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

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General discussion

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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.

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 reactions have been reported. For example subclinical proteinuria is observed in patients treated with 2OMePS AONs but also for patients treated with PMO AONs. Upon subcutaneous administration of 2OMePS AONs injection site reactions and occasionally severe thrombocytopenia have been observed. Upon intravenous administration of PMO AONs vomiting, balance disorders and catheter site pain have been observed (1,2) (www.sarepta.com). Unfortunately, no significant clinical benefit (improvement of 6MWT) has been shown for AON in humans (2OMePS and PMO). While in September 2016 a PMO has been approved by the FDA, this was based on dystrophin restoration (increases of 0.2-0.9% of normal in a subset of patients), and FDA explicitly specified that functional effects still need to be confirmed in future studies. So far results are suggestive of a slower decline in the 6MWT compared to natural history data (chapter 2). Since there is no treatment available besides symptomatic care (chapter 1.3.1), a drug that appears to slow down disease progression is a big achievement where many patients would like to benefit from.

Chemical modifications are an interesting approach to increase the efficacy of AON for DMD. In Chapter 5, fully modified 2FPS AON have been investigated for the first time for DMD (2'-deoxy-2'-fluoro RNA modification (2F)). 2F modified AON resulted in increased exon skipping levels *in vitro* as well as *in vivo* in a model for SMA (3). I observed these findings as well in cultured human myotubes (chapter 6). However, upon systemic administration of *mdx* mice, no exon skipping levels were found in any of the analysed tissues and 2FPS AONs were not well tolerated as suggested by increased spleen weight and decreased body weight compared to 2OMePS AONs. Later, Shen *et al*, found that cells treated with 2F modified oligonucleotides have elevated levels of double stranded breaks (DSB) and more cell death compared to 2'-O-methoxyethyl (2MOE) oligonucleotides (4). They show that the DSB pathway is likely disturbed upon 2F oligonucleotide treatment due to the loss of p54nrb, PSF and PSPC1

proteins, and ultimately leads to a loss of cellular proteins (even limiting the 2F modification to just 4 nucleotides resulted in down regulation of P54nrb). These findings likely explain the lack in weight gain and increased spleen weight observed in *mdx* mice treated with 2FPS AONs. Together these results show that the 2F modification leads to severe toxicity and should not be used in future experiments. Even though there is still room for improvement of AON by chemical modifications, they will not make the AON muscle specific (although they might result in improved muscle uptake due to increased binding affinity).

In my opinion, the lack of significant clinical benefit (meaning at least 30 m differences in 6MWT compared to placebo) is the result of inefficient drug delivery to the target. Since the human body consists of 30-40% of muscle, efficient drug delivery is a major challenge (chapter 1.3.5). Upon systemic administration a large proportion of the administered AONs ends up in liver and or kidney and is lost for targeting muscle. Nevertheless skeletal muscles in DMD patients have leaky membranes, which facilitates the uptake of AONs compared to healthy muscle tissue, yet efficient uptake in cardiac muscle remains challenging as here membranes are intact. Many approaches have been investigated to improve the delivery of AON (chapter 1.3.6) but only a few of them have applied a muscle specific approach (chapter 1.5). I believe this is the preferred option since AON by itself can already distribute throughout the body and be taken up by various tissues (chapter 1.3.5). Improving delivery to and uptake specifically for skeletal and cardiac muscle tissue is key.

To improve the delivery and uptake of AON, muscle specific homing peptides could be ideal candidates to accomplish this. However, the identification of muscle homing peptides from combinatorial phage display peptide libraries is not without challenges e.g. phage display biopanning selections are dominated by parasite sequences (chapter 1.5). In chapter 4 we describe for the first time the implementation of Illumina next generation sequencing (NGS) to analyse the outcome of phage display biopanning selections using the Ph.D.-7™ peptide library (expressing linear 7-mer peptides). We found that sequencing the naïve library after one round of bacterial amplification is a useful tool to identify parasite sequences with a growth advantage. Further, by using NGS a single round of biopanning is enough to identify potential candidate peptides. Databases like Pepbank (5) and Sarotup (6) are versatile tools to check whether your candidate peptide has been found before for a different target (meaning it is not specific for your target), or to inform you whether this is a parasite sequence known from literature (e.g. known to bind albumin, plastic or having a growth advantage). Our NGS results led to a new tool in Sarotup database, PhD7faster. Now other researchers can use this improved tool to filter their data for known 7-mer parasite sequences with a growth advantage (7).

In the last few years Matochko *et al.*, performed more detailed analyses of the peptide libraries, Ph.D.-7TM, Ph.D.-C7CTM and Ph.D.-12TM library (8). They illustrate with statistical analyses how to identify parasite sequences in the naïve library and their behaviour upon bacterial amplification. These results are comparable to ours (Ph.D.-7TM and Ph.D.-C7CTM unpublished). What I found interesting in their analyses compared to ours, are the very high frequency counts found in the naïve library before and after bacterial amplification. Their top 30 peptides have frequency counts between 947 and 5.548 before and between 14.489 and 74.758 after bacterial amplification, whereas in our data set the top frequency count observed in the naïve library was no more than 29 and after bacterial amplification 323. I believe that the library batch used by Matochko *et al.*, is already biased towards parasite sequences through manufacturing/ amplification by the manufacturer/others, or something did not go well in the preparation of the samples for NGS sequencing or a combinations of the above. We did observe a similar issue for our Ph.D.-C7CTM library where the naïve unamplified library (as provided by the manufacturer) showed a frequency distribution in the range of our Ph.D.-7TM library after amplification and observed even further increased frequency counts after bacterial amplification (top 30 peptides Ph.D.-C7CTM library: naïve 103-775, amplified 179-2,106). However these frequency counts are not even close to the counts observed by Matochko *et al.* Nonetheless these findings demand attention from the manufacturer as they can lead to major issues in the phage display field (e.g. limiting the change of identifying true candidate peptides, which by itself is already challenging). Together this does support our finding that it is crucial to sequence your naïve library before and after one round of bacterial amplification to identify parasite sequences with a growth advantage. I believe one has to do this for each library one uses, even for a new batch of the same library.

Matochko *et al.*, also investigated a new method for phage amplification: emulsion amplification (9,10). Here, individual phages are amplified in droplets preventing competition between different phages. This method suppresses fast growing phages and gives other phages more room to amplify, resulting in a more uniform distribution of amplified phages thereby increasing the change of identifying true binders. I find this a valuable approach that should be used in the future by researchers as well as by manufacturers.

Since more researchers are starting to implement NGS sequencing there is a growing need for improved tools which are capable of handling millions of sequences simultaneously. In this area, just recently, improvements became available like PHASTpep (11) and FASTAptamer (12). Further there is an increase in the number of researchers developing and combining high-throughput sequencing with computational analyses (13,14). We have investigated a computational algorithm to predict secondary structure (% of helical content) of all possible 7-mer peptides (1.28 billion) to further improve the analyses of phage display selections (Jirka *et al.*, unpublished). Combining the structure information with the outcome of our NGS data from the naïve unamplified and amplified Ph.D.-7TM library we found a significant increase of peptide sequences with no predicted secondary structure for the parasitic sequence population. This would mean that sequences without a predicted structure have an increased chance of being a parasitic sequence. This finding is not strange since it can be expected that a phage with a peptide sequence without structure would be able to pass the membrane of bacteria much quicker (thereby amplify faster) than a phage with a peptide sequence which does have a structure. This hypothesis still needs to be further investigated using higher resolution algorithms and more thoroughly analyses. Nevertheless the approach has the potential to improve the analysis of phage display selections further and thereby improve the identification of parasite sequences in not only the fraction sequenced by NGS, but for all possible 7-mer peptides. Additionally this approach could be used to characterize parasite sequences further as despite their growth advantage some could still have the potential to bind the target of interest.

In Chapter 3, I describe the identification of muscle homing peptide P4. This peptide is selected from *in vivo* phage display biopanning selections (up to 4 selection rounds) using the Ph.D.-7TM phage display peptide library (without NGS analyses). P4 conjugated 2OMePS AONs resulted, upon systemic administration in *mdx* mice, in increased delivery and exon 23 skipping levels for all muscle tissues analysed and was significant for diaphragm and cardiac muscle tissue. Although increased uptake is seen for liver and kidney as well, the ratio is favoured towards skeletal and cardiac muscle tissue. An outstanding question is whether the modest increase shown would be enough. Looking at the current clinical trial results for 2OMePS AON, low levels of dystrophin protein appear to slow down disease progression. One can hypothesize that a small increase of these low levels can have a serious impact and by this means even small increases have the possibility to further slowdown disease pathology. Pre-clinical studies have shown that improvements in skeletal muscle pathology alone, resulted in increased deterioration of cardiac muscle pathology (15). The significantly increased exon skipping levels seen in diaphragm and cardiac muscle combined with the fact that most DMD patient die of respiratory or cardiac failure, in this manner, illustrates once more the potential of P4.

Although the improvements by P4 are encouraging, ideally we need a better muscle homing peptide. This could be accomplished by chemical modifications of the peptide. Here one could think of amino acid modifications, using non-natural amino acids and alternative conjugation strategies. Continuing to improve the identification methods, new phage display biopanning selections (*in vitro* and *in vivo*) combined with NGS

analyses (using not more than 2 selections rounds) have been performed using a more potent phage display library, Ph.D.-C7C (Chapter 5). This library expresses 7-mer peptides in cyclic conformation from which is thought to have increased binding affinity compared to the linear expressed peptides. This resulted in the identification of muscle homing peptide CyPep10, which upon conjugation to a 2OMePS AON increased the uptake and exon 23 skipping in all skeletal and cardiac muscle tissues analysed, by 2-fold on average and was well tolerated in *mdx* mice. This is a big improvement compared to the P4 peptide and thereby has a lot more potential. Unfortunately, the increase in uptake is also seen for liver and kidney, this might mean that the peptide is not tissue specific. However comparing the frequency counts (number of phages found) of this particular phage (with CyPep10 sequence) in tissues after the biopanning selections, showed higher frequency counts for skeletal and cardiac muscle than liver and kidney. This indicates that CyPep10 should be preferentially taken up by muscle in favour of liver and kidney. Nonetheless, even if the peptide is not muscle specific it remains a potential candidate because the increased uptake seen in liver and kidney did not lead to safety concerns so far. It is possible that the bulkiness of the AON hampers the function of the peptide. Notably, 3-5 copies of the peptide are expressed on the phage coat used in the biopanning experiments and only one is conjugated to the AON. A solution to this problem is to conjugate more than one peptide to the AON, however this requires further research in how to conjugate peptides to 2OMePS AON and the influence of different “linkers” or “spacers” used to accomplish this. Future research is needed to address all these questions and the potential of CyPep10 further.

Muscle homing peptides are not only interesting to enhance delivery of AON for DMD but potentially could help deliver AON for other muscle diseases as well e.g. myotonic dystrophy (DM) or spinal muscular atrophy (SMA) where uptake of AON in muscle is less straightforward due to the absence of leaky muscle fiber membranes as observed in DMD patients. Muscle homing peptides could be interesting for AAV delivery as well (16). The advantage for AAVs is that muscle homing peptides do not need to be conjugated but can be directly integrated in the vectors genome. Another advantage for AAVs is that one does not have to take the charge of the peptide in to account while for 2OMePS AONs only non-cationic homing peptides can be used.

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.

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