Therapeutic Development of saRNA for Duchenne Muscular Dystrophy by Targeted Activation of Utrophin

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Background

- Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are rare neuromuscular disorders caused by the mutation of DMD gene which encodes dystrophin protein.
- DMD is the largest gene in human body with 79 exons.
- Current disease modifying therapeutic approaches for DMD include antisense oligonucleotides (ASO) mediated exon skipping, gene therapy and gene editing, with exon skipping being the most common strategy.
- Utrophin protein encoded by the gene UTRN is structurally and functionally similar to dystrophin, and its upregulation could potentially compensate the function of dystrophin and treat all DMD and BMD patients regardless of the location of DMD mutation. However, previous attempts involving the use of epigenetic modifying agents such as histone deacetylase inhibitor (HDACi) to activate UTRN have largely failed in clinical trials, apparently due to lack of target specificity.
- saRNA is created to induce human utrophin expression following systemic administration to the skeletal muscles and heart. The present study explores its therapeutic development as a novel RNAa modality for the treatment of DMD.

Overview of therapeutic strategy

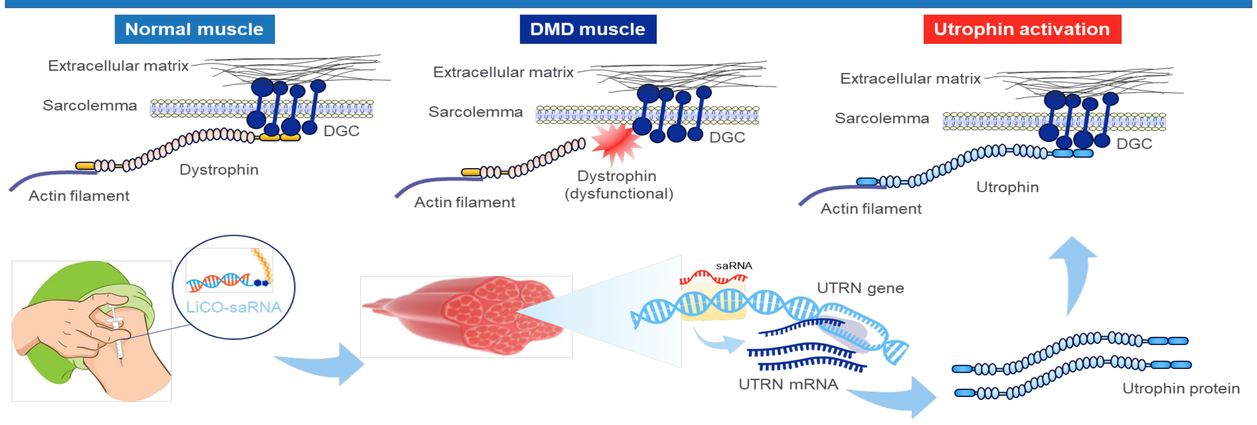


Figure 1. Therapeutic concept-Targeted activation of utrophin by saRNA rescues the phenotype of dystrophin loss.
A small activating RNA (saRNA) designed to target human UTRN promoter to induce the expression of UTRN at the transcriptional level is conjugated to our proprietary LiCO™ delivery system and administrated to patients by a systemic route (e.g., subcutaneous). Increased utrophin protein level would compensate for the loss of dystrophin and ameliorate muscle damage in DMD/BMD patients.

Creation of hUTRNp KI/+ and hUTRNp KI/KI × MDX mice

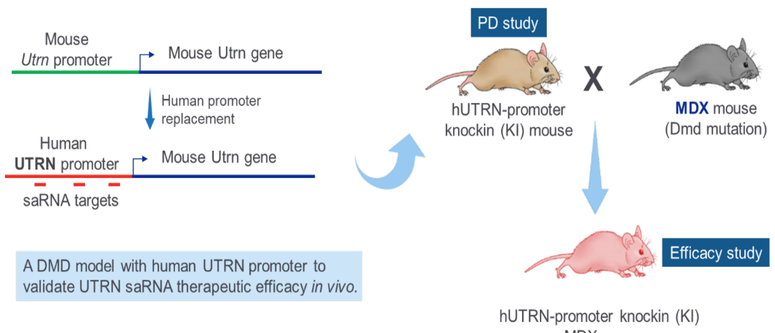
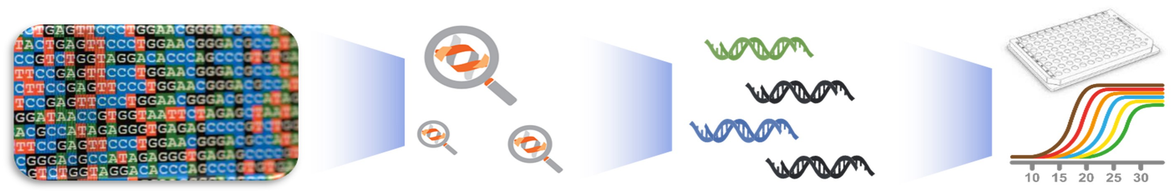


Figure 2. Creation of hUTRNp KI/+ and hUTRNp KI/KI × MDX mice.
- hUTRNp KI/+ mice were created by replacing mouse Utrn promoter (~12K upstream of ATG start codon of mouse Utrn) with human UTRN promoter (~11.5K upstream of ATG start codon of human UTRN) via targeting in C57BL/6J embryonic stem (ES) cells.
- The resulted hUTRNp KI/+ mice were used for pharmacodynamic (PD) study and were further crossed to MDX mice to derive hUTRNp KI/KI × MDX mice for efficacy study.

UTRN saRNA discovery



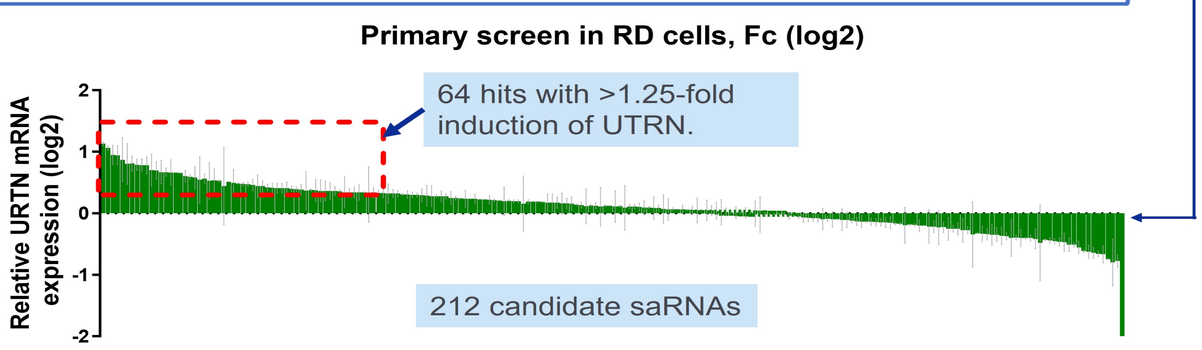


Figure 3. UTRN saRNA design and screen.
- 212 candidate saRNAs were designed on human UTRN promoter using proprietary algorithms, synthesized in house and transfected into RD cells for 72 h at the concentration of 25 nM. UTRN mRNA expression was assessed by RT-qPCR.
- 64 hits were identified with an activity of inducing UTRN mRNA by at least 1.25 fold.

UTRN saRNA lead optimization

Table 1. Chemically and structurally optimized leads and their LiCO™ conjugated version

Lead saRNAs		LiCO™-conjugated saRNA	
Name	Code	Name	Code
saUTRN1	RD-13869	saUTRN1-LiCO	RD-15637
saUTRN2	RD-13870	saUTRN2-LiCO	RD-15638
saUTRN3	RD-14752	saUTRN3-LiCO	RD-15639

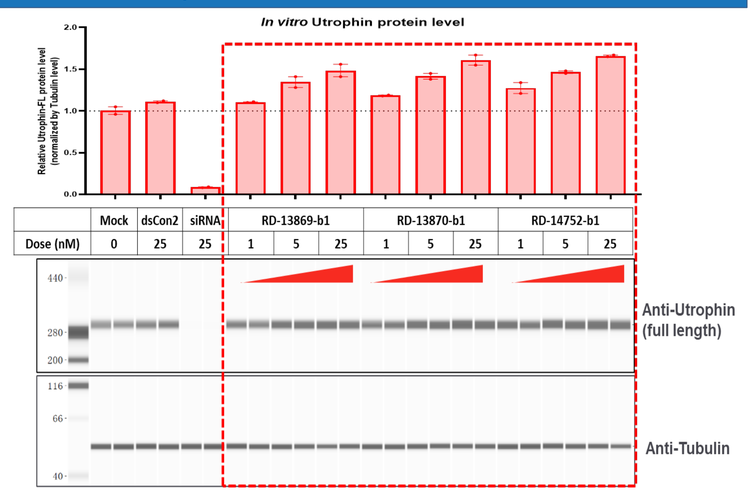


Figure 4. Optimized UTRN saRNA leads dose-dependently induce utrophin protein expression in RD cells.
- Chemically and structurally optimized leads were transfected into RD cells at the indicated concentration for 72 h and utrophin protein expression was assessed by digital western blotting.
- Dose-dependent induction of utrophin was observed in cells transfected by all 3 lead saRNAs.

LiCO™ conjugation of lead saRNAs retained RNAa activity in inducing UTRN expression

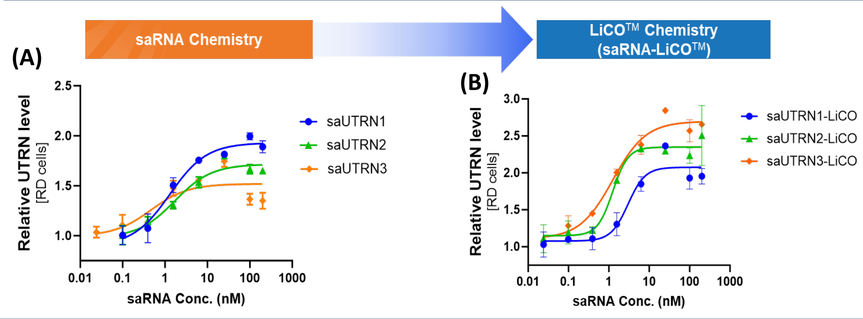


Figure 5. *In vitro* potency of lead saRNAs and LiCO-conjugated leads.
- Dose-dependent activity of 3 leads (A) and LiCO™-conjugated leads (B) in UTRN mRNA induction was assessed in RD cells by transfecting leads for 72 h.
- LiCO™-conjugated leads (B) retained RNAa activity with a Emax of at least a 2-fold induction.

LiCO™ (lipid-conjugated oligonucleotide) delivers duplex RNA into skeletal muscle and heart with potent and durable activity

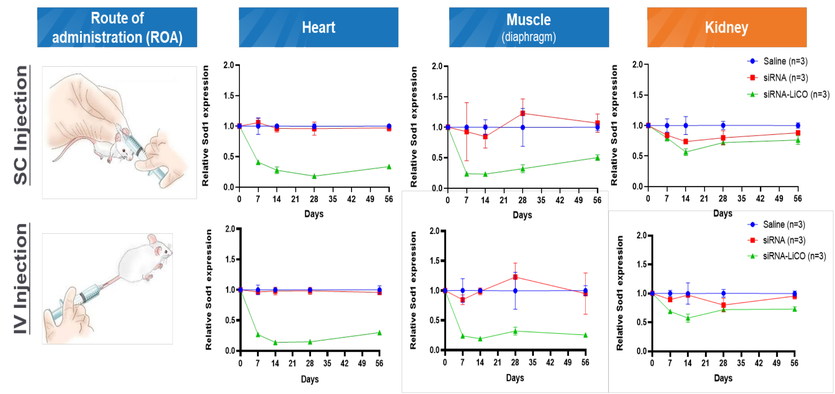


Figure 6. Validation of LiCO delivery system for the muscle and heart.
- A Sod1 siRNA was LiCO™ conjugated and was s.c. or i.v. dosed to mice at 20 mg/kg and animals were sacrificed at day 7, 14, 28 and 56 postdosing. Sod1 mRNA was assessed by RT-qPCR.
- Both s.c. and i.v. administration yielded profound Sod1 knockdown activity in the muscle and heart with a knockdown efficiency over 80% compared to saline or unconjugated Sod1 siRNA.
- "Productive" delivery avoided the kidney.

In vivo induction of utrophin mRNA and protein by lead saRNAs in the skeletal muscle and heart

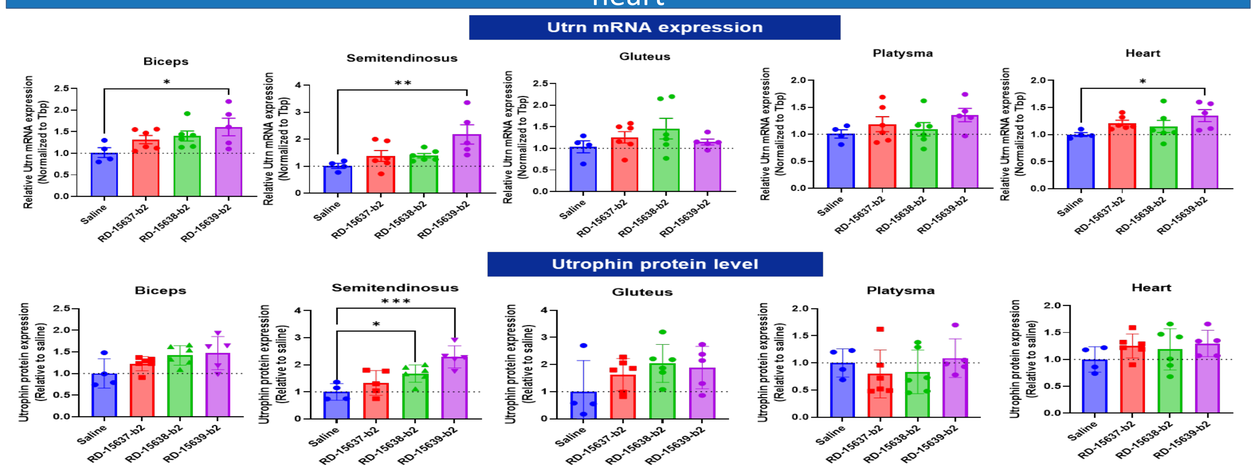


Figure 7. Lead saRNA induced utrophin mRNA and protein expression in hUTRNp KI/+ mice.
- saRNAs were dosed at 50 mg/kg by i.v. injection on day 1 and day 7 into 7 weeks old hUTRNp KI/+ mice which were sacrificed on day 14 after the first dose.
- mRNA and protein expression were assessed by RT-qPCR and digital western blotting.
- Utrophin mRNA induction was obvious especially by saUTRN3-LiCO (RD-15639) in most muscle parts and the heart.
- Utrophin protein was consistently induced in most muscle parts except the platysma.

Treatment of hUTRNp KI/KI × MDX mice with a lead saRNA ameliorated muscle damage in a proof-of-concept efficacy study

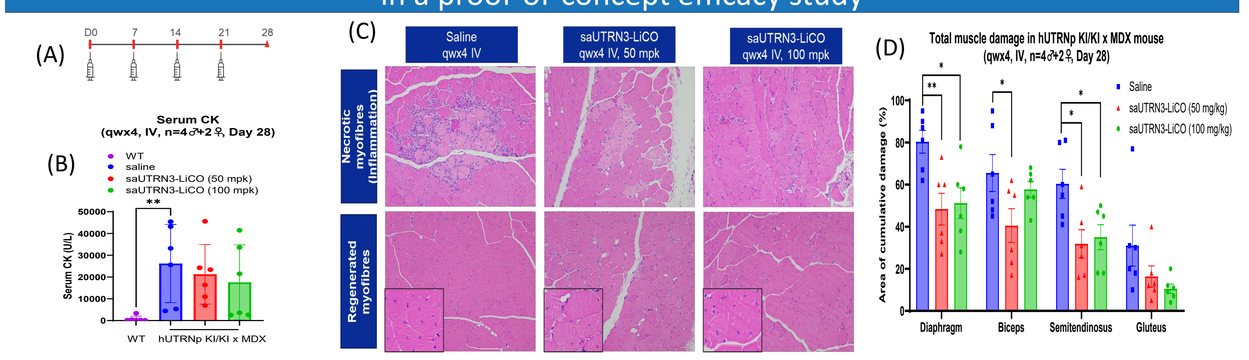


Figure 8. UTRN saRNA treatment ameliorated muscle damage in hUTRNp KI/KI × MDX mice.
- (A) 9 weeks old hUTRNp KI/KI × MDX mice were treated with weekly dose of saUTRN3-LiCO (RD-15639) for 4 weeks and sacrificed on day 28.
- (B) Serum CK level was significantly higher in model mice than wild-type mice which indicates successful DMD model creation. saUTRN3-LiCO treated mice exhibited decreased serum CK compared to saline group, although the difference was not statistically significant.
- (C) Representative histological features of muscle damages including degeneration (necrotic myofibres with inflammatory cells infiltration) and regeneration (regenerated myofibres with central nuclei) were shown in a tissue section of semitendinosus from saline treated mouse, especially. Muscle damage was obviously decreased in mice receiving saUTRN3-LiCO treatment dose-dependently compared with saline treatment.
- (D) Muscle damage was significantly decreased in diaphragm, biceps, and semitendinosus by saUTRN3-LiCO treatment. The muscle damage in gluteus was also reduced but with no statistical significance.
- Data was statistically analyzed using one-way ANOVA via GraphPad Prism, * P<0.05, ** P<0.01.

Pilot acute toxicity study in SD rats

- 4-6 weeks old SD rats (n=3 per group) were treated with single dose of saUTRN3-LiCO at indicated dose levels (i.e., 60, 120, and 180 mg/kg) through intravenous injection and sacrificed on day 14.
- No obvious abnormal changes were noted in body weight, clinical signs, FOB, and clinical pathology.
- Histopathological changes of note were limited to occasional vacuolar degeneration of cardiomyocytes at ≥ 120 mg/kg and occasional centralization of myonuclei and/or interstitial inflammation in the remaining muscle tissues at all doses without clear dose response.
- Maximum tolerated dose was considered to be 180 mg/kg in rats.

Summary & conclusion

- Human utrophin could be induced by promoter targeted saRNAs at both the mRNA and protein levels in cell lines derived from skeletal muscle.
- LiCO™ conjugation of duplex RNAs rendered very durable activity in skeletal muscle and heart.
- Utrophin induction activity of human saRNA leads could be validated in mice bearing human UTRN promoter.
- Treatment of hUTRNp KI/KI × MDX mice with a lead saRNA ameliorated muscle damage.
- In summary, our work provides initial evidence that saRNAs could be delivered to skeletal muscles and heart to activate endogenous utrophin gene. Treatment of MDX mice bearing human UTRN promoter with a lead saRNA ameliorated muscle damage. Our approach could represent a novel and translatable therapeutic strategy for DMD and BMD caused by any mutation of the DMD gene.

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Declaration of interests:

All authors are employed by Ractigen Therapeutics.

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