

# Identification Of Compounds Enhancing Utrophin Expression In Primary Human Skeletal Muscle Cells

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## Introduction

### Charley's Fund

Charley's Fund is a charity (<http://www.charleysfund.com/>) founded by Tracy and Benjamin Seckler who's son is affected by Duchenne Muscular Dystrophy (DMD). The charity is dedicated to funding research to cure or treat children with DMD. Charley's Fund invests primarily in translational research that focuses on moving science from the laboratory into human clinical trials.

### Duchenne Muscular Dystrophy

DMD is the most prevalent, genetically inherited neuromuscular disorder worldwide and affects 1 in 3500 young males. This disorder is caused by mutations or deletions in the gene encoding dystrophin that prevent the synthesis of full-length dystrophin molecules in skeletal muscle fibers. The lack of dystrophin in muscles of DMD patients leads to sarcolemmal instability and disruption of the dystrophin-associated protein complex (DAPC). Together with dystrophin, DAPC forms a link between the extracellular matrix and the intracellular actin cytoskeleton, thereby providing structural integrity to muscle fibers. In DMD patients, the regenerative potential of dystrophic muscle fibers diminishes over time, resulting in progressively severe muscle necrosis and wasting. This loss of muscle mass causes patients to be confined to wheelchairs in their early teenage years and to die by the second or third decade of life as a result of cardiac or respiratory failure.

Although the molecular defect responsible for DMD was identified 20 years ago, there is still no effective treatment available for this devastating disease. Doctors recommend daily stretching and the use of corticosteroids that provides minimal short term benefits.

### Aim

A promising pharmacological treatment for DMD aims to increase levels of utrophin, a fetal homologue of dystrophin, in muscle fibers of affected patients to compensate for the absence of dystrophin. Studies have indicated that expression of utrophin in adult murine muscle cells in mouse models for dystrophy prevents dystrophy pathology. Thus, induction of utrophin in muscle cells may be of therapeutic value in DMD patients. To this aim, BioFocus DPI developed a cell-based assay for Charley's Fund in which the effect of chemical compounds on utrophin up-regulation could be quantified. The assay was subsequently used to screen the NIH Clinical Collection (NCC), a plated array of 446 small molecules that have a history of use in phase I-III of human clinical trials.

## Materials and Methods

### Cells

The Clonetics skeletal muscle myoblast cell system was obtained from Lonza Verviers SPRL (Belgium). It includes normal human muscle myoblasts (HSMM) originating from a 16 years old caucasian male and the appropriate culturing medium. Cryopreserved HSMM were shipped from Lonza at passage 2 and sub-cultured for 2 passages prior to differentiation. Fifty percent confluent myoblasts were switched from culturing medium to differentiation medium (DMEM-F12 supplemented with 2% horse serum) that promoted myoblasts fusion into multinucleated myotubes over 5 days. Differentiation was confirmed by myosin immunostaining and cells morphological assessment using titin immunostaining combined with DAPI nuclear staining.

### Utrophin quantification assay

BioFocus DPI has successfully developed a cell-based assay measuring utrophin upregulation by human primary myotubes in response to 2 different stimuli: a recombinant growth factor or a specific phosphatase inhibitor. Briefly, myotubes were stimulated over myoblast differentiation with a concentration range of the stimulating agent. Cells were lysed and utrophin was subsequently measured in the lysates on the Mesoscale Discovery (MSD) platform. The MSD assay was optimized as a sandwich immunoassay using utrophin present in lysates of the murine skeletal muscle cell line Sol8. In the assay, utrophin was specifically captured by a polyclonal antibody and detected by a murine monoclonal antibody in combination with an anti-mouse labelled with MSD SULFO-TAG<sup>TM</sup> label.

### Screen of the NIH Clinical Collection

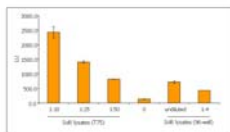
The NIH Clinical Collection (NCC) contains 446 small molecules that have been tested in human clinical trials and have highly developed properties of drug-likeness, such as bioavailability and stability and well-characterized safety profiles. The 446 compounds are arrayed in six 96-well plates and a 50 µl aliquot is supplied for each compound of an approximately 10 nM solution in 100% DMSO.

The NCC screen was performed in the assay described above. Each compound was tested in biological duplicate at two concentrations: 10 and 5 µM in differentiation medium containing a final 0.1% DMSO. A dilution range of the recombinant growth factor (500, 100 and 10 nM) and of the phosphatase inhibitor (7.5, 5 and 2.5 nM) selected as positive controls were included on each assay plate with the appropriate vehicle. Two control plates containing a wider dilution range of the two positive controls and Sol8 lysates and lysates originating from skeletal muscle cells before and after cell differentiation were processed as first plates of the screen.

## Results

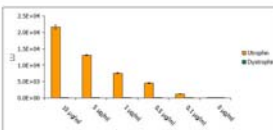
### Utrophin quantification assay

#### Assay readout optimization



The assay was optimized on the MSD platform by selecting a combination of two anti-utrophin antibodies on the basis of their ability to detect utrophin in Sol8 lysate. After optimization of the assay parameters, utrophin was specifically detected in 96-well format as indicated by the dose-response effect.

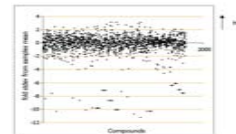
#### Assay readout specificity



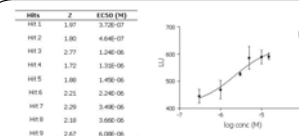
A dilution range of purified utrophin and dystrophin were tested in the assay. Utrophin was specifically detected as shown by the dose-response obtained between 10 and 0.1 µg/ml and the immunoassay did not cross-react with dystrophin.

## Results

### Screen



Data analysis was performed per plate, each readout value (RV) was normalized as follows:  
 $Normalized\ RV = Z = (RV - plate\ average) / plate\ standard\ deviation$   
 The cutoff value ( $Z = 1.5$ ) was set according to controls performance: > 80% of positive controls scoring positive and >90% of negative controls scoring negative. Out of the 446 tested compounds, eighteen hits were identified.



The half maximal effective concentration (EC50) was determined for 18 hits identified during screening. For each compound, a range of 8 concentrations were tested in biological duplicate. EC50 could be reliably determined for 9 compounds with values ranging from 3.72E-07 M to 1.30E-05 M.

## Conclusion

BioFocus DPI has developed a 96-well, cell-based assay in primary human skeletal muscle cells in combination with a novel MSD readout to quantify utrophin upregulation in response to compounds. The full NCC compound collection was screened in the assay leading to the identification of 9 compounds able to induce utrophin up-regulation in primary human skeletal myotubes. The activity of these compounds was confirmed by the determination of their EC50. Two compounds were active in the nM range. These compounds might be considered as potential drugs for the treatment of Duchenne's Muscular Dystrophy where utrophin upregulation would compensate for the loss of dystrophin.

## Acknowledgements

### Financial support:

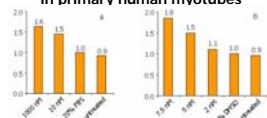
This work is supported by Charley's Fund Inc.



### Contribution of materials:

We would like to thank Dr. James Ervasti for the supply of purified utrophin and purified dystrophin.

### Stimulation of utrophin up-regulation in primary human myotubes



Utrophin up-regulation in response to myotubes stimulation by a growth factor (A) or a specific phosphatase inhibitor (B) was measured using the utrophin quantification assay in 96-well format. Utrophin induction window was calculated as the ratio between triplicate readout values obtained with stimulated and vehicle treated myotubes.