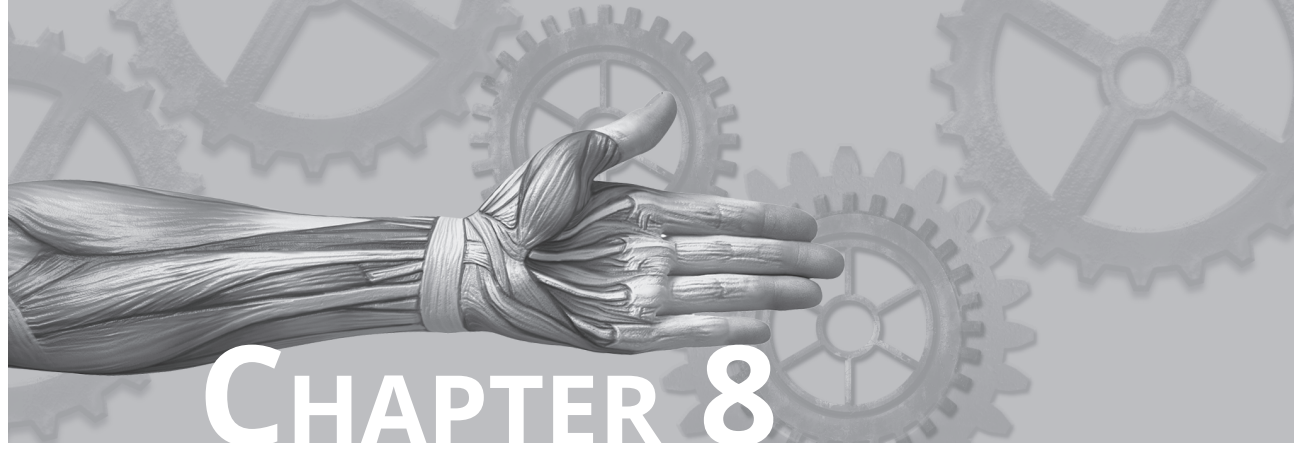
Developing tissue specific antisense oligonucleotide-delivery to refine treatment for Duchenne muscular dystrophy

Jirka, S.



Chapter 8

Summary

Efficient drug delivery is a key issue for any potential drug in development. A drug can have a high efficacy in vitro but when it fails to reach its target properly in vivo, the drug is of no use for humans. Furthermore, a drug needs to be safe and well tolerated. Drugs with severe toxicities or where the side effects are more severe than the disease, are doing more harm than good. A balance between efficient delivery, safety and tolerability will result in a drug with the required efficacy that is suitable to use for humans.

Duchenne muscular dystrophy (DMD) is a severe progressive muscle wasting disorder affecting 1 in 5,000 new born boys. DMD is caused by reading frame disrupting mutations in the *DMD* gene resulting is an absence of the dystrophin protein. Dystrophin is an important muscle protein as it provides stability upon muscle fiber contraction. Without this protein muscle fibers are easily damaged and chronic damage leads to muscle fibrosis which in turn results in a loss of muscle function. In general DMD patients are generally diagnosed before the age of five and become wheel chair dependent around the age of 12. Patients need assisted ventilation around the age of 20, an age where in most patients cardiomyopathy is prevalent as well. DMD patients have a life expectancy of around 30 years of age in the western world where respiratory and cardiac failure is the main cause of death. In contrast to DMD, Becker muscular dystrophy (BMD) is a muscle wasting disorder, caused by mutations in the *DMD* gene that not affect the reading frame but result in an internally deleted but partly functional dystrophin protein. The symptoms of BMD are much milder and ranges from mild to moderately severe. The life expectancy of these patients varies from 40-50 years for the more severely affected patients to a nearly normal life expectancy for the milder affected patients.

Currently there is no therapy for the majority of the DMD patients. As part of the standard of care patient receive symptomatic treatment e.g. corticosteroids, respiratory and cardiac support. Various therapeutic approached are currently under development and in general can be grouped in two main groups. One is focussed on the genetic defect, aiming to restore dystrophin production. The other is focussed on secondary pathology, for example reducing fibrosis and inflammation or improving muscle growth and strength. Most advanced therapeutic approach is aimed at restoring dystrophin production: exon skipping. Here AON are designed to bind a specific exon in the pre-mRNA of the dystrophin transcript. Upon binding the AON hides the exon from the splicing machinery, it is no longer recognized as an exon and thereby spliced out together with the other introns (chapter 1, figure 4c). This results in restoration of the reading frame and the production of a partly functional dystrophin protein as seen in BMD patients (chapter 1, figure 1).

This thesis focusses on delivery of AON to skeletal and cardiac muscle for DMD. Since one cannot treat each muscle individually, systemic treatment

is necessary. From pre-clinical studies it is known that this is feasible. Clinical studies have shown that AON (2OMePS and PMO AONs) are safe for humans, however mild to moderate side effects have been reported. On September 19th 2016 the FDA conditionally approved eteplirsen (a PMO targeting DMD exon 51) for the treatment of DMD patients that benefit from exon 51 skipping. However, upon systemic administration of AON, a large proportion of the administrated AONs ends up in liver (2OMePS) and or kidney (2OMePS and PMO) and are lost for targeting muscle.

Strategies taken to improve AON therapy are chemical modification of the AON to enhance nuclease resistance or improve thermodynamic stability, affinity for the target, bio-availability and tissue half-life. In chapter 6 2FPS (substitution of a Fluoro (F) at the 2' position) and isosequential 2OMePS AON counterparts have been compared for DMD. While *in vitro* 2FPS AON resulted in increased exon skipping levels, the modification appeared less effective *in vivo* and was found to be toxic in *mdx* mice (a mouse model for DMD).

Other strategies taken to improve AON therapy are more focussed on the use of a delivery system. Such delivery system should first of all not interfere with the function of the AON, should have a good safety profile and good biostability. Secondly, the delivery system should be small in size to allow efficient uptake by muscle cells and promote endosomal escape. Third, preferably the delivery system is muscle specific thereby limiting or preventing uptake by e.g. liver, kidney and spleen. In this thesis the conjugation of muscle homing peptides, selected from phage display experiments, to 2OMePS AON is described as a delivery system (chapters 3, 4 and 5).

Phage display is a well described, powerful technique to identify peptides, antibodies or other proteins with target specific binding properties from phage libraries. Such a selection is called biopanning and the basic principles are the same for various targets. The phage library is exposed to the target for binding. When all non-binders are removed binding phages are recovered by elution, amplified and prepared for a next biopanning round. After several rounds of biopanning, binding phages are identified by for example sequencing of candidate phage DNA (chapter 1, figure 7). The use of muscle specific homing peptides potentially leads to a delivery system which is safe, small in size, promotes endosomal escape and limits uptake by e.g. liver and kidney.

Focussing on the 7-mer phage display peptide library (Ph.D.-7), the first potential muscle homing peptide for 2OMePS AON, selected from *in vivo* biopanning selections in *mdx* mice, is peptide P4 (7-mer: LGAQSNF). P4 conjugated 2OMePS AONs resulted in small but significantly increased exon skipping levels in diaphragm and cardiac muscle tissue compared to unconjugated AONs, after subcutaneous administration in *mdx* mice

(chapter 3). Despite an increase in AON levels in all tissues was observed, the uptake was favorable for muscle.

In the last couple of years, technology used to analyze phage display outcomes rapidly improved. We integrated for the first time Illumina next generation sequencing (NGS) to analyze phage display biopanning selections (chapter 4). We showed that high throughput sequencing of the naïve library after one round of bacterial amplification is a powerful tool to identify parasite sequences with a growth advantage. We also showed that by using NGS a single biopanning round is enough to identify candidate peptides.

In search for better muscle homing peptides we used NGS sequencing to analyze new *in vitro/in vivo* phage display selection. This, combined with the use of a 7-mer cyclic peptide library (here peptides are more restrained in conformation resulting in higher binding affinity) allowed the identification of new candidates. From these candidates the lead peptide CyPep10 (Cyclic 7-mer: CQLFPLFRC) resulted, upon conjugation to 2OMePS AONs, in 2-3 fold increase in AON levels in all tissues analyzed. Despite increase in AON levels in liver and kidney is seen as well the conjugate has a high potential as it resulted in a significant 2-2.5 fold increase in exon skipping levels in all muscle tissues analyzed (chapter 5).

In conclusion, muscle specific homing peptides have the potential to improve the delivery of AON and other compounds towards muscle. By this means they have the potency to improve the balance between delivery, safety and tolerability resulting in an optimized drug with the required efficacy that is necessary for optimal treatment of DMD patients.