SRP-4053 Induces Exon Skipping Leading to Sarcolemmal Dystrophin Expression in Duchenne Muscular Dystrophy Patients Amenable to Exon 53 Skipping

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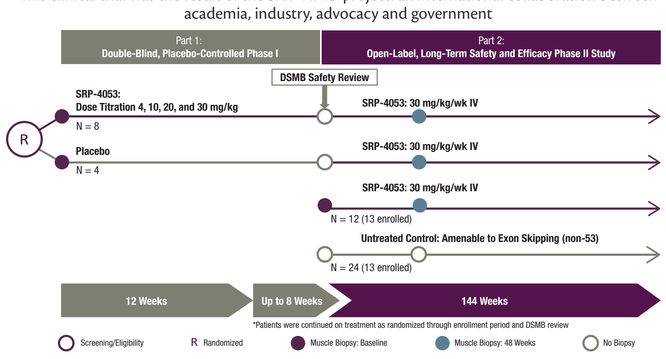
BACKGROUND

Study 4053-101 is a clinical study of SRP-4053 conducted in Europe to assess the safety, tolerability, pharmacokinetics, and efficacy of SRP-4053 in 25 DMD patients with confirmed mutations amenable to correction of the mutation by skipping exon 53.

- SRP-4053 (golodirsen) is a phosphorodiamidate morpholino oligomer (PMO) designed to promote skipping of exon 53. This modification is intended to result in restoration of the reading frame and production of internally deleted dystrophin
- Induction of novel dystrophin expression by SRP-4053 was evaluated in muscle biopsy samples obtained from Study 4053-101
  - Semi-quantitative RT-PCR was used to measure the amount of dystrophin mRNA with exon 53 skipped to assess the expected mechanism of action of SRP-4053
  - Expression of novel dystrophin protein in total muscle protein homogenates was measured using a quantitative Western blot assay
  - A novel automated image analysis method MuscleMap™ assessed the proper localization of the novel dystrophin at the sarcolemma and provided additional fiber morphometric information
- A lack of dystrophin is the direct cause of DMD
  - Historically, accurate measure of novel dystrophin expression in clinical trials has been challenging
  - We describe efforts to generate complementary validated assays that standardize appropriate measures of novel dystrophin expression levels and meet stringent regulatory expectations to support drug development

FIGURE 1. STUDY 4053-101: A COLLABORATIVE STUDY

This clinical trial was the result of the SKIP-NMD project: an international collaboration between academia, industry, advocacy and government



Population
- Age: 6-15 yrs
- 6MWT ≥250m
- NSAA total >17 and/or Rise time <7 sec

Part 2 Primary Endpoints:
- 6MWT at W144
- Western blot at W48

Secondary Endpoints:
- Pulmonary Function Tests
- Dystrophin
  - Intensity
  - PDPF
  - Exon skipping

METHODS

BIOPSY SURGERY (CLINICAL SITES)

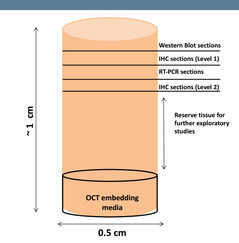
Biopsy muscle specimens were collected from contralateral biceps brachii at Baseline and Week 48 of Study Part 2 ("On-treatment"). Mounted biopsy specimens were frozen in isopentane, and stored in liquid nitrogen at University College London until all samples were allocated for analysis.

TISSUE ALLOCATION AND BLINDING (UNIVERSITY COLLEGE LONDON)

Tissue sections allocated for RT-PCR, Western Blot and MuscleMap analysis were collected and labeled with blinded ID. Laboratory analysts of all dystrophin assays were blinded to patient ID and treatment status throughout analysis.

- For each biopsy surgery, two pieces of muscle were excised, A block and B block, which were analyzed separately
- All biopsies were of high quality and generated reportable data

FIGURE 2. MUSCLE BIOPSY METHODS



Western
4 replicates: A and B, duplicate gels

PCR
8 replicates: A and B, quadruplicate reactions

IHC
4 replicates: A and B, level 1 and 2 slides

METHOD FOR EXON SKIPPING ANALYSIS USING RT-PCR

- A qualified, semi-quantitative end-point RT-PCR method was developed for the quantification of exon 53 skipping (Sarepta Therapeutics)
- RT-PCR performed under GCLP-compliant conditions
  - Three primer pairs were designed to address the deletions (exons 45-52, 48-52, 49-52, 50-52, and 52) with amplicons ranging from 94 to 594 bp
  - Acceptance criteria required presence of an unskipped band with at least three of the unskipped bands in the quadruplicate reactions agreeing within an RSD <50%
  - Percent skipping was calculated as [molarity of skipped band/(sum of the molarity of the skipped + unskipped bands)]*100

WESTERN BLOT METHOD FOR MEASURING TOTAL DYSTROPHIN

A validated quantitative Western blot method was developed and used to accurately assess dystrophin protein (Sarepta Therapeutics)

- Muscle sections were homogenized to lysates in ice-cold buffer containing 4% sodium dodecyl sulfate, 4 M urea, 125 mM tris-hydrochloride (pH 6.8), and complete mini protease inhibitor tablet
- Samples were prepared to contain 40 µg total protein and run on 3-8% 12-well Tris-Acetate gels. Transfer was conducted for 75 minutes and dystrophin was detected with Dys1 primary antibody (Leica) and Sheep anti-Mouse-HRP secondary antibody (GE Healthcare). Membranes were incubated in ECL Prime Western detection kit buffer and exposed to film
- A 5-point calibration curve was generated for each gel using a pooled lysate from 11 non-DMD/BMD normal controls (NC) that showed equivalent dystrophin to previous dystrophin analyses for eteplirsen
- Percent of normal dystrophin was calculated from the log-transformed standard curve linear regression using the equation: 10^((LOG(band intensity)-y intercept)/slope)
- BLOQ= 0.25. ULOQ= 4.0. All data presented using as-measured values

Western blot performed under GCLP-compliant conditions:

- Film exposures were standardized to ensure accurate quantification: The film was exposed for 5, 10, and 15 minutes and dystrophin reported using the exposure that met acceptance criteria as follows:
  - Coefficient of variation for the linear regression of the standard curve >0.90 and the highest slope of the three exposures
  - DMD only control lane reports a lower dystrophin band intensity than the 0.25% standard

METHODS (cont'd)

MUSCLEMAP™ METHOD FOR MEASURING SARCOLEMMAL DYSTROPHIN

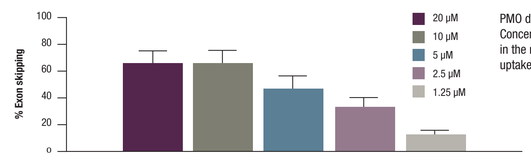
Immunofluorescent Labeling (University of Iowa)
Microscope slides of frozen tissue sections were labeled with anti-dystrophin (1:25, MANDYS106, clone 2C6, obtained directly from Glenn Morris, Wolfson Centre for Inherited Neuromuscular Disease) and anti-merosin (1:400, ab11576, Clone 4H8, Abcam) antibodies.

Whole-slide Image Capture (Flagship Biosciences)
Slides were scanned at 20x in the FITC and TRITC channels on a 3DHISTECH Pannoramic MIDI fluorescent scanner (PANMIDI, PerkinElmer) at a fixed exposure time. The MuscleMap™ algorithm (MM) was used to define muscle fibers based on morphometric and FITC channel labeling with merosin antibody. Based on the FITC signal, a membrane mask was built to allow quantification of dystrophin per TRITC labeling in membrane proximal areas only. Primary data outputs included mean membrane intensity (MI) for each fiber in a biopsy and classification of individual fibers as positive or negative based on a predetermined dystrophin intensity threshold.

Analysis of sarcolemmal dystrophin was performed using validated algorithm and detection methods under Flagship's clinical quality systems

RESULTS

FIGURE 3. SRP-4053 INDUCTION OF EXON 53 SKIPPING IN HUMAN MYOBLAST CELL CULTURES



PMO delivered via nucleofection: Concentrations are of SRP-4053 in the nucleofection cuvette during uptake into cells.

- SRP-4053 PMO was delivered using nucleofection at the indicated concentrations

TABLE 1. HIGH QUALITY OF DMD MUSCLE BIOPSY TISSUE

Both Baseline and On-treatment biopsies were of good quality and had comparable and relatively high percentage of muscle tissue for a DMD patient cohort.

Timepoint	Mean % Muscle	SD
Baseline	46.58	10.93
On-treatment	46.19	9.83
Combined	46.39	10.29

- Images from MuscleMap samples were analyzed for the percent tissue section area that contained muscle fibers
- Normal, non-dystrophic muscle biopsies have muscle fiber area >95%

TABLE 2. SUMMARY OF WESTERN BLOT AND EXON SKIPPING

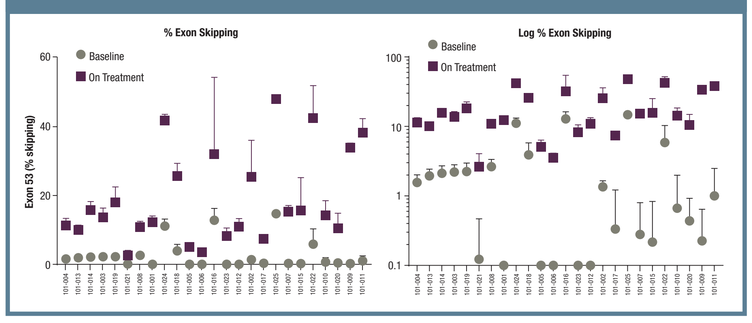
Method	Baseline MEAN % (SD)	On-treatment MEAN % (SD)	Mean change from baseline (SD)	*p*-value	Fold increase
Western blot % dystrophin	0.095 (0.0680)	1.019 (1.0328)	+ 0.924 (1.0129)	< 0.001	10.7
RT-PCR % exon skipping	2.590 (4.0864)	18.953 (13.2245)	+16.363 (10.6223)	< 0.001	7.3

EXON SKIPPING TO ASSESS MECHANISM OF ACTION

All 25 patients displayed an increase in the exon 53 skipped band ($p < 0.001$) over baseline levels, representing a 100% response rate.

- Mean percentage point increase from baseline = +16.363% (SD 10.6223)
  - Mean Baseline = 2.590% (SD 4.0864)
  - Mean On-treatment = 18.953% (SD 13.2245)
- Range of responses observed across individual patient biopsies (Min 2.50%, Max 37.32%)

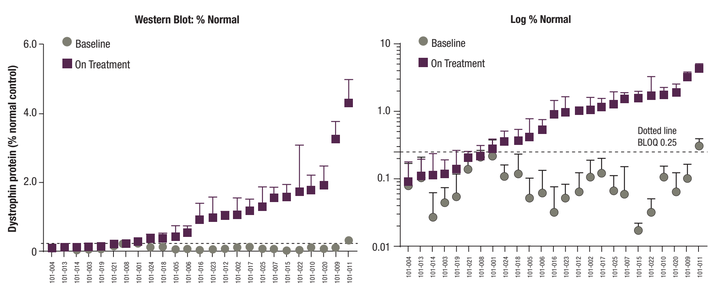
FIGURE 4. EXON SKIPPING BY RT-PCR



WESTERN BLOT: SIGNIFICANT INCREASE IN DYSTROPHIN PROTEIN OVER BASELINE

- A percentage point increase of +0.924% (10.7 fold) in novel dystrophin protein was observed
  - Mean Baseline = 0.095% normal (SD 0.0680)
  - Mean On-treatment = 1.019% normal (SD 1.0328)
  - Range of responses observed across on-treatment individual patient biopsies (Min 0.09%, Max 4.30%)
- Untreated DMD biopsies have very low but detectable levels of dystrophin (only one baseline biopsy>BLOQ)

FIGURE 5. DYSTROPHIN BY WESTERN BLOT

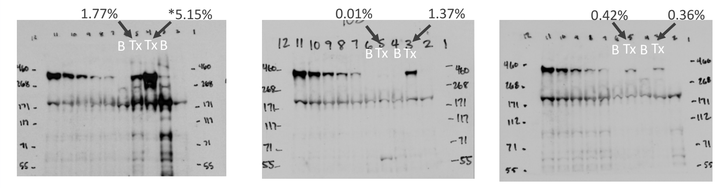


SD in mean is reflective of range in response of individual patient biopsies.

RESULTS (cont'd)

FIGURE 6. HIGHLY REPRODUCIBLE WESTERN BLOTS

- Relative Standard Deviation (RSD) for the raw dystrophin band intensity of the standard curves from all 50 gels: 13-49%
- The standard curve average R^2= 0.98 for all exposures that passed acceptance criteria



B, Baseline sample.
Tx, paired patient On-treatment sample; *above 4% upper limit of quantitation.

FIGURE 7. SIGNIFICANT POSITIVE CORRELATION BETWEEN WESTERN BLOT AND EXON SKIPPING

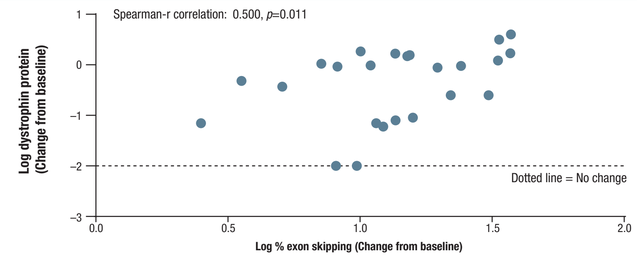
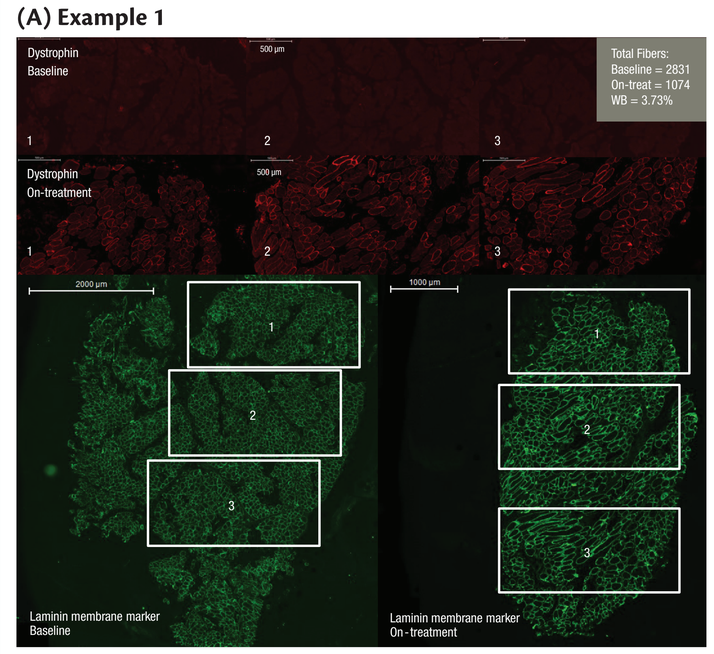


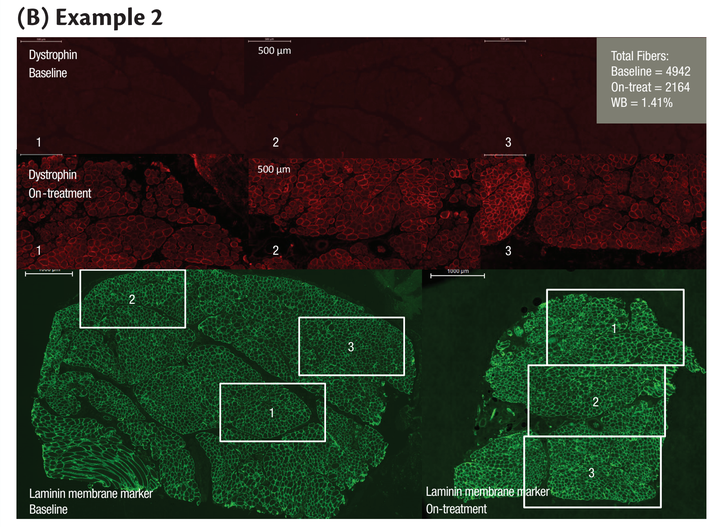
FIGURE 8. MUSCLEMAP: NOVEL DYSTROPHIN CORRECTLY LOCALIZED TO SARCOLEMMA

- Stained biopsies were of consistently high quality and documented an increase in dystrophin staining localized at the sarcolemma
- Quantitation and further analysis of pathology and morphometrics is ongoing
  - MuscleMap identifies each individual fiber in the whole-slide scan image enabling detailed analysis by cTA™ (computational Tissue Analysis)

(A) Example 1



(B) Example 2



CONCLUSIONS

- Analysis of muscle biopsy samples from Study 4053-101 demonstrated an increase in novel dystrophin
  - Statistically significant increase in mean dystrophin to 1.019 percent of normal (+10.7-fold) by Western blot, achieving the primary biological endpoint in the study
  - Increase in skipping of exon 53 observed in all 25 patients; statistically significant mean increase in exon skipping observed
  - Increase in novel dystrophin localized to the sarcolemma, which is required for dystrophin activity
- SRP-4053 (golodirsen) represents the second PMO that has demonstrated an ability to increase dystrophin expression in humans
- Progress in advancing the rigor of methods to measure dystrophin expression aids ongoing efforts to understand the relationship between dystrophin and clinical outcome measures

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