Drug Discovery for Duchenne Muscular Dystrophy via Utrophin Promoter Activation Screening

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Abstract

Background: Duchenne muscular dystrophy (DMD) is a devastating muscle wasting disease caused by mutations in dystrophin, a muscle cytoskeletal protein. Utrophin is a homologue of dystrophin that can functionally compensate for its absence when expressed at increased levels in the myofibre, as shown by studies in dystrophin-deficient mice. Utrophin upregulation is therefore a promising therapeutic approach for DMD. The use of a small, drug-like molecule to achieve utrophin upregulation offers obvious advantages in terms of delivery and bioavailability. Furthermore, much of the time and expense involved in the development of a new drug can be eliminated by screening molecules that are already approved for clinical use.

Methodology/Principal Findings: We developed and validated a cell-based, high-throughput screening assay for utrophin promoter activation, and used it to screen the Prestwick Chemical Library of marketed drugs and natural compounds. Initial screening produced 20 hit molecules, 14 of which exhibited dose-dependent activation of the utrophin promoter and were confirmed as hits. Independent validation demonstrated that one of these compounds, nabumetone, is able to upregulate endogenous utrophin mRNA and protein, in C2C12 muscle cells.

Conclusions/Significance: We have developed a cell-based, high-throughput screening utrophin promoter assay. Using this assay, we identified and validated a utrophin promoter-activating drug, nabumetone, for which pharmacokinetics and safety in humans are already well described, and which represents a lead compound for utrophin upregulation as a therapy for DMD.

Introduction

Duchenne muscular dystrophy (DMD) is a devastating X-linked muscle wasting disease, caused by mutations in the dystrophin gene [1,2]. Dystrophin provides structural integrity to skeletal and cardiac muscle by linking the subsarcolemmal actin cytoskeleton to the extracellular matrix, via the dystrophin associated protein complex (DAPC). In the absence of dystrophin, the entire DAPC is lost from the sarcolema [3]. Muscles are unable to transmit force efficiently and become susceptible to damage during contraction, leading to cycles of degeneration and regeneration. Eventually, regeneration fails and muscle fibres are replaced by fatty and fibrous tissue [2]. Calcium misregulation and chronic inflammation are also thought to contribute to the phenotype [4,5,6]. For patients, DMD leads to progressive muscle weakness, dependence on a wheelchair, respiratory and cardiac complications and a shortened lifespan [7,8]. There is currently no effective treatment available.

Utrophin is an autosomal homologue of dystrophin that can also bind to proteins of the DAPC [9,10,11,12]. Dystrophin and utrophin share 74% similarity at the amino acid level and have very similar domain structures [12,13]. Utrophin is expressed in place of dystrophin in foetal muscle, but in adult myofibres is confined to the neuromuscular and myotendinous junctions. Utrophin is also expressed in other tissues including lung, kidney and liver [9,14]. There are two isoforms of utrophin, A and B, that are transcribed from different promoters [15]. Utrophin A is the predominant isoform in the myofibre [16]. Studies in mdx mice, a
model for DMD, have shown that utrophin, when overexpressed in myofibres by viral vector-mediated delivery or by transgenic means, can compensate for the absence of dystrophin, restoring normal muscle function [17,18]. It is also worth noting that, because utrophin is expressed in foetal muscle and in various non-muscle tissues in the adult [9,10], its overexpression in the muscles of people with DMD is unlikely to provoke an immune response. Utophin upregulation is therefore an attractive therapeutic approach for DMD. Preclinical investigations of utrophin-upregulating treatments, such as heregulin, L-arginine, viral delivery of an artificial transcription factor targeting the utrophin promoter or direct administration of a TAT-tagged ‘microutrophin’ protein have yielded promising improvements in the mdx phenotype [19,20,21,22,23]. However, no utrophin upregulation therapy is yet available for clinical use in DMD patients.

In contrast to protein or virus-based therapeutics, a small-compound drug for utrophin upregulation would avoid potential obstacles in terms of delivery, safety and regulatory body approval. The process of drug discovery, from high-throughput screening through lead optimisation, in vivo studies, clinical trials and eventual approval for patient use, is protracted and expensive, with high failure rates [24,25,26]. An accelerated passage to the clinic and an improved chance of success could be achieved by screening compounds that are already approved for other indications [27,28,29]. Indeed, this approach was successful in identifying β-lactam antibiotics as potential new drugs for amyotrophic lateral sclerosis [30]. With this in mind, we developed a cell-based, high-throughput assay for utrophin A promoter activation, and used it to screen the Prestwick Chemical Library, which comprises 90% approved drugs and 10% natural compounds. Initial screening generated 20 hits out of 1120 compounds (1.8%). Further testing for dose-dependent utrophin promoter activation confirmed 14 molecules as hits, one of which, nabumetone, was shown to upregulate endogenous utrophin A mRNA and protein, in independent validation experiments using the C2C12 muscle cell line. This drug, for which pharmacokinetics, bioavailability and safety in humans are already well described, represents a potential therapeutic candidate for DMD.

Methods

Chemicals

Trichostatin A (TSA) was purchased from Wako; a stock solution of 0.1 mg/ml (331 μM) was prepared by dissolving in methanol. Heregulin-β1 EGF domain was purchased from R&D systems; a 1.25 μM stock solution was prepared by dissolving in sterile PBS supplemented with 0.1% bovine serum albumin. L-arginine was purchased from Sigma; a 100 mg/ml (574 mM) stock solution was made by dissolving in sterile water. Okadaic acid was purchased from Sigma; a stock solution of 20 mM was made by dissolving in DMSO. The Prestwick Chemical Library was purchased from Prestwick Chemical. The 1120 compounds were supplied at 2 μg/ml in DMSO, in 96-well format. For screening, the library was reformatted to 384-well format.

Cell Culture

C2C12 cells (purchased from ATCC) were cultured in high glucose DMEM with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cell culture reagents were purchased from Gibco. For the C2C12utrn stable cell line, 250 mg/ml hygromycin B (Roche) was added to the media. Cells were plated at 1200 cells/well in 384-well plates, 3000 cells/well in 96-well plates or 60 000 cells/well in 6-well plates. For 384-well plates, cells were seeded using a Matrix WellMate (Thermo Scientific).

Luciferase Assays

Luciferase assays were done using the BrightGlo assay (Promega), following the manufacturer’s instructions. For assays done in 96-well format, luminescence was recorded using a Luminoskan Ascent luminometer (Thermo Labsystems). For assays done in 384-well format, luminescence was recorded using an Envision plate reader (Perkin Elmer).

Cell Interference Assay

Quantilum recombinant luciferase (Promega) diluted in C2C12 media was added to 96-well plates with or without C2C12 cells at concentrations of 10^{-5} to 10^{-14} M (50 μl/well). Luciferase activity was assayed as described above. Statistical analysis was done by two-way ANOVA, using GraphPad Prism 4 (GraphPad Software, Inc).

Generation of pGL4:14/utrnAprom Construct

The 2.3 kb human utrophin A promoter fragment was amplified by PCR from CHORI BAC clone PR1-9124 (EMBL accession no. AL024474), using the primers 5’-TCAAAACCTGCAATGTTGCCCTTAATATCTA-3’ and 5’-TAAAGGTCTGGAGACAGACAGACAC-3’. The PCR product was TA-cloned into the pCR2.1-TOPO vector (Invitrogen) and completely sequenced before subcloning into the multiple cloning site of pGL4:14 (Promega) using the restriction enzymes KpnI and EcoRI, to generate the construct pGL4:14/utrnAprom.

Generation of C2C12utrn Stable Cell Line

C2C12 cells were transfected with the pGL4:14/utrnAprom construct using Lipofectamine2000 (Invitrogen), following the manufacturer’s instructions. After 24 hours, media was changed and after 48 hours, cells were trypsinised and re-plated in media supplemented with 250 mg/ml hygromycin B. Resistant colonies were removed using filter paper and cells were re-plated in 96-well plates with sequential dilution so that cell numbers decreased to 0 across multiple wells. From the wells with the lowest starting cell number in which cells survived and multiplied, cells were harvested and subjected to a second round of plating with sequential dilution. The wells with the lowest starting cell number were again selected and clones were expanded into 24-well plates.

Validation of C2C12utrn Stable Cell Line

C2C12utrn clones were first validated using the BrightGlo luciferase assay (Promega). From the four clones with the highest luciferase activity, genomic DNA was isolated using the Archive-Pure DNA Cell/Tissue Kit (5Prime, Inc). For PCR validation, the primers 5’-ACTCTGGAGCGCGCAGCAGCAGAC-3’ and 5’-CGCCCTCTGAGGCGTCGCGGCTC-3’, which bind specifically to the utrophin A promoter, were used for amplification from 300 ng of genomic DNA. The validated clone with the highest luciferase activity was used for all subsequent experiments.

DMSO Toxicity and Positive Control Evaluation

For evaluation of sensitivity to DMSO, C2C12utrn cells were seeded in 96-well plates and treated with DMSO (Sigma) at final concentrations of 0–0.5%. Luciferase activity was assayed after 48 hours, as described above.

For evaluation of potential positive controls, C2C12utrn cells were seeded in 96-well plates 24 hours prior to compound treatment. Cells were exposed to L-arginine (2 mM), TSA.
(50 nM), okadaic acid (50 nM) or heregulin (2 nM) for 24 or 48 hours before assaying luciferase activity. Differences between treatments were tested by one-way ANOVA using Statview software (SAS Institute, Inc). Statistical robustness was assessed by Z-factor, defined as $1 - \frac{(3 \times SD_{control} + SD_{treated})}{\text{mean treated} + \text{mean control}}$ and percentage covariance (% CV).

For generation of the TSA dose-response curve, C2C12utrn cells were plated in 384-well plates 24 hours prior to treatment with TSA. Five dilutions of TSA from 0.3 to 5000 μM were prepared in 100% DMSO. TSA was added to cells at final concentrations ranging from 0.3 to 500 nM using an Evolution P3 robot liquid handling system (Perkin Elmer). The final DMSO concentration was 0.1%. Luciferase activity was assayed after 24 hours. Sigmoidal dose-response curve-fitting and EC50 calculation was done by non-linear regression using GraphPad Prism 4 (GraphPad Software, Inc).

**Initial Screen**

C2C12utrn cells were plated in 384-well plates 24 hours prior to treatment with compounds. Compounds were added at a final concentration of 2 μg/ml (approximately 5 μM based on an average molecular weight of 400) using the Evolution P3 robot liquid handling system (Perkin Elmer). The final DMSO concentration was 0.1%. Negative controls were treated with 0.1% DMSO only. Quality control (QC) plates were treated with 5 nM TSA. After 24 hours compound exposure, luciferase activity was assayed as described above. QC plates were run at the beginning and end of the assay. An algorithm for cross-talk correction was applied. Data analysis was done using ActivityBase software (IDBS). The threshold for hits was set at 20% upregulation (approximately 3 times the % CV of the negative controls).

**Hit Confirmation and Dose Optimisation**

For hit confirmation (dose-response I), C2C12utrn cells were treated with each of the hits from the initial screen at 15 concentrations from 0.5 ng/ml to 8 μg/ml (approximately 1.6 nM to 25 μM based on an average molecular weight of these 20 compounds of 316), to generate dose-response curves. The final DMSO concentration remained constant at 0.4%. Otherwise, the protocol was as described for the initial screen. For dose optimisation (dose-response II), compounds were obtained in greater quantity and dissolved in DMSO at 0.1%. The final DMSO concentration was 0.1%. The protocol was otherwise as for the initial screen.

**Validation by Quantitative Real-Time PCR**

C2C12 cells were plated in 6-well plates 24 hours prior to treatment with nabumetone (25 μM). The final DMSO concentration was 0.05%. Control cells were treated with 0.05% DMSO only. After 24 hours compound treatment, cells were lysed and RNA isolated using an RNeasy kit (Qiagen), following the manufacturer’s protocol. RNA was reverse-transcribed using a Superscript II First-Strand Synthesis kit (Invitrogen). A custom TaqMan quantitative real-time PCR (qRT-PCR) assay for utrophin A [31] was performed using 500 nM each of primers 5'-ACGAATCTAGTGATCATGATGAGTC-3' and 5'-ATCTTTTTTGAGGGTCTTCG-3' and 250 nM of FAM-labelled MGB probe with sequence ATCTTTTTTGAGGGTCTTCG-3' and 250 nM of TAMRA-labeled MGB probe. The reaction mixture contained 2 μL of a reaction volume of 25 μL. As an endogenous control, 18S rRNA was amplified using pre-mixed reagents from Applied Biosystems (Eukaryotic 18S rRNA Endogenous Control (VIC/MGB probe, primer limited)), with 0.8 ng cDNA in a reaction volume of 50 μl. Other reaction components were provided by Applied Biosystems TaqMan Universal Mastermix. TaqMan qRT-PCR reactions were carried out in 96-well plates using a 7300 Real-Time PCR System (Applied Biosystems) and default thermocycler program. Analysis was done using the ΔΔCt method, having previously validated the equal efficiencies of the two primer sets. Statistical analysis of multiple independent experiments was done by one-way ANOVA using Statview software (SAS Institute, Inc).

**Validation by Western Blotting**

Cells were plated in 60 mm dishes that confluence was approximately 25% the following day, at which point they were treated with 25 μM nabumetone or vehicle (DMSO) only (0.1%) for 4 days. After 2 days, cells were passaged and re-seeded in fresh media with nabumetone or DMSO. After 4 days, cells were trypsinised and resuspended in 300 μl TNEC lysis buffer (1.5 mM Tris-HCl pH 8, 2.15 mM NaCl, 3.1% Igepal CA630, 4.2 mM EDTA with Complete protease inhibitors (Roche)). Lysates were incubated on ice for 20 minutes, centrifuged at maximum speed for 10 minutes in a benchtop centrifuge at 4 °C and supernatants removed and stored at −20 °C. The DC protein assay (Bio-Rad) was used to determine total protein concentration. For Western blotting, lysates containing 30 μg protein were combined with LDS sample buffer and NuPAGE reducing agent and heated to 99°C for 5 minutes, then separated on 3–8% Tris-Acetate gels (Invitrogen) with 4% running buffer for 2 hours 15 minutes at 80 V. Proteins were transferred to PVDF membranes for 2 hours at 80 V in ice-cooled transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 20% methanol, 0.05% sodium dodecyl sulphate). Membranes were blocked overnight at 4°C in 5% non-fat milk in TBS (50 mM Tris pH 7.5, 150 mM NaCl), then probed for utrophin with mouse monoclonal anti-utrophin antibody mancho 3 clone 8A4 (developed by Glenn E. Morris and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa Department of Biology) diluted 1:20 in 5% non-fat milk in TBST (TBS with 0.05% Tween 20), for 1 hour at room temperature. Blots were washed in 3 changes of TBST for 10 minutes each, then incubated with HRP-conjugated goat-anti-mouse IgG (Jackson ImmunoResearch), diluted 1:4000 in 5% non-fat milk in TBS, for 1 hour. TBST washes were repeated, then bands were visualised using SuperSignal West Chemiluminescent Substrate (Thermo Scientific) and images obtained using an LAS-3000 Imager (Fujifilm). Band densities were quantified using ImageJ (http://rsweb.nih.gov/ij/index.html). Statistical analysis of multiple independent experiments was done by Student’s T test using GraphPad Prism 5 (GraphPad Software, Inc).

**Results**

**Generation and Validation of C2C12utrn Stable Cell Line**

The C2C12 mouse muscle cell line was selected for generation of a stable cell line containing the human utrophin A promoter linked to a luciferase reporter. Before making the cell line, luciferase assays were performed with a known range of concentrations of recombinant luciferase, in the presence or absence of C2C12 cells. The presence of C2C12 cells had no significant effect on the measured luciferase activity (Fig. S1A). C2C12 cells were then transfected with a construct containing a 2.3 kb utrophin A promoter region linked to a luciferase reporter gene. Hygromycin (250 mg/ml) was used to select stably transfected cells, and resistant colonies were subjected to 2 rounds of sub-cloning to obtain homogenous lines. Clones were validated.
using both a luciferase assay and PCR with utrophin A promoter-specific primers and genomic DNA as template (data not shown). The clone with the greatest luciferase activity (named C2C12utrn) was selected for development of a cell-based utrophin promoter activation assay.

Utrophin Promoter Activation Assay Development

C2C12utrn cell number was optimised to 3000 cells per well, which resulted in 70% confluence at the time of luciferase quantification. DMSO is typically used as a solvent when screening chemical libraries. To determine the tolerance of the C2C12utrn cell line to DMSO, cells were treated with a range of DMSO concentrations from 0–0.5% and their luciferase activity was assayed. Concentrations of DMSO up to 0.2% had little effect on measured luciferase activity. Above 0.2% DMSO, luciferase activity declined but at 0.5% DMSO the activity was still 75% that of cells without DMSO (Fig. S1B).

To find a positive control to assist in assay optimisation and to examine the effects of different compound exposure times, C2C12utrn cells were treated with four compounds previously demonstrated to upregulate utrophin: L-arginine (2 mM) [32], okadaic acid (50 nM) [33], heregulin (2 nM) [34] and trichostatin A (TSA; 50 nM) (Bogdanovich et al., manuscript in preparation), for 24 or 48 hours, before assaying for luciferase activity. Incubation with TSA for 24 hours gave the greatest upregulation of luciferase activity. For the other positive controls, the treatment time did not affect the degree of promoter activation (Fig. 1A). To determine the statistical robustness of the observed upregulation, Z-factors were calculated for each positive control. Only TSA, with 24 hours incubation, had a Z-factor indicative of suitability for high-throughput screening (0.6; acceptable range 0.5–1) [35]. Additionally, the percentage covariance (% CV) was under 10% and lower than for most other treatments (Table 1). Based on these results, a compound exposure time of 24 hours was chosen for the assay and TSA was selected as a positive control for further assay development.

To further characterise the effect of TSA on C2C12utrn luciferase activity and to confirm that the assay would translate to
high-throughput format, a dose-response curve was constructed for TSA in 384-well format using concentrations from 0.5 to 5000 nM. Non-linear regression was used to fit a sigmoidal dose-response curve. From this, the EC50 was calculated to be 1.7 nM. Peak luciferase activity occurred at 20 nM, above which the activity declined, presumably due to cellular toxicity (Fig. 1B). Cells are typically more susceptible to toxic effects in smaller sized plate wells (unpublished observations). Z-factors and % CVs calculated for TSA in 384-well format were similar to those obtained in 96-well format (Table 2), confirming that the assay would perform robustly in high-throughput screening.

Primary Screen of Prestwick Chemical Library

The utrophin promoter activation assay was used to screen the Prestwick Chemical Library of approved drugs and natural compounds. The compounds were screened at 2 μg/ml (approximately 5 μM based on an average molecular weight of 400). The control % CVs for the four plates were between 5.3 and 6.8%. The threshold for hits was set at 20% upregulation (approximately 3 times the % CV of the controls, estimated to be low enough to capture all true positive hits). Out of 1120 compounds, 20 hits were obtained (1.8% of the library) with upregulation up to 80% (1.8-fold; Fig. 2 and Table 3).

To confirm these hits, high-throughput dose-response curves were generated, using 15 concentrations from 0.5 ng/ml to 8 μg/ml (approximately 1.6 nM to 25 μM based on an average molecular weight for these 20 compounds of 316). Of the 20 initial compounds, 14 showed dose-dependent activation of the utrophin A promoter, confirming them as hits (dose-response I; Fig. 3 and Table 3). These included 7 approved drugs and 7 natural compounds. Maximum fold-changes in utrophin A promoter activity obtained during dose-response testing ranged from 1.2 to 1.9 (Table 3). Dose-response curves were also generated using a lower throughput, 96-well format (not shown). Dose-dependent activity was confirmed, and fold-changes in promoter activity between 1.9 and 3.5 were achieved.

For confirmed hits, stock solutions of higher concentration (100 or 200 nM) were obtained, and dose-response testing repeated using concentrations up to 200 μM (dose-response II; Fig. 4 and Table 3). Based on this, nabumetone, an FDA-approved drug that showed high fold-changes and a lack of cellular toxicity (indicated by a drop in luciferase activity at higher concentrations, e.g. piperine, Fig. 4) was selected for independent validation.

| Table 1. Determination of Z-factor and % CV for positive controls. |
|---------------------------------------------------------------|
| **Positive control** | 24 h treatment | 48 h treatment | 24 h treatment | 48 h treatment |
|--------------------|----------------|----------------|----------------|----------------|
|                    | Z-factor | % CV | Z-factor | % CV |
| L-arginine         | 1       | 10   | 0.3      | 7.6  |
| Trichostatin A     | 0.6     | 3.6  | 8        | 7.8  |
| Okadaic acid       | 0.1     | 7.5  | 0.06     | 4.1  |
| Heregulin          | 0.3     | 7.5  | 0.08     | 3.5  |

C2C12utm cells cultured in 96-well plates were exposed to L-arginine (2 mM), TSA (50 nM), okadaic acid (50 nM) or heregulin (2 nM) for 24 or 48 hours before assaying luciferase activity. Statistical robustness was assessed by calculating the Z-factor and percentage covariance (% CV). Z-factors between 0.5 and 1 predict good performance in high-throughput screening.

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Independent Validation

To confirm the effect of nabumetone on endogenous utrophin promoter activity, normal C2C12 cells were treated with nabumetone at its optimum concentration based on dose-response testing, and utrophin A mRNA levels measured using a TaqMan qRT-PCR assay. Treatment with nabumetone resulted in a statistically significant, 1.8-fold increase in endogenous utrophin A mRNA, compared to DMSO only controls (Fig. 5A).

As further validation, C2C12 cells were treated with nabumetone for 4 days, and utrophin protein levels were measured by Western blotting. As shown in Fig. 5B-C, nabumetone treatment resulted in a 1.2-fold increase in utrophin protein, confirming that the observed upregulation of utrophin mRNA led to an increase in protein levels.

Discussion

In this study, we present a novel utrophin promoter activation assay, which we have used to screen a library of approved drugs and natural compounds. After initial screening, hit confirmation and independent validation, we have identified a lead compound, nabumetone, that is a potential therapeutic candidate for DMD.

The utrophin promoter activation assay performed well in tests of robustness, with a Z-factor of 0.6 and % CV under 10%. The number of hits as a percentage of the library (1.8%) was comparable to other published screens [36,37,38], suggesting that the assay was specific with a low number of false positives. Nevertheless, we had set the initial screen threshold of 20% upregulation low enough to avoid false negatives, in the expectation that some of the initial hits would be false positives due to ‘statistical noise’. Consistent with this, only 14 out of 20 initial hits (70%) were confirmed upon dose-response testing.

In screening assays that use luciferase as a reporter, false positives can arise from compounds that act as luciferase inhibitors. These compounds bind to and stabilise luciferase in cells, increasing its levels, and are then competed off by the substrate in the luciferase assay reagent, such that an artifactually high luciferase activity is produced [39]. Thus, it is important to independently confirm the effects of the hit compounds on the endogenous utrophin A promoter, mRNA and protein. We did this for one candidate, nabumetone, using a TaqMan qRT-PCR assay for utrophin A mRNA and Western blotting for utrophin protein. Validation of the remaining compounds is ongoing. However, definitive in vitro validation experiments are challenging, in part due to the differences in utrophin protein expression compared to the in vivo situation, where utrophin is enriched at specific locations, such as neuromuscular junctions [40,41,42], that do not exist in cultured cells. To move our findings closer to the
clinic, it will be essential to determine the efficacy of the compounds in vivo, using animal models of DMD. Previous studies suggest that an increase of approximately 2-fold in utrophin protein in muscle is sufficient for correction of the dystrophic phenotype in mice [18]. In our study, we identified several compounds that could upregulate the utrophin A promoter up to 3.5-fold. Independent validation of nabumetone showed that it could increase endogenous utrophin A mRNA levels approximately 2-fold, and increase utrophin protein by 1.2-fold. This is extremely promising given that even a very small upregulation of utrophin appears to have a beneficial effect in dystrophin deficient mice [18].

A variety of potential therapies for DMD are being investigated, and some have reached clinical trials (http://www.clinicaltrials.gov/). While this is greatly encouraging, there are still many obstacles to be overcome before all patients with DMD can be treated successfully. In many cases, problems of delivery, safety and large-scale, cost-effective manufacture have not yet been resolved. Some approaches, such as antisense oligonucleotide-mediated exon-skipping and nonsense codon suppression, are only applicable to subsets of patients with particular types of dystrophin mutations [43,44]. Regulatory body approval may also be more complicated for new kinds of drug molecules such as proteins and oligonucleotides. For example, under current regulation, each of the potentially hundreds [43] of mutation-specific exon-skipping oligonucleotides would be treated as separate drugs [45]. It is also important to consider that, initially at least, combinations of treatments may be needed in order to achieve therapeutic efficacy. Therefore, the continuation of research along multiple therapeutic avenues, including utrophin upregulation, is of great importance to ensure the development of effective therapies for all patients with DMD.

There are a number of advantages to small molecule-mediated utrophin upregulation that make it both a strong candidate for DMD therapy and a complimentary approach to those discussed above. The introduction of dystrophin protein into the body of a DMD patient where it has never been expressed could provoke an immune reaction against the protein, which might be recognised...
Dose-response curves were generated in 384-well format for the 20 compounds identified in the initial screen, using concentrations from 0.5 ng/ml to 8 μg/ml. Fourteen molecules (nabumetone, chrysin, piperine, apigenin, riluzole HCl, phenazopyridine HCl, resveratrol, tiabendazole, hesperetin, leflunomide, kawain, kaempferol, clorgyline HCl and equilin) showed dose-dependent activity and were confirmed as hits. HCl, hydrochloride. RLU, relative luminescence units.

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Figure 4. Dose optimisation: dose-response II. Dose-response curves were generated in 384-well format for the 14 confirmed hits using concentrations from 3.1 nM to 200 μM HCl, hydrochloride. RLU, relative luminescence units. doi:10.1371/journal.pone.0026169.g004
As ‘non-self’. Utrophin is expressed in muscle in the foetus and at high levels in other tissues such as liver, lung and kidney throughout life in DMD patients (as well as healthy people) [9,10], so increasing its production therapeutically in muscle would not risk inciting an immune response. Additionally, the utilisation of the endogenous utrophin gene provides an elegant solution to problems arising from the large size of the dystrophin coding sequence (14 kilobases) [46], which makes it difficult to incorporate into viral vectors, except in truncated form. Finally, the use of a traditional ‘drug-like’ small molecule, with favourable absorption, distribution, metabolism and excretion properties [47,48], to upregulate utrophin offers obvious advantages in terms of delivery, stability and bioavailability.

Drug repositioning, the exploitation of existing drugs for new applications, is becoming an increasingly important part of research and development for the pharmaceutical industry [27,28,29]. Our approach of screening a library of regulatory body-approved drugs and natural compounds offers a distinct advantage in terms of the speed and efficiency of future therapy development. All the hits identified in our screen are compounds that have been shown to be safe in humans, and for which pharmacokinetic data is available. This eliminates a significant proportion of the time and expense required when developing a novel compound as a drug, and gives the potential for a rapid progression from the lab to the clinic.

To date, we have validated one drug, nabumetone, at the mRNA and protein level, in C2C12 cells. Nabumetone is a COX-1/COX-2 inhibitor that shows a preference for COX-2 inhibition in vitro [49]. It is used for the management of pain and inflammation in osteoarthritis and rheumatoid arthritis [50]. Nabumetone is generally well-tolerated by patients [50], and its anti-inflammatory activity might be beneficial in DMD, since inflammation is a component of the disease [6,51,52]. There is some evidence that the use of selective COX-2 inhibitors may increase the risk of adverse cardiovascular events, especially in patients who already have an increased risk [53,54,55]. This is less of a concern with COX-1/COX-2 inhibitors, possibly because COX-1 inhibition has an antiplatelet effect, which may protect against thrombotic events [56]. Nonetheless, because of the involvement of the heart in DMD pathology, the safety of nabumetone use in this group would need to be carefully evaluated.

In developing our assay, we used as positive controls a number of substances already known to upregulate utrophin: heregulin, TSA, okadaic acid and L-arginine. Of these, only L-arginine has been used in human beings, as a supplement. There are some safety concerns about its use, particularly at high dosages [57,58]. Its use in DMD patients has not been investigated.

In terms of doses, it is not possible to directly compare in vitro and in vivo doses without considering pharmacokinetics; however, using a crude calculation based on an average total human body fluid volume of 42 l, the optimum dose for nabumetone determined in cell culture (25 μM) lies far below that used in human beings (equivalent to approximately 100–200 μM). As a comparison, L-arginine was effective in activating our utrophin upregulation assay at 2 mM, whereas doses used in humans would correspond roughly to 0.2–5 μM.

In our experiments, we observed a smaller increase in utrophin at the protein level than at the mRNA level. Although these experiments were done at different time points to allow for the expected slow synthesis of the large utrophin protein (approximately 400 kDa), this difference may also reflect the regulation of utrophin at the translational level. Indeed, it is known that utrophin expression is influenced by post-transcriptional mecha-
nisms, acting via the 5’- and 3’-untranslated regions (UTRs) of the utrophin mRNA [59,60,61,62]. It may be that by combining drugs that activate the utrophin promoter with therapies targeting points of post-transcriptional expression control, or therapeutic substances such as bighycan that promote localisation and stabilisation of utrophin in the sarcoplemma [63], a far greater upregulation of utrophin can be achieved.

In conclusion, we have taken a novel approach to the problem of DMD therapy by screening existing drugs for utrophin promoter activation. Following the successful screening project and independent validation presented here, the lead compound nabumetone will be tested in preclinical trials for its ability to improve and improve the phenotype of dystrophic mdx mice. This venture offers great promise for the rapid development of an effective drug therapy for DMD. However, we caution that although nabumetone is an FDA-approved drug that is used safely in human beings, it will still be important to conduct thorough preclinical studies in animals, as well as clinical trials, to determine the safety and efficacy of its long-term use in DMD.

**Supporting Information**

**Figure S1 Development of utrophin promoter activation assay. A.** A standard curve with increasing concentrations of recombinant luciferase was generated in the presence or absence of normal C2C12 muscle cells. The presence of C2C12 cells had no effect on luciferase activity, as tested by two-way ANOVA. Error bars represent standard deviation. **B.** C2C12-transfected mdx 1441–1444.

**Author Contributions**

Conceived and designed the experiments: CM OL NS ADN SLD TSK. Performed the experiments: CM OL NS. Analyzed the data: CM OL NS. ADN. Contributed reagents/materials/analysis tools: ADN SLD TSK. Wrote the paper: CM.

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