P.206

Evaluation of the Lipid-Binding and Stability Properties of Recombinant Dystrophin Spectrin-Like Repeat Constructs

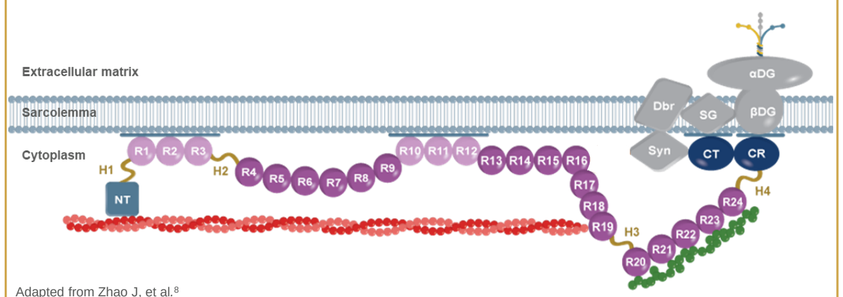
Grace Cooper-Olson, Rachael A. Potter, and Louise R. Rodino-Klapac

Sarepta Therapeutics, Inc., Cambridge, Massachusetts, USA

BACKGROUND

- Duchenne muscular dystrophy (DMD) is a rare, X-linked, fatal, neuromuscular disease caused by mutations in the DMD gene that disrupt the production of functional dystrophin protein.[1,2]
- Gene therapy has emerged as a promising approach for monogenetic diseases such as DMD, and is being evaluated as a therapeutic strategy to restore production of functional dystrophin.
- Systemic adeno-associated virus (AAV)-based gene therapy for DMD faces challenges that require careful design, given the need to target cardiac muscle and broadly distributed skeletal muscle tissue, and the package limitation of AAV.[3,4]
- Early clinical data showed that large deletions in the dystrophin gene that do not disrupt the open reading frame in patients with milder Becker muscular dystrophy result in partially normal muscle structure and function.[5]
- Identification of the optimal shortened dystrophin construct that most normalizes muscle function is a key translation question that could overcome the constraints of the AAV cloning capacity. Rational design of shorter dystrophin constructs demonstrated that some domains seem to be essential.[6]
- Dystrophin has four main functional domains: an actin-binding amino-terminal domain; a central rod domain composed of spectrin-like repeat units and hinges; a cysteine-rich domain (CR), and a carboxyl-terminus (CT).[7] Interaction with the sarcolemma is central to how dystrophin protects the muscle (**Figure 1**).[8]
  - Molecular, biochemical and structural studies have shown that the CR domain, the spectrin-like repeats R1-3 and R10-12, and the CT are the anchor points that bind dystrophin to the membrane.[8]
  - In vivo, delivery of shortened dystrophin genes via AAV has shown:
    - The CT may be non-essential due to redundant protein–protein interaction domains within the dystrophin-associated protein complex.[9]
    - The R1-3 domain has a role in modulating radial force transmission and mechanical vulnerability and might impact shortened dystrophin functionality.[10]

Figure 1. Model of dystrophin–sarcolemma interaction



Adapted from Zhao J, et al.[8]

Figure 1. Binding to the sarcolemma is essential for dystrophin to protect muscle from contraction-induced injury, and in vivo evidence suggests that dystrophin contains four membrane-binding domains, including spectrin-like repeats (R)1-3, R10-12, cysteine-rich (CR) domain, and C-terminus (CT)[8]

OBJECTIVE

- The purpose of this study is to evaluate the binding affinity of several spectrin-like repeats of dystrophin protein to lipid vesicles in vitro in order to identify differences that might be necessary to consider in designing of a micro-dystrophin construct for AAV gene therapy in DMD patients.

METHODS

Preparation of lipid vesicles

- Lipid mixtures were dried and resuspended in EDTA/TRIS buffer or PBS buffer
- After freeze/thaw, an extruder was used to create vesicles of appropriate size
- Prepared vesicles were stored at 4°C until use

Incubation of peptides with vesicles

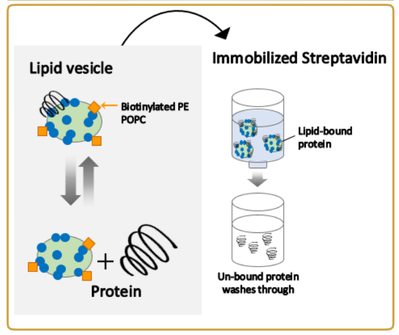
- Peptide concentration varied from 1:100 to 1:5, peptide:vesicle, room temperature (RT) for 1–2 hours

METHODS (CONT'D)

Assessment of lipid binding

- Biotinylated Lipid Kd determination methodology is shown in **Figure 2**
- Peptide/lipid mixtures were applied to a streptavidin column and incubated 10 minutes RT
- Columns were washed with 5 column volumes (cv) of PBS
- Protein was eluted with 5 cv of elution buffer (8M guanidine HCl pH 1.5)
- Eluted sample was neutralized with buffer A (1M Tris pH 9.0)

Figure 2. Biotinylated lipid Kd determination



Quantification of unbound protein using nanodrop

- Wash and eluate were concentrated; protein concentrations were determined using nanodrop absorbance at 280nm

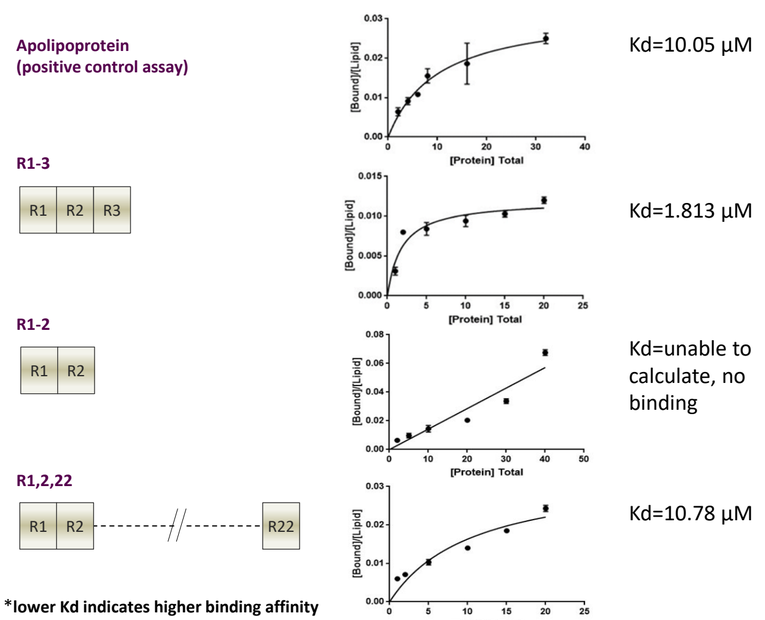
Analysis

- The lipid-binding affinity of 3 peptide constructs containing different R modules (R1-3; R1-2; and R1,2,22) was measured by determination of the equilibrium dissociation constant (Kd; lower Kd indicates higher binding affinity)
- Apolipoprotein was used as a positive control assay

RESULTS

- Previous studies show that the clinical concentrations of micro-dystrophin protein in biopsies demonstrate a range from approximately 0.8-14 µM when converted to µM concentration.
- Results demonstrate differences in lipid-binding affinities in peptide modules containing different R modules (**Figures 3 and 4**)
  - Apolipoprotein (Kd = 10.05 µM ± 2.352)
  - R1-3 (highest lipid-binding affinity, Kd=1.813 ± 0.486 µM)
  - R1-2 (Kd unable to calculate, no binding)
  - R1,2,22 (Kd=10.78 ± 3.997 µM)
- Notably, R module R1-3 showed the highest lipid-binding affinity with the lowest Kd value out of the three peptide constructs.

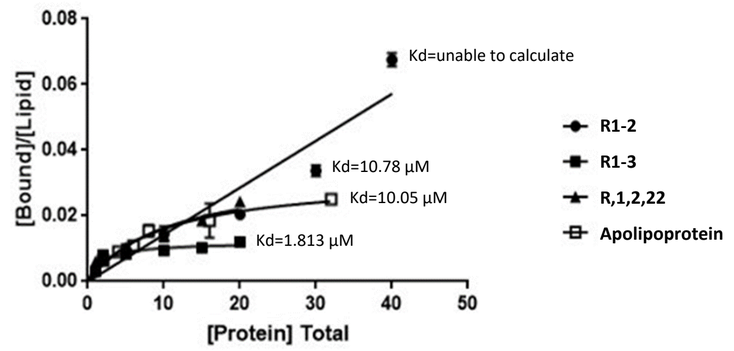
Figure 3. Lipid-binding affinity of 3 peptide constructs containing different R modules.*



*lower Kd indicates higher binding affinity

RESULTS (CONT'D)

Figure 4. Combined lipid-binding affinity of 3 peptide constructs containing different R modules



	Kd µM (SE)	Bmax (SE)	Bmax
Apolipoprotein	10.05 (2.352)	0.03212 (0.0033)	0.9099
R1-3	1.813 (0.4864)	0.01203 (0.0008)	0.849
R1-2	Unable to calculate	Unable to calculate	Unable to calculate
R1,2,22	10.78 (3.997)	0.03395 (0.0059)	0.8958

CONCLUSIONS

- Previous studies have shown that the R1-R3 region binds to the lipid component of the sarcolemma in vivo,[8] and studies in *mdx* mice have shown that the inclusion of spectrin-like repeats R1–3 is important for protection against eccentric contraction-induced force drop.[10]
- This study demonstrates the high affinity of the module R1-3 to bind to the lipid vesicles in vitro. This property is disrupted when R3 is not present, suggesting that the complete R1-3 region may play an important role in dystrophin functionality.
- We hypothesize that R1-3 may be a structural region that contributes to maintaining structural integrity as an anchor point to the sarcolemma, which may provide a functional benefit when included in the design of a micro-dystrophin construct for AAV gene therapy in DMD patients.

REFERENCES

1. Hoffman EP, et al. *Cell*. 1987;51(6):919-928.
2. Koenig M, et al. *Cell*. 1987;50(3):509-517.
3. Mendell JR, et al. *Neurosci Lett*. 2012;572(2):90-99.
4. Duan D. *Mol Ther*. 2018;26(10):2337-2356.
5. England SB, et al, *Nature* 1990; 343: 180-182.
6. Harper SQ, et al. *Nature Med* 2002;8:253.
7. Gao Q et al. *Compr Physiol*. 2015 July 1; 5(3):1223.
8. Zhao J, et al. *Hum Mol Genet*. 2016;25(17):3647-3653.
9. Ramos JN, et al. Mol Ther 2019; 27(3):623-635.
10. Nelson DM, et al. *Hum Mol Genet*. 2018;27(12):2090-2100.

ACKNOWLEDGEMENTS & DISCLOSURES

This study was sponsored by Sarepta Therapeutics, Inc. Authors are employees of Sarepta Therapeutics and may have stock options. Medical writing and editorial support was provided by Health & Wellness Partners, LLC, funded by Sarepta Therapeutics.