

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

4.1 Abstract

Duchenne Muscular Dystrophy (DMD) is a severe X-linked genetic disorder, caused due to mutation in the largest human gene dystrophin. Current therapeutic strategies mainly focusses on symptomatic management of DMD pathophysiology by corticosteroid treatment, as exon skipping strategies to treat DMD is inaccessible to majority of DMD population due to its high treatment cost. Corticosteroid treatment is the part of the standard care for DMD but such treatment for long time is associated with several side effects such as weight gain, cataract, osteoporosis, behavioural changes, and vertebral compression fractures. Herein, we developed a library of peptides, and lead candidate identified for promoting myogenesis, E.M.P-2, was further engineered for mitochondrial targeting. E.M.P-2 showcased potent myogenic activity by upregulating myogenic markers and downregulating fibrosis at a nanomolar concentration of 0.15 nM. Furthermore, E.M.P-2 effectively chelates calcium ions, maintaining mitochondrial calcium homeostasis. E.M.P-2 also shows its potency in significantly downregulating expression of IL-6 in DMD patient derived cells which shows significant baseline IL-6 expression. Overall this study introduces E.M.P-2 as the peptide-based therapeutic with the ability to manage DMD secondary pathological mechanisms, offering a promising platform for managing DMD disease progression.

4.2 Introduction

Duchenne muscular dystrophy (DMD) is a rare and progressive genetic disorder, arising from mutations in the dystrophin gene as detailed in introduction chapter under segment 1.1 and 1.2.¹⁻⁵ This chapter focuses on the development of a novel peptide-based therapeutic approach aimed at addressing the secondary pathological complications associated with DMD progression. The objective is to provide an alternative to the current standard of care, which primarily relies on corticosteroids for symptomatic management.

The absence of dystrophin disrupts the DAPC, leading to a cascade of progressive secondary pathological changes. These include disruptions in calcium homeostasis, activation of pro-inflammatory cytokines and proteases, mitochondrial dysfunction, and chronic inflammation. This cycle contributes to fibrosis, repeated necrosis, impaired muscle regeneration, and compromised vascular adaptation. The resulting damage makes muscle fibers highly susceptible to contraction-induced injuries, ultimately leading to muscle degeneration, fatty tissue replacement, and premature death.⁶ Despite advancements in clinical management and the use of corticosteroids, an effective cure for DMD remains unavailable. Recent progress in

genetic therapies offers promise, but the options are limited.⁷ To date, only one exon-skipping drug, Exondys51 has received conditional approval from the FDA. These treatments are mutation-specific and highly expensive, rendering them unaffordable to the majority of patients.⁸ Therefore, there is an urgent need for an affordable therapeutic approach that can effectively address the secondary pathologies of DMD and be universally applicable to the entire DMD population. Such strategy would also aim to minimize the dependence on long-term corticosteroid therapy and its associated adverse effects.

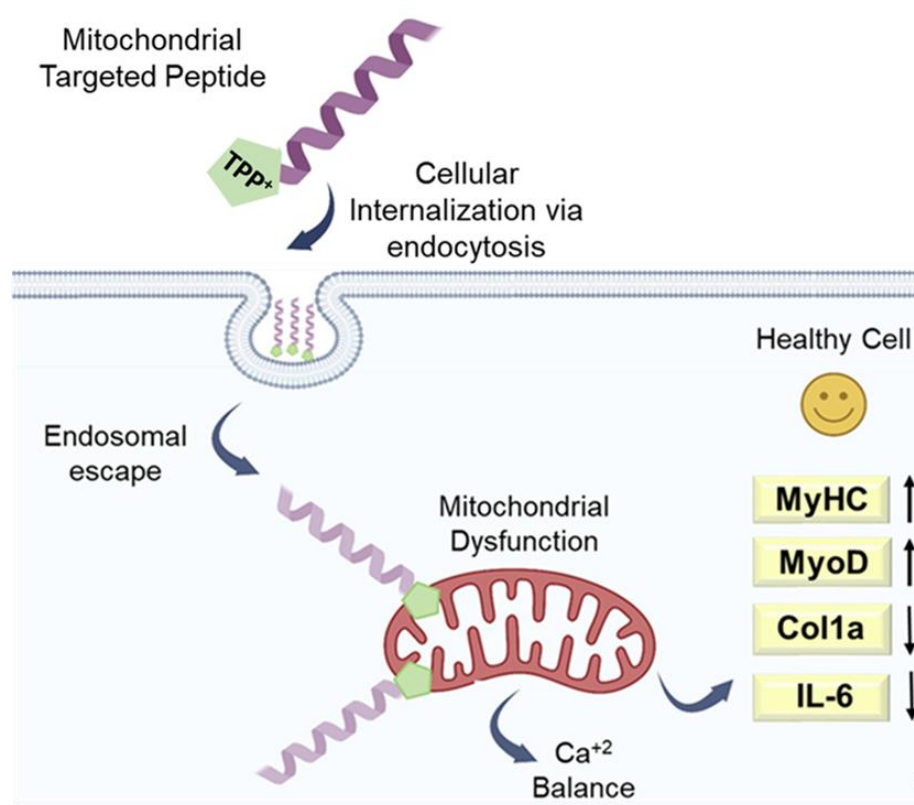


Figure 4.1: Schematic representation of the role of E.M.P-2 in addressing secondary pathological hallmarks within DMD cellular environment

In this study, we designed and synthesized a library of peptides derived from a 56-amino acid mitochondrial micropeptide, referred to as 'MPM'. (Figure 4.2) Notably, MPM localizes to mitochondria and exhibits increased expression during C2C12 myoblast differentiation *in vitro*, as well as during early postnatal skeletal muscle development and regeneration following cardiotoxin (CTX)-induced injury *in vivo*. Conversely, its expression is significantly downregulated in the muscle tissues of DMD patients.⁹ MPM plays a crucial role in promoting myogenesis, a fundamental process for muscle development and regeneration, which is markedly impaired during DMD progression. Prior literature revealed that silencing MPM impaired the differentiation of C2C12 myoblasts into mature myotubes, suggesting its essential role in myogenic progression and thus implicates its potential role for muscular dystrophy therapy.⁹⁻¹¹ In this study, to harness the therapeutic potential of MPM into a peptide-based treatment strategy to manage the progression of DMD we systematically truncated and synthesized a library of six overlapping 10-mer peptides from full-length 56-amino acid micropeptide (MPM), to identify the functional regions within the entire stretch

of MPM. This approach enabled us to pinpoint the key amino acid sequences critical for promoting myogenesis and muscle regeneration. Following the synthesis and screening of the peptide library using In-Cell ELISA, we identified a lead candidate, M.P-2, based on its ability to enhance myogenic marker MyHC at nanomolar doses. To improve its therapeutic efficacy, M.P-2 was further engineered into E.M.P-2 by incorporating two key functional modifications, firstly a triphenylphosphonium (TPP⁺) moiety was attached at the N-terminus to facilitate mitochondrial targeting, reflecting MPM's native mitochondrial localization, and an "EKK" motif was attached at the C-terminus to confer calcium-chelating properties, addressing calcium dysregulation, a hallmark of DMD disease progression.

Additionally, E.M.P-2 also effectively mitigated fibrosis, a hallmark of DMD pathology,¹² by downregulating collagen type I (COL1A1) expression. Importantly, the peptide also contributed to maintaining calcium homeostasis, which is disrupted in the mitochondrial environment due to muscle tears during disease progression.¹³ E.M.P-2 also plays active role in managing pro-inflammatory cytokine interleukin-6(IL-6) which is typically overexpressed in DMD patients.

Overall, the mitochondria-targeted peptide E.M.P-2 represents a novel therapeutic approach with dual efficacy in promoting myogenesis and reducing fibrosis within nanomolar doses. Thus, positioning E.M.P-2 as a promising peptide-based therapeutic candidate to serve the universal DMD patient population, by addressing the secondary pathological mechanisms associated with DMD, and thereby filling a critical gap in current treatment strategies.

4.3 Results and Discussion

4.3.1 Design and synthesis of mitochondria targeted peptides

To address the current limitations of managing disease progression for the universal DMD population we solely rely on the use of corticosteroids, but it has associated side effects on long time exposure.¹⁴⁻¹⁵ So, considering the secondary factors associated with disease progression herein we focus to improve myogenesis, which gets disrupted in DMD patients¹¹ by peptide based therapeutic strategy. Myogenesis is an important event required for muscle Development and regeneration, and its dysfunction is associated with DMD disease progression.¹⁶ In that regard we identified that till date two micropeptides have been reported to regulate myogenic differentiation.¹⁷ In prior art, it was observed that micropeptide in mitochondria (MPM), a muscle-enriched and mitochondria-localized micropeptide, was upregulated during myogenic differentiation in vitro and in vivo, and promoted myogenic differentiation of myoblasts into myotubes. Also, MPM^{-/-} mice exhibited smaller skeletal muscle fibers, worse muscle performance, and compromised muscle regeneration.¹⁸ In this study to harness the therapeutic potential of the micropeptide 'MPM', and to identify the key amino acids involved in the process of myogenesis we truncated the MPM micropeptide, each comprising of ten to twelve amino acids. Library of peptide synthesized was further screened to evaluate its myogenic potential via MyHC upregulation through In-Cell ELISA technique, and identified our lead peptide M.P-2. The lead peptide 'M.P-2' was further modified by attaching triphenyl phosphine motif (TPP) unit to its N-terminal end to enhance its mitochondrial target ability,¹⁹ as inspired from its source of MPM's localization.¹⁸

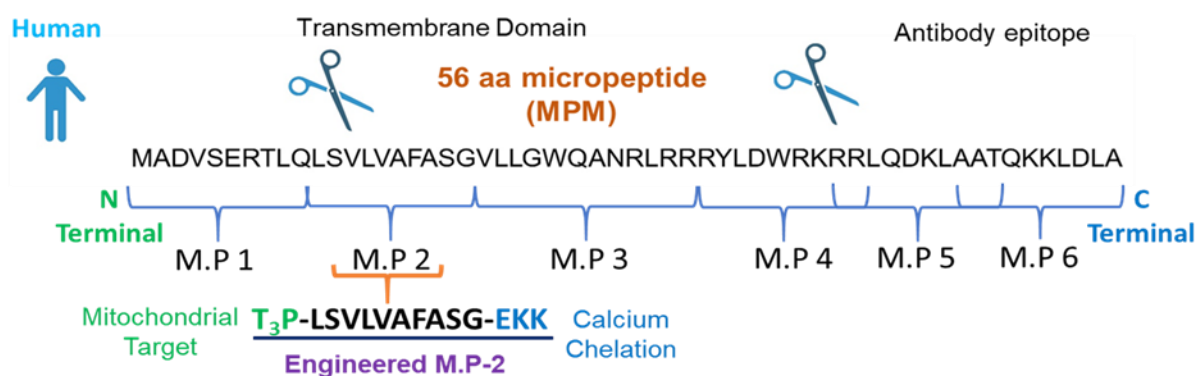


Figure 4.2: Amino acid sequence alignment of MPM micropeptide in human. Library of peptide synthesized by truncation of MPM micropeptide.

Additionally, we appended an "EKK" sequence to the C-terminus of peptide sequence to impart calcium chelation ability, addressing calcium imbalance another hallmark of DMD associated mitochondrial dysfunction.²⁰ The presence of gly (G) and glu (E) amino acid consecutively within the peptide sequence significantly enhance the calcium chelating ability for the peptide.²¹ (Figure 4.2) Next, the potency of the engineered peptide E.M.P-2 was further evaluated for its role in managing fibrosis, a critical pathological feature of DMD.¹² It was observed that E.M.P-2 could significantly mitigate fibrosis by downregulating COL1A1 expression. Overall, the engineered peptide E.M.P-2 offers a promising peptide based therapeutic strategy to mitigate DMD progression by managing secondary downstream pathological mechanism.

4.3.2 Screening of Peptide Library by High-throughput In-Cell ELISA Technique

In-Cell ELISA is a highly sensitive and quantitative method designed to measure intracellular protein levels directly within intact cells, eliminating the need for cell lysis. Herein, it was employed to screen the synthesized peptide library. To check the potency of the peptide candidates in improving myogenesis, here expression of the myogenic marker Myosin Heavy chain (MyHC) was assessed, and to determine the optimal concentration at which the MyHC expression is high. DMD patient derived cell line (KM1328) were treated with one of the peptide (M.P-2) at different concentrations ranging from 10 μ M to 0.15 μ M for 72 hr, and it was observed that at 0.15 μ M concentration MyHC protein expression was significantly higher (Figure 4.3A). So, for further screening of our remaining peptide library, we selected 0.15 μ M as the optimal concentration (Figure 4.3B). In this study, retinoic acid was chosen as our positive control at 5nM concentration.²² The M.P-2 displayed the highest MyHC upregulation of 2.13 fold at 0.15 μ M among all the other peptides screened at the same concentration in comparison to the positive control retinoic acid, which demonstrated 1.3-fold increase in MyHC protein at a concentration of 5 nM (Figure 4.3A&B). M.P-2 was identified as lead peptide candidate and selected for further modification and validation to assess its role in myogenesis and managing other secondary pathological mechanism during disease progression.

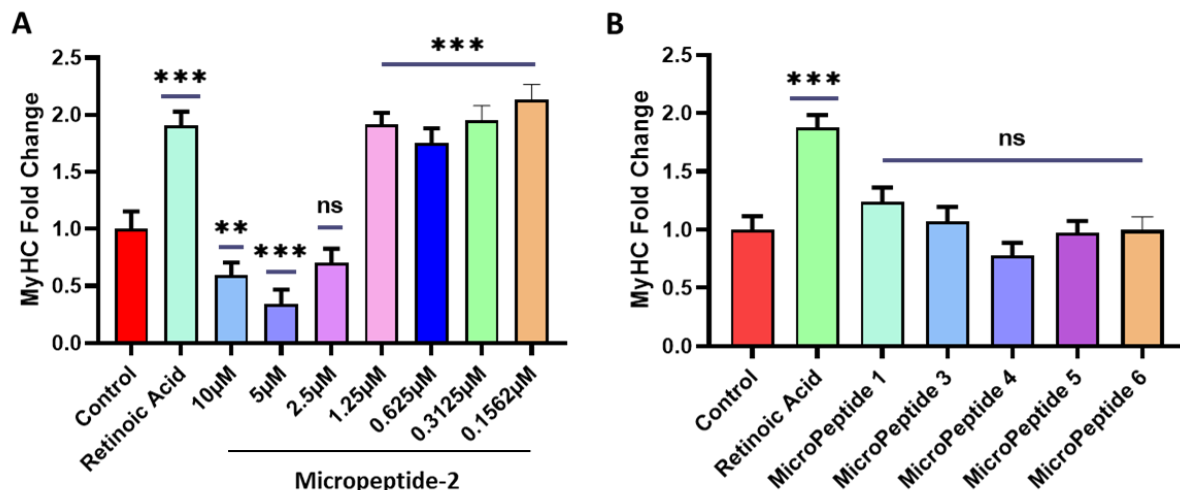


Figure 4.3: A) In-cell ELISA screening of E.M.P-2 for determination of effective screening concentration ranging from 10 μ M to 0.15 μ M. B) Screening of remaining micropeptides library at optimum concentration of 0.15 μ M via high throughput In-Cell ELISA technique. In all data sets, significance was calculated using one-way ANOVA, *** p <0.001.

4.3.3 Mechanistic pathway behind cellular internalization and mitochondrial localization

Peptides internalize into cells through two primary mechanisms firstly direct translocation across the cellular membrane, an energy-independent process, and secondly endocytosis, an energy-dependent process. To investigate the uptake kinetics of the E.M.P-2, flow cytometry was employed. C2C12 cells were treated with 5-carboxyfluorescein (FAM)-conjugated E.M.P-2 and incubated at 37 °C and 4 °C for 4 hours. Flow cytometry analysis revealed a significantly higher uptake of FAM tagged E.M.P-2 at 37 °C compared to 4 °C, consistent with the involvement of energy-dependent endocytosis in the internalization process.^{23, 24} (Figure 4.4 A&B). Further, conjugation of TPP motif at the N-terminal of the engineered E.M.P-2 enhances mitochondrial target ability of the peptide.¹⁹ This modification was inspired by the localization of the parent micropeptide MPM within the inner mitochondrial membrane to regulate myogenic differentiation, a process critically impaired in DMD patient. Another objective of conjugating the TPP moiety to the engineered peptide E.M.P-2 was to support the restoration of calcium homeostasis within the mitochondrial environment, which gets disrupted in DMD patients. The TPP group with its high positive charge density, enables efficient interaction with the mitochondria's negative membrane potential. This electrostatic attraction facilitates the translocation of the TPP-conjugated peptide easily inside the mitochondrial compartment and executing its therapeutic effect.

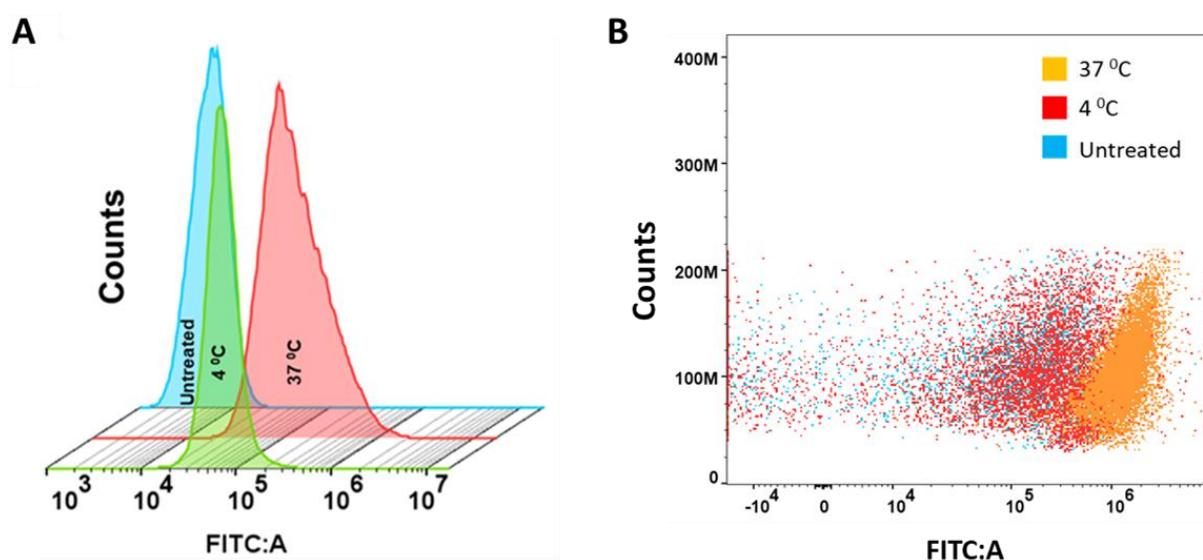


Figure 4.4: A cellular uptake analysis of 5 μ M FAM-E.M.P-2 suggests that the uptake occurs through an energy dependent endocytosis pathway, as evidenced by significantly higher uptake at 37°C compared to 4°C. A&B) Comparative histogram and dot blot representations.

Additionally, TPP-based mitochondrial targeting offers several advantages over other delivery approaches, including the stability of the TPP moiety in biological systems. These attributes highlights the potential of TPP conjugation as a robust strategy for mitochondrial targeting in therapeutic applications. To evaluate the mitochondrial targeting capability of the TPP-conjugated peptide (E.M.P-2), the peptide was labelled with FAM dye at the N-terminal lysine side chain. This was achieved using Fmoc-Lys(dde)-OH, where the dde protecting group on the lysine side chain amine was selectively removed under nucleophilic conditions, fully orthogonal to Fmoc deprotection chemistry as detailed in Section 4.3.2.2.

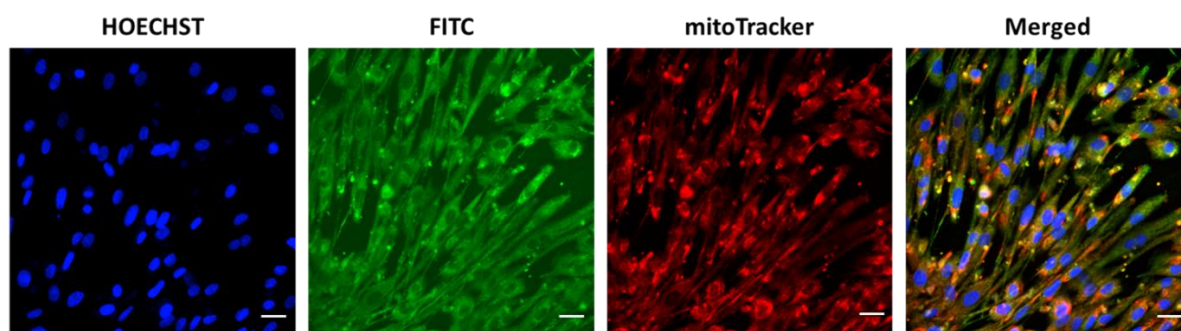


Figure 4.5: Mitochondrial target ability of TPP conjugated engineered E.M.P-2, were FAM tagged engineered peptide co-localizes within mitochondrial compartment of the cell labelled by mitoTracker. Scale bar corresponds to 100 μ m.

Following FAM tagging, TPP was conjugated to the N-terminal amine of the peptide. The engineered FAM-labelled peptide (green fluorescence) was then examined for mitochondrial localization in a human DMD cell line using fluorescence microscopy. After four hours of incubation, the peptide was observed to co-localize with mitochondria labelled by mitoTracker (red fluorescence), confirming its mitochondrial targeting capability. This co-

localization study validates the engineered E.M.P-2 as an effective mitochondrial-targeting therapeutic candidate. (Figure 4.5)

4.3.4 Fusion Assay

Impaired myogenesis is critical contributor to the progression of DMD. The fusion of myoblasts into mature myotubes is essential for muscle development and repair. However, this process is disrupted in dystrophic muscles.¹⁰

The dystrophin-associated protein complex (DAPC) plays a pivotal role in muscle regeneration, but its disassembly due to the loss of dystrophin compromises with the myogenic commitment of activated satellite cells. This impairment leads to defective muscle regeneration, ultimately resulting in progressive muscle degeneration and wasting.²⁵ To evaluate the myogenic potential of the engineered peptide E.M.P-2, derived from its source peptide MPM,¹⁸ we checked the potential of E.M.P-2 in upregulation of key myogenic marker MyoD²⁶ and further calculated the fusion index using immunocytochemistry in a DMD patient-derived cell line. MyoD, a member of the myogenic regulatory factor (MRF) family, is a crucial protein for myogenic determination. It serves as a master regulator, initiating the transcription of critical myogenic genes, including MYOG and MyHC, which play essential roles in muscle differentiation.²⁷⁻²⁸

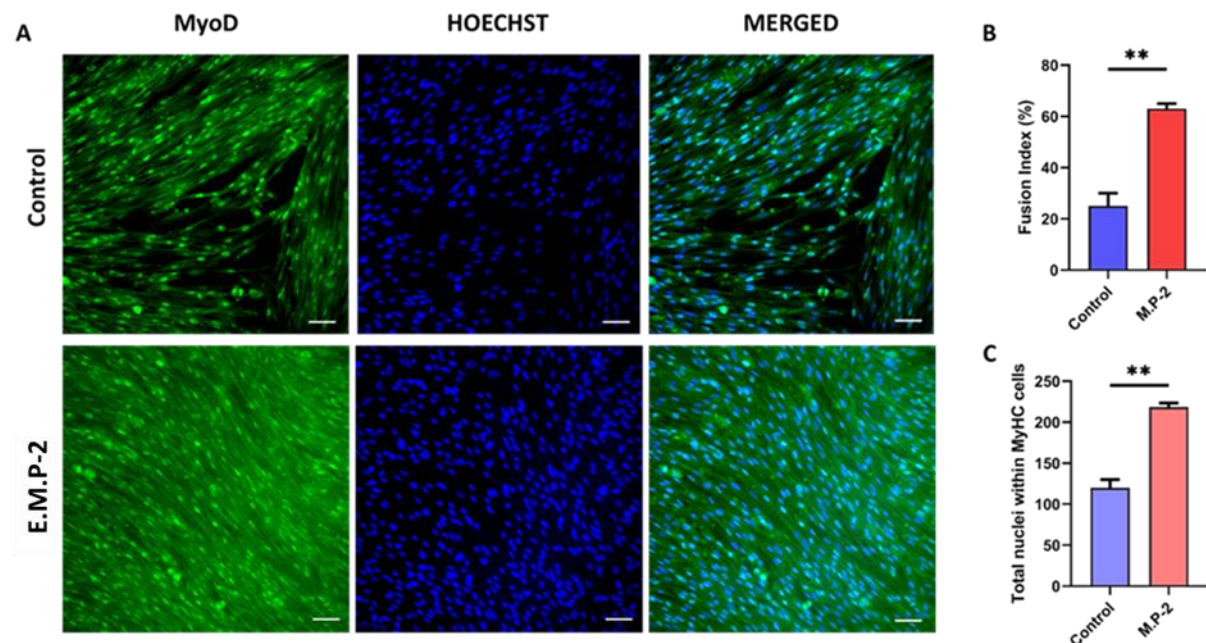


Figure 4.6: Myoblast Fusion. A) Immunofluorescent image of fused cells, where the cells were labelled with primary antibody to MyoD and secondary Alexa 488 (green) and Hoechst 33258 (blue). Scale bar corresponds to 100µm. Myoblast fusion is observed in the group treated with compound M.P-02 (150 nM) 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.

Considering the pivotal roles of MyoD in muscle differentiation, this myogenic marker was chosen to assess the efficacy of E.M.P-2 in promoting myogenic differentiation. Our findings revealed that treatment with engineered E.M.P-2 markedly enhanced the fusion of myoblast cells into mature myotubes in DMD patient-derived cell lines. This enhancement in myogenesis was validated through immunocytochemistry analysis, which exhibit increased expression of MyoD by 2.5 fold (Figure 4.6 A-C). Remarkably, the improvements in myogenesis were observed after three days of treatment, highlighting the potential of E.M.P-2 to support muscle repair and regeneration in DMD pathology. These results positioned the therapeutic efficacy of E.M.P-2 in addressing impaired myogenesis and promoting muscle regeneration in DMD.

4.3.5 Protein and mRNA expression of E.M.P-2

Addressing secondary pathological factors associated with DMD is a crucial component of therapeutic strategies aimed to halt disease progression. These approaches can also complement treatments targeting the primary genetic defect, thereby providing a more comprehensive management plan for the disease. In DMD patients, continuous muscle damage, loss of regenerative capacity and chronic inflammation leads to fibrosis during disease progression, which results in diminished muscle functionality and contractility, which ultimately results in scar tissue formation.¹² Another key pathological factor in DMD is impaired myogenesis, the process by which myoblasts differentiate and fuse to form mature myotubes during embryonic development and in response to muscle injury. Myogenic impairment hinder effective muscle regeneration and contributes to progressive muscle degeneration and weakness.¹⁰

Herein, after thorough screening and identification of lead peptide, potency of the E.M.P-2 was evaluated by assessing the mRNA and protein expression of key proteins involved in myogenesis and mitigating fibrosis. Expression profile of myogenic marker MyoD and MyHC were evaluated to check the potency of E.M.P-2 to restore impaired myogenesis, while the expression of COL1A1 was analysed to evaluate its potential in reducing fibrosis. MyoD, a master transcriptional regulator, coordinates the initiation of myogenic gene expression, including the activation of Myogenin (MYOG) and MyHC, which are crucial for muscle differentiation. MyoD also governs the expression of late-stage myogenic markers such as myogenin, MyHC, and dystrophin, facilitating progression through the advanced stages of myogenesis.^{27,28} Enhancing myogenic differentiation through therapeutic intervention could able to restore the expression of critical myogenic markers like MyoD and MyHC,²⁹⁻³⁰ thereby improving muscle function. Consequently, the expression levels of MyoD and MyHC were assessed to determine the efficacy of E.M.P-2 in promoting myogenesis.

Simultaneously COL1A1 is expressed in dystrophic muscle, and increased collagen levels can contribute to fibrosis in DMD, thus therapeutic strategies able to downregulate COL1A1 expression could mitigate fibrosis,³¹⁻³² addressing one of the critical secondary pathological features of DMD. Thus, expression profile of COL1A1 was thoroughly evaluated to check the potency of E.M.P-2 in mitigating fibrosis.

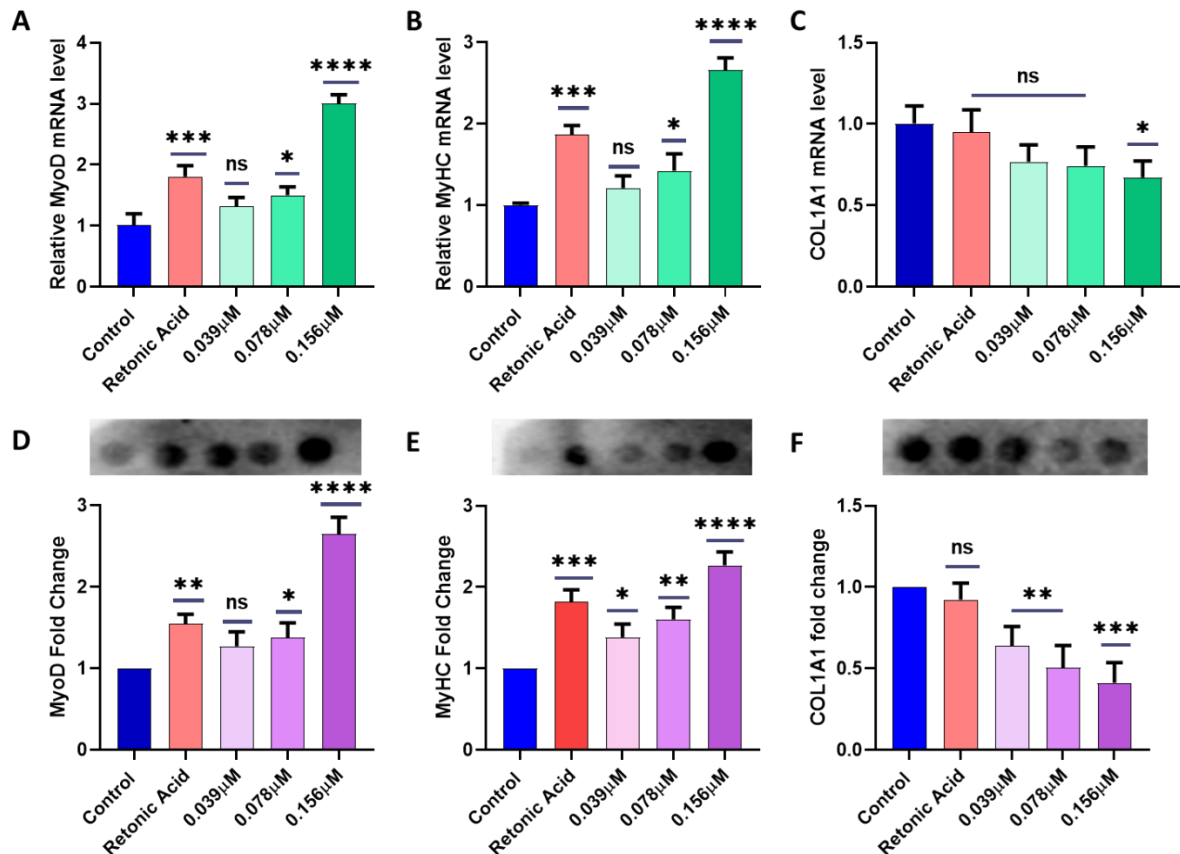


Figure 4.7: qRT-PCR assay and Dot blot results, after treatment of human DMD patient derived cells with lead peptide E.M.P-2 at 0.156 μM concentration for 72 hr compared to retinoic acid at 5 nM and untreated control (A) MyoD mRNA expression in dose dependent manner. (B) MyHC mRNA expression in dose dependent manner (C) COL1A1 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 COL1A1 in a dose-dependent manner. In all data sets, significance was calculated using one-way ANOVA, * $p < 0.1$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$.

qRT-PCR and dot blot analyses were conducted to evaluate the mRNA and protein expression profile of key markers involved for myogenesis and fibrosis by therapeutic lead E.M.P-2 in a dose-dependent manner ranging from 0.039 to 0.156 μM in a clinically relevant DMD patient-derived cell line. These experiments were performed relative to the positive control, retinoic acid, at 5 nM,²² with a treatment duration of 72 hours. The results demonstrated that E.M.P-2 significantly upregulated the mRNA expression of the myogenic marker MyoD, achieving a 3-fold increase at the optimal concentration of 0.156 μM , compared to a 1.8-fold upregulation observed with retinoic acid. Similarly, E.M.P-2 enhanced the mRNA expression of MyHC, another key myogenic marker, by 2.6-fold at the same concentration, in comparison to 1.86-fold increase induced by retinoic acid. Moreover, E.M.P-2 effectively downregulated the mRNA expression of COL1A1, a critical fibrosis marker, by 30% at 0.156 μM , whereas retinoic acid induced 5% reduction (Figure 4.7 A-C). Considering the protein-level analysis using dot blot assays exhibits that E.M.P-2 induced a dose-dependent upregulation of MyoD protein expression, achieving a 2.6-fold increase at 0.156 μM , compared to a 1.5-fold increase observed with retinoic acid. Similarly, MyHC protein expression was elevated by 2.26-fold at 0.156 μM

in the presence of E.M.P-2, in comparison to 1.8-fold increase observed with retinoic acid. Simultaneously, E.M.P-2 shows a significant reduction in COL1A1 protein expression, decreasing it by 60% at the optimal concentration of 0.156 μ M, while retinoic acid only achieved an approximately 10% reduction (Figure 4.7 D-F).

These findings highlight the therapeutic potential of E.M.P-2 in simultaneously addressing impaired myogenesis and fibrosis, two critical pathological features associated with DMD. By significantly upregulating MyoD and MyHC, E.M.P-2 effectively enhances myogenesis, a critical process impaired in DMD. Importantly, MyoD's ability to reprogram fibroblasts into induced myogenic progenitor cells (iMPCs), which can restore dystrophin expression in dystrophic muscle,³³ highlights its relevance in therapeutic applications. Additionally, the significant downregulation of COL1A1 by E.M.P-2 demonstrates its efficacy in mitigating fibrosis, a key contributor to DMD pathology. Collectively, these results establish E.M.P-2 as a promising therapeutic candidate with the potential to address both muscle degeneration and fibrosis.

4.3.6 Maintaining Calcium homeostasis by E.M.P-2

In DMD patients, increased membrane instability and the formation of ribs in the muscle membrane lead to an excessive influx of calcium ions, resulting in elevated intracellular calcium levels and disrupted calcium homeostasis.²⁰ This instability further contributes to mitochondrial calcium overload, this impaired calcium balance in mitochondria leads to a marked reduction in Adenosine triphosphate (ATP) production and compromises the mitochondrial membrane potential, thereby worsens cellular dysfunction.³⁴ The increase in cytosolic calcium levels within dystrophic muscle fibers is a critical hallmark of disease progression. Calcium-mediated changes in mitochondrial function further intensify muscle damage by creating an environment unfavourable for mitochondrial homeostasis.³⁵ Thus, therapeutics capable of maintaining intracellular calcium balance in DMD patients have the potential to mitigate calcium dysregulation, thereby addressing a key pathological mechanism and halting disease progression.

In this regard, to check the potency of our engineered E.M.P-2 in maintaining calcium homeostasis within cytoplasm and mitochondria, the calcium chelating activity of E.M.P-2 was evaluated using UV-Vis, and fluorescence spectroscopy. The UV-Vis absorption spectra are shown in Figure. 4.8A. E.M.P-2 exhibited two absorption peaks between 190–210 nm corresponding to the amino acids residues in the peptide. Following the chelation reaction with calcium ions, a red shift in the maximum absorption peak of E.M.P-2 in the range of 190–195 nm along with decrease in absorbance was observed. This spectral change indicates a specific interaction between E.M.P-2 and calcium ions, as measured across varying volume ratios of CaCl₂ to E.M.P-2, ranging from 1:10 to 1:1.³⁶

Moreover, the UV absorption spectrum of the calcium-chelated peptide (E.M.P-2-Ca²⁺) also exhibited a broader profile compared to unmodified E.M.P-2, with a weak peak emerging in the range of 200–215 nm. This can be attributed to the presence of metal-chelating functional groups, such as the -COOH group of glutamic acid within the peptide sequence, supporting the evidence for chelation. This spectral shift suggests that the chelation process altered the

electron transitions and spatial structure of the peptide, which suggests that peptides successfully chelated with Ca^{+2} ions.³⁷

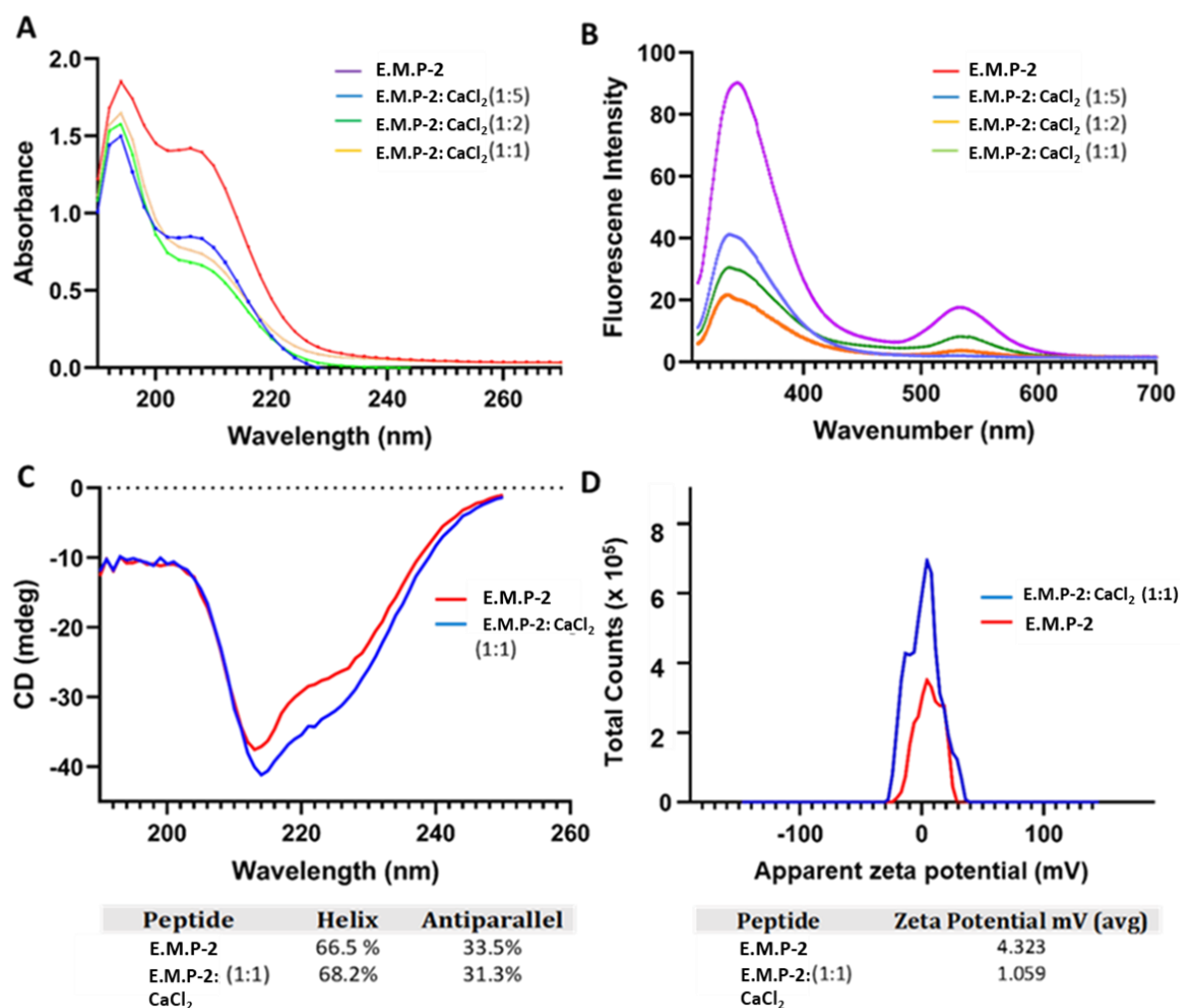


Figure 4.8: UV-Vis and fluorescence spectra (A) UV-Vis spectra of peptide E.M.P-2 and calcium chelated peptide (E.M.P-2:CaCl₂), at different peptide to CaCl₂ volume ratios of 1:1, 1:2, and 1:5. (B) Fluorescence spectra of peptide E.M.P-2 and calcium chelated peptide (E.M.P-2:CaCl₂), at different peptide to CaCl₂ volume ratios of 1:1, 1:2, and 1:5. C) CD spectra of peptide E.M.P-2 and E.M.P-2:CaCl₂ at 1:1 volume ratio of peptide to CaCl₂. D) Comparative Zeta potential of peptide E.M.P-2 and E.M.P-2:CaCl₂ at 1:1 volume ratio of peptide to CaCl₂.

Similarly, in fluorescence spectroscopy (Figure. 4.8B), the fluorescence intensity of E.M.P-2- Ca^{2+} decreased sharply in the range of 230–245 nm and almost flattened between 530 and 540 nm in comparison to unmodified peptide E.M.P-2. The reduced fluorescence intensity reflects structural alterations in E.M.P-2 upon binding with calcium ions. This reduction in fluorescence intensity might be because of fluorescence quenching by calcium ions binding to the peptide,³⁶ and secondly, due to the formation of a new structural configuration involving chromophores interacting with calcium ions. These interactions alter the energy of the excited state, leading to a decrease in fluorescence intensity.³⁸ The interaction between E.M.P-2 and calcium ions was measured using varying CaCl₂ to E.M.P-2 volume ratios, ranging from 1:10 to 1:1.

Further to determine the secondary structure of E.M.P-2 and its effect on binding to Ca^{2+} , a circular dichroism (CD) experiment was performed using CaCl_2 . As shown in Figure 4.8C, the addition of CaCl_2 induces a slight change in the CD curve at Ca^{2+} :E.M.P-2 volume ratio of 1:1, where helical content increases from 66.5 % to 68.2 % and decrease in antiparallel content from 33.5% to 31.3 % on calcium chelation to E.M.P-2 suggesting a delicate increase in the helical content and other structural adjustments upon calcium chelation. Also, the identical spectra of Ca^{2+} -loaded and Ca^{2+} free peptide indicates no significant damage to the secondary structure of the peptide E.M.P-2 on calcium chelation.³⁹

Next, the surface charge of E.M.P-2, both before and after chelation with Ca^{2+} was determined through zeta potential measurement. At a 1:1 molar ratio of E.M.P-2 to calcium ions, the zeta potential of E.M.P-2 decreased significantly from +4.323 mV to +1.059 mV, as shown in Figure 4.8D. This reduction in zeta potential suggests an electron transfer and the formation of coordination bonds between E.M.P-2 and Ca^{2+} , indicating successful interaction and chelation.⁴⁰ Collectively, the changes observed in UV-Vis spectroscopy, fluorescence spectroscopy, circular dichroism (CD), and zeta potential measurements confirms the successful chelation of calcium ions by E.M.P-2 and thus highlight the therapeutic potential of engineered E.M.P-2 confirms the successful chelation of calcium ions by E.M.P-2 and thus highlight the therapeutic potential of engineered E.M.P-2 in maintaining intracellular calcium homeostasis within cytoplasm and mitochondria, thus addressing the another key pathological hallmark associated with disease progression along with promoting myogenesis and mitigating fibrosis.

4.3.7 Downregulation of pro-inflammatory marker IL-6, and fibrosis marker COL1A1 by E.M.P-2

To evaluate the efficacy of E.M.P-2 in downregulating the inflammatory markers interleukin-6 (IL-6) which is directly linked with DMD pathophysiology.⁴¹ Firstly, DMD patient-derived human cell lines were treated with E.M.P-2, to assess its therapeutic potential in downregulating IL-6. Elevated IL-6 levels play a pivotal role in DMD pathogenesis by promoting chronic inflammation, supporting repeated cycles of muscle degeneration and regeneration, and leading to the eventual exhaustion of satellite cell populations.⁴¹

Increased IL-6 levels impair the dystrophic muscle phenotype and worsen disease progression. Some studies also claims that IL-6 downregulation has been linked to promote myogenesis,⁴² highlighting it as a critical therapeutic target in DMD management. Notably, glucocorticosteroids, the current gold standard treatment for DMD, exert their therapeutic effects in part by reducing IL-6 levels, thereby delaying disease progression.⁴³ In this study, significant baseline IL-6 expression was observed in untreated DMD patient-derived cells, thus there was no requirement for forced expression of IL-6 by lipopolysaccharide (LPS), and next E.M.P-2 treatment exhibited a significant reduction in IL-6 expression at an optimal concentration of 0.156 μM , observed after 24 hours of treatment. These findings highlights the ability of E.M.P-2 to effectively suppress IL-6 expression, thereby modulating the inflammatory environment within dystrophic muscle. Overall, inhibition of IL-6 by E.M.P-2 promotes anti-inflammatory responses, enhances muscle repair, and contributes to the functional homeostasis and maintenance of dystrophic muscle, positioning E.M.P-2 as a promising candidate for DMD therapy. (Figure 4.9 A&B)

Next, the role of COL1A1 in DMD progression and the ability of E.M.P-2 to downregulate its expression at both mRNA and protein levels have already been discussed in detail under section 4.2.5. To further confirm these findings, immunocytochemistry was employed to validate the COL1A1 downregulation by E.M.P-2 after 24 hours of treatment. As, the cells derived from DMD patients displayed inherently elevated baseline levels of COL1A1, which aligns with their characteristic pathological phenotype, thus no induction of COL1A1 overexpression was required. The results shows parity with earlier observations, reinforcing the potency of E.M.P-2 in suppressing COL1A1 deposition. (Figure 4.9 A&C)

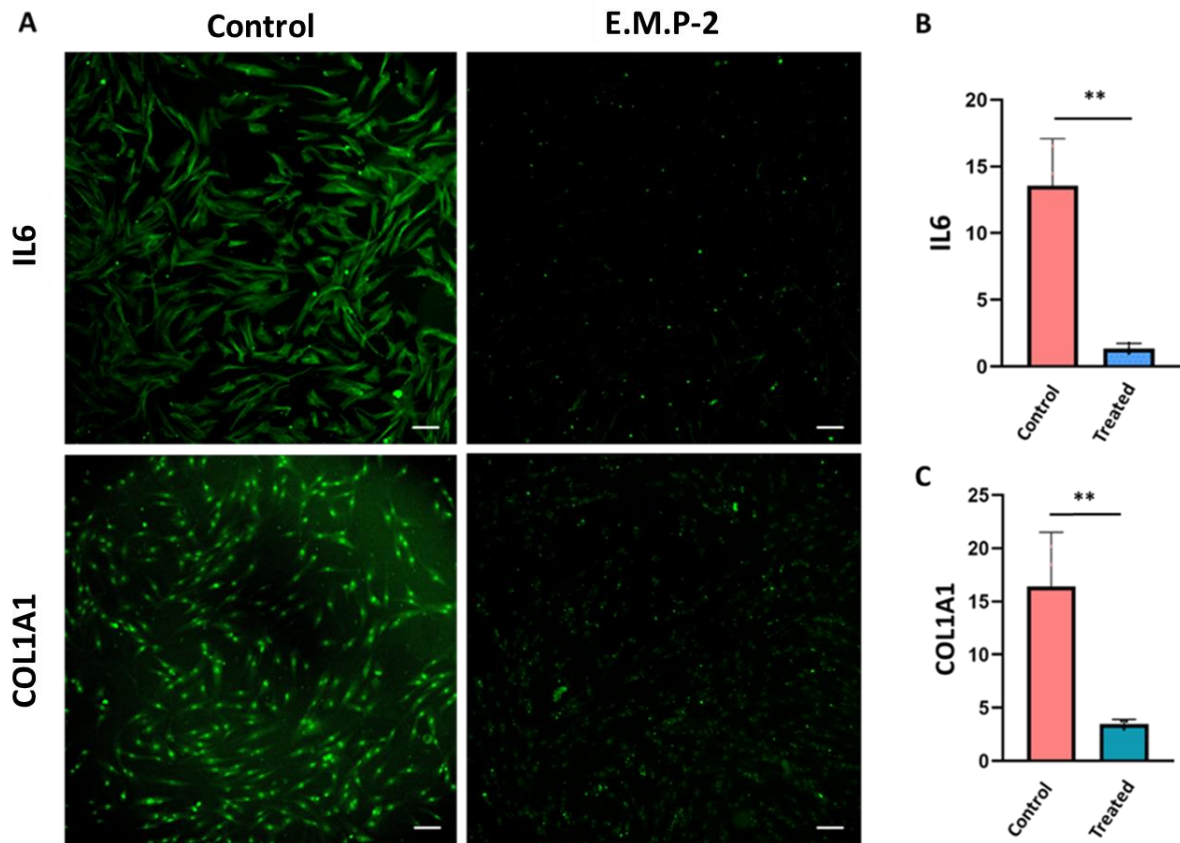


Figure 4.9: Immunocytochemistry analysis. A) Downregulation of pro inflammatory cytokine IL-6 significantly (Upper panel), similarly significant downregulation of COL1A1 fibrosis marker. B&C) Bar diagram representing IL-6 & COL1A1 downregulation. Scale bar corresponds to 100µm. In all data sets, significance was calculated using t test **p<0.01.

4.3.8 Cytotoxicity and serum stability of the lead engineered peptide E.M.P-2

Cytotoxicity of the lead engineered peptide was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay after treatment of human DMD myoblast cells (KM1328) with peptide at varied concentrations for 24 h.

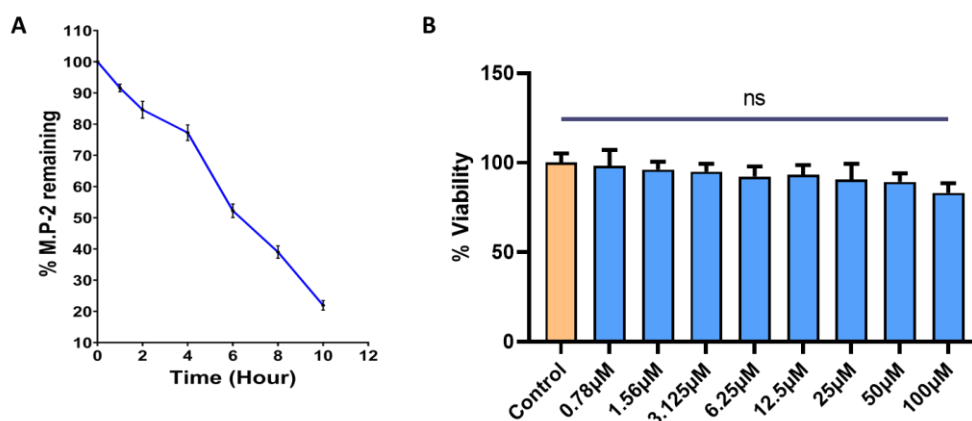


Figure 4.10: A) Serum stability of lead engineered peptide E.M.P-2 monitored for 10 hours in human plasma. B) Cell survival percentage after treatment of KM1328 cells with the lead peptide identified E.M.P-2 in an increasing gradient of doses (3.125-100 μ M) for 24 h, n=3. In all data sets, significance was calculated using one-way ANOVA.

The obtained results, as illustrated in (Figure 4.10 A) shows that the administered peptide molecules exhibited negligible toxicity even at higher concentrations.

These findings suggest that the lead molecules possess a favourable safety profile, particularly at therapeutic concentrations, making them promising candidates for further development. Further, to understand the involvement of plasma enzymes in the metabolism of compounds is crucial for assessing their stability and potential for therapeutic efficacy. We performed a human plasma stability assay to assess the stability of our lead molecule, engineered E.M.P-2. Incubation of peptide with human serum over various time intervals revealed that the compound remained stable up to fifty percent after 6 hr of incubation. This study emphasizes the compound's resilience within the plasma environment. (Figure 4.10 B)

4.4 Materials and Methods

4.4.1 Materials

Chemistry

All starting materials, reagents, and solvents were purchased from commercial suppliers and used without further purification. Fmoc-Rink Amide AM resin (8.55004.0025), Fmoc-amino acid was used for peptide synthesis. The compounds underwent routine analysis through LCMS for determining mass of synthesized peptides. Purity of the peptides synthesized were measured through analytical HPLC. Mass spectra were recorded utilizing an Agilent Technologies electrospray ionization ESI; Q-TOF mass spectrometer instrument (G6530C). Infrared spectra were acquired by using Bruker FT-IR spectrometer. For high-performance liquid chromatography (HPLC) analysis, a Shimadzu C18 column (150 mm $\times$ 4.6 mm $\times$ 5 μ m) was employed. UV-Vis and fluorescence were recorded in JASCO FP-8300. CD spectra were

recorded in a Jasco J-815 spectropolarimeter. Zeta potential were measured in a Malvern Panalytical Zetasizer.

Biology

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (M2128, Sigma), Triton X-100 (BB031, Himedia), Bovin Serum Albumin (BSA) (83803, SRL), Antimycotic antibiotic solution (Himedia A002), Fetal Bovine Serum (Invitrogen 2023-01-30), DMEM, high glucose, Penicillin Streptomycin solution (Invitrogen 15140-122), Trypsin EDTA Solution (Sigma T4174), Formaldehyde solution (Sigma-Aldrich 47608), Anti α -tubulin antibody (ab18251, Abcam, 1:1000), Hoechst 33258 (ab228550, Abcam), Alexa fluor 594 (ab150116, Abcam, 1:500), cDNA Synthesis Kit (Cat. No. #1708890, Bio-Rad), IL-6 (sc-57315), COL1A1 (sc-59772), mitoTracker CMXROS, anti mouse alexa fluor 488 secondary antibody (A11029).

4.4.2 Methods

Synthesis of designed peptide library were performed via CEM-LIBERTY manual solid phase peptide synthesizer with rink amide resin as solid support. Next, the steps involving conjugation of TPP motif at N-terminal end of the peptide and attachment of 5-carboxy fluorescein to the synthesized peptide were performed outside peptide synthesizer, by using a screw capped glass peptide vessel.

4.4.2.1 Synthesis of Peptide Library

The peptides were synthesized employing microwave supported solid phase peptide synthesis technique utilizing CEM-LIBERTY manual peptide synthesizer as per established protocol.⁴¹ Fmoc protected Rink Amide resin was taken as the solid support. A solution containing 20%v/v piperidine in DMF was used as deprotection step to remove Fmoc-protecting group of resin. After deprotection step, DIPEA (10 eq) and HBTU (5 eq) and Fmoc-protected amino acid (5 eq) were taken in DMF and stirred in vortex to make a homogeneous mixture and added in resin to allow it to couple for first amino acid. Then sequentially the deprotection and protection step were performed simultaneously until the last amino acid was attached to the resin. After final deprotection, the resin was washed and subjected to cleavage under standard conditions using a cleavage cocktail consist of TFA, H₂O, ethanedithiol (EDT), and thioanisole in a 94:2:2:2 volume ratio. The mixture was stirred at room temperature for 4 hours. Following cleavage, excess TFA was removed under a stream of air until the volume was minimized. The resulting solution was then poured into ice-cold diethyl ether and stored overnight at 4°C to allow for complete precipitation. The precipitate was collected by centrifugation at 4000 rpm for 5 minutes, dissolved in a 1:1 volume ratio of acetonitrile and water, and lyophilized to obtain the final peptide product.⁴⁴

4.4.2.2 Synthesis of TPP conjugated lead peptide E.M.P-2

To conjugate the TPP motif to the N-terminus of the lead peptide identified to induce mitochondrial target ability. The resin-bound peptide having free N-terminal amine after final deprotection step was subsequently removed from the microwave synthesizer, washed thoroughly with DMF and DCM, and air-dried. The peptide-resin was then transferred to a screw-capped glass peptide vessel, followed by the addition of (2-Carboxyethyl)triphenylphosphonium bromide having TPP moiety, along with the coupling reagents N,N'-Diisopropylcarbodiimide (DIC, 5 eq) and HOBT (5 eq). The reaction mixture was gently agitated on a rocker for 24 hours for complete conjugation. Upon completion of the coupling reaction, the resin was washed and subjected to cleavage under standard conditions using a cleavage cocktail as discussed in previous section.

4.4.2.3 Synthesis of 5-carboxy fluorescein conjugated TPP-E.M.P-2

To achieve the synthesis of 5-carboxy fluorescein conjugated N-terminal TPP -E.M.P-2, Fmoc-Lys (dde)-OH was taken. As, dde protection group on the side chain amine of lysine was selectively cleaved under nucleophilic conditions which is fully orthogonal to Fmoc deprotection conditions. This synthesis of TPP-E.M.P-2 was initiated on a solid-phase Rink amide resin under a microwave-assisted peptide synthesizer, beginning with the first amino acid Fmoc-Lys(dde)-OH instead of Fmoc-Lys(Boc)-OH. The remaining coupling processes were similar to those discussed in section 4.3.2.1. After the deprotection of the Fmoc group from the final amino acid and its subsequent conjugation to the TPP moiety, as detailed in Section 4.3.2.2, the dde protecting group was selectively removed using a deprotection mixture under standard conditions conducted outside the microwave synthesizer. The deprotection mixture was prepared by taking 1.25 gm of hydroxylamine hydrochloride and 0.918 gm of imidazole in 5 mL of NMP solvent, followed by vortex until complete dissolution. After this 1 mL of DCM was added prior to the reaction, and then resin attached peptide was gently shaken in a screw-capped glass peptide vessel with this freshly prepared deprotection mixture on a rocker for 3 hours.⁴⁵ Next, the resin was filtered off and washed with DMF and DCM (5 mL each). For FAM conjugation at side chain free amine group of lysine, 5-carboxy fluorescein was taken (5eq) in DMF (5 mL) followed by addition of DIC (10 eq) and HOBT (5 eq) and allowed to activate it for fifteen minutes. Following activation, the FAM containing acid was allowed to shake with resin attached peptide for next 72 hours under dark condition for conjugation of FAM. Next the solid support resin was washed with DMF and DCM, and it was treated with a 20% piperidine solution in DMF for N-terminal Fmoc deprotection. This N-terminal amine was then converted to azide by treating it with azide transfer reagent, following the procedure outlined in section 3.3.2.3.

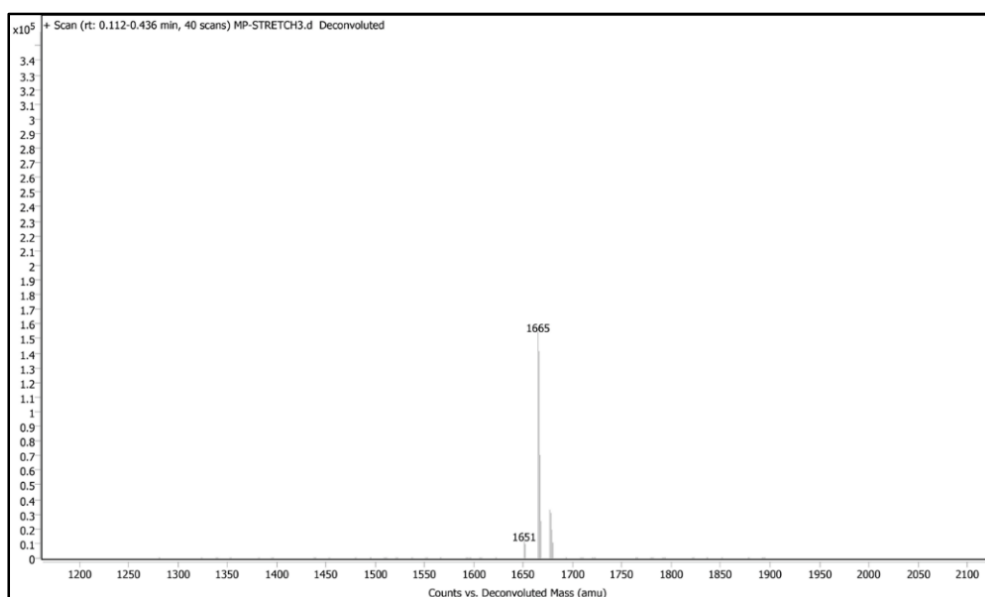


Figure 4.11: Mass Spectra of the engineered lead peptide E.M.P-2

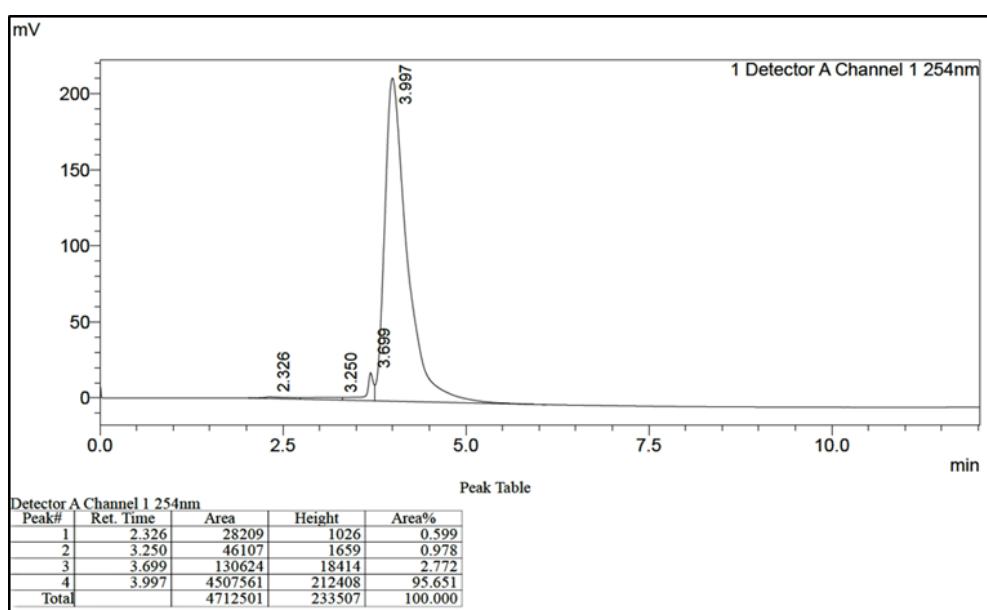


Figure 4.12: HPLC Spectra of the engineered lead peptide E.M.P-2.

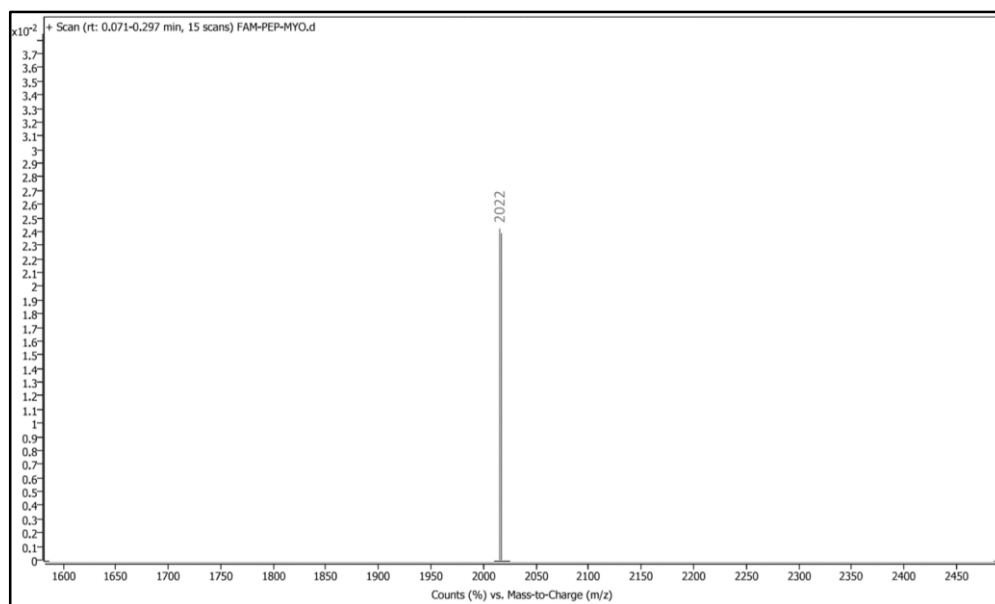


Figure 4.13: Mass Spectra of the FAM labelled engineered lead peptide E.M.P-2

4.4.2.4 UV-Vis fluorescence spectroscopy

E.M.P-2 was first dissolved in a minimal volume of DMSO and subsequently diluted with deionized water to a final concentration of 0.1 mg/mL. A CaCl_2 solution was prepared in deionized water at a concentration of 1 mol/L. The E.M.P-2 and CaCl_2 solutions were then combined at volume ratios of 1:10, 1:5, 1:2, and 1:1. The resulting mixtures were incubated at 37°C for 1 hour in a shaker incubator. Post-incubation, the absorbance of the samples was measured over the wavelength range of 190–480 nm using a JASCO UV-Vis spectrophotometer.³⁷

4.4.2.5 Fluorescence spectroscopy

Peptide–calcium complexes were prepared in the same volume ratio as described in the UV-Vis analysis above and incubated for 1 hour at 37 °C. After incubation, the emission spectra of the samples were analysed between 280 and 500 nm with a slit width of 5 nm, and the excitation wavelength was set at 275 nm by using JASCO FP-8300 fluorescence spectrophotometer.⁴⁶

4.4.2.6 CD Spectroscopy

Peptide–calcium complexes were prepared at a 1:1 volume ratio, as detailed in the UV-Vis analysis protocol, and incubated at 37°C for 1 hour. Following incubation, circular dichroism (CD) spectra were recorded using a Jasco J-815 spectropolarimeter. The measurements were conducted over a wavelength range of 190–250 nm with a step size of 1.0 nm bandwidth at 37 °C for both E.M.P-2 and the E.M.P-2: CaCl_2 complex (1:1 volume ratio).⁴⁷

4.4.2.7 Zeta Potential

The zeta potential of the peptide and its calcium complex was determined using a Malvern Zetasizer particle size analyzer. Measurements were conducted for peptide–calcium complexes prepared at a 1:1 volume ratio, as described in the UV-Vis analysis protocol. After incubation at 37°C for 1 hour and vortexing for 5 minutes, the samples were transferred to a U-shaped cell and equilibrated for 60 seconds at 25°C prior to analysis.⁴⁸

4.4.2.8 Cytotoxicity Study

The cytotoxicity of all the peptides were evaluated on the clinically relevant human DMD patient derived cell line (KM1328) using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. KM1328 cells were seeded at a density of 5×10^5 cells per well in a 96-well culture plate and incubated for 24 hours. Subsequently, the cells were treated with various concentration of peptides followed by incubation at 37 °C in a 5% CO₂ environment for 24 hours. After treatment, the cells were incubated with MTT solution (5 mg/mL) in filtered PBS for 4 hours. After 4 hours, the culture medium was discarded, and the formed formazan crystals were solubilized using a solution of DMSO and methanol in a 1:1 ratio. The absorbance was measured at 550 nm using a multiplate reader.⁴⁹

4.4.2.9 Serum stability assay for lead engineered peptide E.M.P-2

To evaluate the stability of the lead engineered Peptide E.M.P-2 in human plasma, a serum stability assay was performed. Initially, a stock solution of the E.M.P-2 at a concentration of 10 mM in DMSO was prepared. A test compound stock at 50 μ M concentration was prepared by diluting the stock solution in acetonitrile. The frozen plasma samples were thawed at ambient temperature and then centrifuged at 14,000 revolutions per minute at 4 °C for 15 minutes. The resulting clear supernatant, approximately 90% of the total volume, was transferred to a separate tube along with 10% PBS for use in the assay. The plasma was then pre-incubated at 37 °C for 15 minutes, after which the test compound was added. Samples of 100 μ L were withdrawn from the incubated mixture at time points of 0 minutes, 30 minutes, and subsequently every 30 minutes up to 7 hours. Immediately after withdrawal, each sample was precipitated with 100 μ L of ice-cold acetonitrile. The mixture was then vortexed for 1 minute and centrifuged at 12,000 revolutions per minute for 10 minutes. The resulting supernatant was collected and stored at -20 °C for subsequent analysis using HPLC techniques.

4.4.2.10 Flow Cytometry Studies

Human DMD patient derived myoblast cells (KM1328) were seeded in 6-well culture plates and allowed to incubate overnight. Human DMD patient-derived cells were seeded into two 6-well culture plates. After treatment with the dye-labeled peptide, the cells were incubated

at two different temperatures, 4°C and 37°C, respectively. After treatment, the cells were washed to remove any residual culture medium and then trypsinized followed by centrifuge to pellet down the cells. The resulting cell pellet was re-suspended in PBS (0.2 mL) maintaining appropriate cell density. Then the cells were analyzed by BD LSR- FORTSEA flow cytometer to measure the intensity of green fluorescence associated with E.M.P-2.²³

4.4.2.11 In Cell ELISA

The In-Cell ELISA assay was used to screen the peptide library. Cells KM1328 were seeded into a white, 96-well plate at a density of 5×10^3 cells per well. The cells were incubated for a period of 24 hours at a temperature of 37 °C in a humidified environment enriched with 5% CO₂. After this incubation period, cells were treated with peptides at a concentration of 0.156 µM for 72 hours. Subsequently, the cells were fixed using 4% paraformaldehyde for 15 minutes and then washed twice using 1× PBS and permeabilized by 1× permeabilization buffer for 30 minutes at 300 rpm on a shaker. Blocking was done using a 2% BSA blocking buffer for 2 hours at room temperature. After blocking, the cells were washed once with 1× wash buffer and incubated overnight at 4 °C with a primary antibody specific to MyHC. The next day, the cells were washed three times with 1× wash buffer and incubated with an HRP-conjugated secondary antibody for 1 hour at room temperature. After incubation washed with the washing buffer three times then incubated in the OPD solution and incubated at 37°C for 15 minutes. Afterward, the reaction was stopped by adding 8% H₂SO₄. The absorbance was read at 492 nm by a microplate reader (Thermo Scientific Varioskan LUX), and to normalize the number of cells, the staining Janus Green B was employed and absorbance is taken at 595 nm.⁵⁰

4.4.2.12 RNA extraction and quantitative reverse transcriptase- PCR (RT-qPCR)

Total RNA was extracted from the treated KM1328 cells using the PureLink RNA Mini Kit (Cat. No. 12183018A, Invitrogen) according to the manufacturer's instructions. The concentration and the purity of the RNA (A₂₆₀/A₂₈₀ > 1.8) were determined by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Using 300 ng of total RNA, cDNA was synthesized using the iScript cDNA Synthesis Kit (Cat. No. #1708890, Bio-Rad) according to the manufacturer's instructions. Diluted the cDNA 10-fold with nuclease-free water for RT-qPCR analysis. A reaction mix of 10 µL was prepared for the assay using 5 µL of iTaq™ Universal SYBR Green Supermix (2x), 0.5 µL of each specific primer of a concentration of 10 µM, and 4 µL of the diluted cDNA. The PCR was conducted using a standard fast protocol. This includes 95 °C for 20 s for initial heating followed by 40 cycles of amplification that comprised of the following: 95 °C for 3 s and annealing temperature of 60 °C for 30s followed by 70 °C for 5s. The expression levels were calculated against GAPDH and used as an endogenous control. Relative levels of mRNA expression were estimated by the $2^{-\Delta\Delta C_t}$ method.⁵¹

Gene	Forward 5'-3'	Reverse 5'-3'	Accession No.
MyH3	GCTTGTGGGCGGAGGTC T	AGCAGATATGCCGAA CACTT	NM_002470.4
MyoD1	CCGACGGCATGATGGA CTA	CAGTCTAGGCTCGACA CCG	NM_002478.5
COL1A1	CCCCGAGGCTCTGAAGG T	GCAATACCAGGAGCA CCATTG	NM_000088.4
GAPDH	CTAGGCGCTCACTGTTC TCT	GCCCAATACGACCAA ATCCGT	NM_001289746.2

Table 4.1: Forward and reverse primer for MyHC, MyoD and COL1A1.

4.4.2.13 Dot blot for protein expression

To determine the protein expression level, we executed the dot blot assay. First, we incubated the cell lysate with beta-mercaptoethanol (5%) and heated it at 95 °C for 5 minutes. After the incubation of the lysate were loaded on the nitrocellulose membranes. When the membranes got dry, we put it for blocking with 5% BSA, and then, the membranes were treated with respective primary antibodies, MyoD, MyHC and COL1A1 for overnight. After washing, each membrane was treated with the HRP-conjugated secondary antibody, and the chemiluminescence intensity of the spots imaged using the G-Box Chemi XRQ system (Syngene, UK) and compared with the control experiment.⁵²

4.4.2.14 Mitotracker

In order to check the localization of Peptide-2 to mitochondria, we tagged the peptide-2 with FAM. Then KM1328 cells were treated with Peptide-2 (0.156µM) for 72 hours. After that cells were incubated with with 200 nM of mitoTracker CMXRos red probe following the manufacturer's instruction prepared in colourless DMEM for 10 minutes in the dark. After fixing with 4% paraformaldehyde nucleus is stained with Hoechst. The cells were visualization under a fluorescence microscope (Olympus IX83). It is observed that there is a complete overlap between green and red channels which confirm the localization of micro peptide-2 to the mitochondria.⁵³

4.4.2.15 Immunocytochemistry

KM1328 Human DMD patient derived cells were seeded in 12 well plate at a cell density of 3×10^4 cells and the cells were incubated for a period of 24 hours at a temperature of 37 °C in a humidified environment enriched with 5% CO₂. After this incubation period, cells were treated with peptides at a concentration of 0.156 µM for 72 hours. Subsequently, the cells were fixed using 4% paraformaldehyde for 15 minutes and then washed twice using 1× PBS and permeabilized by 1× permeabilization buffer for 30 minutes at 300 rpm on a shaker. Blocking

was done using a 2% BSA blocking buffer for 2 hours at room temperature. After blocking, the cells were washed once with 1× wash buffer and incubated overnight at 4 °C with a primary antibody specific to IL-6 (sc-57315) and COL1A1 (sc-59772). The next day, the cells were washed three times with 1× wash buffer and incubated with an anti mouse alexa fluor 488 secondary antibody (A11029) incubated for 2 hours for 2 hour at room temperature. Cells were then washed again and image were taken at 20x using a fluorescence microscope (Olympus IX83)

4.5 Conclusion

Addressing DMD secondary pathological progression would be a significant therapeutic strategy to slow down the onset of the disease. These approaches can also complement treatments targeting the primary genetic defect, thereby providing a more comprehensive management plan for the disease. The current therapeutic regime to manage secondary symptoms relies on the use of corticosteroids, but its long time use in DMD patients is associated with severe side effects such as weight gain, cataract, osteoporosis, behavioural changes, and vertebral compression fractures. The majority of the DMD population solely depends on the use of corticosteroids as drugs targeting exon skipping addressing primary defects of the disease is out of reach for the majority of the population because of its high cost.

In this regard, our mitochondria-targeted peptide, designed from mitochondria-localized micropeptides (MPM), has the potential to be the first peptide-based therapeutic for managing secondary pathophysiological symptoms of DMD and delaying disease progression. This novel peptide therapy could serve as an alternative to standard corticosteroid treatment, offering a promising therapeutic platform for managing secondary pathological symptoms of DMD.

These newly truncated and further engineered lead peptide stretch E.M.P-2 have significant potential in promoting myogenesis by elevating the expression of myogenic markers MyoD and MyHC up to two fold within lower optimum dose of 150 nM. Apart from promoting myogenesis M.P-2 also simultaneously downregulated COL1A1 a fibrosis marker, up to 60%, a key pathological hallmark associated with disease progression. These findings of myogenic markers upregulation and reduction in fibrosis by E.M.P-2 was mediated through the suppression of TGFβ expression, a pro-fibrotic cytokine known to hinder muscle regeneration and drive fibrotic tissue accumulation in DMD.

E.M.P-2 subsequently also maintains impaired calcium homeostasis within the mitochondrial compartment because of the peptide calcium chelating ability. Maintaining calcium balance within the cellular environment further intensifies the potential of E.M.P-2 in managing DMD disease progression.

Inflammation is another key defect engaged with disease progression, overexpression of pro-inflammatory cytokines IL-6 in DMD patient's leads to promoting chronic inflammation, supporting repeated cycles of muscle degeneration and regeneration. E.M.P-2 also shows its potency in significantly downregulating the expression of IL-6 in DMD patient derived cells which shows significant baseline IL-6 expression. Treatment of E.M.P-2 significantly reduced IL-6 mRNA levels by ~30%, over 3 days at 0.156 μM. These results suggest that E.M.P-2

effectively mitigates IL-6 expression, potentially improving the inflammatory profile in dystrophic muscle.

Overall, our newly designed E.M.P-2 would be the first of its kind peptide based therapeutic platform to manage key secondary pathological symptoms of DMD. By promoting myogenesis, mitigating fibrosis, maintaining calcium homeostasis, and managing pro-inflammatory cytokine IL-6, E.M.P-2 positioned itself as a future alternative to corticosteroids use in DMD patients.

References

1. Duan, D.; Goemans, N.; Takeda, S. i.; Mercuri, E.; Aartsma-Rus, A., Duchenne muscular dystrophy. *Nature Reviews Disease Primers* 2021, 7 (1), 13.
2. Nowak, K. J.; Davies, K. E., Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. *EMBO reports* 2004, 5 (9), 872-6.
3. Bach, J. R.; O'Brien, J.; Krotenberg, R.; Alba, A. S., Management of end stage respiratory failure in Duchenne muscular dystrophy. *Muscle & nerve* 1987, 10 (2), 177-82.
4. England, S. B.; Nicholson, L. V.; Johnson, M. A.; Forrest, S. M.; Love, D. R.; Zubrzycka-Gaarn, E. E.; Bulman, D. E.; Harris, J. B.; Davies, K. E., Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. *Nature* 1990, 343 (6254), 180-2.
5. Guiraud, S.; Aartsma-Rus, A.; Vieira, N. M.; Davies, K. E.; van Ommen, G. J.; Kunkel, L. M., The Pathogenesis and Therapy of Muscular Dystrophies. *Annual review of genomics and human genetics* 2015, 16, 281-308.
6. Emery, A. E. H., The muscular dystrophies. *The Lancet* 2002, 359 (9307), 687-695.
7. Guiraud, S.; Chen, H.; Burns, D. T.; Davies, K. E., Advances in genetic therapeutic strategies for Duchenne muscular dystrophy. *Experimental physiology* 2015, 100 (12), 1458-67.
8. Niks, E. H.; Aartsma-Rus, A., Exon skipping: a first in class strategy for Duchenne muscular dystrophy. *Expert opinion on biological therapy* 2017, 17 (2), 225-236.
9. Wang, L.; Fan, J.; Han, L.; Qi, H.; Wang, Y.; Wang, H.; Chen, S.; Du, L.; Li, S.; Zhang, Y.; Tang, W.; Ge, G.; Pan, W.; Hu, P.; Cheng, H., The micropeptide LEMP plays an evolutionarily conserved role in myogenesis. *Cell Death & Disease* 2020, 11 (5), 357.
10. Choi, I. Y.; Lim, H.; Estrellas, K.; Mula, J.; Cohen, T. V.; Zhang, Y.; Donnelly, C. J.; Richard, J. P.; Kim, Y. J.; Kim, H.; Kazuki, Y.; Oshimura, M.; Li, H. L.; Hotta, A.; Rothstein, J.; Maragakis, N.; Wagner, K. R.; Lee, G., Concordant but Varied Phenotypes among Duchenne Muscular Dystrophy Patient-Specific Myoblasts Derived using a Human iPSC-Based Model. *Cell reports* 2016, 15 (10), 2301-2312.
11. Heslop, L.; Morgan, J. E.; Partridge, T. A., Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. *Journal of cell science* 2000, 113 (Pt 12), 2299-308.
12. Kharraz, Y.; Guerra, J.; Pessina, P.; Serrano, A. L.; Muñoz-Cánoves, P., Understanding the process of fibrosis in Duchenne muscular dystrophy. *BioMed research international* 2014, 2014, 965631.
13. Blake, D. J.; Weir, A.; Newey, S. E.; Davies, K. E., Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiological reviews* 2002, 82 (2), 291-329.

14. Matthews, E.; Brassington, R.; Kuntzer, T.; Jichi, F.; Manzur, A. Y., Corticosteroids for the treatment of Duchenne muscular dystrophy. The Cochrane database of systematic reviews 2016, 2016 (5), Cd003725.
15. McDonald, C. M.; Henricson, E. K.; Abresch, R. T.; Duong, T.; Joyce, N. C.; Hu, F.; Clemens, P. R.; Hoffman, E. P.; Cnaan, A.; Gordish-Dressman, H.; Vishwanathan, V.; Chidambaramathan, S.; Biggar, W. D.; McAdam, L. C.; Mah, J. K.; Tulinius, M.; Cnaan, A.; Morgenroth, L. P.; Leshner, R.; Tesi-Rocha, C.; Thangarajh, M.; Duong, T.; Kornberg, A.; Ryan, M.; Nevo, Y.; Dubrovsky, A.; Clemens, P. R.; Abdel-Hamid, H.; Connolly, A. M.; Pestronk, A.; Teasley, J.; Bertorin, T. E.; Webster, R.; Kolski, H.; Kuntz, N.; Driscoll, S.; Bodensteiner, J. B.; Carlo, J.; Gorni, K.; Lotze, T.; Day, J. W.; Karachunski, P.; Henricson, E. K.; Abresch, R. T.; Joyce, N. C.; McDonald, C. M., Long-term effects of glucocorticoids on function, quality of life, and survival in patients with Duchenne muscular dystrophy: a prospective cohort study. The Lancet 2018, 391 (10119), 451-461.
16. Jiang, C.; Wen, Y.; Kuroda, K.; Hannon, K.; Rudnicki, M. A.; Kuang, S., Notch signaling deficiency underlies age-dependent depletion of satellite cells in muscular dystrophy. Disease models & mechanisms 2014, 7 (8), 997-1004.
17. Matsumoto, A.; Pasut, A.; Matsumoto, M.; Yamashita, R.; Fung, J.; Monteleone, E.; Saghatelian, A.; Nakayama, K. I.; Clohessy, J. G.; Pandolfi, P. P., mTORC1 and muscle regeneration are regulated by the LINC00961-encoded SPAR polypeptide. Nature 2017, 541 (7636), 228-232.
18. Lin, Y.-F.; Xiao, M.-H.; Chen, H.-X.; Meng, Y.; Zhao, N.; Yang, L.; Tang, H.; Wang, J.-L.; Liu, X.; Zhu, Y.; Zhuang, S.-M., A novel mitochondrial micropeptide MPM enhances mitochondrial respiratory activity and promotes myogenic differentiation. Cell Death & Disease 2019, 10 (7), 528.
19. Zielonka, J.; Joseph, J.; Sikora, A.; Hardy, M.; Ouari, O.; Vasquez-Vivar, J.; Cheng, G.; Lopez, M.; Kalyanaraman, B., Mitochondria-Targeted Triphenylphosphonium-Based Compounds: Syntheses, Mechanisms of Action, and Therapeutic and Diagnostic Applications. Chem Rev 2017, 117 (15), 10043-10120.
20. Hopf, F. W.; Turner, P. R.; Denetclaw, W. F., Jr.; Reddy, P.; Steinhardt, R. A., A critical evaluation of resting intracellular free calcium regulation in dystrophic mdx muscle. The American journal of physiology 1996, 271 (4 Pt 1), C1325-39.
21. Ke, H.; Ma, R.; Liu, X.; Xie, Y.; Chen, J., Highly effective peptide-calcium chelate prepared from aquatic products processing wastes: Stickwater and oyster shells. LWT 2022, 168, 113947.
22. Lamarche, É.; Lala-Tabbert, N.; Gunanayagam, A.; St-Louis, C.; Wiper-Bergeron, N., Retinoic acid promotes myogenesis in myoblasts by antagonizing transforming growth factor-beta signaling via C/EBPβ. Skelet Muscle 2015, 5 (1), 8.
23. Bhunia, D.; Mondal, P.; Das, G.; Saha, A.; Sengupta, P.; Jana, J.; Mohapatra, S.; Chatterjee, S.; Ghosh, S., Spatial Position Regulates Power of Tryptophan: Discovery of a Major-Groove-Specific Nuclear-Localizing, Cell-Penetrating Tetrapeptide. Journal of the American Chemical Society 2018, 140 (5), 1697-1714.
24. Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chernomordik, L. V.; Lebleu, B., Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. The Journal of biological chemistry 2003, 278 (1), 585-90.
25. Duan, D.; Goemans, N.; Takeda, S.; Mercuri, E.; Aartsma-Rus, A., Duchenne muscular dystrophy. Nature reviews. Disease primers 2021, 7 (1), 13.

26. Wood, W. M.; Etemad, S.; Yamamoto, M.; Goldhamer, D. J., MyoD-expressing progenitors are essential for skeletal myogenesis and satellite cell development. *Developmental biology* 2013, 384 (1), 114-27.
27. Kim, J. A.; Shon, Y. H.; Lim, J. O.; Yoo, J. J.; Shin, H.-I.; Park, E. K., MYOD mediates skeletal myogenic differentiation of human amniotic fluid stem cells and regeneration of muscle injury. *Stem Cell Research & Therapy* 2013, 4 (6), 147.
28. Zammit, P. S., Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. *Seminars in cell & developmental biology* 2017, 72, 19-32.
29. Morgoulis, D.; Berenstein, P.; Cazacu, S.; Kazimirsky, G.; Dori, A.; Barnea, E. R.; Brodie, C., sPIF promotes myoblast differentiation and utrophin expression while inhibiting fibrosis in Duchenne muscular dystrophy via the H19/miR-675/let-7 and miR-21 pathways. *Cell Death & Disease* 2019, 10 (2), 82.
30. Sabourin, L. A.; Rudnicki, M. A., The molecular regulation of myogenesis. *Clinical genetics* 2000, 57 (1), 16-25.
31. Brashear, S. E.; Wohlgemuth, R. P.; Gonzalez, G.; Smith, L. R., Passive stiffness of fibrotic skeletal muscle in mdx mice relates to collagen architecture. *The Journal of physiology* 2021, 599 (3), 943-962.
32. Lieber, R. L.; Ward, S. R., Cellular mechanisms of tissue fibrosis. 4. Structural and functional consequences of skeletal muscle fibrosis. *American journal of physiology. Cell physiology* 2013, 305 (3), C241-52.
33. Bar-Nur, O.; Gerli, M. F. M.; Di Stefano, B.; Almada, A. E.; Galvin, A.; Coffey, A.; Huebner, A. J.; Feige, P.; Verheul, C.; Cheung, P.; Payzin-Dogru, D.; Paisant, S.; Anselmo, A.; Sadreyev, R. I.; Ott, H. C.; Tajbakhsh, S.; Rudnicki, M. A.; Wagers, A. J.; Hochedlinger, K., Direct Reprogramming of Mouse Fibroblasts into Functional Skeletal Muscle Progenitors. *Stem Cell Reports* 2018, 10 (5), 1505-1521.
34. Kyrychenko, V.; Poláková, E.; Janíček, R.; Shirokova, N., Mitochondrial dysfunctions during progression of dystrophic cardiomyopathy. *Cell calcium* 2015, 58 (2), 186-95.
35. Allen, D. G.; Whitehead, N. P.; Froehner, S. C., Absence of Dystrophin Disrupts Skeletal Muscle Signaling: Roles of Ca²⁺, Reactive Oxygen Species, and Nitric Oxide in the Development of Muscular Dystrophy. *Physiological reviews* 2016, 96 (1), 253-305.
36. Luo, J.; Yao, X.; Soladoye, O. P.; Zhang, Y.; Fu, Y., Phosphorylation modification of collagen peptides from fish bone enhances their calcium-chelating and antioxidant activity. *LWT* 2022, 155, 112978.
37. Yuan, F.; Fu, Y.; Ma, L.; Zhu, H.; Yu, Y.; Feng, X.; Sun, Y.; Dai, H.; Liu, X.; Liu, Z.; Zhang, Y., Calcium-chelating peptides from rabbit bone collagen: characterization, identification and mechanism elucidation. *Food Science and Human Wellness* 2024, 13 (3), 1485-1493.
38. Wang, X.; Gao, A.; Chen, Y.; Zhang, X.; Li, S.; Chen, Y., Preparation of cucumber seed peptide-calcium chelate by liquid state fermentation and its characterization. *Food Chemistry* 2017, 229, 487-494.
39. Venyaminov, S. Y.; Klimtchuk, E. S.; Bajzer, Z.; Craig, T. A., Changes in structure and stability of calbindin-D(28K) upon calcium binding. *Analytical biochemistry* 2004, 334 (1), 97-105.

40. Sun, N.; Jin, Z.; Li, D.; Yin, H.; Lin, S., An Exploration of the Calcium-Binding Mode of Egg White Peptide, Asp-His-Thr-Lys-Glu, and In Vitro Calcium Absorption Studies of Peptide-Calcium Complex. *Journal of Agricultural and Food Chemistry* 2017, 65 (44), 9782-9789.
41. Pelosi, L., Berardinelli, M.G., Forcina, L., Spelta, E., Rizzuto, E., Nicoletti, C., Camilli, C., Testa, E., Catizone, A., De Benedetti, F. and Musarò, A., 2015. Increased levels of interleukin-6 exacerbate the dystrophic phenotype in mdx mice. *Human molecular genetics*, 24(21), 6041-6053.
42. Wada, E., Tanihata, J., Iwamura, A., Takeda, S.I., Hayashi, Y.K. and Matsuda, R., 2017. Treatment with the anti-IL-6 receptor antibody attenuates muscular dystrophy via promoting skeletal muscle regeneration in dystrophin-/utrophin-deficient mice. *Skeletal muscle*, 7, pp.1-15.
43. Mammen, A.L. and Sartorelli, V., 2015. IL-6 Blockade as a therapeutic approach for duchenne muscular dystrophy. *EBioMedicine*, 2(4), pp.274-275.
44. Adak, A.; Das, G.; Barman, S.; Mohapatra, S.; Bhunia, D.; Jana, B.; Ghosh, S., Biodegradable Neuro-Compatible Peptide Hydrogel Promotes Neurite Outgrowth, Shows Significant Neuroprotection, and Delivers Anti-Alzheimer Drug. *ACS Appl. Mater. Interfaces*. **2017**, 9 (6), 5067-5076.
45. Klabenkova, K.; Fokina, A.; Stetsenko, D. Chemistry of Peptide-Oligonucleotide Conjugates: A Review. *Mol.*, 2021, 26, 5420.
46. Han, L., Li, Y., Hu, B., Wang, W., Guo, J., Yang, J., Dong, N., Li, Y. and Li, T., 2024. Enhancement of Calcium Chelating Activity in Peptides from Sea Cucumber Ovum through Phosphorylation Modification. *Foods*, 13(12), 1943.
47. Topchiy, E. and Lehmann, T., 2014. Chelation of Ca²⁺ ions by a peptide from the repeat region of the Plasmodium falciparum circumsporozoite protein. *Malaria Journal*, 13, 1-11.
48. Wang, Z., Zhai, X., Xiao, X., Xia, P., Chen, X., Li, Y., Hao, L. and Zhang, Y., 2024. Novel peptides with calcium-binding capacity from antler bone hydrolysate, its bioactivity on MC3T3-E1 cells, and the possible chelating mode. *Food Science & Nutrition*.
49. Jana, A., Garg, S., Ghosh, S., Khan, J., Roy, R., Mukherjee, N., Jash, M., Gupta, V., Nayak, P. and Ghosh, S., 2024. Generation of Functional Neurons from Mesenchymal Stem Cells Using Neural Differentiator and Engineered Peptide Hydrogel: Potential Therapeutic Lead for Traumatic Brain Injury. *ACS Applied Materials & Interfaces*.
50. Ghosh, S., Arshi, M.U., Ghosh, S., Jash, M., Sen, S., Mamchaoui, K., Bhattacharyya, S., Rana, N.K. and Ghosh, S., 2024. Discovery of Quinazoline and Quinoline-Based Small Molecules as Utrophin Upregulators via AhR Antagonism for the Treatment of Duchenne Muscular Dystrophy. *Journal of Medicinal Chemistry*.
51. Garg, S., Jana, A., Gupta, S., Arshi, M.U., Gharai, P.K., Khan, J., Roy, R. and Ghosh, S., 2024. Discovery of Gallic Acid-Based Mitochondriotropic Antioxidant Attenuates LPS-Induced Neuroinflammation. *Free Radical Biology and Medicine*.