1	Name and designation of ICMR- IF	Dr Shashikant Ray
2	Address	Department of Biotechnology, Mahatma Gandhi Central University, Motihari, Bihar-845401
3	Frontline area of research in which training/research was carried out	Muscular Dystrophy
4	Name & address of Professor and host institute	Prof. Krishna M.G. Mallela, Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, Aurora, Denver, USA
5	Duration of fellowship with exact date	One Year (8th February 2023 to 7th February 2024)
6	Highlights of work conducted	

i)Technique/expertise acquired

Training Obtained:

I have undergone training to create the mini-dystrophin construct for subsequent characterization in gene therapy. Additionally, I have conducted a characterization of the disease-causing mutations on the structure-function of the CT domain of dystrophin.

Protocol or Assay Developed:

1. Purification of different disease-causing mutant proteins of CT domain of dystrophin.
2. Purification of syntrophin unique domain (SUD) protein.

Techniques Learnt:

1. Gene cloning in a mammalian system.
2. Gene cloning in a prokaryotic system.
3. Cell culture techniques for the isolation of mammalian proteins.
4. Transfection for the isolation of mammalian proteins from non-adherent HEK cell lines.
5. Protein purification from transfected HEK cell lines.
6. Monitoring protein stability through thermal denaturation using Circular Dichroism.
7. Monitoring protein stability through urea denaturation using Circular Dichroism.
8. Monitoring protein interactions using isothermal calibration.
9. Site-directed mutagenesis.
10. Fluorimetry.

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Summary of work done:

Title: Elucidating the effect of disease-causing mutations on the structure-function of the CT domain of dystrophin

Duchenne muscular dystrophy (DMD) is the most common and lethal type of MD caused by deletions, duplications, or point mutations in the dystrophin gene. Dystrophin protein consists of four domains: N-terminal domain which interacts with actin, a rod domain consisting of 24 spectrin domains, a cysteine rich domain and C-terminal (CT) domain. The CT domain encompasses two polypeptide chain that folds into secondary structure similar to the spectrin repeats into α-helical coiled coils structure. It is known to interact with other proteins by using their coiled coil protein motifs. The CT-domain provides binding sites for dystrobrevin and syntrophins. Numerous single amino acid substitution mutations in CT-domain reported to cause either DMD/BMD. Here, we have examined the effect of six disease-causing mutations on the CT domain of dystrophin. Surprisingly, these mutants exhibited similar secondary structures and thermal stability compared to the wild-type CT domain. Moreover, our investigation into the interaction of these mutant proteins with α-dystrobrevin revealed an intriguing finding that there was no discernible alteration in the strength of interaction compared to the wild-type CT. However, their functional implications and how these mutations affect protein interactions and muscle function remain subjects of ongoing research. Understanding the structure-function relationships of these mutant proteins is critical for shedding light on the molecular mechanisms underlying DMD and may provide insights for potential therapeutic interventions in the future.

Title: Mini-dystrophin-based gene therapy for Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) results from mutations in the dystrophin gene, and the potential cure lies in gene therapy by replacing the faulty gene with a normal one. Currently undergoing clinical trials, this gene therapy employs Adeno-associated viral vectors (AAV) to deliver the dystrophin gene. However, a key limitation of AAV-based therapy is the constraint on the gene size accommodated by the virus. Earlier attempts at utilizing truncated dystrophins for gene therapy failed due to issues like reduced stability, diminished function, increased aggregation, and heightened proteolytic susceptibility in patients. Therefore, the development and characterization of mini-dystrophins, based on a profound understanding of dystrophin's structure-function relationship, become crucial for successful gene therapy.

In our study, we designed a mini-dystrophin within the specified size range for gene therapy. We synthesized a codon-optimized cDNA sequence of the mini-dystrophin, placed under the BamH1 and Xho1 restriction sites. The purified cDNA was then digested with BamH1 and Xho1, and the desired cDNA was isolated using an agarose gel extraction kit. Subsequently, the cDNA was ligated with a pre-digested pcDNA™ 3.4 TOPO™ vector with BamH1 and Xho1 restriction enzymes and transformed into DH5α competent cells. Putative clones were selected, grown in LB media with ampicillin, and their plasmid DNA was isolated and confirmed by DNA sequencing.

To assess the expression and purification of mini-dystrophin, a maxi prep was performed to obtain a maximum yield of plasmid DNA for transfection into non-adherent HEK cell

	lines. Transfection involved the use of 30 µg of plasmid DNA with PEIMAX (1 mg/mL) in a cell culture environment adjusted to 2.5-3 X 10^6 cells/mL with pre-warmed Expi293 expression media. Cells were incubated for 4-6 days, and the expression of protein was monitored after 3, 4 and 5 days of incubation. Post-incubation, cells were centrifuged, supernatant collected, and filtered with a 0.22 µm filter. The filtered solution underwent Ni-column purification, with impurities washed using 30 and 50-mM imidazole in ULP1 buffer. The desired protein was eluted with 250 mM imidazole in ULP1 buffer, and the protein's purity was assessed using SDS-PAGE. This comprehensive approach integrates gene synthesis, vector design, cloning, transfection, and protein purification, contributing to the ongoing pursuit of effective gene therapy for DMD. **ii)Research results, including any papers, prepared/submitted for publication.** **Publication as First Author:** **Shashikant Ray**, Vaibhav Upadhyay, Sudipta Panja, Jeffrey Kearns, and Krishna M.G. Mallela. Elucidating the effect of disease-causing mutations on the structure-function of the CT domain of dystrophin **(Manuscript Under preparation)** **Poster Presentation:** **Shashikant Ray**, Vaibhav Upadhyay, Sudipta Panja, Jeffrey Kearns, and Krishna M.G. Mallela. Elucidating the effect of disease-causing mutations on the structure-function of the CT domain of dystrophin (Gibbs37 conference, at the Touch of Nature Outdoor Education Center in Carbondale, IL, from 14th October 2023 to 17th October 2023) **iii)Proposed utilization of the experience in India:** The cutting-edge techniques assimilated will prove instrumental for the seamless and impactful execution of ongoing research endeavors at the institute, specifically focusing on muscular dystrophy. The overarching research proficiency and advanced experimental skills cultivated during my tenure at the Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, Aurora, Denver, USA, will serve as a cornerstone for the inception and advancement of a pioneering research program in the realm of gene therapy for muscular dystrophy at MGCU, Motihari, Bihar.

REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

Shashikant Ray

Signature of ICMR-IF

ICMR Sanction No. INDO/FRC/452/(Y-45)/2022-23-IH &HRD

Dated 19 October 2022