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„The development of Splice-Switching-Oligonucleotides in In-vivo studies on the basis of disease-causing mutations“

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1. Introduction

1.1. RNA-Splicing

Pre-messenger RNA (Pre-RNA) consists of non-coding RNA sequences, called introns, and coding RNA sequences, called exons. To obtain a mature mRNA that represents the template for protein translation, the introns have to be removed from the pre-mRNA and the exons have to be connected. This process is called pre-mRNA splicing (Havens *et al.*, 2013; Lee e Rio, 2015). This pre-mRNA splicing process depends on precision and accuracy ensuring that the correct open reading frame is maintained for efficacious protein production during translation. Achieving this high fidelity is caused by sequences and structures within the RNA transcript that direct the binding of splicing proteins that aid in positioning the RNA in a manner that facilitates the correct cleavage and ligation reactions of splicing (Havens e Hastings, 2016). A complex macromolecular machine, the spliceosome, enables this delicate reaction. The spliceosome consists of small nuclear RNA (snRNA) components of five ribonucleoproteins (snRNPs), U1, U2, U4, U5, and U6. The snRNPs form specific RNA-RNA base pairs with the core splice site elements consists of the 5' splice site (5'ss), the branchpoint sequence (BPS), the polypyrimidine (Py) tract, and the 3' splice site (3'ss), as well as with proteins. The splicing reaction starts by U1 snRNP binding to the 5' splice site, followed by U2 snRNP interactions at the BPS and eventually U4, U5, and U6 snRNP interactions near the 5' and 3' splice sites (Fig. 1)(Lee e Rio, 2015).

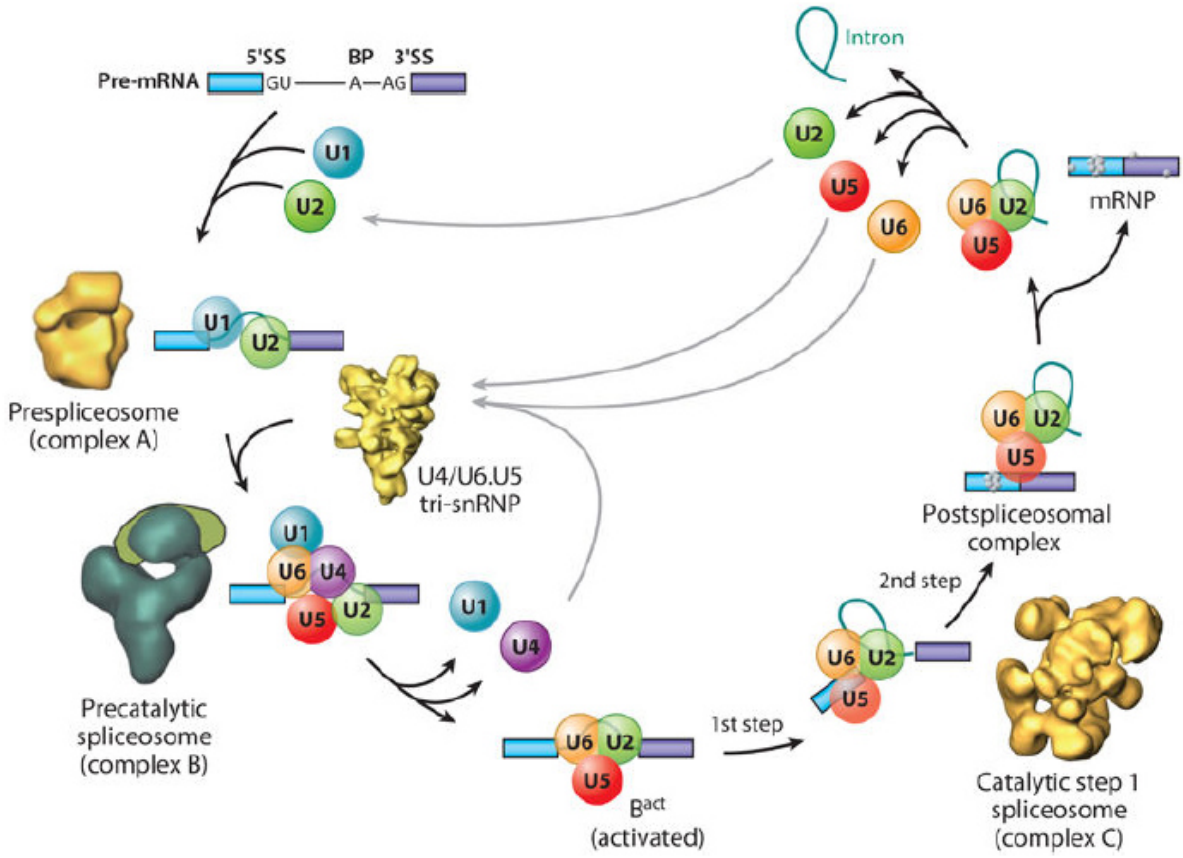

Figure 1. The process of removing an intron from a pre-mRNA containing two exons (blue and purple) by snRNPs. Figure from Lee e Rio, 2015.

1.2. Alternative splicing

In the whole RNA splicing process variability can occur, enabling alternative splicing events. Alternative splicing displays an essential mechanism to sustain the phenotypic diversity of higher eukaryotes that it expands gene expression complexity without an elevation at the large number of genes (Havens *et al.*, 2013). Up to 59% of human genes produce multiple mRNAs by alternative splicing and about 80% of alternative splicing leads to alterations in the encoded protein (Lee e Rio, 2015). Alternative splicing creates segments of mRNA variability that can include or exclude amino acids, shift the reading frame, or insert a termination codon. The alternative splicing process also manipulates gene expression by excluding or introducing regulatory elements controlling translation, mRNA stability, or localization (Faustino e Cooper, 2003). Common alternative splicing events are skipping of exons and joining of different 5'ss and 3'ss. Differential protein binding to cis-acting

sequences in the pre-mRNA transcript mainly directs the regulation of alternative splicing. Cis-regulatory sequences consist of exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs). Depending on the function and location of the binding sites relative to other splicing signals, those splicing factor proteins are able to either favour or to impede splicing at a particular site. Splicing enhancer, when bound by its cognate protein, enables the splicing of a nearby exon, whereas a splicing silencer, when bound by its cognate protein, blocks or inhibits splicing at a particular site (Havens e Hastings, 2016). Trans-acting factors are characterized by binding to splicing enhancers and silencers and insert heterogeneous nuclear ribonucleoprotein (hnRNP) proteins and tissue-specific factors. Those factors can either enhance or inhibit the use of splice sites or some of them can act in both ways depending on the sequence and position of the target site in the pre-mRNA (Fig. 2). Proteins binding splicing enhancer and silencer elements usually bind in a sequence-specific manner to single-stranded RNA. RNA secondary structures and chromatin structures are also able to affect alternative splicing (Havens e Hastings, 2016).

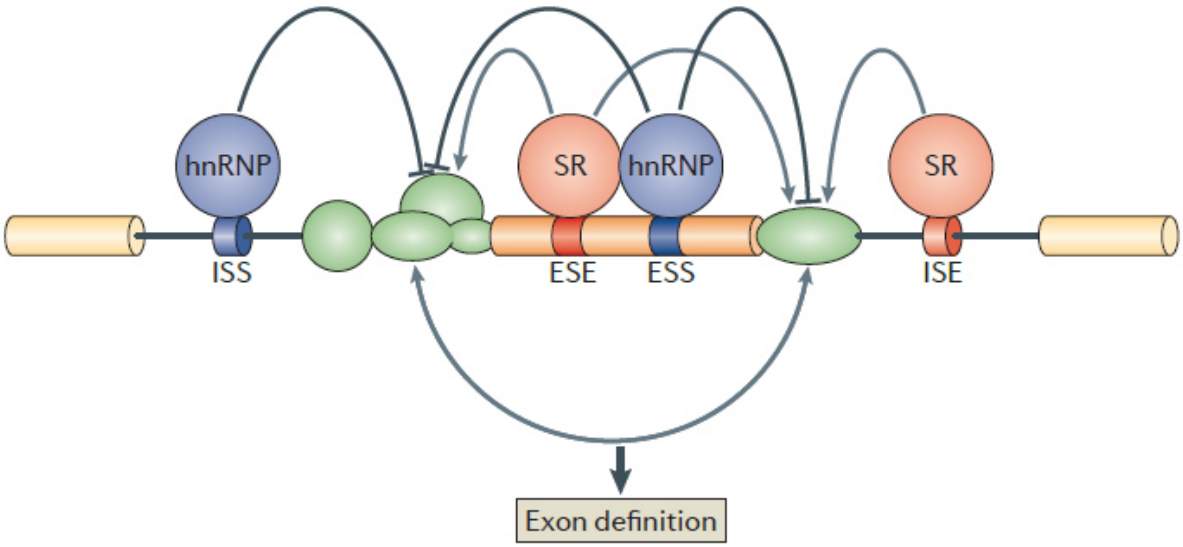

Figure 2. Splicing is conducted by cis-regulatory sequences in the pre-mRNA (ESEs, ESSs, ISEs and ISSs) and two main families of alterative splicing regulatory proteins, Ser/Arg-rich proteins (SRs) and hnRNPs. These regulatory proteins aim at components of the spliceosome that associate with the 5'ss and the 3'ss marking the alternative exon and is able to either enabling or inhibiting the use of that site. Figure from Kornblihtt et al. 2013.

2. Alternative splicing and diseases

Nearly one tenth of all disease-causing mutations are led back to mutations that alter normal splicing (Havens *et al.*, 2013). Disease-associated mutations that appear within introns alter splicing pattern, but do not change coding sequences. Mutations in introns are able to halt the core splice sites resulting in the skipping of one or more exons upstream or downstream of the mutated splice site or in the retention of the intron. Mutations that occur within coding exons can lead to disruption on an RNA secondary structure that has a regulatory function, or they intermit a splicing silencer or enhancer so that the site cannot be recognized by the sequence-specific RNA-binding protein, which is essential for splicing at a particular site. Mutations in the splicing process generally lead to formation of an aberrant mRNA or quantitative alterations in alternative mRNA isoform abundance and loss of normal protein expression (Havens *et al.*, 2013). Well investigated diseases caused by gene mutations are Duchenne Muscular Dystrophy (DMD), Spinal Muscular Atrophy (SMA), X-linked agammaglobulinemia (XLA) and dilated cardiomyopathy (DCM).

Duchenne Muscular Dystrophy

One of the best studied diseases caused by mutations in the splicing process is Duchenne Muscular Dystrophy (DMD). DMD is an X-linked, inherited and progressive muscle-wasting disease, affecting around 1 in 5,000 newborn boys. Patients, who are diagnosed with DMD, are typically between the ages of 3 and 5 years (Heemskerk *et al.*, 2010). The gene where mutations occur is the dystrophin-encoding DMD gene. Dystrophin offers a scaffolding function in muscle fibers and is responsible for the ligation of the actin cytoskeleton and the extracellular matrix. Thus it can take in the force which is generated during muscle fibers contraction. Mutations in the dystrophin-encoding DMD gene are mostly deletions of one or more exons. Since the DMD gene is extremely large, many different mutations can be found in patients with the majority carrying a deletion located between exon 43-55. These deletions disrupt the open reading frame and therefore only pre-maturely truncated proteins can be built and the ligation between the action cytoskeleton and the extracellular

matrix can not occur. This results in continuously damaged fibers during normal exercise and as a consequence in complete loss of muscle fibers and muscle function (Aartsma-Rus, 2010). Patients suffer severe disability, loss of ambulation and often die before the age of 30 years due to respiratory and/or cardiac failure (Heemskerk *et al.*, 2010).

A clinically milder disorder is Becker muscular dystrophy (BMD), which is provoked by in-frame dystrophin deletions that enable the production of a truncated but partially functional dystrophin. Although there is a large variation in the clinical severity of BMD patients, the progression of the disease is slower and patients often remain ambulant and experience a nearly normal life and lifespan (Anthony *et al.*, 2011).

Spinal muscular atrophy

Spinal muscular atrophy (SMA) is a neuromuscular disorder with severe muscle weakness and atrophy of the voluntary muscles of the limbs and trunk, eventually leading to paralysis as a result of the degeneration of motor neurons in the anterior horn of the spinal cord (Hua *et al.*, 2010). In its most severe form, it can also lead to infant death. The disease is caused by insufficient production of the survival of motor neuron (SMN) protein which results from deletion in the SMN1 gene. The main function of SMN is to induce the assembly of snRNPs (Hua *et al.*, 2010). The SMN2 gene is a paralog of SMN1 and is able to modify SMA. Most SMN protein in the cell is generated from the SMN1 gene. Patients with SMA do not own a functional version of SMN1 mostly because of the deletion of parts of the gene. SMN2 differs from SMN1 in a single nucleotide difference in exon 7, which disrupts splicing and leads to skipping of exon 7 in most SMN2 mRNA transcripts. This SMN2 exon 7-skipped mRNA isoform produces an SMN protein isoform that is not stable and does not operate in the same manner as the full-length SMN isoform. Patients with a large number of multiple SMN2 (Type 2, 3, 4) copies display a less severe form of the disease, whereas those with the fewest copies often die within the first months of life (Type 1) (Rigo *et al.*, 2014; Havens e Hastings, 2016).

X-linked agammaglobulinemia

X-linked agammaglobulinemia (XLA) belongs to the group of inherited agammaglobulinemia. It is characterized by a B cell lineage developmental block, resulting in an essential lack of mature B and plasma cells and decrease of immunoglobulin levels. XLA is caused by splicing defects in the gene which is responsible for the expression of Bruton's tyrosine kinase (BTK), a cytoplasmic, nonreceptor tyrosine kinase. The splicing defect is led back to an A-to-T transition in intron 4 of the BTK gene inducing a novel 5'ss and together with a preexisting cryptic 3'ss upstream in the same intron, lead to the inclusion of the cryptic exon 4a between exons 4 and 5 in the mRNA. Thus, the reading frame is altered and the BTK protein can not be expressed. XLA patients often suffer recurrent infections by pyrogenic bacteria or enteroviruses, which cause dermatomyositis and fatal chronic encephalomyelitis (Bestas *et al.*, 2014).

Dilated cardiomyopathy

Diltated cardiomyopathy (DCM) is caused mostly by truncating mutations in the TTN gene encoding titin. Titin is a modular sarcomeric protein and functions as a stretch sensor transmitting signals from its anchor at the Z-disk to its carboxyterminal kinase (TK) domain at the M-band (Gramlich *et al.*, 2015). The human mutation in TTN is located in exon 326 and is responsible for a frameshift with a premature stop codon in A-band titin. Patients with this mutation suffer from heart diseases characterized by ventricular dilation, systolic dysfunction, and progressive heart failure (Gramlich *et al.*, 2015).

Since there have hardly been any specific treatment options in the above mentioned diseases, there has been great interest in identifying drugs that can specifically modulate splicing in ways that may work to treat disease symptoms. Especially one approach has proved to offer great potential in specifically modulating splicing at any given site – short antisense oligonucleotides (ASOs). ASOs are able to manipulate splicing by base-pairing in an antisense orientation to a specific pre-mRNA sequence (Havens e Hastings, 2016).

2.1. Antisense mechanisms

There are a lot of definitions what ASOs might be. The one I think is the most appropriate is that ASOs are oligonucleotides, consisting of 8 to 50 nucleotides, binding through Watson-Crick base pairing and upon binding to RNA, modulating the function of the targeted RNA. Watson-Crick base pairing is regulated by specific hydrogen bonding interactions between bases on the ASOs and the target RNA strand and hydrophobic interactions resulting from base shape complementarity and coaxial base stacking (Bennett e Swayze, 2010). ASOs that specifically target splicing are called splice-switching oligonucleotides (SSOs). SSO base-pairing to a target RNA induces an altered recognition of splice sites by the spliceosome, resulting in an alteration of normal splicing of the targeted transcript.

By binding to an exonic splicing enhancer (ESE) sequence SSOs induce a steric block to the binding of the stimulatory splicing factor to its cognate enhancer binding site resulting in exon skipping. Through binding of an SSO to an intronic splicing silencer (ISS) sequence though, a block of its activity is created by hindering binding of a negatively acting splicing factor. Disruption of the binding of splicing inhibitory proteins to its cognate binding sequence induces splicing at the splice site and results in exon inclusion (Fig. 2) (Havens e Hastings, 2016).

SSOs are vulnerable to degradation by the RNA-cleaving enzyme RNase H. This enzyme forces the cleavage of the RNA in an RNA-DNA heteroduplex resulting in release of the intact DNA strand. To protect SSOs from degradation by RNase H, modifications of the nucleotides of an SSO have been crucial (Bennett e Swayze, 2010). Moreover modifications improve the binding affinity and delivery efficiency of SSOs.

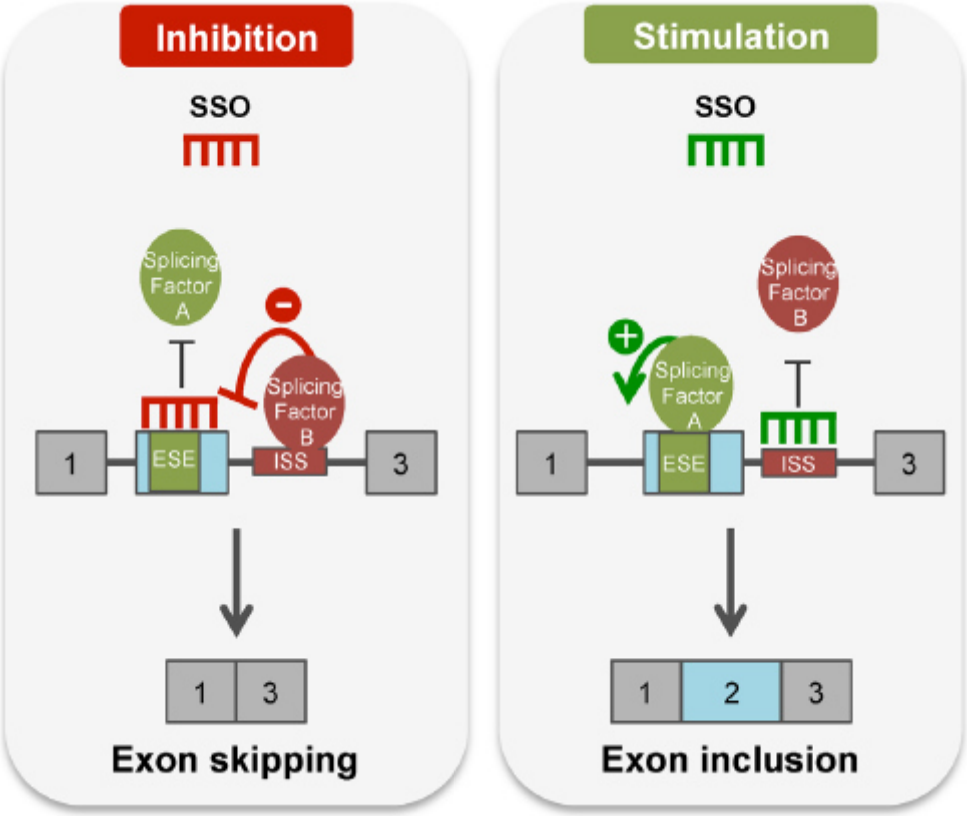

Figure 3. Normally exonic splicing enhancer (ESE) induces the splicing of exons and therefore exon inclusion while intronic splicing silencer (ISS) inhibits the splicing of exons resulting in exon skipping. (left panel) When binding of an SSO to an exonic splicing enhancer results in exon skipping. (right panel) Binding of an SSO to an intronic splicing silencer induces the inclusion on an exon. Figure from Havens e Hastings, 2016.

3. Chemistry of splice switching oligonucleotides

The binding affinity, stability and pharmacodynamic properties of oligonucleotides can be enhanced by chemical modifications. For improving the delivery efficiency, chemical oligonucleotide backbone modifications, covalent conjugation with transport vehicles and supramolecular assembly into nanosized formulations are considered (Lächelt e Wagner, 2015).

Since phosphodiester linkage displays an inherent instability to nucleases, the oligonucleotide backbone offers a perfect target for improvements with chemical modification (Bennett e Swayze, 2010). Changes to the phosphate backbone can lower the negative charge of phosphodiester bond, elevate nuclease resistance, enhance pharmacokinetic characteristics in vivo and alleviate cell uptake. Backbone modifications contain phosphorothiate (PS) and peptide nucleic acids (PNAs). Sugar modifications of oligonucleotides are supposed to reduce immunogenicity and toxicity, elevate RNA binding affinity and enhance stability and bioavailability. 2'-O-methyl (2'-OMe), 2'-O-methoxyethyl (2'-MOE) and locked nucleic acid (LNA) are the most common representatives of sugar modifications (Chen *et al.*, 2018). These naked oligonucleotides can be conjugated with several components to improve their delivery, such as cell-penetrating peptides (CPPs). The conjugated oligonucleotides include vivo-morpholinos (VPMO) or peptide phosphorodiamidate morpholino oligomers (PPMOs) (Godfrey *et al.*, 2017).

3.1. Chemical modifications

Phosphorothiate-containing oligonucleotides

Phosphorothiate (PS)-containing modification was the first analogue that was used in clinical applications (Flierl *et al.*, 2015). By substitution of one of the non-bridging phosphate oxygen atoms with a sulfur atom, the chemical structure of PS-containing oligonucleotides differs from the structure of natural nucleic acids (Fig. 4). This replacement of sulfur for oxygen in the phosphate ester improves stability in vivo and increases resistance towards nucleases. The PS modification of oligonucleotides also enhances the binding of SSOs to plasma proteins and thus inhibits rapid renal excretion and allows binding to other acceptor sites that facilitate uptake into tissues (Bennett e Swayze, 2010). Since SSOs with a PS backbone are not resistant to RNAse H, additional modifications to the molecule are required to create a steric blocking SSO for splice-switching applications (Havens e Hastings, 2016).

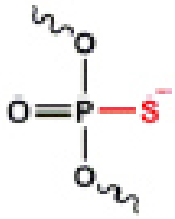

Figure 4. Chemical structure of PS. Figure from Lächelt e Wagner, 2015.

Peptide nucleic acid backbone modification

Peptide nucleic acids (PNAs) are nucleobase oligomers that contain N-(2-aminoethyl) glycine units as replacement for the sugar phosphate backbone but still maintain the ability to Watson-Crick base pair with complementary RNA and DNA (Shakeel *et al.*, 2006). Because of this substitution, PNAs are chemically stable and resistant to degradation by nucleases and

proteases and do not induce RNase H. Despite their neutral peptide backbone, PNAs can not easily pass cell membranes. To improve tissue distribution PNAs have been conjugated with short peptides or charged amino acids, like lysine, at their C terminus (Bennett e Swayze, 2010).

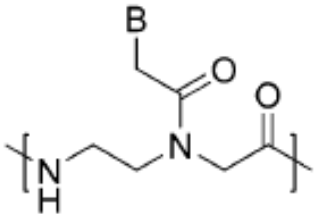

Figure 5. Chemical structure of PNA. Figure from Dragulescu-Andrasi et al., 2006.

Sugar modifications

Oligonucleotides that are fully modified at the 2' sugar position display RNAse H-resistance and are generally employed as SSOs. Modifications to the 2'-position enhances binding affinity and the proximity of the 2'-substituent to the 3'-phosphate in an oligonucleotide causes 2'-modified oligonucleotides to improve RNase H-resistance. However, this increased RNase H-resistance leads to reduction or complete inhibition of the ability to cleave the RNA strand opposite the modification. To minimize this limitation a gapmer strategy has been used, where regions of 2'-modified residues flank a central DNA region of the oligonucleotide. The 2'-modified wings enhance affinity and nuclease resistance, whereas the central gap region facilitates RNase H-mediated cleavage of the target RNA (Bennett e Swayze, 2010).

Especially 2'-O-methyl (2'-OMe) and 2'-O-methoxyethyl (2'-MOE) are used as alterations at the 2' position. 2'-MOE is currently the most advanced modification and has entered clinical trials for multiple indications, for example in the treatment of DMD and SMA (Fig. 6). 2'-MOE increases melting temperature by about +2°C per modification versus RNAs and enhances resistance to nucleases. Moreover 2'-MOE limits certain nonspecific protein binding resulting in reduced toxicities (Bennett e Swayze, 2010).

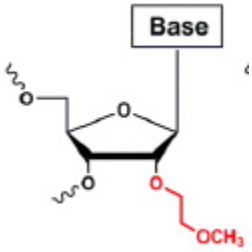

Figure 6. Chemical structure of 2'-MOE. Figure from Lächelt e Wagner, 2015.

SSOs employing 2'-O-methyl nucleosides (2'-OMe) have already advanced to human clinical trials (Fig. 7). 2'-OMe is considered as well tolerated and can limit off-target effects due to reducing their ability to serve as microRNA agonists (Bennett e Swayze, 2010).

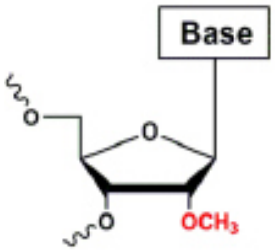

Figure 7. Chemical structure of 2'-OMe. Figure from Lächelt e Wagner, 2015.

Locked nucleic acid (LNA) chemistry is another modification of the sugar position, which implies bridging of the furanose ring (Fig. 8). The LNA modification can be considered as a constrained analog of 2'-OMe RNA, where the 2'-substituent is conjugated to the 4'-C atom.

This modification results in a major benefit from a dramatically improved binding affinity of LNA modified SSOs and therefore allowing the use of shorter SSO sequences. Thus, a shorter sequence can lower the likelihood of binding to an incorrect site as a result of partial sequence complementarity to another sequence and can reduce the risk of undesirable off-target effects (Havens e Hastings, 2016). The improved binding affinity can be explained by an increase in melting temperature caused by the binding of an LNA base to DNA. As more LNA bases are added, the increase of the melting temperature per LNA base drops. This indicates that short LNA oligomers combine high affinity binding with minimal size (Braasch e Corey, 2001).

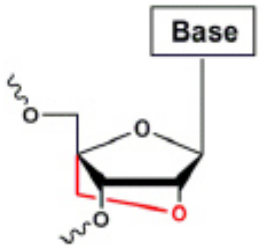

Figure 8. Chemical structure of LNA. Figure from Lächelt e Wagner, 2015.

Each of these mentioned modifications can be combined to successfully target splicing in ways predicted to be therapeutic for a number of different pathological conditions.

Phosphorodiamidate morpholinos (PMOs) are another version of modified oligonucleotide that has been applied widely to modify splicing (Fig. 9). PMOs consist of a morpholine ring instead of the furanose, with a phosphorodiamidate linkage connecting the morpholine nitrogen atom with the hydroxyl group of the 3'-side residue. The phosphorodiamidate linkage is the reason for the neutral charge of PMOs. This neutral charge leads to low binding of plasma proteins, which enhance tolerability in vivo. However, they are also quickly cleared by the kidney and therefore demonstrate reduced accumulation in tissues compared to a charged PS backbone. Moreover they do not induce RNase H. PMO is besides 2'-OMePS the second AON chemistry that is currently being studied extensively toward clinical application in the treatment of DMD (Heemskerk *et al.*, 2010).

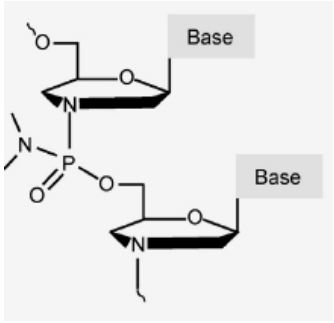

Figure 9. Chemical structure of PMO. Figure from Havens e Hastings, 2016.

Hammond et al. describe a novel SSO non-base modifier to improve splice-switching efficacy. The modifier, N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine (dubbed "ZEN™"), can be introduced to any anionic oligonucleotide to improve its binding affinity by elevating the melting temperature. ZEN has been successfully employed in anti-microRNA oligonucleotide designs, markedly enhancing the potency of 2'OMe-based antagomirs in vitro (Hammond *et al.*, 2014).

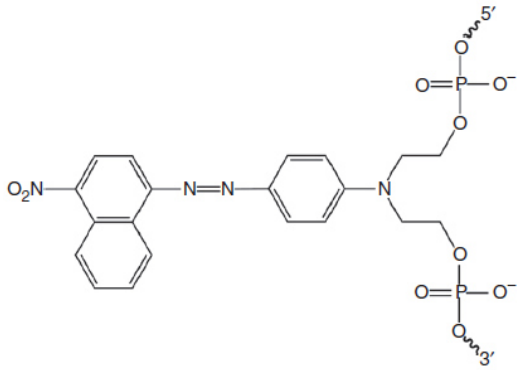

Figure 10. Chemical structure of ZEN. Figure from Hammond et al., 2014.

3.2. Covalent conjugation

Oligonucleotides can be conjugated to ligands, such as cell-penetrating peptides (CPPs), that can bind to various acceptor sites which are expected to alter the pharmacokinetic properties of the oligonucleotide in hope to improve tissue and cellular uptake. Conjugation of PMO to an arginine-rich cell penetrating sequence, termed PPMO, can significantly reduce the PMO dose (Fig. 11) (Clayton *et al.*, 2014). Further studies also confirmed that therapeutic effects of PPMOs have been far more efficient compared to the unconjugated SSOs, PMO and 2'-OMePS (Lu-Nguyen *et al.*, 2015). PMOs can also be conjugated to arginine-based peptides with a 6-aminohexanoic-spaced oligoarginine to enhance the uptake into nucleus and cytosol in vitro and in vivo. It is assumed that the active parts of arginine peptides are the guanidinium head groups of the arginine residues. The guanidinium head groups have been predicted to interact with phosphates of phospholipids both by electrostatic attraction and multiple hydrogen bonds and, as such, each guanidinium head group is capable of a charge-charge interaction plus hydrogen bonding. For most efficient uptake a total of eight guanidine head groups on two of the side chains of a triazine core have been installed, termed Vivo-Morpholinos (VPMOs) (Morcos *et al.*, 2008).

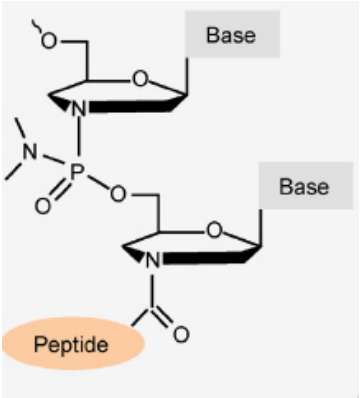

Figure 12. Chemical structure of PPMO. Figure from Havens e Hastings, 2016.

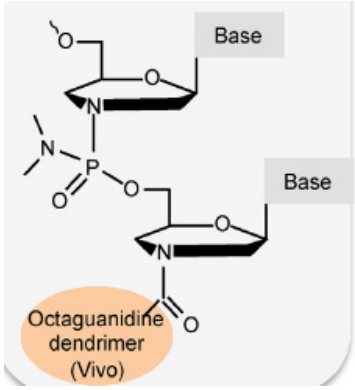

Figure 11. Chemical structure of VPMO. Figure from Havens e Hastings, 2016.

3.3. Supramolecular assemblies

In spite of all these chemical modifications, it is still difficult to deliver oligonucleotides into cells as easily as successful small drug compounds. Therefore, covalent conjugation with delivery moieties such as targeting or membrane translocating compounds displays a second encouraging delivery option for oligonucleotides. Especially noncovalent complexation and encapsulation into carrier nanoparticles are considered as effective delivery agents. Lipid-based nanoparticles include liposomal encapsulations or nucleic acid complexes with cationic liposomes, called lipoplexes. Polymer-based nanosystems imbed polymerosomes and polymer micelles, hydrogels, and complexes of nucleic acids with cationic polymers, called polyplexes.

The polyplex formation process is based on the entropy-driven ionic interaction between the polyanionic nucleic acid and the multivalent cationic polymers. This formation process is able to yield nanoparticles containing the nucleic acid payload in compacted and protected form. The size of polyplexes can reach from small sizes of 6 nm to larger sizes of one to several hundreds of nanometers (Lächelt e Wagner, 2015). The polyplex size has a tremendous impact on systemic administration, biodistribution and pharmacokinetics. Small nanoparticles with a hydrodynamic diameter of below 6 nm are quickly cleared by the kidneys (Lächelt e Wagner, 2015). Nanoparticles with sizes of up to 400 nm though can enable accumulation in highly vasularized solid tumors (Lächelt e Wagner, 2015).

Since the stability of polyplexes in blood and other biological fluids are seen as critical, more stable packaging of polyplexes and shielding the positive surface charges against unwanted specific interactions with the bioenvironment are essential measures to overcome this problem.

Cationic core polymers

Cationic polymers can be considered as fitting noncovalent interaction partners of oligonucleotides. They are able to bind, condense, protect and release the oligonucleotides. Moreover they can trigger intracellular uptake and mediate endosomal membrane

destabilization required for cytosolic release and delivery to the intended site of action. However, within the bioenvironment cationic polymers are faced with many unwanted interactions, leading to loss of activity and onset of toxicity (Lächelt e Wagner, 2015).

Polyethylenimine

Polyethylenimine (PEI) is considered as a very potent and commonly used as transfection polymer. The transfection efficiency is relatively high both in vitro and in vivo at dosages where the polymer shows no or only slight cytotoxicity. PEIs can be synthesized with branched and linear topology of different molecular weight ranges, which allows modulate polyplex stability, delivery efficacy and toxicity. Since the oligonucleotides have to be liberated at the intracellular site of action, the flexible linear PEI (LPEI) polyplexes have proved to be better in intranuclear delivery in nondividing cells than branched PEI (BPEI) polyplexes, which require dividing cells in or before mitosis for efficient transfection. The cytotoxicity of polyplexes can be decreased by conjugation of LPEI or BPEI with the nature-derived polymer beta-cyclodextrin (CD) without reducing the transfection efficiency (Lächelt e Wagner, 2015).

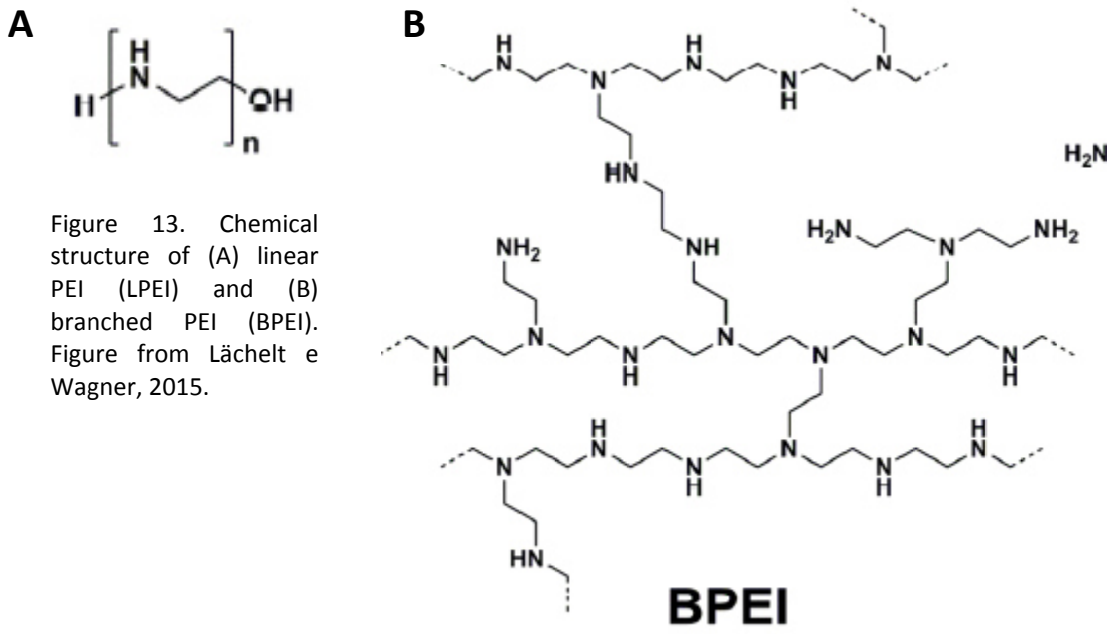

Figure 13. Chemical structure of (A) linear PEI (LPEI) and (B) branched PEI (BPEI). Figure from Lächelt e Wagner, 2015.

Dendritic polyamidoamines

Dendritic polyamidoamines (PAMAM dendrimers) exhibit proton sponge activity because of their high charge density but also show cytotoxicity (Fig. 14). The proton sponge activity is based upon the large buffering capacity of polycations which is considered to increase the pH. The efficacy of PAMAM dendrimers can also be enhanced by conjugation with alpha-, beta-, and gamma-CDs (Lächelt e Wagner, 2015).

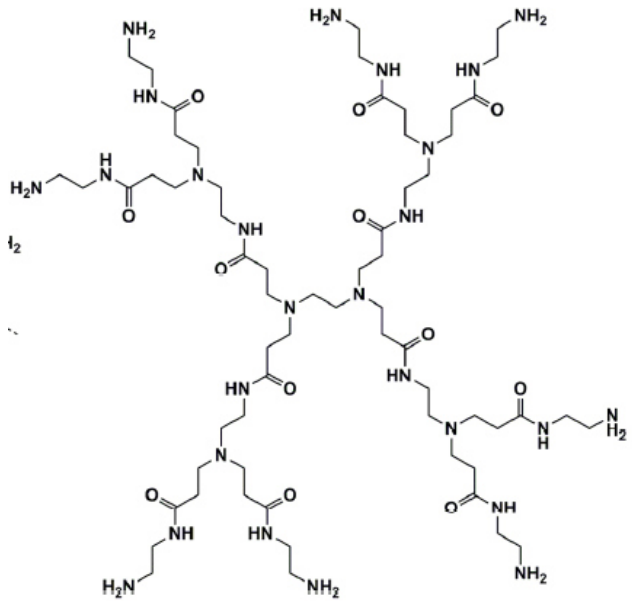

Figure 14. Chemical structure of PAMAM. Figure from Lächelt e Wagner, 2015.

Shielding domains

Efficient complexation of oligonucleotides with remnants of polycations normally leads to formation of nanoparticles with positive zeta potential. Although this positive charge favors binding of negatively charged cell surfaces and helps in endosomal escape, within the extracellular space this positive surface charge induces many unwanted reactions, mostly after intravenous injections (Lächelt e Wagner, 2015). Acute toxicity provoked by partial dissociation and aggregation of nanoparticles and blood cells, unspecific interactions with complement and other blood components or nontarget cells can be lowered by providing

polyplexes with a coat of hydrophilic macromolecules, which shield the surface potential from the exterior environment. Polyethylenglycol (PEG) displays the most common shielding agent. Shielding domains strongly enhance biocompatibility, shielding from unspecific extracellular interactions and markedly extend blood circulation time. Nevertheless, stable polyplex shields may also lower intracellular efficacy when transfecting the target cells (Lächelt e Wagner, 2015).

Lipoplexe/liposomes

Another way to increase efficiency of delivering SSOs into the cells after systemic administration can be effectuated by incorporating the SSOs into liposomes. Encapsulation of SSOs in lipid nanoparticles maintains the SSOs intact and biologically active after administration. Lipid nanoparticles are able to exhibit extended blood circulation profiles, which is particularly important for tumor-targeted SSO delivery. Due to increased circulation half-life the therapeutics are enabled to accumulate in the tumor tissue through a tumor's leaky vasculature referred as enhanced permeability and retention effect (EPR effect). Since lipoplexes cannot pass tight endothelial junctions of normal blood vessels, they exploit the abnormalities of tumor vasculature like the aberrant vascular architecture and extensive production of vascular permeability factors inducing extravasation within tumor tissues. Once lipoplexes have penetrated endothelial junctions of tumor tissues they accumulate there due to the lack of lymphatic drainage (Fang *et al.*, 2011). Encapsulation of SSOs in cationic liposomes is based on electrostatic interaction between the cationic lipid headgroup and the anionic backbone of oligonucleotides. Lipoplexes are assembled of a higher order multilamellar structure with SSOs sandwiched between cationic bilayers (Wang *et al.*, 2015).
Especially cationic liposomes are employed for SSOs delivery since the multiple cationic groups on the surface of the liposomes are able to interact with polyanionic oligonucleotides and form lipoplexes. Cationic lipids can be divided into three major categories based on the head group structure: monovalent lipids such as N (1-(2,3-dioleyloxy) propyl)-N,N,N-trimethylammonium chloride (DOTMA) and 1,2-dioleyl-3-trimethylammonium-propane (DOTAP), multivalent lipids such as dioctadecylamidoglycylspermine (DOGS) and cationic lipid derivatives such as 3β-(N-(N',N'-dimethylaminoethane)-carbamoyl) cholesterol (DC-

Chol) (Wang *et al.*, 2015). The hydrophobic myristoyl (C14) chain seems to be perfect for transfection, whereas longer chains (C16 and C18) elevate the phase transition temperature and lower the fluidity of the lipid membrane, which is unfavorable for lipid membrane fusion and ion-pair formation. Although there are different types of lipoplexes, they have some structural similarities in common such as i) amide linkages, ii) more than two alkyl tails, iii) an 8-12 carbon chain tail and iv) one tail that is not substituted by amine reactants and includes one secondary amine (Wang *et al.*, 2015).

Similar to formulated SSOs and polyplexes, lipoplexes also face the same critical issues that hinder the in vivo application of lipoplexes such as fast serum clearance and a major distribution in the lung, liver and spleen after intravenous injection. Thus, cationic liposomes are also endowed with PEG displaying better colloidal stability as consequence of low charge and surface steric hindrance that hinders the adsorption of serum proteins.

Alternative approaches

Another interesting approach of enhancing the pharmacological effects of antisense SSOs demonstrates Ming et al. in their work. The substances they used, termed Retro compounds, were identified after a high throughput screen for small molecules that reduce the harmful actions of bacterial and plant toxins (Fig. 15). Ming et al. hypothesized that the Retro compound might beneficially influence the intracellular trafficking of oligonucleotides so as to enhance their pharmacological effects. This effect appears at the level of intracellular trafficking or processing and is correlated with elevated oligonucleotide accumulation in the nucleus, but does not embrace the pertubation of lysosomal compartments. Retro-1 induces a rapid partial release of SSOs from a subset of endomembrane compartments, where they have accumulated but are pharmacologically inert, and therefore enable SSOs to enter into the cytosol and then the nucleus (Ming *et al.*, 2013).

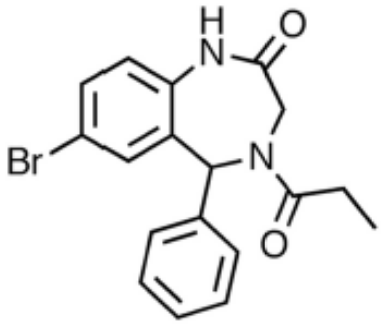

Figure 15. Chemical structure of Retro-1. Figure from Ming et al., 2013.

	Type	Structure	Pros	Cons
Backbone modification	Peptide nucleic acid (PNA)	N-(2-aminoethyl) glycine units as replacement for the sugar phosphate backbone conjugation with short peptides or charged amino acids	• maintain ability to Watson-Crick base pair with complementary RNA and DNA • chemically stable • resistant to degradation by nucleases and proteases • do not induce RNase H	• can not easily pass cell membranes
Backbone modification	Phosphorothiate (PS) containing modification	substitution of one of the nonbridging phosphate oxygen atoms with a sulfur atom	• improved in vivo stability • enhanced nuclease resistance • enhanced binding affinity to plasma proteins • inhibition of rapid renal excretion	• degradable by RNase H
Sugar modification	2'-O-methoxyethyl nucleotide (2'-MOE)	integration of methoxyethyl remnant at 2' sugar position	• improved RNase H resistance • limitation of nonspecific protein binding • enhanced binding affinity	• reduced cleavage of target RNA
Sugar modification	2'-O-methyl nucleotide (2'-OMe)	integration of methyl remnant at 2' sugar position	• well tolerated • can limit off-target effects due to reducing their ability to serve as microRNA agonists	• reduced cleavage of target RNA
Sugar modification	Locked nucleic acid (LNA)	bridging of the furanose ring	• well improved binding affinity allowing the use of shorter SSO sequences • reduced risk of undesirable off-target effects	

Sugar modification	Phosphorodiamidate morpholino	substitution of a morpholino ring instead of the furanose ring, with a phosphorodiamidate linkage connecting the morpholine nitrogen atom with the hydroxyl group of the 3'-side residue	• enhanced tolerability in vivo	• rapid renal clearance • reduced accumulation in tissues • do not induce RNase H
	N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine (ZEN)	can be introduced into any anionic oligonucleotide	• improves binding affinity of conjugated SSOs	
Covalent conjugation	PMO conjugated to cell-penetrating peptides (PPMO)	conjugation of arginine-rich cell penetrating sequence	• reduces PMO dose • enhanced cellular uptake • enhanced endosomal release	
Covalent conjugation	PMO conjugated to octaguanidine dendrimers (VPMO)	installing of 8 guanidine head groups on 2 of the side chains of a triazine core	• reduces PMO dose • enhanced cellular uptake • enhanced endosomal release	

Table 1. Characteristics of chemically modified SSOs.

4. Routes of administration

As SSO drug design finally aims at the efficient treatment of human diseases, efficient delivery to target tissues and –cells is indispensable. Intraperitoneal (i.p.), subcutaneous (s.c.) or intravenous (i.v.) administrations lead to exposure of many peripheral tissues to the oligonucleotide. More tissue-specific delivery has been tried to achieve by other approaches, such as the intramuscular (i.m.), intratumoral (i.t.m.), subconjunctival (s.c.j.) or intravitreal (i.v.i.) injection of SSOs. Since SSOs do not easily cross the blood brain barrier when administered peripherally, intracerebroventricular (i.c.v.) or intrathecal (i.t.) administration has been shown to result in therapeutic doses of SSOs throughout the CNS (Fig. 16) (Havens e Hastings, 2016).

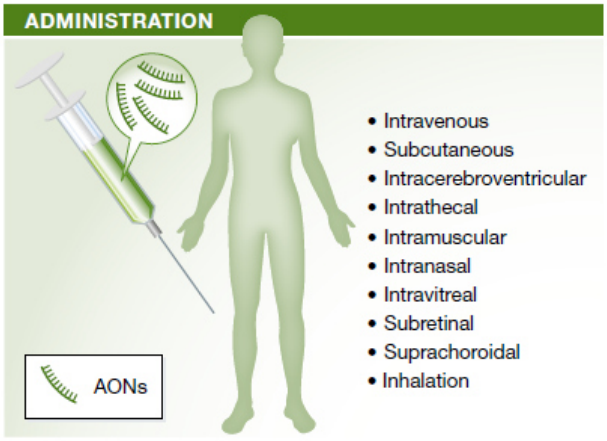

Figure 16. Different administration routes of SSOs. Figure from Havens e Hastings, 2016.

5. In vivo studies with different types of splice-switching oligonucleotides

Transgenic EGFP mouse model

For many in vivo studies testing SSOs transgenic mice have been applied as test model. The transgenic mouse model provides a convenient platform for comparison since it is a positive readout test for the activity, delivery, and distribution of SSOs. The transgenic mice express a truncated gene (EGFP-654) due to aberrantly spliced mutated intron of the human β-globin gene (Sazani *et al.*, 2002). Normally the gene encodes the enhanced green fluorescence protein (EGFP), but because of the aberrant splicing the expression of EGFP-654 is prevented. Studies have now been testing SSOs in restoring correct splicing and therefore upregulation of the EGFP-654 expression. After a disruption of the EGFP coding sequence caused by a mutated intron 2 of the human β-globin aberrant splice sites are induced. This results in retention of the intron fragment in spliced mRNA and translation of EGFP is inhibited. SSOs are considered to restore correct pre-mRNA splicing by blocking the aberrant splice site and increase EGFP expression (Sazani *et al.*, 2002). The upregulation of EGFP provides direct quantitative measurement of the sequence-specific antisense activity of the tested SSOs.

For testing the efficiency of different ways of administration and biodistribution of LNA modified SSOs, this transgenic mouse model was used. When a dose of 25 mg/kg was injected i.p. and i.v., LNAs mainly functioned in the liver, small intestine, and colon, where they induced significant increase in correctly spliced EGFP. The effect was sequence specific, meaning that LNAs with mismatches to the target pre-mRNA are ineffective. Even lower doses of LNAs correctly spliced EGFP in liver and small intestine (Roberts *et al.*, 2006). This might result from the superior serum stability of LNAs, allowing more intact molecules to reach target.

Compared to 2'-OMe oligonucleotides there are additional advantages for the use of LNA oligomers. LNA phosphorothiates may also have slower plasma and renal clearance, allowing

elevated blood concentrations to be achieved and therefore increasing the effective dose of the oligonucleotides. Slower plasma clearance also allows more time for target tissues to take up the drug. Moreover, LNA oligonucleotides have a markedly higher melting temperature for target sequences and therefore greater affinity for their target site of action. The antisense effect of LNA modified SSOs persisted for at least 3 weeks postinjection, while 2'-OMe oligonucleotides loose potency in less than 2 weeks. LNA modified SSOs have also been the first chemical modification of antisense oligonucleotides, which have been applied by oral administration, suggsting that smaller sized nucleotides pass the intestinal barrier and gain entry into the bloodstream. However, oral gavage of transgenic mice at a dose of 50 mg/kg resulted in a slight elevation in splicing pattern in the same tissues as by i.v. and i.p. administration (Roberts *et al.*, 2006). This suggests that a modest fraction of the orally administered oligonucleotide is passing the digestive tract and is able to reach the bloodstream as a functional molecule. High potency, persistence, and tissue specificity make LNAs attractive candidates for drugs that act via modulation of aberrant and/or alternative splicing.

When testing the efficiency of other chemically modified oligonucleotides, 2'-MOE-PS, PNAs and PMOs, higher dosages (50 mg/kg) compared to LNAs, are needed. PNAs are additionally carrying one or four lysines at their C terminus, which confer a positive charge whereas the remaining two are negatively charged or neutral. Since those antisense oligonucleotides are rapidly cleared from ciruculation by i.v. injection, i.p. injection is the preferred way of administration. 2'-MOE-PS and PNA-4K induced high levels of EGFP in the cardiac muscle in the cortex of the kidney and in the liver hepatocytes as well as in the lung (Sazani *et al.*, 2002). This shows that the oligomers are distributed by the lymphatic system and bloodstream throughout the mouse. The EGFP fluorescence was also induced in several intestinal structures, including the villi, the lamina propria and the smooth muscle lining, indicating that the oligomers are taken up and distributed throughout these tissues. Although the morpholino oligomers produced similar effects they were only detectable after four days of treatment. This could be due to fast distribution to and elimination of these compounds by the kidney. Since the administration of PNA-1K had no antisense effects in any tissue after four daily injections, this suggests that the four-lysine moiety contributes substantially to the in vivo activity of the PNA-4K oligomer. EGFP fluorescence was barely detectable in brain, skin, and stomach. For the brain, this was expected, because the

blood/brain barrier should prevent access of the oligomers to the cortex. In skin, it has been indicated by others that intact phosphorothiate oligonucleotides do not accumulate in the dermal layers of the skin, and that direct application is a more efficient route of oligonucleotide administration if antisense activity in the skin is desired. The superiority of the PNA-4K oligomer was also detected in the lung and muscle. The uncharged morpholino oligomer had low activity in all tested tissues (Sazani *et al.*, 2002). A reason could be the rapid distribution to and elimination of these compounds by the kidney, similar to the data obtained with neutral methylphosphonate and morpholino oligomers. The same mechanism may apply to the PNA oligomer with only one lysine (PNA-1K), which indicated no detectable levels of correction in any tested tissues. Pharmacokinetic studies indicated that 90% of uncharged PNA oligomers were secreted in urine. The remainder was distributed at low levels primarily to the liver and kidney as well as other tissues (Sazani *et al.*, 2002).

5.1. Targeting splicing in diseases

The chemical modifications of SSOs can be used to target splicing in ways predicted to be therapeutically active for a number of different pathological conditions. Therapeutic RNA splicing interventions are considered to correct aberrant splicing that is associated with diseases, or to induce aberrant splicing to prevent disease phenotypes.

5.1.1. Duchenne Muscular Dystrophy

Especially in the treatment of DMD, SSOs show promising results. In the last couple of years, progress has been made toward the development of SSOs as a potential therapy targeting the underlying genetic defect of DMD. Since DMD is caused by mutations in the dystrophin-encoding DMD gene, eventually leading to loss of muscle fibers and muscle functions, the use of SSOs aims to restore the reading frame or bypass a small mutation by skipping one or more exons. This results in the continuation of the translation and the formation of a largely functional protein, which is also found in the related, but much milder, BMD (Verhaart *et al.*, 2014).

The in vivo studies have mostly been conducted in transgenic mdx mice which do not express dystrophin due to a premature stop codon in exon 23 (Heemskerk *et al.*, 2010). Different chemical modifications of SSOs, as well as various forms of administration and dosages have been tested.

The SSO chemistry 2'-OMe-PS is currently studied extensively toward clinical application. When administering 2'-OMe-PS SSOs intravenously into mdx mice, gastrocnemius and triceps muscles showed four and eight times higher exon 23 skipping levels. The exon 23 levels in heart were significantly lower but still 2.5 times higher in mdx mice than in treated wild-type mice. Better results were obtained by i.m. injections, which showed 4-20 times higher exon 23 skipping levels in mdx mice compared to wild-type mice (Heemskerk *et al.*, 2010).

Placing the modifier N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine, termed ZEN, near the ends of 2'-OMe-PS increases the binding affinity by elevating the melting temperature. To further improve the delivery, lipid transfection can be used. While intramuscular administration of ZEN-modified SSOs led to a higher potency compared to non-ZEN-modified SSOs, i.v. administration only showed a low level of dystrophin protein restoration in diaphragm, quadriceps, tibialis anterior and heart (Hammond *et al.*, 2014). It is interesting to note that the use of naked i.v. administration of 2'-OMe-PS led to a significant increase in dystrophin protein within quadriceps muscle with an average of 3.75% dystrophin protein compared to only 1.12% generated by ZEN-modified 2'-OMe-PS. A possible reason for the lower in vivo efficacy could be the higher molecular weight of ZEN modified 2'-OMe-PS over non-modified 2'-OMe-PS. Although in vitro studies showed promising results for ZEN modified 2'-OMe-PS, these results could not be confirmed by in vivo studies, displaying no advantage to 2'-OMe-PS (Hammond *et al.*, 2014).

Since myostatin is a negative regulator of muscle mass, the blockage of myostatin offers another strategy for counteracting muscle-wasting conditions. Exon skipping of myostatin pre-mRNA enhances muscle hypertrophy and lower muscle necrosis offering a positive effect on the process of DMD (Lu-Nguyen *et al.*, 2015). SSOs are able to induce skipping of exon 2 of myostatin resulting in an out-of-phase splicing of exons 1 and 3, and knockdown of myostatin due to truncation of the open reading frame and nonsense-mediated mRNA decay (Kang *et al.*, 2011).

PMOs are the second type of antisense oligonucleotides which have entered clinical trials in the treatment of DMD and can also knockdown myostatin at the transcript level (Scully *et al.*, 2013). To enhance delivery and efficiency of exon skipping, PMOs can be conjugated with octa-guanidine dendrimers, called VPMOs. The guanidinium head groups are expected to interact with phosphates of phospholipids by electrostatic attraction and multiple hydrogen bonds. Intravenous injections of VPMOs increase the muscle mass and the level of exon skipping of myostatin in the soleus but do not influence the extensior digitorum longus (EDL) (Kang *et al.*, 2011). It is also notable that the efficiency of exon skipping by i.v. administration of VPMO in mdx mice is dose dependent. A higher dose is able to restore dystrophin protein levels similar to that of normal mice (Morcos *et al.*, 2008). PMOs can also be combined with arginin-rich B peptides, termed PPMOs, also demonstrating more efficient therapeutic effects compared to unconjugated PMOs when administered the same dose in neonatal mdx mice. Intraperitoneal injections of PPMOs showed dystrophin expression in body-wide skeletal plus cardiac muscles, whereas the highest level of exon skipping of dystrophin pre-mRNA was noticed in the diaphragm. The highest skipping levels of myostatin were seen in the soleus whereas the diaphragm showed the lowest skipping rate (Lu-Nguyen *et al.*, 2015).
Since the exon-skipping strategy is a sequence-specific therapeutic approach, targeting the human DMD (hDMD) gene offers another promising approach. Therefore, transgenic hDMD mice were established, which carry an integrated copy of the full-length human DMD gene. 2'-OMe-PS, which is able to specifically skip exon 46, was complexed to the delivery agent ExGen 500 (linear PEI) and was injected intramuscularly into the gastrocnemius muscle of the hDMD mice. One week after the injection skipping of the targeted exons 44, 46, and 49 from the human DMD transcript was noticed. Although PEI enhanced delivery of SSOs into the myofibers by lower doses, it still induced extensive fiber degeneration and regeneration, with dose-dependent infiltration of cytotoxic and helper T cells (Bremmer-Bout *et al.*, 2004).
Since most PEIs display a poor pharmacokinetic profile and high toxicity, mostly due to high molecular weight, decreasing the molecular weight of PEI below 2 kDa, referred as oligoethylenimines (OEIs), modified the in vivo distribution in mice after tail vein injection from liver to kidney accumulation and did not show any cytotoxicity, even at high doses (Chiper *et al.*, 2017).
However, OEIs do not exhibit nucleic acid delivery abilities on their own, probably due to their low molecular weight. To enhance the transfection efficacy, 1.8 kDa branched OEI was

modified with salicylamide and further equipped with methoxyethoxypolyethyleneglycol 550 (OEG) for polyplex shielding, referred to as SaOEI. SaOEI was complexed to 2'-OMe-PS, targeting exon 23 skipping, and injected into the tiabilis anterior muscle of mdx mice. Analysing the muscle fibers displayed that there was no increase in the number of dystrophin-positive fibers induced by SaOEI treatment compared to untreated muscles. Due to this disappointing result SaOEI was once again equipped with methoxyethoxypolyethyleneglycol 550 (OEG) to see if this designed SaOEIOEG was able to induce more dystrophin-positive fibers. Analysis showed that the SaOEIOEG complex led to about 260 dystrophin-positive fibers, corresponding to a correction of 13% of the total fiber number (Chiper *et al.*, 2017).

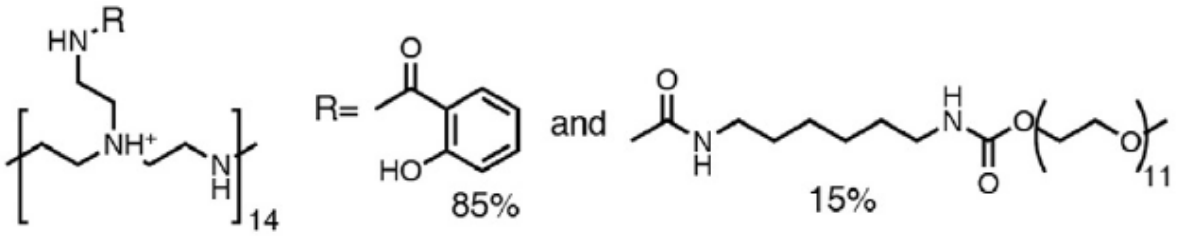

Figure 17. Chemical structure of SaOEIOEG.Figure from Chiper et al., 2017.

5.1.2. Spinal Muscular Atrophy

SMA is caused by inactivation of *SMN1* gene, resulting in significantly decreased SMN protein expression. Although the remaining *SMN1* paralog *SMN2* is also able to generate SMN protein (termed SMN2), this isoform is unstable and does not function in the same manner as the SMN protein expressed from *SMN1*. The instability of SMN2 protein is due to skipping of exon 7 in the SMN2 pre-mRNA. Since SMN2 is a genetic modifier of SMA, elevation of SMN2 expression and consequently increasing full-length SMN protein production from the gene has been a major focus of SMA therapeutic strategies. Because skipping of exon 7 is the major cause of the low SMN production from *SMN2*, SSOs which target exon 7 splicing and induce its inclusion to enhance expression of functional SMN protein have been tested in in vivo studies (Havens e Hastings, 2016).

2’-MOE-PS has proved to be the most potent SSO in central nervous system (CNS) tissues of adult mice, compared to other chemically modified SSOs. The 18mer 2’-MOE-PS targets a site in intron 7, that represses SMN2 exon 7 inclusion. 2’-MOE-PS can be administered into the CSF of adult mice by i.c.v. infusion and i.c.v. bolus injection. Results showed that both methods of delivery led to a dose-dependent increase in SMN2 splicing correction, observed as an increase in transcripts including exon 7 and a decrease in transcripts excluding exon 7, in the spinal cord and brain. The correction effect of SMN2 remained similar for over two months based on the observed level of correction in the spinal cord and brain on day two and 71 after i.c.v. infusion.. Also, the more efficient method of delivery proved to be the i.c.v. bolus injection, based on the half-maximal effective dose (EC_{50}). The EC_{50} for SMN2 splicing correction represents an indicator of how much drug has been taken up into tissues via a productive uptake route. The lower EC_{50} in CNS tissues indicates that the delivery of 2’-MOE-PS after i.c.v. bolus injection results in higher productive uptake compared to i.c.v. infusion (Rigo *et al.*, 2014).

Testing the relationship between the amount of 2’-MOE-PS in CNS tissue and the degree of SMN2 splicing correction after both i.c.v. infusion and i.c.v. bolus injection showed again, that the i.c.v. bolus injection of 2’-MOE-PS was more efficiently delivered when compared to the i.c.v. infusion. 2’-MOE-PS after i.c.v. bolus injection was broadly distributed throughout the spinal cord and brain with accumulation in cortical, striatal, hippocampal, and motor neurons. Both routes of administrations showed a long lasting effect after administration. After i.c.v. infusion for 7 days, SMN2 splicing correction in the spinal cord and brain was maintained for at least one year with no signs of microglial activation. Administration of a single i.c.v. bolus injection of 2’-MOE-PS also led to a prolonged pharmacological activity that was maintained for at least 36 weeks after the last injection in the spinal cord and brain (Hua *et al.*, 2010; Rigo *et al.*, 2014).

Since SMA already affects newborns, 2’-MOE-PS was also administered into embryos of mice. Type III SMA mice do not show any muscle weakness, but they do have progressive tail necrosis beginning at three weeks postnatally, and ear necrosis at 4-5 weeks (Hua *et al.*, 2010). In untreated mice, the tail is completely lost at an age of 5-6 weeks, or a short stub of dead tissue remains for several days, in addition to gradual loss of most of the ear tissue. To prevent or delay the onset of necrosis in type III SMA mice it is important to treat the mice as early as possible. Since i.c.v. infusion is only suitable for adult mice, 2’-MOE-PS was

administered by i.c.v. injection into embryos. 2'-MOE-PS markedly induced exon 7 inclusion but the effect dropped after 30 weeks. This result for a single embryonic injection was expected because with the increase in body size and cell proliferation, the effective concentration of 2'-MOE-PS will be reduced. I.c.v. injection of 2'-MOE-PS into embryos also remarkably rescued both the tail and ear necrosis of newborn mice. Mice treated with a high dosage showed no sign of necrosis and had intact tails at 2 months whereas mice with low dosage indicated a delay in the onset of tail and ear necrosis but had less phenotypical improvement (Fig. 18). However, compared to untreated wild-type mice the tails of treated mice were still shorter probably because SMN is essential for embryonic development and therefore the stage at which high levels of SMN are restored is important. High levels of SMN administered into the embryo may still not be as effective as normal levels at the beginning of embryo formation. Although tail necrosis could be clearly delayed for two months of age, it still occurred due to the limited concentration of 2'-MOE-PS in the growing mice. Performing an i.c.v. injection of 2'-MOE-PS in type III SMA neonates resulted in a delayed onset of tail necrosis for 2 weeks, and part of the tail still remained after 2 months (Hua *et al.*, 2010). The reduced phenotypic rescue in neonates-treated compared to embryonic-treated mice is a reflection of the timing rather than the extent of splicing correction. This suggests that earlier intervention is probably more effective.

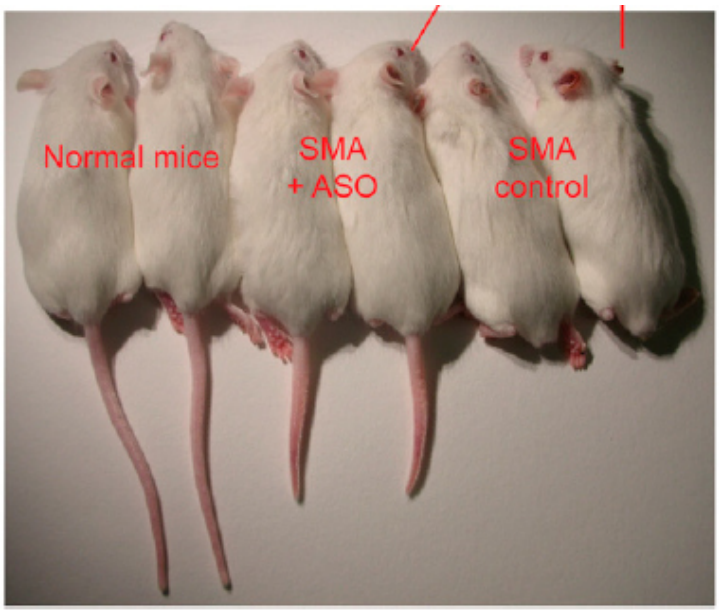

Figure 18. Rescue of type III SMA mice after a single embryonic i.c.v. injection of 2'-MOE-PS. Figure from Hua et al., 2010.

Administration of 2'-MOE-PS in neonatal mice by s.c. administration showed a dose-dependent increase in exon 7 inclusion in spinal cord, brain, liver, heart, kidney, and skeletal muscle, with the greatest effect appearing in the liver, whereas i.c.v. injection led to a much more robust change in exon 7 inclusion in brain and spinal cord tissues, but very limited effects in peripheral tissues. The median level of SMN2 exon 7 splicing in the spinal cord was way longer by s.c. administration with 108 days, compared with i.c.v. injection with only 1-2 weeks suggesting that systemic administration might be more effective than i.c.v. administration (Hua *et al.*, 2011). As i.c.v. injection, earlier s.c. administration led to better results on long-term survival, highlighting the importance of early-postnatal therapeutic intervention.

Comparing SSOs with different chemistries in the efficiency of exon 7 inclusion further confirms the high potency of 2'-MOE-PS in the treatment of SMA. Administration of 2'-OMe-PS did not show any effect on SMN2 splicing correction but rather led to a proinflammatory effect in the brain and the spinal cord. Administration of PMO led to a dose-dependent elevation of SMN2 splicing correction in spinal cord and brain, however 2'-MOE-PS was 3- to 5-fold more potent when administered to adult SMN2 transgenic mice (Rigo *et al.*, 2014).

5.1.3. Dilated cardiomyopathy

DCM is caused by truncating mutations in the Ttn gene encoding titin. The human mutation in Ttn is located in exon 326 and is responsible for a frameshift with a premature stop codon in A-band titin. Since vivo-morpholino-modified (VPMO) SSOs displayed good penetration of heart tissue, in vivo studies used them to skip of Ttn exon 326 in the heart of mice carrying an autosomal-frameshift mutation in titin exon 326. The animals received i.p. injection of a 28-mer VPMO. Echocardiographic analysis of systolic and diastolic function showed hypertrophic response one week after Ang II infusion, with decreased left ventricular end-diastolic diameters (LVEDD) and increased left ventricular ejection fraction (LV-EF) and wall thicknesses. After a two weeks treatment with the cardiac stressor protein Angiotensin II (Ang II), the mice treated with VPMO did not develop DCM, whereas animals treated with saline displayed a DCM-like phenotype characterized by a reduction in LV-EF and wall thicknesses and by an increase in LVEDD. Fluorescence in situ hybridization (FISH) with a

specific probe complementary to the VPMO sequence indicated widespread tissue penetration with VPMO in the heart (Gramlich *et al.*, 2015). These results suggests exon skipping as a potential treatment approach for numerous DCM patients.

5.1.4. X-linked agammaglobulinemia

XLA is caused by splicing defects in the gene which is responsible for the expression of Bruton's tyrosine kinase (BTK), a cytoplasmic, nonreceptor tyrosine kinase. The splicing defect is due to an A-to-T transition in intron 4 of the BTK gene inducing a novel 5'ss. Together with a preexisting cryptic 3'ss upstream in the same intron this leads to the inclusion of the cryptic exon 4a between exons 4 and 5 in the mRNA. Thus, the reading frame is altered and the BTK protein cannot be expressed. Since PPMO has already proved to be highly efficient for exon skipping in the mdx mouse model, it was chosen to correct aberrantly spliced BTK. Transgenic mice were employed, which carry the entire human mutant BTK locus and are devoid of endogenously expressed BTK protein. PPMOs were administered intravenously three times every second day during one week, followed by a single subcutaneous dose. A robust therapeutic effect was detected in the bone marrow and in the spleen. All treated animals expressed corrected BTK mRNA and BTK protein (Bestas *et al.*, 2014). Although the duration of treatment for only one week was rather short, the data obtained could show that the splice correction concept might become a viable treatment option for patients with XLA.

5.1.5. β-thalassemia

Another disease where splice switching oligonucleotides could be an effective therapy is β-thalassemia. β-thalassemia is one of the most frequently occuring inherited diseases and is caused by defects in the β-globin, a subunit of hemoglobin. The reason why SSOs are considered to be a possible therapy for this disease is due to the fact that it is caused by mutations which induce aberrant splicing of β-globin pre-mRNA, which then affects proper translation into β-globin protein. The IVS2-654 mutation is responsible for an aberrant 5' splice site and activates a cryptic 3' splice site within intron 2 of the β-globin pre-mRNA,

leading to retention of the intron fragment in the spliced mRNA even though the correct splice sites remain undamaged and potentially functional. The retained fragment prevents proper translation of β-globin, leading to hemoglobin deficiency and β-thalassemia. The study shows that SSOs, which block aberrant splice sites in IVS2-654 and other pre-mRNAs as well as in the coding sequence (HbE) of the β-globin gene, force the splicing machinery to reselect the existing correct splice sites, repairing the splicing pattern of β-globin pre-mRNA (Fig. 19).

In ex vivo studies PPMO showed the best effect in correcting aberrant splicing of IVS2-654 pre-mRNA compared to other chemical modificatios of SSOs. PPMO was injected intravenously into thalassemic IVS2-654 mice. These mice are heterozygotes with one human IVS2-654 thalassemic β-globin gene knocked-in to replace two cis-β-globin genes that code for murine adult β-globin.

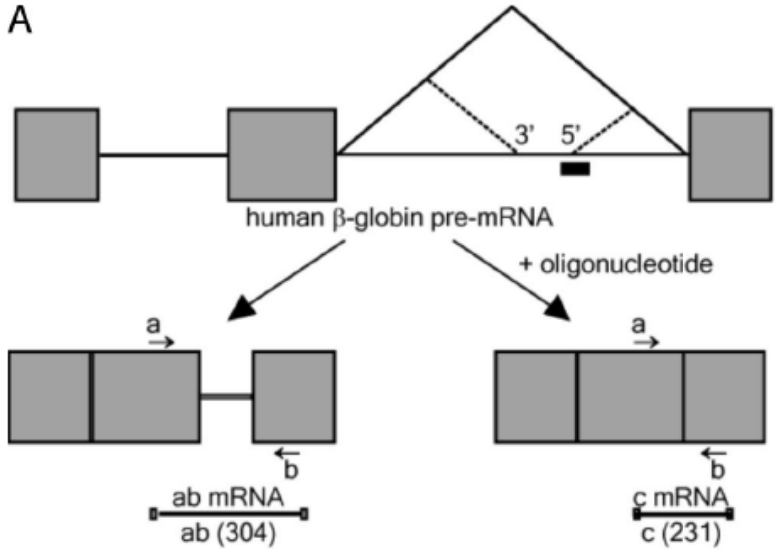

Figure 19. Splicing of IVS2-654 β-globin pre-mRNA and its repair by PPMO.
Figure from Svasti et al., 2009.

PPMO restored correctly spliced human β-globin mRNA in the peripheral blood. The newly repaired β-globin mRNA was properly translated in circulating red blood cells (RBCs). Analysis of RBCs showed that there was an improvement in RBC morphology and the number of abnormal RBCs, including fragmented cells and those with abnormalities in shape and size, decreased, indicating more effective erythropoiesis and less extravascular hemolysis. These results were concordant with a significant reduction of the red cell

distribution width (RDW), an indicator of red cell heterogeneity and increase in total hemoglobin level in the peripheral blood of treated mice. Especially in mice with a lower initial hemoglobin level the increase was more pronounced. I.p. injections into a single IVS2-654 mouse with a low hemoglobin level confirmed this observation. This SSO treatment resulted in elevation of hemoglobin and hematocrit levels. Although a single mouse experiment has no significant value, it further supports the notion that prolonged SSO treatment may be clinically valuable (Svasti *et al.*, 2009).

5.1.6. Pompe disease

Pompe disease is characterized by a reduced amount of acid α-glucosidase (GAA) which is responsible for the catalysis of the breakdown of glycogen to glucose (Clayton *et al.*, 2014). Reduction of GAA leads to progressive lysosomal accumulation of glycogen in skeletal and cardiac muscles resulting in myopathy in young adults and older individuals that is invariably fatal. The *Gys1* gene is responsible for the glycogen synthase activity in skeletal muscle whereas the *Gys2* gene controls the glycogen synthase activity in the liver. When treating Pompe mice with a PPMO targeting a specific sequence in exon 6 of Gys1 mRNA, a dose-dependent reduction of the Gys1 transcript in skeletal muscle and heart but not of the Gys2 transcript in liver was the result. The bioactivity obtained in the heart was only significant at the higher dose tested, a finding consistent with PPMOs tested for exon skipping of dystrophin. Pompe mice received PPMO by tail vein injection. The glycogen synthase protein was markedly increased in the skeletal muscle and in higher doses in the heart. PPMO was able to reduce the amount of glycogen synthase in the quadriceps and diaphragm. Treatment also lowered the elevated levels of glycogen synthase in the Pompe mouse heart, and achieved complete correction at the higher dose but neither dose of PPMO had an effect on the amount of total glycogen synthase in the liver. These results confirmed that PPMO activity at the mRNA level is sequence specific as there was no impact seen on the liver isoform Gys2 (Clayton *et al.*, 2014).

5.1.7. Familial hypercholesterolemia

Familial hypercholesterolemia is a genetic disorder exhibiting high levels of plasma low-density lipoprotein (LDL) caused by defective LDL receptor-apolipoprotein B (APOB) binding (Disterer *et al.*, 2013). APOB displays a key target for lowering high LDL cholesterol levels. One of its isoform, APOB100, is responsible for recycling LDL particles by binding to the LDL receptor. Disruption of this process leads to accumulation of LDL particles in the circulation and hypercholesterolemia. The second isoform of APOB is APOB48, which is assembled into chylomicrons, delivers dietary fat and fat-soluble vitamins from the intestine to the liver. Disruption of intestinal APOB48 expression results in fat intolerance and malabsorption of fat and fat-solube vitamins. Therefore, any treatment aimed at lowering LDL cholsterol by interfering with APOB should be selective for APOB100 and should not effect APOB48 (Disterer *et al.*, 2013).

Disterer et al. used 2'-OMe RNA oligonucleotides that induce skipping of APOB exon 27 to generate APOB87, a shortened APOB isoform (Fig 20). Exclusion of exon 27 by exon skipping results in the APOB87 mRNA with frame-shifted exon 28 and 29 sequences and a premature stop codon. This leads to translation of a truncated APOB protein, APOB87. Subjects who encode for this truncated isoform, showed well below normal cholesterol levels because of greater catabolic rates of particles containing the shortened APOB isoforms (Disterer *et al.*, 2013).

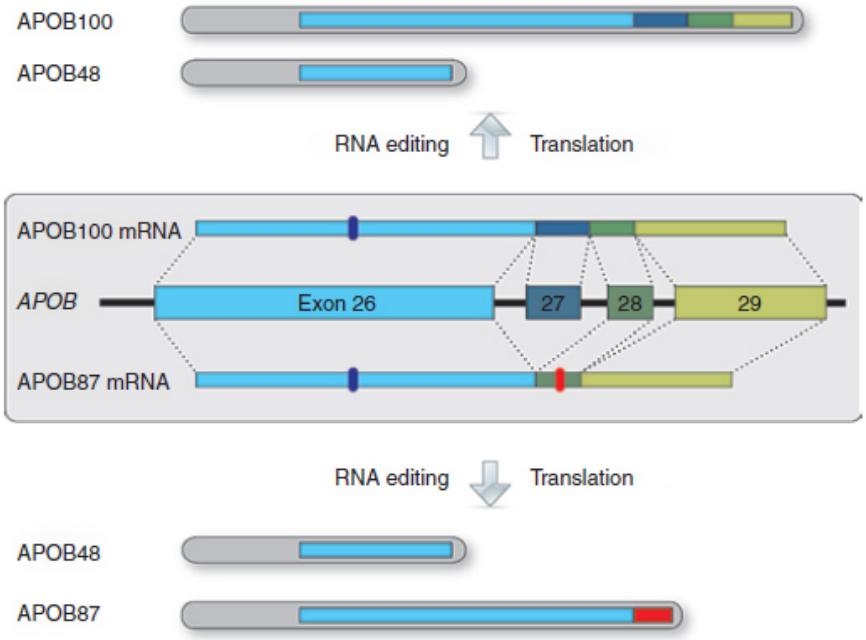

Figure 20. APOB gene structure with normal and exon-skipped protein isoforms. Figure from Disterer et al., 2013.

The designed SSOs consist of a partial PS modification to the 5' and 3' ends and bearing 5' fluorescein or biotin. Partial PS modification enhance exon skipping, whereas full modification lower exon skipping. PS modifications are responsible for oligonucleotide stability to nucleases but reduces binding affinity to RNA. By using a mixed-backbone strategy the reduction of binding affinity to RNA can be overcome. Introduction of fluorescein and biotin has not been used before for other exons. The presence of these groups near the 5' splice site of exon 27 interferes with recognition of this splice site and thus improve efficiency in exon skipping.

To achieve efficient in vivo hepatic uptake of the SSOs, Invivofectamine 2.0 (IVF2.0) was used. IVF2.0 is a cationic complexing reagent which allows efficient delivery of SSOs to hepatocytes. IVF2.0 was injected intravenously into the tail veins of transgenic mice with human APOB. APOB-skipped SSOs induced a sustained 34-51% drop in LDL levels in mice, whereas the exon-skipping efficiency of 6.8% was relatively low (Disterer *et al.*, 2013). This finding can be explained by the similarity of APOB87 to truncated human APOB100 isoforms such as APOB89 and APOB87. This suggests that even low levels of exon skipping can be enough for significant therapeutic effects, which offer clinically relevant effects compared to other oligonucleotide-based therapies in development.

5.1.8. Cancer

The effect of alternative splicing has also been evaluated to modulate apoptotic regulators in tumors (Zammarchi *et al.*, 2011). In vivo studies showed that SSOs are able to target those regulators and induce a shift of expression from an anti-apoptotic to a pro-apoptotic isoform (Zammarchi *et al.*, 2011). In general, delivery of SSOs to tumors is achieved by intravenous or intratumoral administration.

One of those apoptic regulators is signal transducer and activator of transcription 3 (STAT3). STAT 3 belongs to the STAT family of transcription factors involved in cytokine signaling. It is activated by phosphorylation of cytoplasmic monomers and in consequence regulates gene expression. Activated STAT3 is found in most human cancers (Zammarchi *et al.*, 2011). STAT3 consists of a full-length STAT3α and a truncated STAT3β isoform generated by alternative splicing of exon 23. STAT3β overexpression is responsible for the induction of apoptosis and thus the inhibition of tumor growth. VPMOs were used to induce a switch from STAT3α to STAT3β by blocking the α3' splice site and/or nearby a putative exon splicing enhancer of exon 23 and were administered by i.v. or i.t.m. injections into the tumors of mice (Zammarchi *et al.*, 2011). The injections were administered two times during one week but did not lead to any obvious histopathological changes or difference in tumor size. Analysis of the tumor RNAs showed though, that a switch from STAT3α to STAT3β was observed in two-thirds of the tumors treated by i.t.m. injection but not by i.v. injection. Extending the treatment to three weeks using the same dosage displayed a regression in tumor growth. These results are consistent with the data obtained by SSOs inducing a switch from anti-apoptotic splicing variant Bcl-x_L to the pro-apoptotic Bcl-x_L (Bauman *et al.*, 2010). *Bcl-x* is an apoptotic regulator and can express Bcl-x_L and Bcl-x_s by alternative splicing. Especially in cancer, Bcl-x_L is increased leading to a resistance to a broad range of chemotherapeutic drugs. The anti-apoptotic activity of Bcl-x_L and another anti-apoptotic protein, Bcl-2, is antagonized by Bcl-x_s. In vitro experiments showed that Bcl-x splicing from Bcl-x_L to –x_s induced apoptosis and increased chemosensitivity (Bauman *et al.*, 2010). While Zammarchi et al. used VPMO as chemical modification of SSO, Baumann et al. used the chemical structure of 2'-MOE-PS oligonucleotide benefitting from its RNase-H resistance and increased affinity for target mRNA. Moreover, the phosphorothiate inter-nucleotide linkage

enhances the stability and bioavailability in vivo. These features enable the conversion from Bcl-x_L to Bcl-x_S in tumors and therefore represent these SSOs as good candidates for treatment with Bcl-x SSO. To further ensure a successful delivery of SSOs to cancer cells in tumor-bearing mice, the 2'-MOE-PS was combined with a liposome-DNA polycation (LPD) nanoparticle (NP) formulation. For nanoparticle preparation, calf thymus DNA was employed to encapsulate the 2'-MOE-PS within a condensed core. This complex was then coated with cationic liposomes and arranged into equal sized nanoparticles. The enhanced tumor cell-specific uptake of the NP formulation results from surface-modification with anisamide, a ligand of the sigma receptor, which is upregulated in many cancers. The Bcl-x 2'-MOE-PS-NP formulation was administerd by i.c. injections three days in a row. The Bcl-x 2'-MOE-PS-NP formulation was able to induce Bcl-x_S mRNA expression in tumor-bearing mice. As also observed with VPMO, this effect was dose-dependent, as increasing the number of injections resulted in a higher degree of Bcl-s splice switching from Bcl-x_L to Bcl-x_s (Bauman *et al.*, 2010).

Generally, SSOs are conjugated to ligands to improve their pharmacodynamic properties. In contrast, the Retro compounds, termed Retro-1, are not administered complexed to SSOs, but rather injected intravenously 18 hours after i.p. injections of 2'-OMe-PS into tumor-bearing mice. SSOs usually enter the cells via endocytosis before they traffic through membrane-circumscribed vesicular compartments. However, most of the SSOs get stuck in various endomembrane vesicles on the way to the cytosol and nucleus where they can only display their pharmacological activity. The mechanism of the Retro compound is based on its feature to cause a rapid partial release of oligonucleotides from a subset of endomembrane compartments where they have accumluted but are pharmacologically inert, and enables SSOs to gain entry into the cytosol and then into the nucleus. Although the Retro compounds could enhance splice correction of 2'-OMe-PS, the effect is only visible at micromolecular concentrations. Since the effects of Retro-1 in vivo observed are too limited due to its poor water solubility, it may not be useful in the therapeutic context itself (Ming *et al.*, 2013).

Disease	SSO	Route of administration	Dosage used	Splice correction	Organs
EGFP-654 mouse model	• 2'-MOE-PS • PNA-1K PNA-4K • PMO	• i.p.	• i.p.: 50 mg/kg	• EGFP gene	• cardiac muscle • cortex • kidney • liver • lung
	• VPMO	• i.v. • i.t.v.	• i.v.: 12.5 mg/kg • i.t.v.: 12.5 mg/kg	• EGFP gene	• liver • small intestine • colon • skeletal muscle • diaphragm
Duchenne muscular dystrophy	• 2'-OMe-PS	• i.v. • i.m. • i.p. • s.c.	• i.v.: 50-250 /kg • i.m.: 20 µg 30 µg 12,5 mg/kg • i.p.: 25 mg/kg • 250 mg/kg • s.c.: 25-250 mg/kg	• exon 23 skipping	• gastrocnemius, triceps and quadriceps muscle • heart • kidney • liver
	• ZEN20	• i.m. • i.v.	• i.m.: 30 µg • i.v.: 100 mg/kg	• exon 23 skipping	• quadriceps • diaphragm • tibialis anterior
	• VPMO	• i.v.	• i.v.: 6 and 25 mg/kg	• exon 23 skipping	• soleus
	• PPMO	• i.p.	• i.p.: 10 mg/kg	• exon 23 skipping	• skeletal muscle • cardiac muscle
	• 2'-OMe-PS/PEI complex	• i.m.	• i.m.: 0.9-5.4 nmol	• exon 46 skipping	• gastrocnemius muscle
	• 2'-OMe-PS/SaOEI complex	• i.m.	• i.m.: 5 µg	• exon 23 skipping	• tibialis anterior muscle
Spinal muscular atrophy	• 2'-MOE • 2'OMe • PMO	• i.c.v. infusion • i.c.v. bolus injection • s.c.	• i.c.v. infusion: 3-100 µg/day • i.c.v. bolus injection: 6-350 µg • i.c.v. injection (embryo): 2,5-30 µg • i.c.v. injection (neonates: 20 µg • s.c. (neonates: 50 µg/kg	• exon 7 skipping	• spinal cord • brain

Dilated cardiomyopathy	• VPMO	• i.p.	• i.p.: 6 mg/kg	• exon 326 skipping	• ventricular tissue
Tumor	• VPMO	• i.v. • i.t.m.	• i.v.: 15 mg/kg • i.t.m.: 0.12 mg/30 µl	•	• tumor size
	• liposome-DNA-polycation nanoparticle (2'-MOE-PS)	• i.v.	• i.v.: 2.4 mg/kg	• Bcl-x splicing from Bcl-x_L to –x_s	• tumor
	• Retro-1	• i.v.	• i.v.: 40 µl	• Bcl-x splicing from Bcl-x_L to –x_s	• tumor
β-thalassemia	• PPMO	• i.v. • i.p.	• i.v.: 25 mg/kg • i.p.: 50 mg/kg		• red blood cells
Pompe disease	• PPMO	• i.v.	• i.v.: 15 or 30 mg/kg	• exon 6 skipping	• skeletal muscle • heart
X-linked agammaglobulina	• PPMO	• i.v. • s.c.	• i.v.: 30 mg/kg	•	• bone marrow • spleen
Familial hypercholesterolemia	• lipoplex	• i.v.	• i.v.: 12.5 and 25 mg/kg	• exon 27 skipping	• blood

Table 2. Ways of administration and dosages used in in vivo studies.

6. Splice-switching oligonucleotides in clinical trials

Although there are numerous preclinical studies with SSOs, only few clinical trials were conducted so far. The most advanced studies of SSOs as therapeutic opportunities in clinical trials were carried out using 2'-OMe-PS and morpholino for the treatment of DMD and 2'MOE-PS for the treatment of SMA.

One of the first trials conducted investigated the tolerance for Drisapersen, a 2'-OMe-PS oligonucleotide, in DMD patients. Drisapersen induces exon 51 skipping, which is affected in the largest subgroup of DMD patients. Patients (boys, aged 5-16 years) received 6 mg/kg/week s.c. injections of Drisapersen for 188 weeks. Of the 12 patients enrolled in the study, 10 were able to complete the 6 minutes walking test (6MWT), the primary clinical outcome. The 6MWT measures the distance a patient can independently walk in 6 minutes. The study could also prove that long-term treatment with Drisapersen was well tolerated in all patients. However, due to the small number of subjects enrolled and the lack of a placebo control group the study could not prove efficacy of Drisapersen (Goemans *et al.*, 2016).

In a bigger study also conducted by Goemans et al., they wanted to confirm the results already obtained in the first study and further evaluate efficacy of Drisapersen. Compared to the first study with a small number of 12 subjects and without a placebo-control group, this study was a 48-week, randomized, placebo-controlled phase 3 study with a large number of 186 patients. The dose the patients received s.c. was the same with 6 mg/kg/week Drisapersen. However, the outcome of the 6MWT did not show a significant treatment difference in the change from baseline at week 48. The same result was obtained in the secondary endpoints, containing the ambulatory function. This lack of statistical significance was supposedly due to great data variability and subgroup heterogeneity (Goemans *et al.*, 2018). Drisapersen has not been approved for the treatment of DMD patients by the FDA due to safety issues associated with the use of the drug and insufficient evidence of clinical utility (Lim *et al.*, 2017).

The second SSO which has been intensively investigated for the treatment of DMD is PMO and exhibited better results in clinical studies than the 2'-OMe-PS oligonucleotide. Eteplirsen, based on the morpholino structure, is the first and currently only approved drug

for DMD by the US Food and Drug Administration (FDA). It was approved in September 2016 (Lim *et al.*, 2017). Like Drisapersen, Eteplirsen also acts through skipping of exon 51 in defective gene variants. First trials with morpholinos (PMOs) in DMD patients also showed good toleration of the substance in humans and high dystrophin levels after i.m. injections. Although the studies could confirm proof of concept the effect was only limited in local dystrophin synthesis (Heemskerk *et al.*, 2010). Therefore intravenous delivery of SSOs is essential. A systemically delivered morpholino phase Ib/II clinical trial was conducted. Patients expressed uniform and widespread dystrophin-positive fibers (Scully *et al.*, 2013). A Phase IIB randomized, double-blind, placebo-controlled multiple dose efficacy trial showed that Eteplirsen elevated the number of dystrophin in all Eteplirsen-treated patients compared to placebo/delayed treatment over 48 weeks. A significant clinical benefit on the primary clinical outcome measurement, the 6MWT in the higher dose treated group compared to lower dose group and placebo was also noticed (Scully *et al.*, 2013). Although Eteplirsen is approved as treatment opportunity for DMD, it is highly controversial due to its poor efficacy in clinical trials. Indeed, all clinical studies showed an increase in dystrophin levels, these levels only led to a marginal effect on improving DMD clinical manifestations in patients. The studies indicated that Eteplirsen treatment at most led to a delay of disease progression in terms of ambulatory ability as measured by the 6MWT. On the other hand, Eteplirsen was found to positively affect pulmonary function in patients. Eteplirsen treatment significantly slowed progressive decline in forced vital capacity and maximum inspiratory and expiratory pressure predicted percentages. There is still an ongoing, recruiting Phase III trial required by the FDA to support the clinical benefit of the drug. It is an open-label study which consists of two 80-patient cohorts, an Eteplirsen-treated and an untreated cohort (Lim *et al.*, 2017).

The second SSO which has been approved by the FDA three months after the approval of Eteplirsen is Nusinersen, an 18-mer 2'-MOE oligonucleotide, for the treatment of SMA. Nusinersen targets a region in intron 7 that harbors an intronic splicing inhibitor, which is one of the main reasons why exon 7 is skipped in SMN2 transcripts. When this site is blocked, splicing inhibitory factors are prevented from binding. This results in better recognition of exon 7 by the splicing machinery and thus more inclusion into SMN2 mRNA, offering enhanced translation of SMN protein. In contrast to the in vivo studies, where the SSO was administered by i.c.v. injections into mice, in human patients intrathecal (i.t.)

delivery is used to bypass the blood-brain barrier. In several Phase I trials in type II and type III SMA patients, between the age of 2-14 years, this approach was successfully tested (Aartsma-Rus, 2017). The effective uptake upon intrathecal delivery of Nusinersen can be led back to its phosphorothiate backbone, which allows the distribution throughout the brain and spinal cord. Compared to Eteplirsen, the dosing frequency of Nusinersen is much lower with a maintenance dose required every 4 months instead of every week for Eteplirsen. This is due to the benign environment for SSOs in the central nervous system, where the half-life is months rather than days or weeks for systemic organs, which are targeted in the treatment of DMD. Moreover, for the treatment of DMD higher systemic doses are needed to obtain sufficient levels of SSOs in the muscle to achieve minimal levels of exon skipping and dystrophin restoration. Nusinersen is able to apply to all SMA patients across the different types, whereas Eteplirsen applies to only 13%-14% of all DMD patients with a specific mutation (Aartsma-Rus, 2017). The evidence of the very good responders is outstanding. Type I SMA patients using Nusinersen are able to learn walking at an age they would normally have died, and wheelchair-dependent type II and type III patients are able to regain the ability to walk (Aartsma-Rus, 2017).

To investigate the long-term effects of Nusinersen, a phase 1b/2a, 253-day, ascending dose, multiple dose, open-label, multicenter study was conducted. The extension study lasted 715 days and was a single-dose level study. 28 patients with SMA type II or III, aged between 2 and 15 years, were enrolled in this study (Darras *et al.*, 2019). The study showed that clinically meaningful improvements in motor function was observed in patients with SMA type II or type III under the age of 15 years at treatment initiation over the course of about 3 years of follow-up. The results obtained in this study suggest that treatment with Nusinersen prevents motor function deterioration and moreover is able to allow for continued motor function improvement and reversal of motor function loss. This study also supports the results from previous studies demonstrating the clinically meaningful effects of Nusinersen in infants and children with infantile- and later-onset SMA (Aartsma-Rus, 2017; Darras *et al.*, 2019).

7. Conclusion

This thesis reviewed the role of splice switching oligonucleotides as therapeutic opportunity for diseases caused by mutations in the splicing process. Alternative splicing is an important process since it sustains the phenotypic diversity of higher eukaryotes by expanding gene expression complexity without an elevation at the large number of genes. However, in this whole process mutations can occur which lead to formation of an aberrant mRNA or quantitative alterations in alternative mRNA isoform abundance and loss of normal protein expression. SSOs are oligonucleotides that are able to manipulate splicing by base-pairing in an antisense orientation to a specific pre-mRNA sequence. This results in alteration of recognition of splice sites by the spliceosome and alteration of normal splicing of the targeted transcript. Since unmodified SSOs are vulnerable to nuclease degradation in serum and cells and due to their large size, in vivo studies tested different kinds of chemical modifications to improve their binding affinity, stability and pharmacodynamic properties in animal experiments. Therefore the animals, in most of the studies mice, were genetically modified so that they carried the mutated gene, the SSOs were aimed at. Besides different kinds of modifications of SSOs, different ways of administration and dosages have been tested. Despite several promising results in preclinical studies only few SSOs have been enrolled in clinical trials. Drisapersen and Eteplirsen have been intensively investigated in clinical trials for their efficacy for the treatment of Duchenne Muscular Dystrophy and in 2016 Eteplirsen was approved as first drug for DMD patients by the FDA. However, it is still controverisal due to its marginal effect on improving DMD clinical manifestations. Nusinersen is the second SSO which has been approved by the FDA. It is used for the therapy of spinal muscular atrophy. The outstanding responses of patients using Nusinersen offer a promising foundation for further therapeutic possibilities of SSOs.

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11. Appendix

Abstract

Nearly one tenth of all disease-causing mutations are caused by single-base pair substitutions affecting pre-mRNA splicing. Many of these mutations are susceptible to splice switching therapy. In the splice switching process small nucleic acids are conducted toward cis splicing elements within pre-mRNA. These oligonucleotides, referred as splice switching oligonucleotides, can occur through targeted binding to pre-mRNA to enhance or prevent recruitment of splicing factors so as to cause exon inclusion or exclusion and influence alternative splicing. For a successful treatment of diseases caused by splicing abnormalities, delivery of splice switching oligonucleotides to their required cellular site of action is indispensable. Since unmodified DNA and RNA oligonucleotides are vulnerable to nuclease degradation in serum and cells, they are unstable in vivo. To improve binding affinity, stability and pharmacodynamic properties of oligonucleotides, chemical modifications have been applied. The most common modifications used are chemical modifications, covalent conjugation and supramolecular assemblies. In the past decades numerous in vivo studies have been conducted investigating different modifications of splice switching oligonucleotides and their pharmacological effects. In addition to different kinds of modifications, diverse routes of administration and dosages have been tested to achieve optimal results. Although there is a great number of in vivo studies, only few splice switching oligonucleotides overcome those studies and can be enrolled in further clinical trials. The number of splice switching oligonucleotides, which have been approved officially as treatment opportunity for diseases caused by gene mutations, are even much lower.

Kurzfassung

Annähernd jede zehnte Mutation, die zu pathologischen Veränderungen führt, ist auf einen Austausch von Basenpaaren zurückzuführen, welche den Spleißvorgang und dadurch die Proteinbildung beeinflusst. Um solche Krankheiten erfolgreich therapieren zu können, ist es notwendig, in den fehlerhaften Spleißprozess einzugreifen. Oligonukleotide, die in diesen Prozess eingreifen, werden als Splice-switching-Oligonukleotide bezeichnet. Sie können durch gezielte Bindung an prä-mRNA die Rekrutierung von Spleißfaktoren verstärken oder verhindern, bewirken einen Einschluss oder Ausschluss von Exons und beeinflussen somit das alternative Spleißen. Damit die Splice-switching-Oligonukleotide auch in den Spleißvorgang eingreifen können, ist es unerlässlich, dass diese an ihren Wirkort in die Zellen gelangen. In-vivo Studien zeigten, dass DNA- und RNA-Oligonukleotide basierend auf nativen Nukleotiden sehr instabil sind, da diese durch Nukleasen im Serum rasch abgebaut werden können. Um die pharmakodynamischen und –kinetischen Eigenschaften der Oligonukleotide zu verbessern, wurden daher Modifikationen an der Molekülstruktur angewendet. Dabei handelt es sich sowohl um chemische Modifikationen, kovalente Konjugationen als auch supramolekulare Anordnungen. In den letzten Jahrzehnten wurden zahlreiche Versuche und In-vivo Studien durchgeführt, in denen die verschiedenen Modifikationen von Splice-switching-Oligonukleotiden und deren pharmakologischen Wirkungen untersucht wurden. Neben verschiedenen Arten von Modifikationen wurden auch die verschiedenen Verabreichungsformen und Dosierungen getestet, um optimale Ergebnisse zu erzielen. Trotz der großen Anzahl an In-vivo Studien, wird nur ein Bruchteil dieser Oligonukleotide in weitere klinische Studien aufgenommen. Bis zum heutigen Zeitpunkt gibt es nur wenig zugelassene Arzneimittel für die Therapie von genetisch bedingten Krankheiten, die auf Splice-switching-Oligonukleotiden basieren.

Abbreviations

2‘-MOE	2’-O-methoxyethyl
2’-MOE-PS	2’-O-methoxyethyl phosphorothiate
2‘-OMe	2’-O-methyl
2’-OMe-PS	2’-O-methyl phosphorothiate
3’ss	3’ splice site
5’ss	5’ splice site
6MWT	6-min walk test
Aif1	allograft inflammatory factor-1
ALP	alkine phosphatase
APOB	apolipoprotein B
ASO	antisense oligonucleotides
BMD	Becker muscular dystrophy
BPEI	branched polyethylenimine
BPS	branchpoint sequence
B-PMO	B-peptide morpholino
BTK	Bruton’s tyrosine kinase
CD	cyclodextrins
cEt	2’-O-ethyl
ChAT	choline acetyltransferase
CHMP	Committee for Human Medicinal Products
CNS	central nervous system
CPP	cell-penetrating peptides
CSF	cerebrospinal fluid
DC-Chol	3β-(N-(N’,N’-dimethylaminoethane)-carbamoyl) cholesterol
DCM	dilated cardiomyopathy
DIA	diaphragm
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid

DOGS	dioctadecylamidoglycylspermine
DOTAP	1,2-dioleyl-3-trimethylammonium-propane
DOTMA	N (1-(2,3-dioleyloxy) propyl)-N,N,N-trimethylammonium chloride
ED	effective dose
EDL	extensor digitorum longus
EGFP	enhanced green fluorescent protein
EMA	European Medicines Agency
EPR effect	enhanced permeability and retention effect
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
FDA	US Food and Drug Administration
FH	familial hypercholesterolemia
FISH	fluorescence in situ and hybridization
GAA	α-glucosidase
GAS	gastrocnemius
i.c.v.	intracerebroventricular
IHC	immunohistochmistry
i.m.	intramuscular
i.p.	intraperitoneal
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
i.t.	intrathecal
i.t.m.	intratumoral
i.v.	intravenous
i.v.i.	intravitreal
LDL	low-density lipoprotein
LNA	locked nucleic acid
LPEI	linear polyethylenimine
LPD	liposome-DNA-polycation
LVEDD	left ventricular end-diastolic diameters
LV-EF	left ventricular ejection fraction
m-RNA	messenger ribonucleic acid

NP	nanoparticle
NOD	non-obese diabetic
OEI	oligoethylenimine
ON	oligonucleotide
PAMAM	polyamidoamine
PEG	polyethylenglycol
PDLO	Peptide Dendrimer/Lipofectin/Oligonucleotide
PNA	peptide nucleic acid
PMO	phosphorodiamidate morpholino
PPMO	peptide phosphorodiamidate morpholino oligomer
PS	phosphorothiate
Py	polypyrimidine
RBC	red blood cell
RNA	ribonucleic acid
RNP	ribonucleinprotein
PEI	polyethylenimine
SaOEI	Salicyl amide conjugated with oligoethylenimine
s.c.	subcutan
s.c.j.	subconjunctival
SMA	muscular atrophy
SMN	survival of motor neuron
snRNA	small nuclear ribonucleic acid
snRNP	small nuclear ribonucleic protein
SOL	soleus
SSO	splice switching oligonucleotide
STAT3	signal transducer and activator of transcription 3
TA	tibialis anterior
VPMO	vivo-morpholinos
WB	Western blotting
XLA	X-linked agammaglobulinemia
ZEN™	N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine