Novel approaches to automated utrophin imaging in cells and tissue for Duchenne muscular dystrophy drug discovery

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Introduction:
Utrophin is an autosomal analogue of dystrophin shown to functionally substitute for the loss of dystrophin in preclinical models of DMD. Ezutromid is a first-in-class small molecule that modulates the expression of utrophin in both DMD patient derived muscle cells and the dystrophin deficient mdx mouse model. Here we report the development and validation of cell and tissue based imaging approaches to help determine ezutromid’s mechanism of action and facilitate the identification of additional utrophin modulators. A high content cell based imaging approach has enabled the accurate measurement of utrophin protein in healthy and DMD patient derived muscle cell cultures. It also revealed a possible mechanism promoting differentiation and/or fusion in myoblasts treated with various utrophin modulators. In the mdx mouse, as with DMD patients, the regenerating skeletal muscle fibres express utrophin, making it difficult to distinguish drug derived utrophin modulation from utrophin expressed naturally during regeneration without employing additional pathology endpoints in parallel. We have developed an automated multiplexed immunohistochemical (IHC) assay with whole section digital tissue image analysis for the robust quantification of utrophin protein expression (intensity and % utrophin positive fibres). This approach distinguishes between utrophin expression due to drug modulation relative to that measured in control animal muscle. Combining the utrophin analysis with additional tissue morphometrics also provided a more informative measure of the effectiveness of utrophin modulating compounds. This is analogous to the muscle biopsy analysis performed by Flagship Bioscience in samples from Summit Therapeutics’ phase 2 clinical trial (PhaseOut DMD) evaluating ezutromid. Combining these preclinical approaches will assist us to better predict translational efficacy of utrophin modulation approaches in vivo and in the mdx mouse efficacy model.

Automated utrophin quantification in mouse skeletal muscle sections:
Skeletal muscle sections from mice with differing utrophin protein expression profiles (wild-type (WT), BL10 mdx (mdx), and mdx Fiona; transgenic mdx mice that constitutively over-express utrophin from birth) were used to develop and validate an automated utrophin quantification technique. Frozen transverse quad muscle sections underwent automated IHC co-staining using antibodies for utrophin and laminin-ß2, and each slide was scanned to generate a digital image for analysis. Definirs developed a bespoke automated imaging algorithm to quantify the utrophin protein expression (intensity and % utrophin positive fibres) and tissue morphometrics (fibre diameter) at the sarcolemma membrane of each individual muscle fibre in an entire transverse section. A subsequent variability assessment study was performed in quad muscle sections from 20 individual mdx mice to compare the utrophin intensity profiles and percentage utrophin positive fibres. The results are reported below.

Utrophin high-content imaging:
Figure 1. Characterization of an immortalized DMD cell line. Myoblasts isolated from the tensor fasciae latae muscle of a 10 year old DMD (del 52) patient and immortalised with hTert and CDK4 were a gift from Dr. Vincent Mouly. Myoblast cultures were stained with an antibody to desmin. After five days of differentiation, myotubes cultures were stained for either utrophin or dystrophin and myosin heavy chain (MHC). Cultures from a healthy donor were used as a positive control for dystrophin staining. Nuclei were stained with DAPI (blue). All images were captured with a Perkin Elmer Operaetta high-content analysis system using a 10x objective.

Figure 2. Measurement of utrophin and fusion index in treated DMD cultures. A) Immortalized DMD myoblasts were treated with utrophin modulating compounds or vehicle (0.1% DMSO) during five days of differentiation then fixed and stained for utrophin, MHC and the nuclei were stained with DAPI (blue). Measurement of the corrected spot intensity of utrophin in the MHC positive or negative regions, fusion index and nuclei count relative to the vehicle control are shown (**p<0.001, ***p<0.0001). B) Dose response curves (n=5/treatment) of utrophin positive in the MHC negative regions, fusion index and nuclei count, relative to the DMSO control, are shown. The EC50 of ezutromid was 93.4 nM for utrophin modulation and 104.4 nM for fusion index. The EC50 for hengelulin was 0.9 nM for utrophin modulation and 0.4 nM for fusion index.

Figure 3. Individual muscle fibre segmentation. Individual muscle fibres were identified by laminin-ß2 staining (yellow), and the Definirs automated imaging algorithm measured the average utrophin staining intensity (blue) at the sarcolemma membrane of each individual muscle fibre.

Figure 4. Utrophin staining intensity profiles. Three distinct utrophin staining intensity profiles were identified in WT, mdx, and mdx Fiona mouse quad muscle sections, reflecting the differing levels of muscle fibre regeneration in WT and mdx mice, and continuous utrophin protein expression in Fiona mice.

Figure 5. Minimum Feret’s diameter plots. The diameter of each individual muscle fibre was determined by the minimum Feret’s diameter. In mdx mice the greatest proportion of fibres are smaller diameter (<10 µm) regenerating fibres, in contrast to WT and mdx Fiona mice which have larger more stable fibres owing to improved stability and lower levels of regeneration.

Figure 6. Percentage utrophin fibre positivity. Three distinct utrophin fibre positivity profiles were demonstrated in WT, mdx, and mdx Fiona mouse quad muscle sections. The results in the mdx mice demonstrate the greatest amount of muscle fibre regeneration, whilst still maintaining a sufficient window in which to measure utrophin modulation.

Summary of key findings:
• Developed and validated cell and tissue-based imaging approaches to identify utrophin modulating compounds and quantify the associated increase in utrophin protein expression.
• Automated utrophin quantification in skeletal muscle sections, using a translatable approach to the clinic, will enable identification of utrophin modulating compounds for further assessment in efficacy studies with a functional endpoint.

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