MTS/PMS cell viability assay, *significant ($P<0.05$), **very significant ($P<0.01$)

4.2.2.FP-6

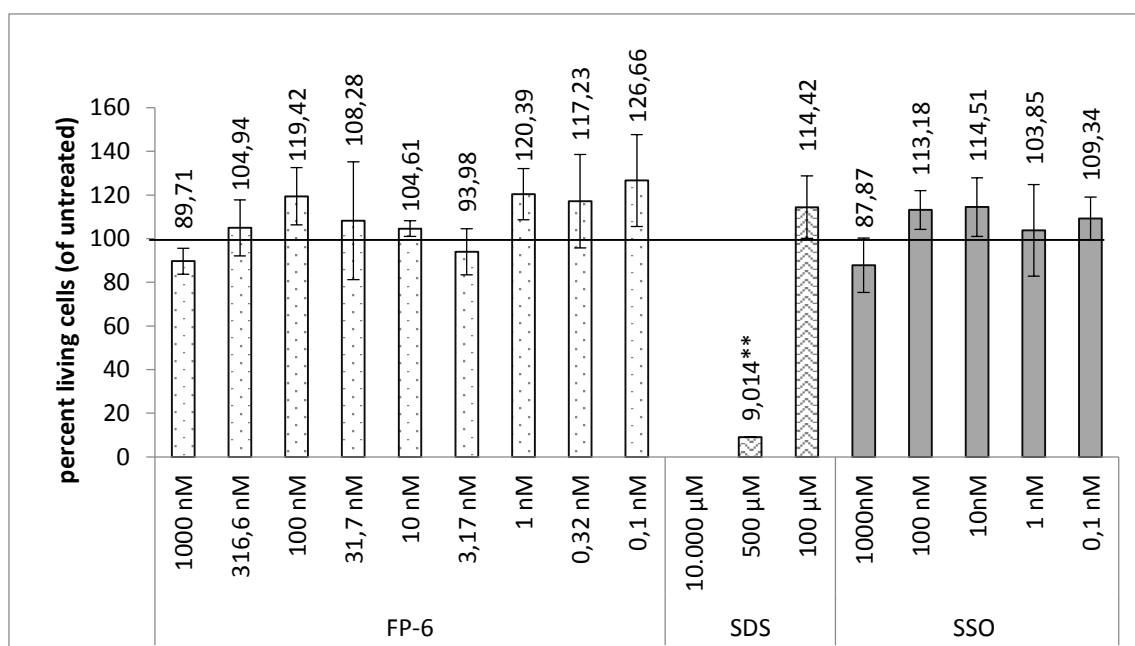


Figure 33 MTS/PMS cell viability assay, *significant ($P < 0.05$), **very significant ($P < 0.01$)

4.2.3.Summary

Overall there was no significant toxic effect detected during the cell viability assays. The viability of the cells treated with the SSO (GSK) were not significantly more or less viable than the untreated cells. The cells treated with SDS were significantly less viable, since SDS was used as a negative control. The cells treated with the modified SSO preparation were overall not more viable than the untreated cell. Testing the substances FP-3 and FP-4 there was a significant higher viability than the cells treated with SSO.

4.3. Particle size distribution

The measurement of size distribution provided following results:

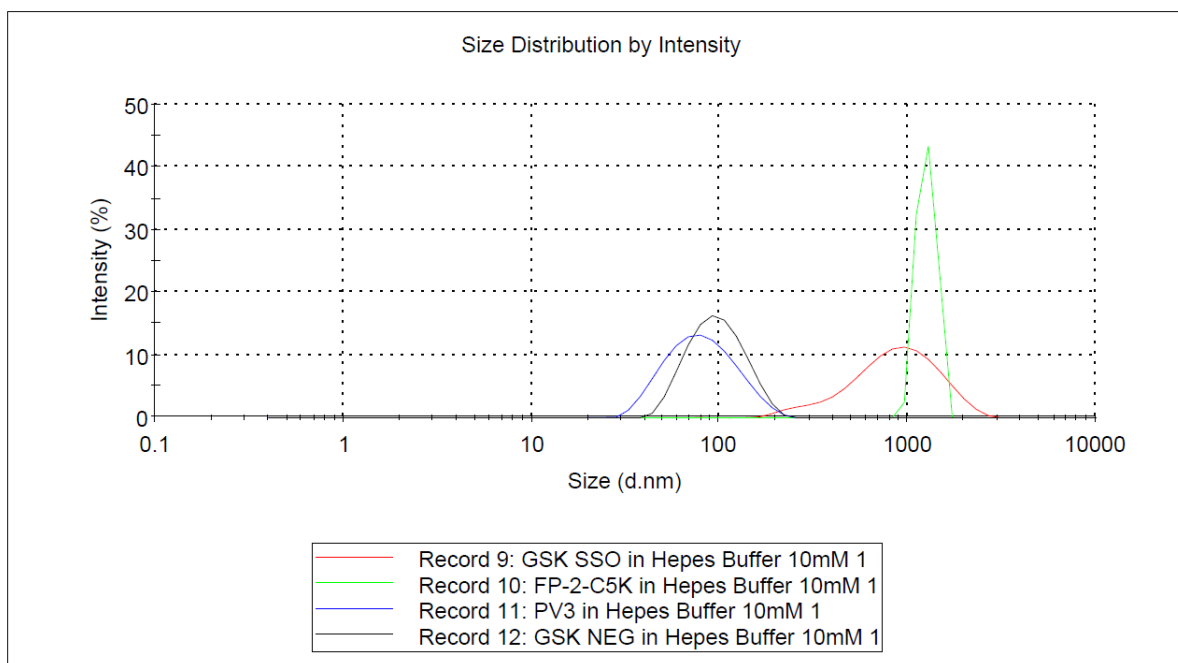


Figure 34 particle size distribution with malvern light scattering

The difference between the NEG = negative control substance and the PV3 is not significant. This proves that the synthesis and the purification process work sufficiently and the products show similar qualities and results as the standardized, bought products.

The difference between the SSO positive control and the Substance FP-2 is also negligible. This proves that the preparation with lipofectamine produces consistent particles sizes both with the standard and the modified SSO.

4.4. Circular dichroism and melting curve

For further characterization of the preparations used in the cell culture assays, the spectra of the substances were measured with circular dichroism.

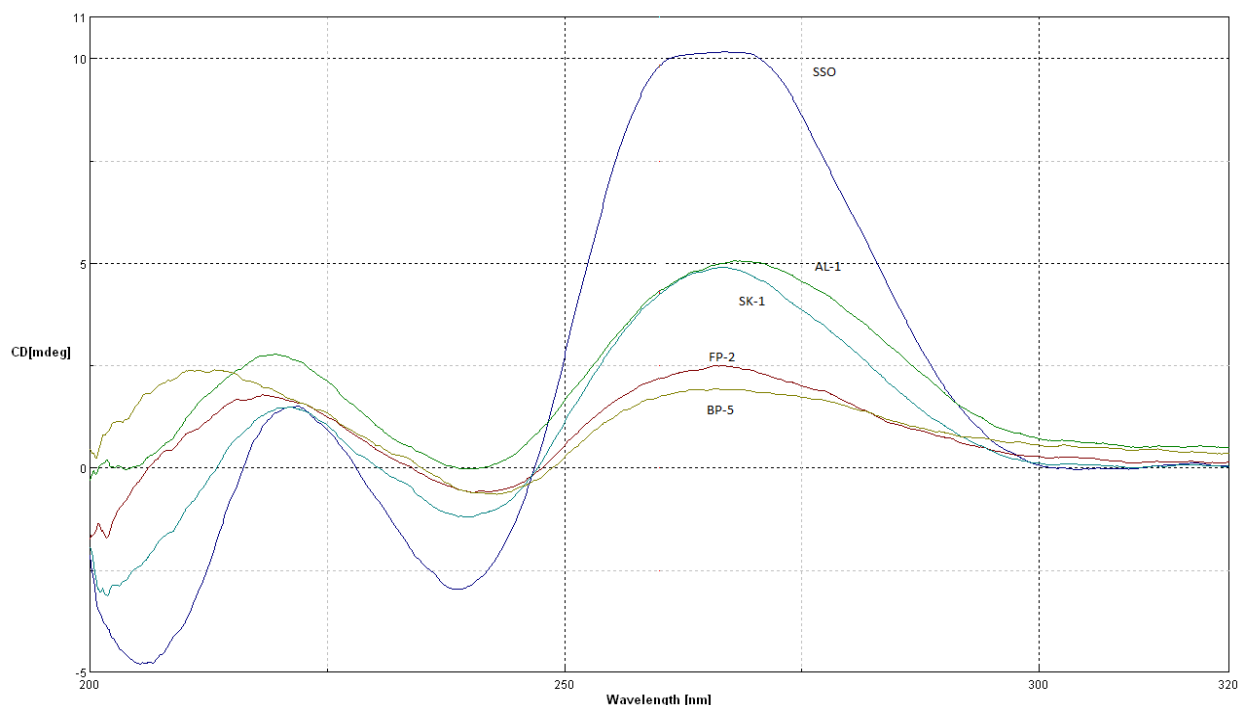


Figure 35 CD-spectra of SSO, AL-1, SK-1, FP-2 and BP-5 duplexes with complimentary strand

To measure duplexes and their melting curves a complimentary, antiparallel single DNA strand was synthesized, which was then mixed with the substances to form DNA/RNA duplexes. The spectra were measured for every single strand and every duplex.

The individual peak point of the spectrum was then used as wave length for the measurement with the variable temperature program.

Variable temperature

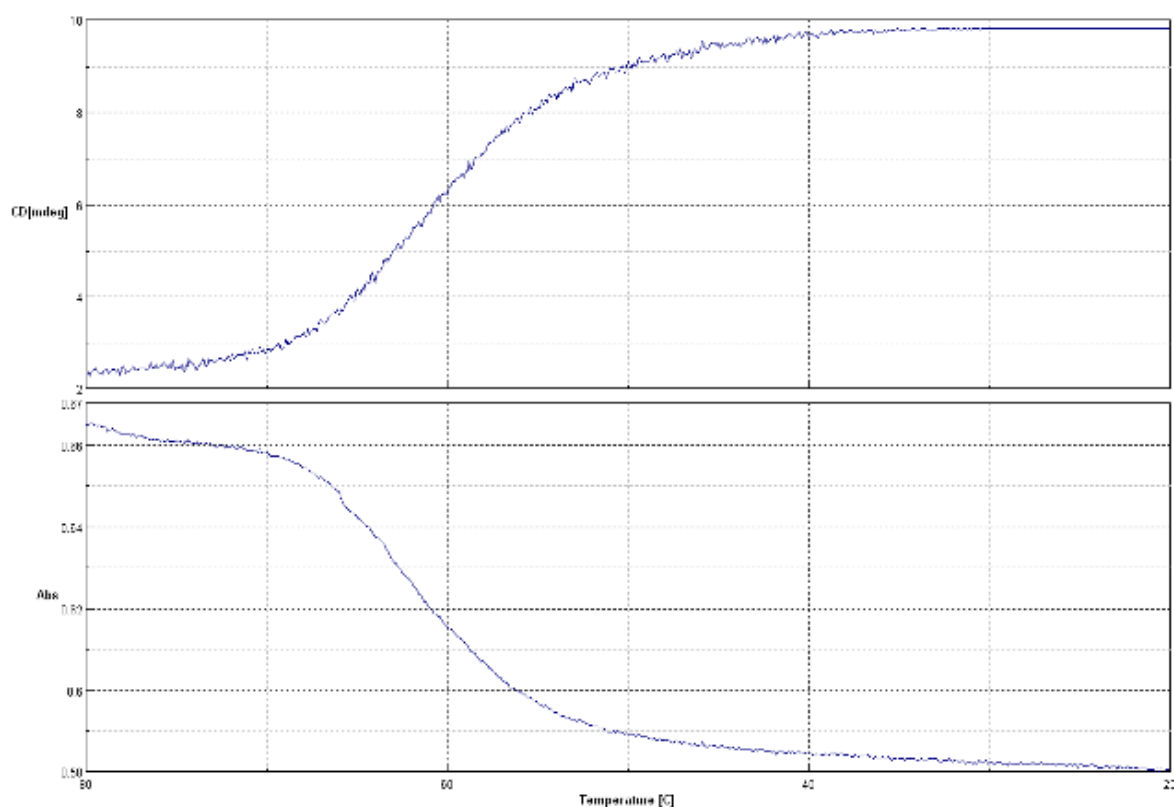


Figure 36 The melting curve of the standard SSO in duplex with the complimentary DNA strand.

Substance	Melting point
SSO duplex	66,6°C
SK-1 duplex	63,4°C

Of the four substances analyzed, only the SK-1 showed an analyzable, sigmoid melting curve. The melting points of the other substances can only be estimated between 50°C and 60°C for AL-1 and FP-2, and between 40°C and 50°C for BP-5.

5. Discussion

5.1. The assay

The optimization of the cell-culture assay lead to a very reliable set up. The chosen incubation time was 48 hours because of different parameters. Firstly the up regulation of luciferase was higher than after 24 hours, secondly the optical evaluation of the cell density suggested 48 hours and thirdly, the standard deviation was lower after 48 hours than after 72 hours.

The cell count of the cells seeded was chosen to be 10.000 after the evaluation of 30.000, 15.000 and 5.000 cells. The 30.000 cells produced high up regulation of luciferase genes, but the optical evaluation suggested that the cells were already growing too dense and it was possible that they would die. This would lead to an adulteration of the normalization via protein quantification according to Bradford. The 5.000 cells lead to a high extent of up regulation of the luciferase gene, but suffered from a higher standard deviation than the 15.000 cells. To exploit the high luminescence values and the stable density and standard deviation, the cell count was set at 10.000 cells per well for all consecutive experiments.

The amount of substances was determined to be between 100 nM and 25 nM for the substances combined with lipofectamine, and 500 nM for the substances, which were tested without lipofectamine. (23)

For the evaluation of the modified splice switching-oligonucleotides it was crucial to select the right substances for standardization because of the imminent shortage of some test substances.

The unmodified SSO was used as reference substances for the positive control. Originally it was intended to exchange the SSO for the PV-3 substance, which was ad hoc synthesized in house, but due to unreliable results and very low up regulation the PV-3 substance was later excluded from the analysis. The same happened to PV-4, which was originally intended to substitute the NEG (gsk) negative control substance.

All the substances, test-oligonucleotides and SSO, with and without lipofectamine, were tested in triplicates and compared to the values of untreated cells. If the luminescence level of the tested well was higher than the mean level of the untreated cells, it was counted as up-regulation.

5.2. The substances:

The test substances were divided into two groups: the FP- and SK-series were conjugated with oligolysines, whereas the AL- and BP-series were conjugated with PEG₈.

On the whole it was shown that the substances, conjugated with the oligolysine peptide produces higher luminescence values than the substances which were PEGylated.

After transfection with lipofectamine all of the peptide-conjugated substances, but only four out of seven of the PEGylated compounds resulted in significant induction of luciferase expression.

Those substances of each group that induced up regulation proof that the sequence was suitable and the oligonucleotides were able to form a stable duplex with the target RNA. The attached PEG-chains possibly interfere with the formation of lipoplexes and therefore reduce the efficiency of transfection, or even prevent hybridization to the pre-mRNA complementary strand. Other works (23, 24, 25) showed that PEG attached to either 3'- or 5'-end of oligonucleotides reduced the transfection efficiency only slightly, with minor effects on siRNA- or antisense mediated gene silencing. However, in this work the PEG chains were attached at the 2'-position, which results in an interaction of the PEG ligands in the minor groove of duplexed nucleic acids. Thus, it is possible for the PEG chains to interfere with target binding. Further examination of cellular uptake or target hybridization will be necessary to explain the results.

Concerning the testing of the enhancement of cellular uptake (determined by examining the pharmacological effect in absence of lipofectamine) again the substances with the oligolysines showed better results than the PEGylated ones. Three out of six peptide conjugated substances showed significant up regulation of luciferase genes, whereas of the PEGylated oligonucleotides only one showed significant up regulation compared to untreated cells: BP-2. The significance of the up regulation concerning BP-2 is still not doubtless, since the observed up-regulation was 1.3 fold, and the SSO-induced up-regulation in the same assay was 1.5 fold. Therefore, in comparison to the SSO-induced effect, the up-regulation was found not significant, which leaves the three substances, which were conjugated with the peptide.

The testing of the FP-series revealed that the substances FP-1, FP-3 and FP-4 induced significant luciferase up regulation in HeLa pLuc705 cells without the use of an enhancer like lipofectamine. The substance FP-1 showed 2.1 fold up regulation, the substance FP-3 2.2 fold and the substance FP-4 5.4 fold, all three were coupled with the C5K peptide. No substance coupled to C6K showed similar results, this could be due to the stronger cationic moieties or the slightly bigger molecular size.

Since FP-1 induced the lowest and FP-4 the highest up regulation of luminescence, it leads to the conclusion that the number of peptides conjugated to the oligonucleotide increases the ability of crossing the membranes. At the same time, multiple peptide substitution at the 2'-position does

obviously not impede hybridization to the target strand. From the data gathered during this work, it remains unclear, whether the position of the conjugation influences the efficacy.

5.3. Further characterisation

For further investigation of the properties and eventual changes in the secondary structure circular dichroism spectra were recorded and melting points determined through temperature-dependent measurements of the CD values.

In general, the secondary structure of the duplexes of the modified oligonucleotides was weaker than the one with the standard 2'-O-methylated, fully phosphorothioated SSO. This is likely explained by changes in the duplex secondary structures induced both by the oligolysines as well as the PEGchains through direct interaction with the counter strand. In addition, a certain steric hindrance is possibly caused by the localization of the rather bulky substituents in the minor groove. The evaluation of the CD-spectra leads to the conclusion that duplexes are formed for all tested substances, but they do not fully resemble the duplex consisting of unmodified oligonucleotides. Furthermore it was confirmed that the peptides influence the conformation of the oligonucleotides in a higher extent than the PEGylation. The only sigmoid and thus straightforward evaluable melting curve was of SK-1. This phosphothioate with a modified uridine at the 3' and a modified cytidine at the 5', which were both coupled with the pentalysine C5K, had a melting point 63,4°C and therefore only 3,25°C lower than the one of the standard SSO.

This shows that the target affinity of SK-1 is only slightly lowered by the 2'-conjugation of the C5K peptide. The other substituted oligonucleotides resulted in more gradual decrease of standard duplex structures as measured by CD, therefore complicating a precise determination of a melting temperature.

5.4. Summary

In an in-vitro assay using HeLa pLuc705 cells chemically modified, splice-switching oligonucleotides were tested positive concerning enhanced cellular uptake, and thus up-regulation of luciferase gene expression. The effective substances were modified with 2'-O-thioethylether-uridines, and conjugated with the penta-lysine C5K using said linkers.

All over the evaluation of 2'-peptide and PEG-conjugated oligonucleotides opens up several new questions that demand further investigation. In some cases, for further in depth investigations it would be necessary to synthesize, purify and conjugate a larger sample batch. Then it would be possible to measure the particle size and encapsulation efficiency of the lipofectamine preparations for all substances, which could answer the question if the PEG-chains influence the formation of lipoplexes. Target hybridization and melting temperatures can be more easily examined in higher concentrations. Cellular uptake can be investigated using fluorescently tagged oligonucleotides and give information about uptake routes and intracellular trafficking both in presence and absence of a transfection agent.

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