



CERTIFICATE

This document hereby certifies that the content presented in the thesis titled "*Smart-Engineered Small Molecule-Based Potential Therapeutic Development for Duchenne Muscular Dystrophy* " has been verified and approved. The research conducted by **Surojit Ghosh** has been conducted under my supervision. The content presented in the thesis is original and has not been previously submitted for any other academic qualification.

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Declaration

I hereby affirm that the research titled "*Smart-Engineered Small Molecule-Based Potential Therapeutic Development for Duchenne Muscular Dystrophy*" is the authentic work conducted by me, under the guidance of **Dr. Surajit Ghosh**, Professor, Department of Bioscience and Bioengineering, Indian Institute of Technology Jodhpur, located in Rajasthan, India (postal code: 342030).

I thus affirm that the content included in this thesis is authentic and has not been previously presented for the purpose of obtaining any other academic qualification, such as a degree, certificate, associateship, fellowship, or any comparable designation, from any university or educational institution. The contributions of other authors in this thesis have been duly recognized.

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Acknowledgement

As I stand at the end of completing my Ph.D. journey, I find myself pausing to reflect on the path that brought me here. While writing this acknowledgment, I imagine that years from now, revisiting these lines will serve as a heartfelt reminder of the unwavering support and invaluable contributions of those who stood by me during this incredible chapter of my life. As of all journey, the past five years of Ph.D. have been filled with both highs and lows, making this path not less than an on road off road journey. This thesis is not mine alone, it is a result of the unwavering support, encouragement, and inspiration I have received from those with whom I share deep connections. It is a testimony to their presence in my life, both inside and outside the lab, and their role in keeping me motivated through this demanding journey. While no words can truly capture the gratitude I feel for their contributions, I will make an earnest attempt to express my heartfelt appreciation in the lines.

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Abstract

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. However, in the majority of cases, the current therapeutic paradigm for managing DMD primarily emphasizes on symptomatic treatment. Although these symptomatic and supportive treatments have shown limited success in improving the life expectancy of individuals with DMD, but there is no currently available treatment that can modify the course of the disease and treat the universal DMD population. Our research addresses these challenges with a multifaceted approach. Here, our overall goal is to design, develop and further validate small molecule and peptide based therapeutics targeting different key strategies to combat DMD and providing better life care support for global DMD population.

This study presents a multi-faceted therapeutic approach addressing key challenges in DMD management, including utrophin modulation, antisense oligonucleotide (ASO) delivery, and the mitigation of secondary pathological hallmarks. Firstly, we designed and synthesized a library of seventy novel quinazoline- and quinoline-based small molecules as utrophin modulators. High-throughput In-cell ELISA screening identified SG-02 as the most promising candidate, exhibiting 2.7-fold upregulation of utrophin at a nanomolar concentration doses of 800 nM in a dose-dependent manner. Mechanistic studies performed identified SG-02 as a potent antagonist of the aryl hydrocarbon receptor (AhR). SG-02 exhibited high binding affinity to AhR, with a dissociation constant (K_d) of 41.68 nM, indicating strong target engagement. Furthermore, SG-02 enhanced myogenesis by upregulating myosin heavy chain (MyHC) expression, a key marker of muscle differentiation. These results highlight SG-02 as a promising therapeutic agent for DMD that targets utrophin upregulation through AhR antagonism, offering broad applicability to the DMD patient population.

Second, to address the delivery limitations of antisense oligonucleotides (ASOs) for exon-skipping therapies, we developed a novel short non-cationic cell-penetrating peptide (CPP), ETWWK. This peptide was specifically designed by avoiding richness of positive charged amino acids to enable efficient conjugation with the negatively charged 2'-O-methyl phosphorothioate (2OMePS) ASO backbone using click chemistry. Functional studies demonstrated that the ETWWK-ASO conjugate successfully delivered ASO to the nuclear compartment, its intended site of action, achieving a 2-fold upregulation of dystrophin mRNA expression compared to untagged ASO. These findings mark a significant advancement in ASO delivery systems, addressing prior limitations such as low nuclear access, poor selectivity, and compromised cytotoxicity.

Lastly, we focused on mitigating secondary pathological hallmarks of DMD progression by developing a novel therapeutic peptide, E.M.P-2. Designed with mitochondrial targeting and calcium-chelating

properties, E.M.P-2 promoted myogenesis by upregulating key myogenic markers, myosin heavy chain (MyHC) and myogenic differentiation factor (MyoD), by more than 2-fold at a concentration of 150 nM. In addition, E.M.P-2 effectively mitigated fibrosis by downregulating collagen type I alpha 1 chain (COL1A1), a critical fibrosis marker, by 60%. The peptide also restored impaired calcium homeostasis within mitochondria and the cellular environment, a key factor in muscle regeneration. Furthermore, E.M.P-2 exhibited anti-inflammatory properties by significantly reducing IL-6 expression in DMD patient-derived cells, which display elevated baseline IL-6 levels. Overall, E.M.P-2 represents a first-in-class peptide therapeutic capable of addressing multiple pathological features of DMD, including fibrosis, impaired myogenesis, calcium dysregulation, and inflammation. This peptide holds the potential to replace corticosteroids, the current gold standard for DMD treatment, which are associated with significant long-term side effects.

Together, these findings demonstrate a comprehensive therapeutic strategy for DMD, advancing the field through small-molecule utrophin modulators, innovative CPP-ASO conjugation systems, and peptide-based interventions targeting secondary disease hallmarks. My research endeavours provides a multifaceted therapeutic approach aimed at addressing the needs of the broader DMD population and providing cost-effective supportive care.

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Contents

	<i>page</i>
Certificate	<i>i</i>
Declaration	<i>ii</i>
Acknowledgements	<i>iii-v</i>
Abstract	<i>vi-vii</i>
List of Publications	<i>viii-ix</i>
Contents	<i>x-xiv</i>
List of Figures	<i>xiv-xx</i>
List of Tables	<i>xx</i>
List of Symbols	<i>xx</i>
List of Abbreviations	<i>xxi-xxii</i>

Chapter 1: Introduction

1.1 Duchenne Muscular Dystrophy: A Rare Genetic Disorder.	1-2
1.2 Role of Dystrophin Protein	2
1.3 DIAGNOSIS OF DMD	3
1.4 DISEASE PROGRESSION OF DMD	3
1.4.1 Pathogenesis of DMD	4-6
1.5 DIFFERENT TYPES OF MUTATION IN DMD GENE	6-7
1.6 Therapeutic strategies for DMD	7-9
1.6.1 THERAPEUTIC APPROACHES IN CLINICAL STAGE OF DEVELOPMENT	9
1.6.1.1 Dystrophin restoration therapies via anti-sense mediated exon skipping strategy	9-10
1.6.1.2 Antisense oligonucleotides (ASOs)	10-11
1.6.1.3 Cell Penetrating Peptides (CPPs)	11-14
1.6.1.4 CPP Conjugated ASOs	14-15
1.6.1.5 Utrophin Modulation	15-16
1.7 Research Objectives	16
1.8 Thesis Overlay	17
1.9 Research Contribution	18
1.10 Discussion and Future Prospect	18-19
1.11 References	19-25

Chapter 2: Quinazoline and Quinoline-Based Small Molecules as Utrophin Upregulators for Duchenne Muscular Dystrophy

2.1 Abstract	26
2.2 Introduction	26-28
2.3 Results and Discussion	
2.3.1 Design of Utrophin Modulators Based on Quinoline and Quinazoline scaffold	28-29
2.3.2 In-silico Molecular Docking Analysis	30-31
2.3.3 Chemistry	31-40
2.3.4. Screening of Molecular Library by High-throughput In-Cell ELISA Technique	41-43
2.3.5 Cellular Cytotoxicity	43-44
2.3.6 Structure Activity Relationship	44-49
2.3.7 qRT-PCR and Western Blot of Hit Molecules Identified	49-50
2.3.8 Utrophin Protein Expression in Human Patient Derived DMD Cell Line	50-51
2.3.9 Target Validation for Lead Molecule SG-02	51-52
2.3.10 Fusion Assay	52-54
2.3.11 In-Vitro ADME Study	
2.3.11.1 Aqueous Solubility Assay	54
2.3.11.2 Microsomal Stability Assay	54-55
2.3.11.3 Serum Stability Assay	55
2.3.11.4 Lipophilicity Assay	56
2.4 Experimental Section	
2.4.1. Materials	56-57
2.4.2 Methods	
2.4.2.1 Synthesis of molecular library	57-59
2.4.2.2 Cell Viability	59-60
2.4.2.3 Screening of Molecular Library by High-throughput In-Cell ELISA Technique	60
2.4.2.4 RNA Extraction and Reverse Transcription-Polymerase Chain Reaction	56
2.4.2.5 Protein Isolation and Western Blot Analysis	60-61
2.4.2.6 Fluorescence Quenching Assay of SG-02 with AhR: ARNT	61
2.4.2.7 Immunofluorescence Analysis of AhR Localization	61-62
2.4.2.8 Myoblast Fusion	62
2.4.2.9 MyHC RNA Extraction and Reverse Transcription-Polymerase Chain Reaction	62
2.4.2.10 Protein Isolation from Patient Derived Human Cell Line (KM1328) and Dot Blot Analysis	63

2.4.2.11 Microsomal Stability Assay	63
2.4.2.12 Aqueous Solubility Assay	63
2.4.2.13 Serum Stability Assay	64
2.4.2.14 Lipophilicity assay	64
2.4.2.15 Molecular Docking Studies	64
2.5 Conclusion	65
2.6 References	65-68
Chapter 3: Delivery of Antisense Oligonucleotide through Chemical Conjugation with Designed Short Non-Cationic Cell Penetrating Peptide using Click Chemistry for Duchenne Muscular Dystrophy	
3.1 Abstract	69
3.2 Introduction	69-71
3.3 Results and Discussion	
3.3.1 Design and Synthesis of Cell penetrating peptides	71-73
3.3.2 Conjugation of azide functionalized CPPs with alkyne containing ASO and vice versa	73-76
3.3.3 Conjugation of N-terminal azide functionalized CPPs with active alkyne containing ASO	76-94
3.3.4 Monitoring of click chemistry reaction via Infrared Spectroscopy	95
3.3.5 Mechanistic Pathway associated with uptake of Cell Penetrating Peptide ETWWK	95
3.3.6 Cytotoxicity of the designed Cell penetrating peptides	96-97
3.3.7 Cellular uptake study by immunocytochemistry and flow cytometry	97-99
3.3.8 qRT-PCR of ETWWK-ASO construct	99
3.4. Materials and Methods	
3.4.1 Materials	100
3.4.2 Methods	
3.4.2.1 Synthesis of Cell Penetrating Peptides	101
3.4.2.2 Synthesis of Azo transfer reagent, Imidazole-1-sulfonyl Azide Hydrogen Sulfate	101
3.4.2.3 Solid phase N-terminal amine to azide transfer by utilizing Imidazole-1-sulfonyl Azide. H ₂ SO ₄	101-102
3.4.2.4 Synthesis of an alkyne linker for conjugation to ASO	102
3.4.2.5 Conjugation of synthesized alkyne linker to free amino linker of ASO	102-103
3.4.2.6 Conjugation of CPPs with ASO via Click Chemistry in solid support	
3.4.2.6.1 Conjugation of N-terminal Azide containing CPPs with Alkyne functionalized ASO under ligand free condition	103-104
3.4.2.6.2 Conjugation of N-terminal Azide containing CPPs with Alkyne functionalized ASO in presence of TBTA and (BimH) ₃	104

3.4.2.7. Synthesis of 5-carboxy fluorescein conjugated Cell Penetrating Peptide (ETWWK-FAM)	104-105
3.4.2.8 Conjugation of ETWWK-FAM with ASO	105
3.4.2.9. ESI-MS analysis for the peptide conjugated ASO	105
3.4.2.10 Cytotoxicity Assay	105
3.4.2.11. Cellular imaging and uptake assay	
3.4.2.11.1 Immunocytochemistry	106
3.4.2.11.2 Flow Cytometry Studies	106-107
3.4.2.12 Dystrophin RNA Extraction and Reverse Transcription-Polymerase Chain Reaction	107
3.4.2.13 Serum stability assay for synthesized cell penetrating peptides	107-108
3.5 Conclusion	108-109
3.6 References	110-114

Chapter 4: Mitochondrial Targeted Engineered Peptide Based Therapeutics for Managing Duchenne Muscular Dystrophy Pathophysiology via Enhancing Myogenesis and Mitigating Fibrosis

4.1 Abstract	115
4.2 Introduction	115-117
4.3 Results and Discussion	
4.3.1 Design and synthesis of mitochondria targeted peptides	117-118
4.3.2 Screening of Peptide Library by High-throughput In-Cell ELISA Technique	118-119
4.3.3 Mechanistic pathway behind cellular internalization and mitochondrial localization	119-121
4.3.4 Fusion Assay	121-122
4.3.5 Protein and mRNA expression of E.M.P-2	122-124
4.3.6 Maintaining Calcium homeostasis by E.M.P-2	124-126
4.3.7 Downregulation of pro-inflammatory marker IL-6, and fibrosis marker COL1A1 by E.M.P-2	126-127
4.3.8 Cytotoxicity and serum stability of the lead engineered peptide E.M.P-2	126-128
4.4 Materials and Methods	
4.4.1 Materials	128-129
4.4.2 Methods	
4.4.2.1 Synthesis of Peptide Library	129
4.4.2.2 Synthesis of TPP conjugated lead peptide E.M.P-2	130
4.4.2.3 Synthesis of 5-carboxy fluorescein conjugated TPP-E.M.P-2	130-132
4.4.2.4 UV-Vis fluorescence spectroscopy	132
4.4.2.5 Fluorescence spectroscopy	132
4.4.2.6 CD Spectroscopy	132
4.4.2.7 Zeta Potential	133

4.4.2.8 Cytotoxicity Study	133
4.4.2.9 Serum stability assay for lead engineered peptide E.M.P-2	133
4.4.2.10 Flow Cytometry Studies	133-134
4.4.2.11 In Cell ELISA	134
4.4.2.12 RNA extraction and quantitative reverse transcriptase- PCR (RT-qPCR)	134-135
4.4.2.13 Dot Blot for protein expression	135
4.4.2.14 Mitotracker	135
4.4.2.15 Immunocytochemistry	135
4.5 Conclusion	136-137
4.6 References	137-141
Chapter 5: Summary and Future Perspective	
5.1 Summary	142-146
5.2 FUTURE PERSPECTIVE	146-148
APPENDIX A	149-193
APPENDIX B	194-202

List of Figures

Figures	Title	Page
1.1	Comparative illustration of an intact muscle membrane (top panel) in which dystrophin anchors the dystrophin-glycoprotein complex (DGC) to the actin cytoskeleton, versus a dystrophin-deficient membrane (bottom panel) showing membrane instability associated with DMD. Comparison depicts differences between a healthy muscle cell in a normal boy and a dystrophic muscle cell in a boy with DMD. Image reproduced from Houang et al., Skeletal Muscle, 2018 with copyright permission).	1
1.2	Illustrative representation of the progression of DMD disease starting from deposition of fat and scar tissue to dysfunction of heart muscle.	4
1.3	Different mutations in DMD gene. A) Mutation spectrum of DMD gene in the cohort. B) Deletion mutations in the DMD gene exhibits two hotspot regions: Proximal (exons 1–22) and distal (exons 43–55)	7
1.4	Chemical structures of PMO and 2'OMe-PS subunits	10
1.5	Schematic representation of the cellular uptake mechanisms for CPPs or CPP-Cargo complexes. Two main pathways of internalization are illustrated: direct translocation and endocytosis. Image reproduced from Cell-Penetrating Peptides - Creative Peptides Blog.	14
1.6	Replacement of utrophin with dystrophin during early human development, from 10 to 25 weeks of fetal development	16

1.7	Research Overlay illustrating multifaceted approach to find cure for Duchenne Muscular Dystrophy	17
2.1	Design of tailor-made molecules having potential of utrophin modulation and molecular docking analysis. (A) Design of quinoline based scaffold from the structural features of phenazopyridine and nabumetone. (B) Design of quinazoline based scaffold from the structural features of GNF-351 and nabumetone.	29
2.2	A) Binding model of SG-02 with AhR: ARNT complex protein (PDB ID: 5NJ8) showing possible binding interactions with key amino acids. (B) 2D binding interactions of compound SG-02 with the important amino acid residues. Pink circles reflects π - π stacking interaction, yellow circle represent pi-sulfur interaction and deep green circle indicates hydrogen bonding interaction of amino acid residues with ligand. (C) Blind docking of AhR protein with SG-02, reflects binding of SG-02 within hydrophobic pocket of AhR protein.	30
2.3	A) Binding site of lead molecule SG-02 with AhR:ARNT (PDBID: 5NJ8) protein complex. (B) Amino acid of protein interacting with SG-02.	31
2.4	Routes employed towards synthesis of quinazoline analogues and library of molecules synthesized	32
2.5	Routes employed towards synthesis of quinoline analogues and library of molecules synthesized. Reagents and conditions	33
2.6	^1H NMR spectra of cpd 15 (SG-02) (500 MHz, DMSO-d6)	35
2.7	^{13}C NMR spectra of cpd 15 (SG-02) (125 MHz, DMSO-d6)	35
2.8	HPLC chromatogram of cpd 15 (SG-02)	36
2.9	ESI-MS spectra of cpd 15 (SG-02)	36
2.10	^1H NMR spectra of cpd 18 (SG-39) (500 MHz, DMSO-d6)	37
2.11	^{13}C NMR spectra of cpd 18 (SG-39) (125 MHz, DMSO-d6)	37
2.12	HPLC chromatogram of cpd 15 (SG-02)	38
2.13	ESI-MS spectra of cpd 18 (SG-39)	38
2.14	^1H NMR spectra of cpd 29 (SG-60) (500 MHz, DMSO-d6)	39
2.15	^{13}C NMR spectra of cpd 29 (SG-60) (125 MHz, DMSO-d6)	39
2.16	HPLC chromatogram of cpd 29 (SG-60)	40
2.17	ESI-MS spectra of cpd 29 (SG-60)	40
2.18	(A-D) In-Cell ELISA Data for Determination of Optimal Screening Concentration for cpd 13, 16, 17, 32. In all data sets, significance was calculated using one-way ANOVA, * $p < 0.05$, ** $p < 0.01$	41
2.19	(A-K) In-Cell ELISA Data for Screening of the Molecular Library at 800 nM. In all data sets, significance was calculated using one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.	42-43

2.20	Cell survival percentage after treatment of C2C12 myoblast cells with the three lead molecules (SG-02, SG-39, and SG-60) identified in an increasing gradient of doses (3.125-100 μ M) for 24 h, n=3. In all data sets, significance was calculated using one-way ANOVA, ****p<0.0001	43
2.21	Core scaffold structures and key regions of interest for structure-activity relationships, illustrating the ligand library and variations in aqueous-soluble motifs	44
2.22	qRT-PCR assay. (A) Utrophin mRNA expression in C2C12 myoblast cell after treatment with three lead molecules SG-02, SG-39, and SG-60 at 800 nM concentration for 72 hr compared to piperine at 3 μ M and untreated control. (B & C) Dose-dependent utrophin mRNA Upregulation in SG-02 and SG-39 treated C2C12 Cells for 72 hr compared to piperine at 3 μ M, and untreated control. In all data sets, significance was calculated using one-way ANOVA, *p< 0.05, **p<0.01, ***p<0.001	49
2.23	Western Blot assay. (a) Western blot analysis comparing protein expression levels of utrophin in C2C12 cells treated with SG-02 in a dose-dependent manner, compared to piperine-treated cells (3 μ M), and untreated cells after 72 hr of treatment. (b) Bar diagram representing utrophin expression in terms of fold change for SG-02. (c) Western blot analysis comparing protein expression levels of utrophin in C2C12 cells treated with SG-39 in a dose-dependent manner, compared to piperine-treated cells (3 μ M), and untreated cells after 72 hr of treatment. (d) Bar diagram representing utrophin expression in terms of fold change for SG-39; GAPDH was taken as a loading control for all Western blot experiments. In all data sets, significance was calculated using one-way ANOVA, **p<0.01, ***p<0.001	50
2.24	Dot Blot. Treatment with SG-02 and piperine increases utrophin protein after treating Patient derived human DMD cell line (KM1328) for 72 hr. (a) Relative utrophin expression was assessed by Dot blot analysis for untreated control, piperine (3 μ M) and SG-02 (800 nM) treated cells. (b) Bar diagram representing utrophin expression in terms of fold change. In all data sets, significance was calculated using one-way ANOVA, ***p<0.001	51
2.25	SG-02 behaves as an aryl hydrocarbon antagonist. (A) The subcellular localization of AhR induced by SG-02 treatment as examined using immunofluorescence. In C2C12, AhR was predominantly found in the cytoplasm unlike the agonist 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), which promotes nuclear translocation of AhR, SG-02 did not cause an increase in nuclear translocation. Whereas, co-treatment of ITE (1 μ M) with SG-02 (800 nM) resulted in the retention of AhR in the cytoplasm. (B) Tryptophan fluorescence quenching assay to determine the binding constant between AhR:ARNT complex and SG-02.	52
2.26	Myoblast Fusion. (A) Immunofluorescent image of fused cells, where the cells were labelled with primary antibody to MyHC and secondary Alexa 488 (green) and Hoechst 33258 (blue). Scale bar corresponds to 50 μ m. Myoblast fusion is observed in the group treated with compound SG-02 (800 nM) for 7 Days. (B) MyHC mRNA expression of SG-02 in C2C12 myoblast cell after treatment with SG-02 in 800 nM concentration for 72 hr shows two fold upregulation in comparison to untreated control. (C) The percent (%) fusion index was determined by calculating the ratio of nuclei detected in the MyHC positive area to the total nuclei count. In all data sets, significance was calculated using t test **p<0.01, and ***p<0.001.	53
2.27	Myoblast Fusion Analysis of Lead Molecule SG-02 performed through Myocount software, which processed the captured images based on immunofluorescent image of fused cells. Analysis was calculated in five image per well. Scale bar corresponds to 50 μ m.	54

2.28	Biophysical characterization of lead molecule SG-02. (A) Turbidity assay to determine aqueous solubility of three lead molecules SG-02, SG-39, and SG-60 with pyrene as control. After 2 hours of incubation in PBS, absorbance was measured at 620 nm. (B) Microsomal stability of lead molecule analyzed by microsomal incubation of SG-02 over a period of 60 min. (C) Serum stability of SG-02 displays that SG-02 is intact upto 70% after two hours of incubation.	55
3.1	Schematic representation of ETWWK-ASO construct synthesis and its further application in cellular internalization.	72
3.2	Comparative cellular uptake study of ETWW and ETWWK upon treatment with C2C12 cells for 30 minutes. Scale bar corresponds to 20 μ m.	73
3.3	Deconvoluted Mass spectra of ASO attached with azidoacetic acid	74
3.4	Deconvoluted Mass spectra of ASO attached with pentynoic acid.	75
3.5	Mass spectra of CPP-1 (ETWW) attached with pentynoic acid	75
3.6	Mass spectra of CPP-1 (ETWW) attached with azidoacetic acid	76
3.7	Schematic representation of CPP-ASO coupling via click chemistry, where the ASO is functionalized with an alkyne linker and the CPP is converted to N-terminal azide group	76
3.8	Comparative mass spectra of ASO and ASO attached with linker. Mass peaks observed at M-3H/3, M-4H/4, M-5H/5, M-6H/6, and M-7 H/7.	77
3.9	Comparative mass spectra of ASO-linker, ETWWR conjugated ASO, and ETWWK conjugated ASO	79
3.10	¹ H-NMR spectra of synthesized alkyne linker (500 MHz, CDCl ₃)	81
3.11	¹³ C-NMR spectra of synthesized alkyne linker (125 MHz, CDCl ₃)	82
3.12	Mass spectra of synthesized alkyne linker	82
3.13	HPLC chromatogram of synthesized alkyne linker.	83
3.14	¹ H-NMR spectra of synthesized azo transfer reagent (500 MHz, DMSO-d ₆)	83
3.15	¹³ C-NMR spectra of synthesized azo transfer reagent (125 MHz, DMSO-d ₆).	84
3.16	Mass spectra of synthesized azo transfer reagent.H ₂ SO ₄ salt	84
3.17	Mass spectra of CPP-2 (ETWWK-Azide)	85
3.18	HPLC chromatogram of CPP-2 (ETWWK-AZIDE)	85
3.19	Comparative Deconvoluted spectra between ASO and ASO attached with ASO-Linker.	86
3.20	Comparative Deconvoluted spectra between ASO-Linker and ASO attached with ETWWK.	86
3.21	Comparative Deconvoluted spectra between ASO-Linker and ASO-ETWWR.	87
3.22	Comparative mass spectra between ASO-Linker and ASO attached with ETWW	87

3.23	Comparative Deconvoluted mass spectra between ASO-Linker and ASO-ETWW	88
3.24	Comparative mass spectra between ASO-Linker and ASO attached with ETWWKKR	88
3.25	Comparative Deconvoluted mass spectra between ASO-Linker and ASO-ETWWKKR	89
3.26	Comparative mass spectra between ASO-Linker and ASO attached with ETWWK-FAM	89
3.27	Deconvoluted comparative mass spectra of ASO-Linker and ASO-ETWWK-FAM	90
3.28	Comparative Deconvoluted mass spectra of ASO-Linker and ASO-FAM.	90
3.29	HPLC percentage conversion of ASO and ASO-ETWWR with (BimH) ₃ ligand	91
3.30	HPLC percentage conversion of ASO and ASO-ETWWR with TBTA ligand	91
3.31	HPLC percentage conversion of ASO and ASO-ETWWR under ligand free condition	92
3.32	HPLC percentage conversion of ASO and ASO-ETWWK with TBTA ligand	92
3.33	HPLC percentage conversion of ASO and ASO-ETWW with TBTA ligand	93
3.34	HPLC percentage conversion of ASO and ASO-ETWWKKR with TBTA ligand	93
3.35	HPLC percentage conversion of ASO and ASO-FAM-ETWWK with TBTA ligand	94
3.36	HPLC percentage purity of the lead CPP-ASO construct ASO- ETWWK after purification via NAP-10 desalting column	94
3.37	A) The progression of the reaction was tracked by monitoring the conversion of the azide and alkyne motifs into triazole moiety using IR spectroscopy. B) A cellular uptake analysis of 5 μ M F-ETWWK suggests that the uptake occurs through an endocytosis pathway.	95
3.38	Cell survival percentage after treatment of C2C12 myoblast cells with the four CPP candidates (ETWW, ETWWK, ETWWR and ETWWKKR) identified in an increasing gradient of doses (1.53-200 μ M) for 24 h, n=3. In all data sets, significance was calculated using one-way ANOVA, **p<0.01.	96
3.39	Cell survival percentage. A) After treatment of human DMD patient derived myoblast cells (KM1328) with ETWWK-ASO construct. B) After treatment of human DMD patient derived skin fibroblast cells with ETWWK-ASO construct. In an increasing gradient of doses (3.12-100 μ M) for 24 h, n=3. In all data sets, significance was calculated using one-way ANOVA, ****p $\leq$ 0.0001.	97
3.40	Comparative cellular uptake study between FAM conjugated ASO and FAM conjugated ETWWK tagged ASO via fluorescence microscopy upon treatment with C2C12 cells for 4 hours. Scale bar corresponds to 20 μ m.	97
3.41	Comparative cellular uptake study of FAM tagged ETWWK-ASO construct (Upper panel) and FAM tagged ASO (lower panel) upon treatment with human DMD patient derived myoblast cells (KM1328) for 4 hours. Scale bar corresponds to 50 μ m.	98
3.42	Comparative cellular uptake study between unstained (Blue), FAM conjugated ASO (Green) and FAM conjugated ETWWK tagged ASO (Red) via flow cytometry upon treatment with DMD patient derived diseased muscle cells (KM1328) for 4 hours. A&B)	99

	Comparative histogram and dot blot representations. C) qRT-PCR exhibiting mRNA expression of ETWWK-ASO construct and ASO in comparison to untreated control. In all data sets, significance was calculated using one-way ANOVA, **p<0.01	
3-43	Serum stability of lead peptide ETWWK	108
4.1	Schematic representation of the role of E.M.P-2 in addressing secondary pathological hallmarks within DMD cellular environment	116
4.2	Amino acid sequence alignment of MPM micropeptide in human. Library of peptide synthesized by truncation of MPM micropeptide	118
4.3	A) In-cell ELISA screening of E.M.P-2 for determination of effective screening concentration ranging from 10 μ M to 0.15 μ M. B) Screening of remaining micropeptides library at optimum concentration of 0.15 μ M via high throughput In-Cell ELISA technique. In all data sets, significance was calculated using one-way ANOVA, ***p<0.001.	119
4.4	A cellular uptake analysis of 5 μ M FAM-E.M.P-2 suggests that the uptake occurs through an energy dependent endocytosis pathway, as evidenced by significantly higher uptake at 37°C compared to 4°C. A&B) Comparative histogram and dot blot representations.	120
4.5	Mitochondrial target ability of TPP conjugated engineered E.M.P-2, were FAM tagged engineered peptide co-localizes within mitochondrial compartment of the cell labelled by mitoTracker. Scale bar corresponds to 100 μ m.	120
4.6	Myoblast Fusion. A) Immunofluorescent image of fused cells, where the cells were labelled with primary antibody to MyoD and secondary Alexa 488 (green) and Hoechst 33258 (blue). Scale bar corresponds to 100 μ m. Myoblast fusion is observed in the group treated with compound M.P-02 (150 nM) for 3 Days in comparison to untreated control. (B) The percent (%) fusion index was determined by calculating the ratio of nuclei detected in the MyHC positive area to the total nuclei count. (C) Total nuclei within MyHC cells, were calculated in five image fields per well. This analysis was carried out using Myocount software, which processed the captured images. In all data sets, significance was calculated using t test **p<0.01.	121
4.7	qRT-PCR assay and Dot blot results, after treatment of human DMD patient derived cells with lead peptide E.M.P-2 at 0.156 μ M concentration for 72 hr compared to retinoic acid at 5 nM and untreated control (A) MyoD mRNA expression in dose dependent manner. (B) MyHC mRNA expression in dose dependent manner (C) Col1a mRNA expression in dose dependent manner. (D) Dot blot analysis comparing protein expression levels of MyoD in a dose-dependent manner. (E) Protein expression levels of MyHC in a dose-dependent manner. (F) Protein expression levels of Col1a in a dose-dependent manner. In all data sets, significance was calculated using one-way ANOVA, *p<0.1**p<0.01***p<0.001****p<0.0001.	123
4.8	UV-Vis and fluorescence spectra (A) UV-Vis spectra of peptide E.M.P-2 and calcium chelated peptide (E.M.P-2:CaCl ₂), at different peptide to CaCl ₂ volume ratios of 1:1, 1:2, and 1:5. (B) Fluorescence spectra of peptide E.M.P-2 and calcium chelated peptide (E.M.P-2:CaCl ₂), at different peptide to CaCl ₂ volume ratios of 1:1, 1:2, and 1:5. (C) CD spectra of peptide E.M.P-2 and E.M.P-2:CaCl ₂ at 1:1 volume ratio of peptide to CaCl ₂ . D) Comparative Zeta potential of peptide E.M.P-2 and E.M.P-2:CaCl ₂ at 1:1 volume ratio of peptide to CaCl ₂ .	125
4.9	Immunocytochemistry analysis. A) Downregulation of pro inflammatory cytokine IL-6 significantly (Upper panel), similarly significant downregulation of COL1A fibrosis	127

marker. B&C) Bar diagram representing IL-6 & COL1A1 downregulation. Scale bar corresponds to 100µm. In all data sets, significance was calculated using t test **p<0.01.

4.10	A) Serum stability of lead engineered peptide E.M.P-2 monitored for 10 hours in human plasma. B) Cell survival percentage after treatment of KM1328 cells with the lead peptide identified E.M.P-2 in an increasing gradient of doses (3.125-100 µM) for 24 h, n=3. In all data sets, significance was calculated using one-way ANOVA	128
4.11	Mass Spectra of the engineered lead peptide E.M.P-2	131
4.12	HPLC Spectra of the engineered lead peptide E.M.P-2	131
4.13	Mass Spectra of the FAM labelled engineered lead peptide E.M.P-2	132
5.1	Representative figure summarizing chapter 2	143
5.2	Representative figure summarizing chapter 3	144
5.3	Representative figure summarizing chapter 4	145

List of Tables

1.1	Clinical Trials utilizing exon skipping and stop codon read through for DMD therapy in 2023. Image reproduced from Angulski et al., Frontiers in Physiology, 2023 with copyright permission.	11
2.1	Structure-activity relationships with basic quinazoline scaffold and varying R ₁ and R ₂ substitution with variable chain length of n = 0,1,2. Each data set represent the average of three replicates. Here cpd signifies for compounds.	45
2.2	Structure-activity relationships with basic quinoline scaffold and varying R ₁ and R ₂ substitution with variable chain length of n = 0,1,2. Each data set represent the average of three replicates. Here cpd signifies for compounds.	47
2.3	Structure-activity relationship analyzing regioisomers of aqueous soluble motif for lead molecule SG-02 and SG-39. Each data set represent the average of three replicates Here cpd signifies for compounds.	48
2.4	Preliminary ADME properties such as Solubility, microsomal stability, plasma stability for lead molecule SG-02. ^a Mean % remaining at 2 h in human plasma, ^b Cyclohexane/PBS, ^c Octanol/PBS.	56
3.1	Sequence of ASO and CPP-ASO conjugates with corresponding molecular weights and percentage conversion of product under varied condition and with different CPPs.	80
4.1	Table 4.1: Forward and reverse primer for MyHC, MyoD and COL1A1.	135

List of Symbols

Symbol	Description
α	Alpha

β	Beta
$^{\circ}\text{C}$	Degree centigrade
μ	Micro
$\text{\AA}$	Angstrom
$\pm$	Plus-minus
N_A	Avogadro's number

List of Abbreviations

<i>Abbreviation</i>	<i>Full form</i>
Ph.D.	Doctor of Philosophy
ADME	Absorption, Distribution, Metabolism and Excretion
MyHC	Myosin Heavy Chain
BSA	Bovine serum albumin
ELISA	Enzyme-Linked Immuno Sorbent Assay
qRT-PCR	Real-Time Quantitative Reverse Transcription polymerase chain reaction
ITE	2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester
n-Bu ₄ NCl	Tetrabutylammonium chloride
TMSCl	Trimethylsilyl chloride
t-BuON=O	tert-Butyl Nitrite
K ₂ CO ₃	Potassium carbonate
DMA	Dimethylacetamide;
DCM	Dichloromethane
DMF	Dimethylformamide
RSO ₂ Na	Sodium alkylsulfinate
DMSO	Dimethyl sulfoxide
AlCl ₃	Alumunium chloride
POCl ₃	Phosphorous oxychloride
DMAP	4-(Dimethylamino)pyridine
AhR:ARNT	Aryl hydrocarbon receptor nuclear translocator
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
PBS	Phosphate buffered saline;
TMS	Tetramethylsilane
EDTA	Ethylenediamine tetraacetic acid
TEMED	N,N,N',N' -Tetramethylethylenediamine
PVDF	Polyvinylidene difluoride Membrane
HRP	Horseradish peroxidase;
NaHCO ₃	Sodium Bicarbonate
NaOH	Sodium Hydroxide
EtOAc	Ethyl acetate
CuI	Copper iodide
OPD	o-phenylenediamine dihydrochloride
TBS-T	Tris-buffered saline with 0.1 % Tween® 20 detergent
RIPA Buffer	Radioimmunoprecipitation assay buffer
ASO	Antisense oligonucleotides
CPP	Cell penetrating peptides
PS-ON's	Phosphorothioate based oligonucleotides
ISA.H ₂ SO ₄	Imidazole-1-sulfonyl Azide Hydrogen Sulfate

CuBr.Me ₂ S	Copper(I) bromide dimethyl sulfide complex
HPLC	High performance liquid chromatography
ESI-MS	Electrospray ionization mass spectrometry
NMR	Nuclear magnetic resonance
DAPC	Dystrophin-associated protein complex
DMD	Duchenne Muscular Dystrophy
IL-6	Interlukin-6
FDA	Food and Drug Administration
MPM	Micropeptide in Mitochondria
COL1A1	Collagen type I alpha 1 chain
MyoD	Myoblast determination protein
TPP	Triphenyl phosphine motif
FAM	5-carboxyfluorescein
ICC	Immunocytochemistry
MRF	Myogenic regulatory factor
MYOG	Myogenin
mRNA	messenger Ribonucleic Acid
DNA	Deoxyribonucleic acid
iMPCs	induced myogenic progenitor cells
ATP	Adenosine triphosphate
UV-Vis	Ultraviolet-Visible
CD	Circular dichroism
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)
FT-IR	Fourier Transform Infrared Spectroscopy
BSA	Bovin Serum Albumin
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
EDTA	Ethylenediaminetetraacetic acid
EDT	Ethanedithiol
TFA	Trifluoroacetic acid
DIC	N,N'-Diisopropylcarbodiimide
HOBT	1-Hydroxybenzotriazole
NMP	N-Methylpyrrolidone
Fmoc	9-Fluorenylmethoxycarbonyl
CO ₂	Carbon dioxide
ACN	Acetonitrile
H ₂ SO ₄	Sulfuric acid
OPD	o-phenylenediamine
HRP	Horseradish peroxidase
cDNA	complementary Deoxyribonucleic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
cpd	Compounds
calc.	Calculated
obs.	Observed