ARTICLE

Anisomycin Activates Utrophin Upregulation Through a p38 Signaling Pathway

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Duchenne muscular dystrophy is a recessive X-linked disease characterized by progressive muscle wasting; cardiac or respiratory failure causes death in most patients by the third decade. The disease is caused by mutations in the dystrophin gene that lead to a loss of functional dystrophin protein. Although there are currently few treatments for Duchenne muscular dystrophy, previous reports have shown that upregulating the dystrophin paralog utrophin in Duchenne muscular dystrophy mouse models is a promising therapeutic strategy. We conducted *in silico* mining of the Connectivity Map database for utrophin-inducing agents, identifying the p38-activating antibiotic anisomycin. Treatments of C2C12, undifferentiated murine myoblasts, and *mdx* primary myoblasts with anisomycin conferred increases in utrophin protein levels through p38 pathway activation. Anisomycin also induced utrophin protein levels in the diaphragm of *mdx* mice. Our study shows that repositioning small molecules such as anisomycin may prove to have Duchenne muscular dystrophy clinical utility.

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STUDY HIGHLIGHTS

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
✔ Duchenne muscular dystrophy is caused by mutations in the Duchenne muscular dystrophy gene that lead to a loss of functional dystrophin protein in skeletal and cardiac muscle. This results in progressive muscle wasting and loss of ambulation. Currently, there are no effective treatments for Duchenne muscular dystrophy.

WHAT QUESTION DID THIS STUDY ADDRESS?
✔ As previous reports have shown that upregulating the dystrophin paralog utrophin in DMD mouse models may serve as a therapeutic strategy, we conducted *in silico* mining of the Connectivity Map database searching for utrophin-inducing agents, verifying one of our hits.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
✔ We identified the p38-activating antibiotic anisomycin as a candidate compound. Anisomycin treatments of C2C12, undifferentiated murine myoblasts, and *mdx* primary myoblasts conferred significant increases in utrophin protein levels through p38 pathway activation. We also observed that anisomycin induced utrophin protein levels in *mdx* mice in the diaphragm.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
✔ Our study shows that repositioning small molecules such as anisomycin may prove to have Duchenne muscular dystrophy clinical utility.

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder with a prevalence of 1 in 5,000 to 1 in 6,500 boys in the United States.[1] DMD is caused by mutations in the dystrophin gene (*DMD*) and the resulting reduction of dystrophin protein.[2,3] Dystrophin is a 427 kDa, multidomain protein primarily found in skeletal and cardiac muscle. In muscle, dystrophin is localized to the sarcolemma and forms an essential part of the dystrophin-associated glycoprotein complex (DGC).[4] The function of this protein complex is to connect the extracellular matrix to the intracellular actin cytoskeleton, thereby maintaining sarcolemma stability.[5] Thus, loss of dystrophin in skeletal muscle results in instability of the sarcolemma. Membrane fragility causes the loss of calcium maintenance across the membrane and increased intracellular calcium. This initiates an array of events that lead to contraction-mediated injury, widespread muscle necrosis, and degeneration.[6]

DMD is characterized by progressive muscle wasting, proximal muscle weakness, and significantly elevated serum creatine kinase levels.[7] Increasing lower limb muscle weakness leads to the patient needing a wheelchair for mobility, on average by age 12.[8] The leading cause of DMD mortality is progressive respiratory failure, which usually develops in the second decade.[9] The respiratory muscle most severely affected in DMD is the diaphragm. This may devolve from the body's dependency on the diaphragm to drive ventilation while at rest.[10] Animal models of DMD, such as the *mdx* mouse, show more severe damage in the diaphragm

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compared with other skeletal muscles. Although the mouse phenotype is milder than human DMD, there is a reduction in lifespan, thought to be due to respiratory or cardiac failure.[11] In fact, the diaphragm is abnormal in both structure and function, making it a natural tissue to assess when testing DMD therapies.[12,13]

Despite our growing understanding of the genetic and molecular pathogenesis of the condition, no effective treatment exists for DMD. Pharmacologic upregulation of the structural and functional dystrophin paralog, utrophin, represents one potential therapeutic avenue. Utrophin is a ubiquitously expressed 400 kDa protein that primarily localizes to the neuromuscular junction in normal skeletal muscle. Utrophin upregulation is a promising DMD treatment strategy because, in addition to its functional similarity to dystrophin, when overexpressed a widespread "dystrophin-like" sarcolemmal distribution in muscle is observed in DMD.[14–16] Several studies have established utrophin as a developmental precursor to dystrophin and have shown that increased utrophin partly compensates for low dystrophin at the sarcolemma in DMD.[17,18]

The identification of agents that induce utrophin would clearly be of potential therapeutic value. The advent of massively parallel transcriptional profiling has enabled the creation of system-wide databases of human gene expression changes elicited by compounds including US Food and Drug Administration (FDA)-approved drugs allowing *in silico* drug screens. One such database is the Broad Institute's Connectivity Map (Cmap; https://www.broadinstitute.org/cmap/) which conducted Affymetrix array-based analysis on different cell lines employing 1,600 compounds and is currently the most comprehensive database of clinic-tested drug-induced transcriptional responses in live cells.[19] Using Cmap, we identified a list of small molecule compounds that increased utrophin mRNA. Anisomycin, a p38-activating antibiotic, was among the most promising utrophin mRNA upregulating agents identified.[20] Here we show that anisomycin induces utrophin protein levels both *in vitro* and *in vivo*. We also confirm that p38 MAPK activity is essential to the utrophin-inducing effect of anisomycin.

METHODS

Reagents

Anisomycin and the p38 inhibitor SB239063 (Sigma, St. Louis, MO), utrophin antibody (N-terminus; clone DRP3/20C5) (Vector Labs, Burlingame, CA), tubulin monoclonal antibody (Abcam, Cambridge, MA), phospho-p38 mitogen-activated protein kinase (MAPK; Thr180/Tyr182) rabbit monoclonal antibody, and total-p38 antibody (Cell Signaling Technology, Beverly, MA) were used.

Connectivity map analysis

Cmap is a database of drug–gene interactions employing the AffymetrixGeneChip U133-A microarray (Santa Clara, CA). Three cell lines (HeLa, PC3, and MCF7) were individually treated with ~1,300 drugs for 6 hours at a concentration of 10 μM and RNA was isolated, reverse-transcribed, and used to probe the microarray. Two cDNA probes for utrophin found on the U133-A GeneChip (213022_s_at and 213023_s_at) resulted in two utrophin gene expression lists. We used these two lists, which ranked the 1,300 drugs in terms of utrophin induction potency, to identify the strongest suitable for further validation. In order to reduce the number of false positives, only drugs that were tested a minimum of four times for each probe set were considered as potential utrophin-inducing compounds. Drugs were ranked based on an average of their effect on the utrophin gene between both probe sets.

Tissue culture and drug treatment conditions

C2C12 culture

C2C12 mouse myoblast cells were grown at 37°C with 5% CO_2 in Dulbecco's modified eagle medium (DMEM) with freshly added penicillin, streptomycin, and 10% fetal calf serum. For the utrophin upregulation and p38 inhibition assays, C2C12 cells were plated at a density of 100,000 cells per well and cultured overnight. Cells were treated with anisomycin at 0.1, 1.0, and 10 nM. For the p38 inhibition assay, cells were treated with SB239063 (3 μM) for 2.5 hours and then treated with anisomycin (1 nM) for 24 hours. Following incubation, cells were washed with 1x phosphate-buffered saline (PBS) two times and then lysed using radioimmunoprecipitation assay (RIPA) buffer with phenylmethanesulfonyl fluoride (PMSF), aprotinin, and leupeptin. Antiphosphatase molecules β-glycerol phosphate (5 mM), sodium fluoride (50 mM), sodium orthovanadate (0.2 mM) were added to the RIPA lysis buffer for the p38 phosphorylation assay. Cell lysates were centrifuged at 13,000 rpm for 15 minutes and protein supernatant collected and quantified using Bradford reagent.

Mdx *myoblast culture*

Murine primary myoblasts (*mdx* cells) were isolated from gastrocnemius and quadriceps muscles of adult female *mdx* mice. Cells were cultured in DMEM (ATCC, Rockville, MD) with freshly added penicillin, streptomycin, 20% fetal calf serum, 10% equine serum, 10 ng/ml basic fibroblast growth factor (bFGF; Cell Signaling Technology), and 2 ng/ml hepatocyte growth factor (HGF; PreproTech, Rocky Hill, NJ). Fresh growth factors were added daily to the growth media. Cells were monitored closely for indications of differentiation. *Mdx* cells were plated at a density of 2×10^5 cell per well in 6-well plates. Cells were then treated with 1 nM anisomycin for 24 hours and subsequently processed as outlined for C2C12 culture.

Western blot analysis

Protein samples from cells and murine tissue were run on a biphasic gel system composed of a lower 15% acrylamide and an upper 6% acrylamide gel. Utrophin DRP2 monoclonal antibody (Vector Labs), tubulin, phospho-p38, and total-p38 monoclonal antibodies were used to immunoblot the membranes. Antimouse horseradish peroxidase (HRP)-linked secondary antibody was used for utrophin, tubulin, and total-p38, whereas antirabbit HRP-linked secondary antibody was used for phoshpo-p38. Membranes were exposed using enhanced chemiluminescence-prime (ECL-prime) or ECL (tubulin) and X-ray films. All results were quantified using ImageJ (NIH, Bethesda, MD).

Table 1 Identification of *in vitro* utrophin mRNA inducers from Connectivity Map

No.	Drug	UTRN fold-expression (Cmap data)	p38 MAPK effect	References for p38 effect
1	Anisomycin	1.50	+	Liu *et al.* (2014); Farooq *et al.* (2009); Xiong *et al.* (2006)
2	Emetine	1.47	+	Kim *et al.* (2015); Islam *et al.* (2006)
3	Cycloheximide	1.44	+	Oksvold *et al.* (2012); Itani *et al.* (2003)
4	Chlorzoxazone	1.40	?	
5	Chelidonine	1.38	?	
6	Loxapine	1.38	?	
7	Piperacetazine	1.38	?	
8	Tacrine	1.36	–	Li *et al.* (2005)
9	Alexidine	1.35	–	Zhu *et al.* (2015)
10	Lobeline	1.33	?	

Top 10 hits from the *in silico* screen. Relative expression of utrophin is derived from the three cancer cell lines, MCF7, HeLa, and PC3, treated for 6 hours with 10 μM drug. (+) upregulation, (–) downregulation, (?) not indicated in the literature.[31–34,44–49]

Animals

The Animal Care and Veterinary Services and Ethics Committee of the University of Ottawa approved all protocols and all experiments were performed in accordance with the Canadian Institute of Health Research. Adult male and female C57/BL10ScSn *Dmd*^{mdx} (*mdx*) mice from Jackson Laboratories (Bar Harbor, ME) were used to start a colony yielding 10-day-old mice for the 30-day anisomycin treatment experiments.

Anisomycin administration

For the 30-day trial, 12–14 postnatal day 10 (P10) male *mdx* mice were treated with 2, 20, and 200 μg/kg anisomycin or vehicle (0.2% dimethyl sulfoxide (DMSO) in 1x PBS). These doses were chosen based on pharmacokinetic data of anisomycin serum concentration in mice to approximate the concentration of drug used for *in vitro* studies and are in line with previously published studies on efficacy and tolerability of anisomycin *in vivo*.[37]**37** Mice were dosed daily by intraperitoneal (i.p.) injection using 30½ gauge needles for 30 days until P40. All treatments were diluted so that 2 μl/g was the constant volume administered. At the end of each experiment, mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Tissue samples from diaphragm were flash-frozen in liquid nitrogen for immunoblotting. All tissues were kept at –80°C for long-term storage. Tissue samples were pulverized with a Bessman Tissue Pulverizer and then lysed in RIPA lysis buffer. Protein lysates were extracted and quantified and Western blotting performed as previously described.

RESULTS

Connectivity map analysis; Overrepresentation of p38 pathway modulators

Analysis of the two Cmap utrophin expression builds identified the top 10 utrophin-inducing molecules that were tested a minimum of four times (**Table 1**; ranked by impact on utrophin transcript level). The greatest utrophin-inducing compound was anisomycin, conferring a 1.5-fold upregulation. Interestingly, three of the top 10 utrophin-inducing molecules were p38 activators (anisomycin, ementine, cycloheximide) while two of the top utrophin-inducing molecules were repressors of the p38 pathway (tacrine, alexidine).

In vitro validation: Anisomycin-induced utrophin upregulation

The utrophin upregulating effect of the top-ranking compound from the Cmap screen, anisomycin, was further investigated in C2C12 mouse myoblasts. The Cmap used standardized 10 μM dosing for their drug treatments, however, given the therapeutic serum level of 100 nM for anisomycin, we initially performed a dose curve in these cells to study the effects of anisomycin at low (0.1 and 1 nM) and therapeutic ranges (10, 25 and 75 nM) and the Cmap dose (10 μM). The C2C12 cells appear to be more sensitive to anisomycin at higher doses, as cellular toxicity was observed with the 75 nM dose after 24-hour treatment (data not shown), confirming previous reports in the literature regarding the dose response and toxicity of anisomycin.[34,36] However, a dose–response curve on cells treated with 0.1, 1, and 10 nM anisomycin for 24 hours showed a significant utrophin upregulation with 0.1 and 1 nM anisomycin (**Figure S1a**). Additional biological replicates of C2C12 cells treated with 1 nM anisomycin for 24 hours confirmed an approximate 2.5-fold increase in utrophin protein (**Figure 1a**). The effect of low-dose anisomycin treatment on utrophin protein expression was further studied in *mdx* mouse primary myoblasts. A more modest, 1.4-fold, induction was observed in utrophin protein with 1 nM anisomycin treatment (**Figure 1b**).

p38-MAPK is required for anisomycin-induced utrophin upregulation

Anisomycin is known to activate the p38 MAPK pathway, which has been implicated in the regulation of the utrophin gene.[19,21] Two of the strongest utrophin mRNA-inducing agents identified in the Cmap data, emetine and anisomycin, were p38 MAPK pathway activators (**Table 1**). To explore the potential role of the p38 pathway in the anisomycin-mediated upregulation of utrophin, analysis of the phosphorylation of p38 and a p38 MAPK inhibition assay were performed. C2C12 cells were treated with 1 nM anisomycin and harvested at 0, 2, 4, and 6 hours for Western blot analysis. There was a significant increase in phosphorylation of p38 following 6 hours of anisomycin treatment (**Figure 2a**). The

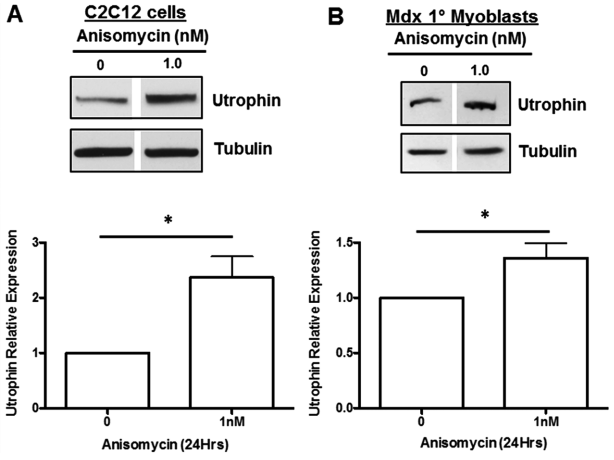

Figure 1 Anisomycin upregulates utrophin protein *in vitro*. (**a**) C2C12 myoblasts treated with 1 nM anisomycin or vehicle (DMSO) and harvested at 24 hours ($n = 9$). (**b**) *Mdx* primary myoblasts treated with 1 nM anisomycin or vehicle (DMSO) and harvested at 24 hours ($n = 4$). Immunoblotting for utrophin shows dramatic upregulation with respect to the housekeeper protein tubulin. Error bars show SEM of average (*$P < 0.01$, two-tailed unpaired Student's *t*-test).

experiments were repeated in the presence of the p38 inhibitor SB239063 to assess the involvement of this pathway in anisomycin-induced utrophin upregulation. SB239063 moderately reversed the anisomycin-induced utrophin induction (**Figure 2b**). Interestingly, the inhibitor alone increased utrophin protein expression as much as did anisomycin treatment, possibly consistent with the presence of p38 inhibitors, tacrine and alexidine, in the Cmap list of utrophin inducers.

In vivo small molecule therapy

The *mdx* mouse is a well-described model of DMD used for *in vivo* testing of potential DMD treatment strategies.[22] For our analysis, we focused on the diaphragm, as it is the most involved muscle in the *mdx* mouse and therefore a good proxy for human DMD.[12] Male *mdx* mice were treated daily with 2, 20, and 200 μg/kg i.p. anisomycin starting at postnatal day 10. After 30 days, mice were sacrificed and diaphragms were collected. Western blot of whole protein lysate extracted from diaphragm showed an ~2-fold upregulation of utrophin protein in mice treated with 20 μg/kg anisomycin (**Figure S1b**). With the addition of nine biological replicates (thus 12 in all), ~1.5-fold utrophin upregulation was observed in *mdx* diaphragm with 20 μg/kg anisomycin treatment for 30 days (**Figure 3a**).

DISCUSSION

DMD is a life-limiting neuromuscular disorder for which there is currently no effective treatment. Respiratory failure is typically the cause of mortality in patients with DMD, due to degeneration of, among other muscles, the diaphragm.[9] Therefore, the restoration or preservation of diaphragmatic function is an important aspect of any effective DMD therapy.

One potential therapeutic approach for DMD is to increase levels of the dystrophin paralog utrophin either through transcriptional induction and/or transcript stabilization. Increased levels of utrophin have been shown to

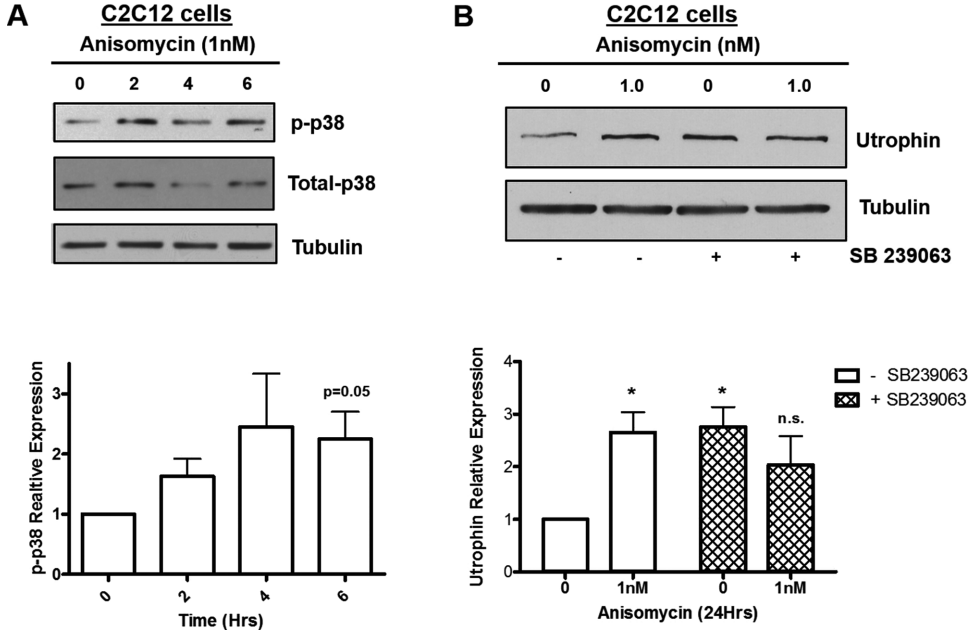

Figure 2 Activation and inhibition of p38-MAPK induces utrophin expression. (**a**) C2C12 myoblasts were treated with 1 nM anisomycin for 0, 2, 4, and 6 hours. Immunoblotting for phospho-p38 and total-p38 indicates upregulation of p38 phosphorylation in comparison to total-p38 ($n = 3$). (**b**) C2C12 myoblasts were treated with 3μM SB239063 (p38 inhibitor) for 2.5 hours. Cells were then treated for 24 hours with 1 nM anisomycin. ($n = 3$) Error bars show SEM of average. The untreated control cells were used as the reference for all treatment groups (*$P < 0.05$, two-tailed unpaired Student's *t*-test).

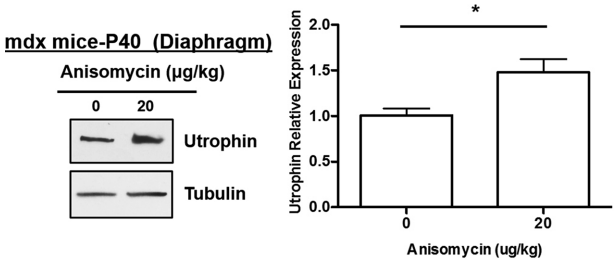

Figure 3 Anisomycin upregulates utrophin protein levels in *mdx* mouse diaphragm. Male *mdx* mice received daily i.p. injections of vehicle (0.2% DMSO in PBS) or anisomcyin (20 μg/kg) from P10 for 30 days. Mice were sacrificed at P40. Representative blots are shown for diaphragm ($n = 12$–14) (*$P < 0.01$, two-tailed unpaired Student's *t*-test).

compensate, in part, for the loss of dystrophin protein and thus reduce *mdx* disease severity.[17,23] Previous *in vivo* studies [15] have shown that very modest inductions of utrophin transcript and protein levels can lead to impressive functional rescue.

In this article we report drug hits mined from the Cmap database; however, given the large variation in transcriptional expression between different tissue culture systems and the rate of false-positives reported by Mears *et al.*,[24] the list of drug hits for utrophin would likely be different if sourced from another database. However, we believe the identification of p38-activating compounds as utrophin-inducing in other databases is still likely given our demonstration here of this relationship as well as previous reports documenting this.

Anisomycin was shown to be the top utrophin-inducing compound identified by *in silico* screening of the Cmap. In this study we documented a 2.5-fold upregulation of utrophin *in vitro* in C2C12 cells and a 1.4-fold upregulation in *mdx* myoblasts with 1 nM anisomycin treatment. A dose of 20 μg/kg in mice resulted in an $\sim$50% increase in utrophin protein levels in the diaphragm. This *in vivo* anisomycin dosing falls well below that used in human trials for infections with amoebas (amoebiasis).[25] Our results are similar to those found in other studies that have been used in preclinical and clinical trials. For example, the nonsteroidal antiinflammatory drug nabumetone upregulates utrophin protein expression 1.2-fold in C2C12 cells.[26] SMTC1100, a drug tested in phase I clinical trials, upregulated utrophin twofold in human DMD cells and showed an $\sim$75% increase in expression in the diaphragm of *mdx* mice.[27]

Previous work has shown that the 3'UTR of utrophin A transcript contains a number of AU-rich elements (AREs) that are crucial to transcript stability.[28,29] These *cis*-acting elements are well-recognized destabilizing sections of mRNA.[30] The interaction of *trans*-acting ARE-binding proteins at these sites can either increase mRNA stability or enhance its degradation.[31] Amirouche *et al.* [21] showed that p38-mediated phosphorylation of the KH-type splicing regulatory protein leads to increased stabilization of utrophin A mRNA that in turn caused an increase in utrophin protein (C2C12 cells). Our lab and others have established anisomycin as a potent p38 activator even at low doses.[32,33]

Interestingly, when we employed the p38 inhibiting molecule SB239063 to establish the pathway of ansomycin-mediated utrophin induction, we found that the inhibitor alone caused significant upregulation of utrophin. This is the first time that inhibition of the p38 pathway has been linked to utrophin upregulation. The Cmap results provide corroborating evidence for a potential link between p38 downregulation and utrophin upregulation, as two of the top 10 utrophin-inducing molecules (tacrine, alexidine) have been linked to p38 inhibition.[34,35] These data indicate that the role of the p38 pathway in utrophin expression may be more complex than previous reports suggest. Further work would be required to elucidate the mechanism that causes utrophin upregulation when the p38 pathway is inhibited.

Several anisomycin doses were used in C2C12 cells to determine the concentration for optimal utrophin induction in cultured myoblasts. The most consistent utrophin protein induction was seen with 1 nM anisomycin in both C2C12 cell and primary *mdx* myoblasts. This concentration is four orders of magnitude lower than the concentration used in the Cmap. Anisomycin doses even two orders of magnitude lower than the dose initially used in the Cmap (10 μM) were highly toxic to C2C12 cells. Other reports have shown that in many cell types anisomycin at micromolar levels completely inhibit protein translation, blocks cell proliferation, and induces apoptosis.[36,37] The apparent bimodal utrophin-inducing nature of anisomycin could be due to a bimodal agonistic effect on the p38 MAPK pathway; at translation-inhibiting levels it acts via the ribotoxic stress response pathway, but may act in a more direct p38-activating fashion at lower than inhibitory concentrations.[38–41] The upregulation of utrophin mRNA observed with 10 μM anisomycin by the Broad Institute was probably caused by ribotoxic stress-mediated p38 induction, whereas the utrophin protein upregulation reported in this work may result from direct p38 activation by low-dose anisomycin, which we have also observed in our previous work.[32]

The use of utrophin as a disease-rescuing dystrophin paralog has been studied in the *mdx* mouse, in which transgene experiments revealed that a twofold increase in sarcolemmal utrophin completely rescues dystrophin-deficient muscle mechanical function and effectively normalizes classical markers of DMD-related muscle damage.[15,42,43] Several reports have also shown that pharmacological induction of utrophin mRNA and protein in *mdx* muscle reduces systemic and muscle-specific hallmarks of DMD.[27,44] In this report we show that daily treatment of *mdx* mice with low-dose anisomycin leads to a significant increase in utrophin protein in the diaphragm. The diaphragm pathology in *mdx* mice most closely resembles the skeletal muscle pathology seen in human DMD patients, and is the best target muscle to test therapeutic agents. Rescuing the diaphragm muscle alone in *mdx* mice can restore cardiac function through increasing both utrophin and dystrophin expression. This finding potentially increases the relevance of our anisomycin findings to treatment of humans, as it may prove to reduce the respiratory and cardiac morbidities seen in DMD patients.[6]

Although our results show a utrophin protein upregulating effect of anisomycin *in vitro* and *in vivo,* it remains to be seen whether this upregulation would lead to physiologically significant results. Behavioral testing to assess grip strength of mice treated with anisomycin and exercise tolerance could be studied as well as the systemic effects of anisomycin by serum creatine kinase detection, which is a marker of muscle damage in DMD patients.

In conclusion, low-dose anisomycin treatment is shown here to be effective for upregulating utrophin protein *in vitro* and at a preclinical level in DMD model mice. Further work will be necessary to determine the efficacy of anisomycin for treating DMD in humans.

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Conflict of Interest. The authors declare no competing interests for this work.

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