

Summary and Future Perspective

5.1 Summary

Duchenne muscular dystrophy (DMD) is a debilitating genetic disorder characterized by progressive muscle degeneration and weakness, predominantly affecting males at a rate of approximately 1 in 3500 births. It arises from mutations in the dystrophin gene on the X chromosome, resulting in deficient or absent dystrophin protein. Current therapeutic strategies focus on specific mutations using exon skipping and stop codon read-through to restore dystrophin function, though limited to certain patient populations. Gene therapy approaches, employing viral vectors and gene editing like CRISPR/Cas9, aim to deliver functional dystrophin genes to muscle cells, yet clinical implementation remains under investigation.

Currently, the only FDA-approved drugs for DMD are the exon-skipping agents Eteplirsen (targeting exon 51) and Golodirsen (targeting exon 53). However, the use of these drugs is highly expensive, placing them out of reach for the majority of the DMD population. Moreover, these therapeutics are mutation-specific, limiting their applicability to a broader, global DMD population. Additionally, concerns remain regarding their limited bioavailability under physiological conditions, as well as their poor cellular and nuclear penetration to reach their intended site of action. These limitations requires frequent dosing, further escalating the cost of ASO-based therapies. As a result, the majority of DMD patients rely on corticosteroids, which are considered the current gold-standard treatment for DMD management. However, prolonged and excessive corticosteroid use is associated with significant side effects, including cataract formation, weight gain, osteoporosis, delayed puberty, and behavioural disturbances. Therefore, there is an urgent need for a cost-effective therapeutic intervention capable of addressing the challenges faced by the global DMD population. Our research endeavours address these challenges with a multifaceted approach. Firstly, we emphasized on elevating utrophin, a dystrophin paralog, which offers an alternative therapeutic strategy for treating DMD. Herein, we discovered a novel series of quinazoline and quinoline based small molecule therapies to enhance utrophin expression, which could treat global DMD population across diverse genetic backgrounds. Secondly, we advanced cell penetrating peptide-conjugated antisense oligonucleotides (ASOs) targeting dystrophin production, optimizing delivery efficiency more precisely to the nucleus with limited cytotoxicity at higher doses for enhanced therapeutic outcomes. Herein, we developed new short 5-mer non-cationic cell-penetrating peptides with enhanced nuclear penetration ability. Additionally, we implemented a straightforward, efficient, and cost-effective strategy for conjugating these peptides with oligonucleotides using click chemistry. We also pioneered a novel mitochondrial-targeted peptide-based therapeutic strategy to promote myogenesis, crucial for muscle development, while reducing fibrosis and maintaining calcium homeostasis. These peptides were designed and crafted from mitochondrial-targeted micropeptides identified for their beneficial effects in enhancing myogenesis. Our findings highlight the potential of these

interventions to improve muscle function, mitigate fibrosis, and enhance the quality of life for individuals with DMD. Overall, our research endeavours provide a multifaceted therapeutic approach aimed at addressing the needs of the broad DMD population and providing cost-effective supportive care.

1. The thesis begins with an introductory **chapter I** that briefly discuss about the background of the disease, pathogenesis associated with the disease progression, current available therapeutics and its limitation. The main findings of the research presented in this thesis are summarized as follows:

2. In **Chapter II** of the thesis I worked on discovery and synthesis of novel quinazoline and quinolone based small molecule library, to identify new Utrophin modulator out of the 'Ezutromid' benzoxazole family. After the failure of the first in class drug for Utrophin modulator 'Ezutromid' from phase-2 clinical trial, there is currently no available therapeutics which could treat global DMD population via Utrophin upregulation.

Herein we present the design, synthesis, and in-vitro validation of novel quinazoline and quinoline core based small molecules distinct from the Ezutromid benzoxazole family, as utrophin upregulators. All together seventy molecules was synthesized from two different schemes. These compounds were crafted through exploring known AhR antagonist ligands and drug molecules having the ability to upregulate utrophin. Notably, our designed series of molecules demonstrates highest utrophin upregulation and also holds promise for myogenesis at lower doses within nanomolar range, offering a potential breakthrough in DMD treatment through utrophin upregulation beyond the benzoxazole scaffold. High-throughput screening via In-cell ELISA in C2C12 myoblasts identified SG-02 as a lead compound. SG-02 demonstrated dose-dependent upregulation of utrophin mRNA (2.11-fold) and protein (up to 2.7-fold) at 800 nM, marking the highest fold change in the nanomolar range observed to date. Evaluation in a DMD patient-derived cell line (KM1328) confirmed SG-02's ability to increase utrophin protein levels by 2.3-fold at the same optimal concentration. Binding studies with AhR protein and co-localization with ITE supported SG-02's role as an AhR antagonist. Additionally, SG-02 exhibited promising potential in promoting myogenesis, crucial for muscle growth and repair. Preliminary ADME assessments highlight SG-02 as a viable oral drug candidate with bioavailability. These findings underscore SG-02 as a dual-action therapy capable of enhancing utrophin expression and supporting myogenesis, suggesting its potential as an effective treatment for DMD.

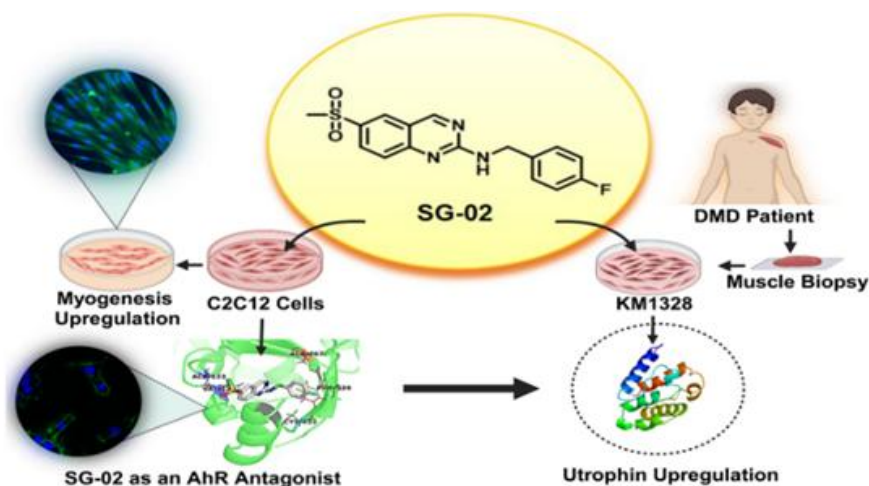


Figure 5.1: Representative figure summarizing chapter 2.

3. In Chapter III, I focussed on advancement of oligonucleotide delivery as current therapeutic strategies for exon skipping using ASOs face challenges, particularly regarding their limited bioavailability, which requires frequent dosing and thus results in high treatment costs. Conjugation of ASOs with CPPs offers a promising avenue to enhance ASO delivery. However, existing CPPs with excellent cell penetration are often enriched with positively charged amino acids, which compromise with cytotoxicity profile and associated with off target localization. Conversely, CPPs lacking positive charge typically exhibit poor cellular uptake. Moreover, the use of negatively charged 2'-OMePS-backbone ASOs presents additional challenges in conjugation with positively charged CPPs. This creates an urgent need for non-cationic CPPs with robust cell penetration and nuclear localization abilities, compatible with negatively charged ASOs. Herein, chapter III focuses on the design and development of a novel short non-cationic CPP with efficient cell-penetrating and nuclear localization capabilities, and its subsequent conjugation with negatively charged 2'-OMePS ASOs with tailored methodology. In this study, a short 5-mer non-cationic CPP, ETWWK, was successfully designed by avoiding the richness of positively charged amino acids within the sequence.

A novel conjugation strategy was developed, employing cost-effective and innovative techniques based on click chemistry. Herein we avoided the use of expensive commercial azide and alkyne linkers commonly utilized in prior studies. Instead, we focused on employing in-house synthesized short and active alkyne linkers, and on the other hand we directly converted the N-terminal amine of CPPs to azide. This method, involving the direct transfer of CPPs N-terminal amine to azide functional motifs, reports the first instance of its application in CPP-ASO conjugation, thereby bypassing the need for costly azide linkers and offering an alternative way-out. In present study we explored an area where little work is done about optimization of conjugation techniques using the 2OMePS ASO backbone, through the utilization of Cu(I)-catalyzed click chemistry. In this study, the conjugation strategy employed results in significant conversion of the unconjugated ASO to peptide conjugated product. The resulting CPP-ASO construct exhibited excellent cellular internalization with precise nuclear localization, as exhibited by uptake studies using flow cytometry and fluorescence microscopy in the clinically relevant DMD patient-derived KM1328 cell line. This study is the

first of its kind to report the design of a short, non-cationic CPP and its successful, cost-effective conjugation with a 2'-OMePS ASO backbone, showcasing its exceptional ability to deliver macromolecular ASOs to the nuclear compartment.

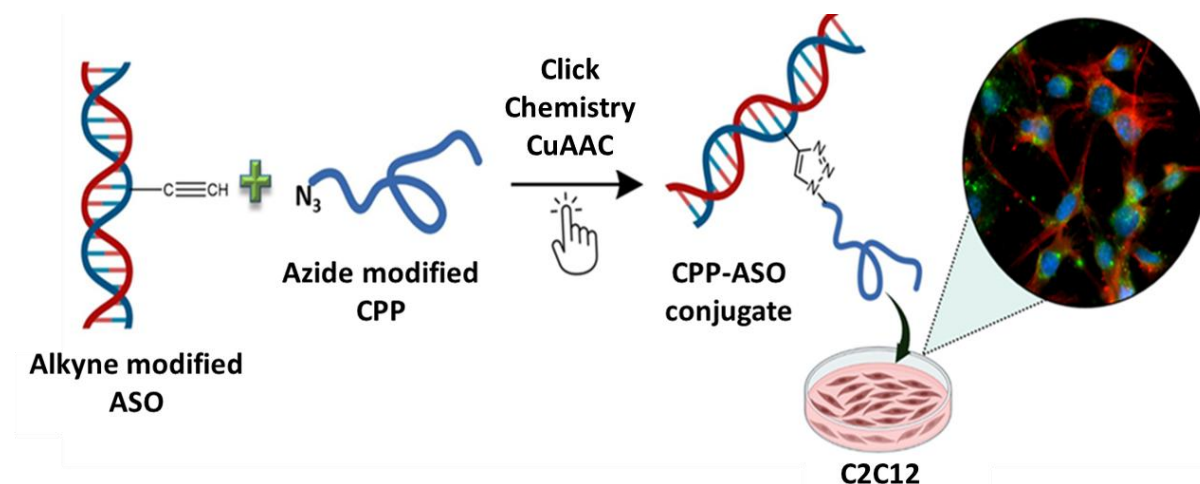


Figure 5.2: Representative figure summarizing chapter 3.

4. Chapter IV emphasized on managing secondary pathology associated with DMD. Use of corticosteroids is considered as the standard care of treatment for DMD till today. As this therapeutic care is easily affordable for the universal DMD population. But, long time exposure to the corticosteroids treatment is associated with several side effects including reduced height, weight gain due to fat deposition, osteoporosis, cataract formation, delayed puberty, and behavioural disturbances. Thus reducing the reliance on corticosteroids would mark a transformative step in redefining the standard of care for DMD patients. In this regard Chapter IV focusses on design and development of mitochondrial targeted peptide to address key pathological features of DMD, aiming to reduce corticosteroid dependency and mitigate associated adverse effects.

Herein, a library of engineered, mitochondria-targeted peptides was developed and synthesized to address key pathological features of DMD, aiming to reduce corticosteroid dependency and mitigate associated adverse effects. The identified lead peptide, E.M.P-2, was able to support myogenesis, reduce fibrosis, and restore cellular homeostasis. Hereby our lead peptide E.M.P-2 can upregulate myogenic markers, as evidenced by a 2.6-fold increase in myogenic marker MyoD and a 2.26-fold increase in MyHC, validated through qRT-PCR and western blot analyses at lower doses within nanomolar range of 150 nM. DMD pathology also involves extensive fibrosis, characterized by excess extracellular matrix (ECM) accumulation and the formation of scar tissue due to repeated muscle injury, chronic inflammation, and disrupted regeneration. The E.M.P-2 peptide has shown significant efficacy in reducing fibrosis, by significant reduction in Col1a protein expression, decreasing it by 60% at the optimal concentration of 0.156 μM thus preserving muscle function and limiting ECM overproduction. E.M.P-2 also maintain calcium homeostasis by chelating calcium ion, as

excess accumulation of calcium within mitochondrial environment leads to disruption of normal mitochondrial function.

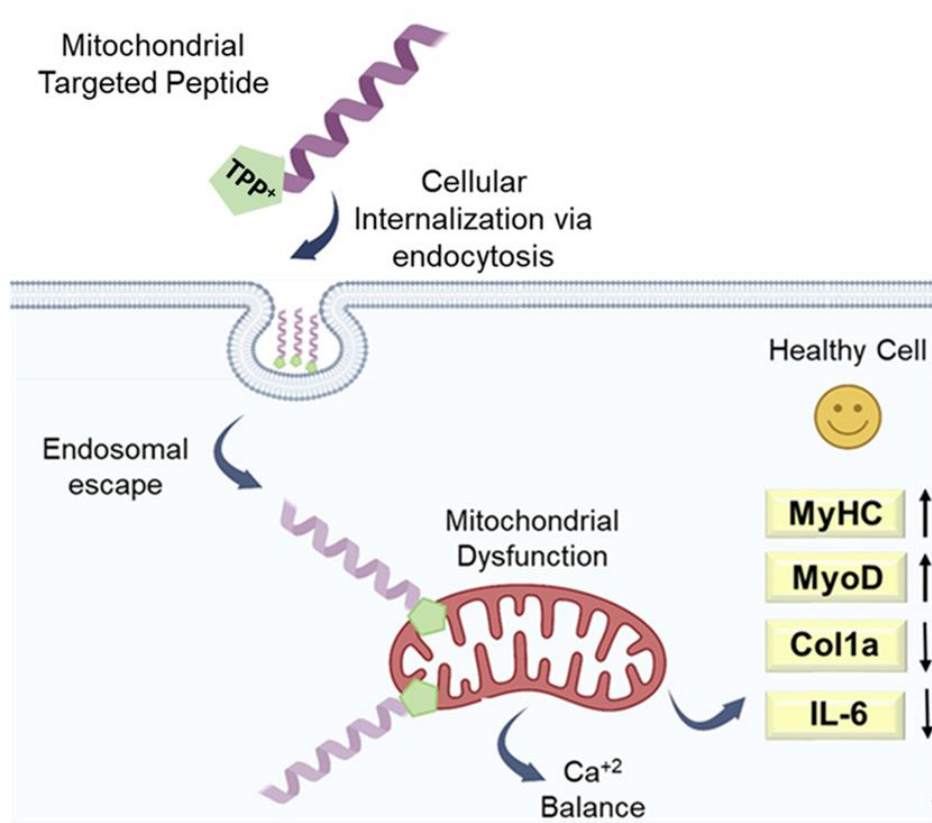


Figure 5.3: Representative figure summarizing chapter 4.

E.M.P-2 offers a holistic approach to DMD treatment by addressing multiple pathogenic pathways. By targeting the root symptoms of DMD, this peptide offers a cost-effective and biocompatible treatment strategy, presenting a promising approach to improve life quality and support for DMD patients across a broader population. This study represents the first instance of peptide-based therapeutics to manage DMD pathophysiology.

5.2 FUTURE PERSPECTIVE

Overall the thesis offers a multifaceted therapeutic strategy to manage DMD, a rare genetic disorder. Three distinct studies are presented in the thesis, each contributing significantly towards development of novel therapeutics solution for DMD.

The first study in **Chapter II** highlight the successful discovery of SG-02, a novel quinazoline-based small molecule, as a promising utrophin modulator for DMD treatment. Given its significant ability to upregulate utrophin expression and promote myogenesis at nanomolar concentrations, SG-02 offers a viable alternative to the failed benzoxazole-based Ezutromid. Moving forward, further preclinical studies, including *in vivo* efficacy assessments in DMD

animal models (mdx mice), are essential to validate the therapeutic potential of SG-02. Future work should focus on advancing SG-02 into preclinical and clinical trials for treating DMD after successful validation of the study in animal model based on its efficacy and toxicological evaluation. Additionally, validation of the mechanistic pathway of SG-02, and its role as an AhR antagonist, could provide deeper insights into its future therapeutic potential to treat other diseases associated with AhR protein. Also, more drugs based on quinazoline and quinoline scaffold should be explored in near future to identify novel drug candidates not only for DMD but also for other AhR-related disorders, such as rheumatoid arthritis and cancer.

The second study in **chapter III** deal with the advancement of oligonucleotide delivery from its prior art by introducing short non cationic cell penetrating peptide attached with negatively charged 2-OMePS oligonucleotides precisely inside the nuclear compartment at its intended site of action. This work addresses critical challenges associated with current oligonucleotide-based exon-skipping therapies. The unique 5-mer non-cationic CPP, ETWWK, avoids the enrichment of positively charged amino acids while maintaining excellent cellular internalization and nuclear targeting efficiency. A novel and cost-effective conjugation strategy was employed here in this study based on Cu(I)-catalyzed click chemistry. This work represents a significant advancement in overcoming the limitations of current exon-skipping strategies, offering a promising pathway to improve oligonucleotide-based therapies for DMD and other genetic disorders. Moving forward, further preclinical studies will be essential to evaluate the *in vivo* efficacy, and safety of the ETWWK-2'-OMePS ASO conjugate. Furthermore, this short, non-cationic CPP can be explored for the delivery of other macromolecular cargoes, including siRNAs, CRISPR-Cas9 components, and other therapeutic leads, broadening its applicability in gene therapy.

The third study in **chapter IV**, presents novel mitochondrial targeted peptide based therapeutics, first of its kind having potential of replacing corticosteroids therapy considered as the standard care for DMD treatment. This work focus on addressing the secondary pathological features of DMD, aiming to address key hallmarks associated with disease progression, including impaired myogenesis, extensive fibrosis, and calcium dysregulation. The identified lead peptide, E.M.P-2, demonstrated significant therapeutic potential by promoting myogenesis, reducing fibrosis, and restoring calcium homeostasis.

Future research should focus on *in vivo* studies to evaluate its therapeutic efficacy, and safety of E.M.P-2 in DMD animal models (mdx mice). A detailed comparative analysis with standard corticosteroid treatment under repeated and long-term dosing regimens in animal models will be essential to evaluate the potential of E.M.P-2 as a viable alternative. These studies will determine whether E.M.P-2 can fully replace corticosteroid therapy as the standard of care for DMD management, offering a safer and more effective therapeutic strategy.

In conclusion, this thesis provide a multimodal therapeutic approach aimed at addressing the needs of the broad DMD population and providing cost-effective supportive care. Collectively, this work significantly advances the therapeutic landscape for DMD by offering novel, targeted, and cost-effective solutions. Firstly development of novel utrophin modulator for treating global DMD population irrespective of mutation type provides a cost effective small molecule based treatment strategy exhibiting significant utrophin upregulation, myogenic potential, and holds oral bioavailability attribute, signifying its candidacy as a

potential therapeutic agent for DMD. Secondly, advancing oligonucleotide delivery by development of novel short non cationic CPP and its conjugation with ASO offers a new therapeutic lead to the evolving landscape of current ASO-based therapeutic regimens most specifically to treat DMD patients by utilizing 2OMePS backbone ASO. These advancement holds promise to reduce the dosing frequency of current ASO-based therapeutics. Lastly, discovery of peptide-Based therapeutics to manage secondary pathological hallmarks associated with disease progression provides a multifaceted strategy to manage key DMD disease progression.

This thesis bridges critical gaps in DMD therapeutics by integrating innovative small molecules for utrophin modulation, advancing oligonucleotides delivery, and novel peptide based therapeutics for managing secondary pathological hallmarks of DMD. Overall, our approach offers a comprehensive framework for transformative DMD management.