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Pharmacological rescue of the dystrophin-glycoprotein complex in Duchenne and Becker skeletal muscle explants by proteasome inhibitor treatment

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Assereto, Stefania, Silvia Stringara, Federica Sotgia, Gloria Bonuccelli, Aldobrando Broccolini, Marina Pedemonte, Monica Traverso, Roberta Biancheri, Federico Zara, Claudio Bruno, Michael P. Lisanti, and Carlo Minetti. Pharmacological rescue of the dystrophin-glycoprotein complex in Duchenne and Becker skeletal muscle explants by proteasome inhibitor treatment. *Am J Physiol Cell Physiol* 290: C577–C582, 2006. First published September 28, 2005; doi:10.1152/ajpcell.00434.2005.—In this report, we have developed a novel method to identify compounds that rescue the dystrophin-glycoprotein complex (DGC) in patients with Duchenne or Becker muscular dystrophy. Briefly, freshly isolated skeletal muscle biopsies (termed skeletal muscle explants) from patients with Duchenne or Becker muscular dystrophy were maintained under defined cell culture conditions for a 24-h period in the absence or presence of a specific candidate compound. Using this approach, we have demonstrated that treatment with a well-characterized proteasome inhibitor, MG-132, is sufficient to rescue the expression of dystrophin, β -dystroglycan, and α -sarcoglycan in skeletal muscle explants from patients with Duchenne or Becker muscular dystrophy. These data are consistent with our previous findings regarding systemic treatment with MG-132 in a dystrophin-deficient *mdx* mouse model (Bonuccelli G, Sotgia F, Schubert W, Park D, Frank PG, Woodman SE, Insabato L, Cammer M, Minetti C, and Lisanti MP. *Am J Pathol* 163: 1663–1675, 2003). Our present results may have important new implications for the possible pharmacological treatment of Duchenne or Becker muscular dystrophy in humans.

muscular dystrophy; membrane proteins; MG-132

DYSTROPHIN, A 427-kDa PROTEIN localized at the plasma membrane (sarcolemma) of muscle cells, is the product of the Duchenne muscular dystrophy (DMD) gene (5, 6, 9). Mutations in the DMD gene lead to two allelic muscular dystrophy disorders, i.e., DMD or the less severe Becker muscular dystrophy (BMD) (1, 10). Dystrophin is absent in the skeletal muscle fibers of patients with DMD as well as in the dystrophin-deficient *mdx* mouse model, whereas dystrophin is reduced or abnormal in size in BMD muscle. DMD is a severe myopathy with an incidence of 1 in 3,500 newborn males. Patients experience progressive muscle weakness, are wheelchair bound before 12 years of age, and often die before the third decade of life. Patients with BMD present with milder

symptoms, often remain ambulatory for >40 years, and have longer life expectancies than do patients with DMD. The severe DMD phenotype is associated with frame-shift mutations in the DMD gene and is correlated with complete dystrophin deficiency. Conversely, the less severe BMD phenotype is caused by mutations in the DMD gene that maintain an intact reading frame and generate shorter, yet partly functional, dystrophin protein products (1, 2, 10).

In normal muscle fibers, dystrophin forms a transmembrane oligomeric protein complex known as the dystrophin-glycoprotein complex (DGC). The DGC is composed of two subcomplexes: the dystroglycan complex (α - and β -subunits) and the sarcoglycan complex (α -, β -, γ -, and δ -subunits) (4, 15). At its NH₂-terminal domain, dystrophin binds to intracellular actin filaments, whereas the dystrophin COOH-terminal domain interacts directly with the membrane-spanning protein β -dystroglycan. As such, dystrophin in contracting muscle cells is thought to provide an essential mechanical link between the intracellular cytoskeleton and the extracellular matrix. The maintenance of the integrity of this structural link is thought to provide stability at the plasma membrane of skeletal muscle fibers (7, 12–14). According to this notion, the loss of dystrophin induces mechanical damage of the cell membrane and perturbs the other members of the DGC, so that they are greatly reduced in DMD and BMD muscle fibers (16, 17).

Despite extensive efforts, no clinically applicable or effective therapy for patients with DMD has been developed (19), although in some cases, a delay in the course of the disease manifestations may be achieved by administering corticosteroid therapy (8, 11).

Recently, using *mdx* mice as a model system, we provided new evidence that dystrophin and other members of the DGC undergo degradation through a proteasome-dependent pathway in dystrophin-deficient skeletal muscle fibers (3). In the present study, we tested the feasibility of using proteasome inhibitors in humans for the possible treatment of DMD and BMD using freshly isolated skeletal muscle explants.

MATERIALS AND METHODS

Incubation with MG-132. As an experimental model, pieces of human skeletal muscle (i.e., explants) were used. All human samples were obtained after patients had signed informed consent forms for publication of their case information and in accordance with the

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guidelines of the G. Gaslini Institute Ethical Committee, including experimental protocols for muscle biopsies. Human explants were isolated from muscle biopsies obtained for diagnostic purposes from six patients with DMD and three patients with BMD. Biopsies were cleaned from fat, connective tissue, and blood; divided into small pieces; and placed into a 60-mm-diameter dish containing F-14 medium (DMEM containing 6 g/l glucose, 15% FBS, 50 μ g/l FGF, 10 μ g/l EGF, 2 mM glutamine, 10 mg/l insulin, 1% penicillin-streptomycin-amphotericin B mixture, and 1 mg/l ATP) at 37°C in a humidified 95% air-5% CO₂ atmosphere in the presence or absence of 20 μ M MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, Z-LLL-CHO, no. 474790; Calbiochem, San Diego, CA) for 24 h. At the end of the incubation period, explants were frozen in liquid nitrogen-cooled isopentane and stored in liquid nitrogen.

Antibodies. MABs directed against β -dystroglycan (NCL- β -DG), α -sarcoglycan (NCL- α -sarco), and dystrophin (NCL-DYS3 and NCL-DYS1) were purchased from Novocastra (Newcastle upon Tyne, UK). We also used the proteasome inhibitor MG-132.

Immunohistochemistry. Unfixed 5- μ m-thick cryosections of skeletal muscle explants were incubated with primary mouse MABs for 2 h at room temperature (or overnight at 4°C). After three washes with PBS (5 min each), sections were incubated with the secondary anti-mouse biotin-conjugated antibody (anti-mouse IgG, biotinylated species-specific whole antibody; Amersham Biosciences, Little Chalfont, UK) diluted 1:100 in common antibody diluent (BioGenex, San Ramon, CA) for 1 h at room temperature. After being washed three times with PBS (5 min each), sections were incubated with fluorescein-conjugated streptavidin (Amersham Biosciences) diluted 1:250 in common antibody diluent for 30 min at room temperature in the dark. Finally, sections were washed three times with PBS (5 min each wash) and mounted with glycerol (glycerol 87% diluted 2:1 in PBS). Slides were observed under a Leica Diaplan microscope.

Western blot analysis. Tissue samples were prepared on ice as follows: five to eight 10- μ m-thick cryosections of skeletal muscle explants were quickly dissolved with 40 μ l of lysis buffer (4% SDS in 4 M urea) by pipetting. Tissue lysates were then sonicated for 10 s on ice and centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were collected, and the protein concentration was determined using the Lowry protein assay method. Equal amounts of protein (30–40 μ g) were mixed with 4 \times SDS gel-loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol), resolved in 6–12% PAGE gel, and run at 80 V for 2–2.30 h in electrophoresis buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS). Proteins were then transferred onto nitrocellulose membranes (Immobilon PVDF; Millipore) and processed for immunoblot analysis using ECL (Amersham Pharmacia Biotech) as previously described (3).

RESULTS

We have previously shown that treatment with MG-132 successfully rescued the expression and cellular localization of dystrophin and dystrophin-related proteins in a mouse model of DMD, the *mdx* mouse. To evaluate whether the pharmacological exposure to proteasome inhibitors may represent a beneficial treatment route in humans, freshly isolated skeletal muscle biopsies (i.e., explants) from a total of nine patients were evaluated, including six patients with DMD and three with BMD. These patients presented with the classic signs and symptoms of muscular dystrophy, such as muscle weakness, calf hypertrophy, and elevated serum creatine kinase levels. Muscle biopsy analysis confirmed the diagnosis of Duchenne or Becker muscular dystrophy with muscle fiber degeneration and hypertrophy, central nuclei, and connective and adipose tissue infiltration. Importantly, initial screening of these pa-

tients revealed an absence or reduction of dystrophin expression. Consistently, β -dystroglycan and α -sarcoglycan were greatly reduced in skeletal muscle biopsies obtained from all of the patients in the study. Finally, we were able to characterize the genetic mutations in the dystrophin (DYS) gene in seven of nine patients (Table 1). Two patients showed neither deletions nor duplications. The fact that the dystrophin gene contains 79 exons impaired our ability to detect point mutations.

Pharmacological treatment with the well-characterized proteasome inhibitor MG-132 yielded interesting results. Figure 1 shows data regarding MG-132 treatment in a representative Duchenne muscle explant (Table 1; *patient 2*). Remarkably, the expression levels of dystrophin, β -dystroglycan, and α -sarcoglycan were strikingly increased at the sarcolemma of skeletal muscle fibers from treated explants compared with control samples (untreated or vehicle alone). The overall morphology of MG-132-treated samples also appeared to improve compared with control samples processed in parallel.

We used a similar pharmacological treatment with MG-132 in freshly isolated skeletal muscle biopsies from patients with BMD. Becker skeletal muscle explants responded to such treatment in a manner similar to that of Duchenne skeletal muscle explants. Figure 2 shows representative results from Becker *patient 1* (see also Table 1). The expression levels of dystrophin, β -dystroglycan, and α -sarcoglycan were markedly increased at the surface of skeletal muscle fibers from MG-132-treated Becker skeletal muscle explants compared with control samples (untreated or vehicle alone).

Table 1 shows that four (~67%) of six Duchenne skeletal muscle explants displayed mild to robust rescue of dystrophin, β -dystroglycan, and α -sarcoglycan expression levels and localization. Consistently, three (100%) of three Becker skeletal muscle explants displayed robust restoration of expression and the distribution of dystrophin, β -dystroglycan, and α -sarcoglycan. Although the number of samples is too small to draw definitive conclusions, these results offer the encouraging

Table 1. MG-132 rescue in skeletal muscle explants

Patients	DYS Mutation	MG-132 Treatment/Rescue		
		DYS	β -DG	α -SG
Duchenne muscular dystrophy				
1	Δ ex45	\pm	+	+
2	Δ ex45	+	+	+
3	No deletions or duplications	–	–	–
4	Δ ex19–24	–	–	–
5	No deletions or duplications	\pm	+	+
6	δ ex19–24	\pm	+	+
Becker muscular dystrophy				
1	Δ ex45–48	+	+	+
2	Δ ex48	+	+	+
3	Δ ex45–47	+	+	+

Data summarize skeletal muscle biopsies (i.e., explants) examined according to type of mutation, if known, and success of MG-132 treatment. Positive results were achieved in 4 (~67%) of 6 Duchenne skeletal muscle explants and in all 3 Becker skeletal muscle explants examined (100%). MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; –, no rescue; + robust rescue; \pm , mild rescue; Δ , deletion; δ , duplication.

Duchenne

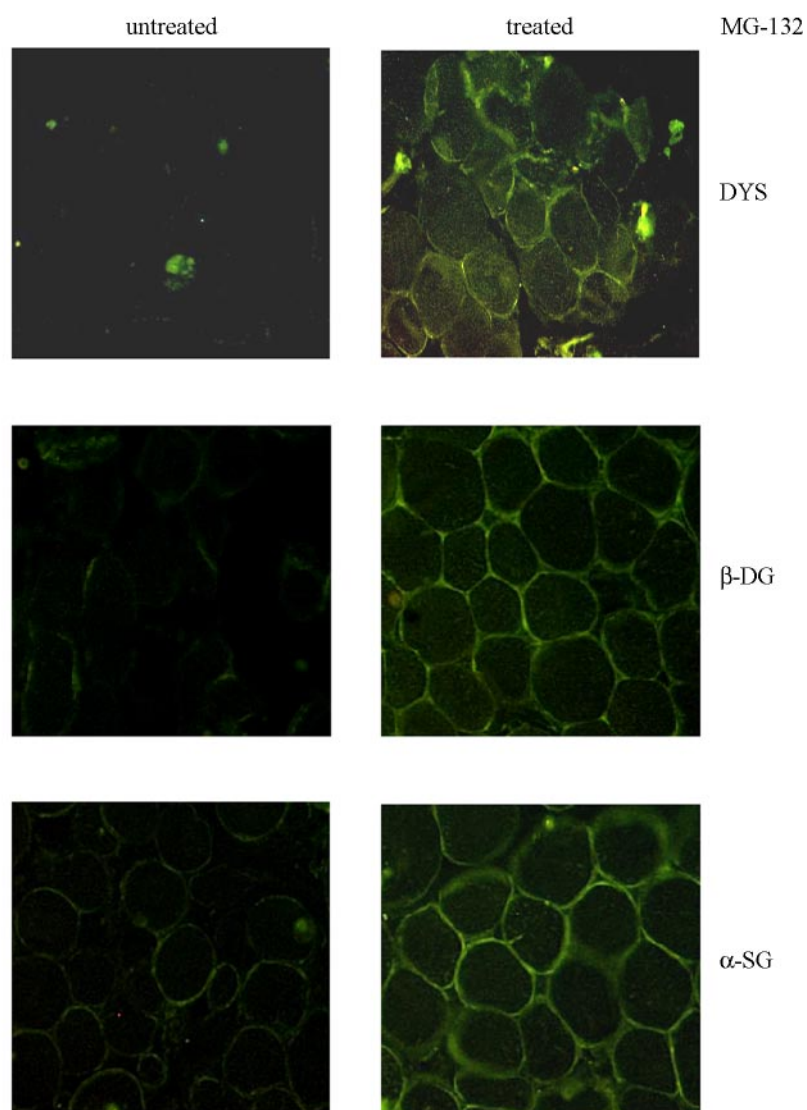


Fig. 1. Rescue of the dystrophin-glycoprotein complex (DGC) in biopsy explants from a patient with Duchenne muscular dystrophy (DMD). Freshly isolated muscle biopsies, termed explants, were incubated for 24 h in culture medium in the presence or absence of MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, Z-LLL-CHO). Then, frozen sections were prepared and immunostained with specific antibodies to monitor the status of the DGC. *Top* row, dystrophin (DYS); *middle* row, β -dystroglycan (β -DG); *bottom* row, α -sarcoglycan (α -SG). Note that MG-132 treatment induced a remarkable increase in all three proteins examined. Results shown are a representative biopsy from a patient with DMD (Duchenne *patient 2*). Original magnification, $\times 40$.

promise of therapies for the treatment of dystrophinopathies in humans.

It is interesting to note that not only the expression levels but also the subcellular localization of dystrophin, β -dystroglycan, and α -sarcoglycan appeared to be restored by MG-132 treatment in Duchenne and Becker skeletal muscle explants. Proper membrane localization might be of vital importance for the restoration of the biological function of these proteins.

Finally, the specific MG-132-induced increases in dystrophin, β -dystroglycan, and α -sarcoglycan observed by immunostaining were independently confirmed at the protein level using Western blot analysis. Figure 3 shows that the expression levels of dystrophin (~ 420 -kDa dystrophin but also a shorter dystrophin isoform, Dp260), β -dystroglycan, and α -sarcoglycan are elevated in MG-132-treated explants compared with control counterparts (untreated or vehicle alone). Equal loading was assessed using Western blot analysis with antibodies directed against the myosin heavy chain.

In this article, we have demonstrated that the exposure of DMD and BMD skeletal muscle explants to the pharmacological proteasome inhibitor MG-132 successfully rescued the expression and plasma membrane localization of dystrophin, β -dystroglycan, and α -sarcoglycan. These results provide important evidence that exposure to proteasome inhibitors may be a valuable treatment route in therapy for patients with muscular dystrophy.

DISCUSSION

We previously demonstrated that local and systemic treatment of *mdx* mice with MG-132, a well-characterized proteasome inhibitor, rescued the expression of DGC (3). Briefly, we treated *mdx* mice with the proteasome inhibitor MG-132 by local injection and systemic administration. Using immunofluorescence and Western blot analysis, we showed that administration of the proteasome inhibitor MG-132 effectively res-

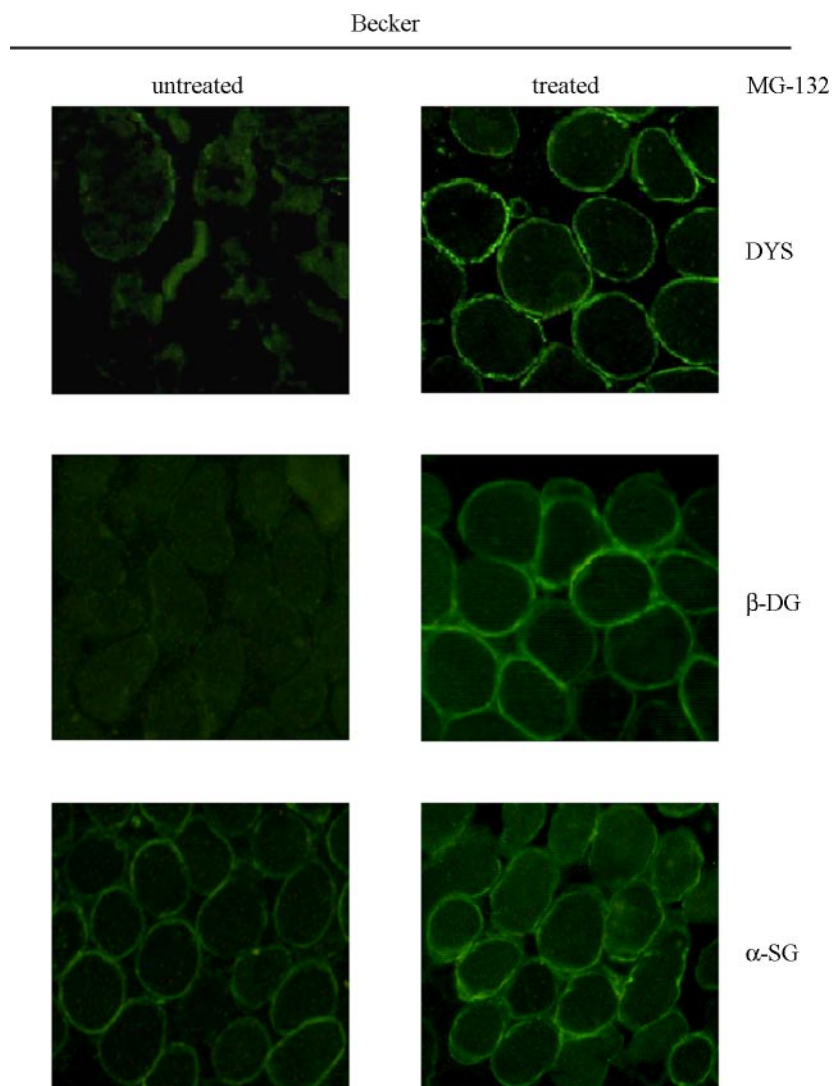


Fig. 2. Rescue of the DGC in biopsy explants from a patient with Becker muscular dystrophy (BMD). Freshly isolated muscle biopsies were incubated for 24 h in culture medium in the presence or absence of MG-132. To monitor the status of the DGC, frozen sections were prepared and subjected to immunostaining with specific antibodies. *Top* row, dystrophin (DYS); *middle* row, β -dystroglycan (β -DG); *bottom* row, α -sarcoglycan (α -SG). Note that dystrophin, β -dystroglycan, and α -sarcoglycan expression levels were greatly increased after MG-132 treatment. Results shown are from a biopsy obtained from a representative patient with BMD (Becker patient 1). Original magnification, $\times 40$.

cued the expression levels and plasma membrane localization of dystrophin, α -dystroglycan, β -dystroglycan, and α -sarcoglycan in skeletal muscle fibers from *mdx* mice. Furthermore, we showed that systemic treatment with MG-132 1) reduced muscle membrane damage as revealed using vital staining (with Evans blue dye) of the diaphragm and gastrocnemius muscle isolated from treated *mdx* mice and 2) ameliorated the histopathological signs of muscular dystrophy as judged using hematoxylin and eosin staining of muscle biopsies from treated *mdx* mice (3).

In the present study, we attempted to evaluate the applicability of these findings systematically in patients with Duchenne or Becker muscular dystrophy (summarized in Fig. 4). To accomplish this goal, freshly isolated skeletal muscle biopsies were maintained under tissue culture conditions as explants. These samples were then treated with MG-132 (20 μ M) or with vehicle alone, or they were left untreated. After 24 h of treatment, explants were quickly frozen and then evaluated for phenotypic changes. Rescue of DGC expression was monitored using 1) immunostaining of frozen sections and 2) Western blot analysis with specific antibodies directed against dystrophin, β -dystroglycan, and α -sarcoglycan.

Interestingly, not all Duchenne muscular dystrophy explants examined showed signs of phenotypic rescue. Only four ($\sim 67\%$) of six patient explants showed clear improvement. Thus the success of MG-132 treatment may depend on the nature of the specific dystrophin mutations, which are listed in Table 1. However, the percentage of DMD and BMD skeletal muscle explants that displayed positive restoration of dystrophin and dystrophin-associated proteins from MG-132 exposure is quite striking, especially in light of the fact that, to date, no real treatment for DMD has been developed.

It is noteworthy that augmentation not only of the ~ 420 -kDa dystrophin isoform but also of a shorter dystrophin isoform, namely Dp260, occurred. Once thought to be retina specific, the Dp260 isoform was recently shown to be expressed in a wide variety of tissues, including brain, cardiac and skeletal muscle, intestine, and kidney, among the others (18). Interestingly, transgenic overexpression of Dp260 in *mdx* mice was demonstrated to rescue the degradation of the DGC and to slow the progression of muscular dystrophy (20). As such, the combined augmentation of dystrophin and the Dp260 isoform in MG-132-treated human tissue explants may cooperate synergistically to restore the expression levels of β -dystroglycan

and α -sarcoglycan. Taken together, this phenomenon may improve the mechanical resistance of the plasma membrane and contribute to reduce fiber degeneration in dystrophic muscle.

To date, no successful treatment has been developed for patients with DMD or BMD. In addition, the high incidence of sporadic mutations suggests that genetic screening will never eradicate this disease, which emphasizes the urgency of developing an effective therapy. Our results indicate that the use of proteasome inhibitors may represent a worthy route toward future treatment of humans with the muscular dystrophy.

Our present results corroborate previous findings in *mdx* mice, showing that treatment with the proteasome inhibitor MG-132 successfully restored the DGC at the cell surface of skeletal muscle fibers. Herein we have shown that treatment of human skeletal muscle biopsies with the same proteasome inhibitor rescued the expression of dystrophin and dystrophin-

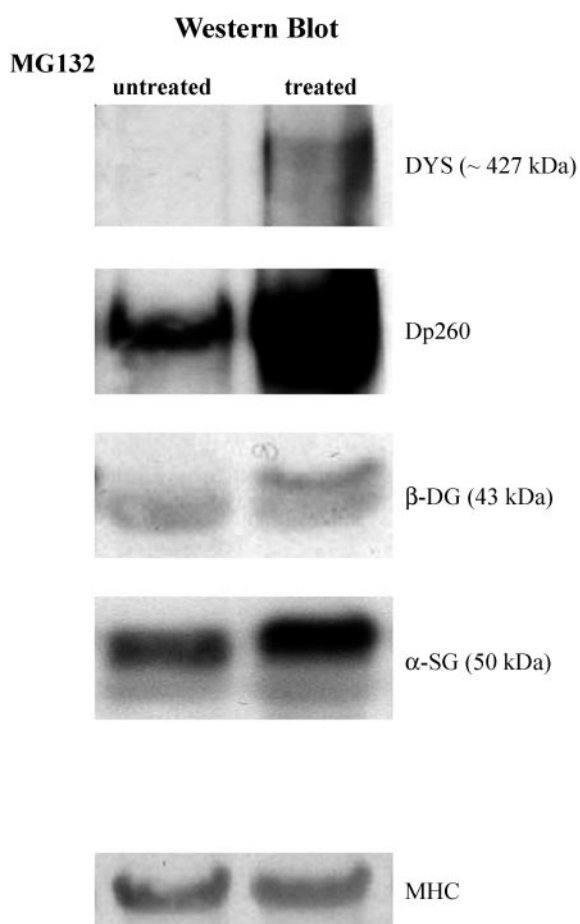


Fig. 3. Western blot analysis showing expression levels of the DGC in a BMD skeletal muscle explant. Freshly isolated muscle biopsies were incubated for 24 h in culture medium in the presence or absence of MG-132. Subsequently, untreated skeletal muscle samples were separated by performing SDS-PAGE, transferred onto nitrocellulose membrane, and subjected to immunoblot analysis with specific antibodies against dystrophin, β -dystroglycan, and α -sarcoglycan. Note that the expression levels of dystrophin, β -dystroglycan, and α -sarcoglycan were significantly augmented in MG-132-treated skeletal muscle explants. Also note the specific augmentation of the Dp260 dystrophin isoform. The amount of the myosin heavy chain (MHC) is comparable in both samples. Representative immunoblots shown are from a biopsy obtained from a patient with BMD (Becker patient 2).

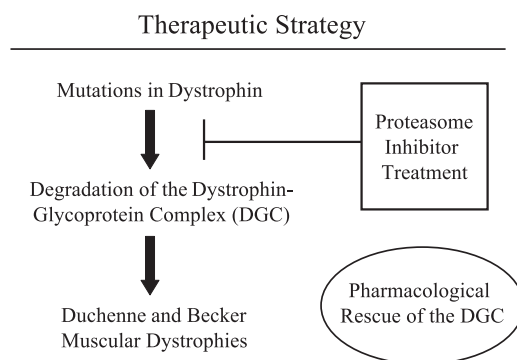


Fig. 4. Schematic diagram summarizing the use of proteasome inhibitor treatment to rescue the DGC. Skeletal muscle explants from patients with Duchenne or Becker muscular dystrophy were treated with MG-132, a well-characterized proteasome inhibitor. Subsequently, we monitored the status of the DGC using immunostaining and Western blot analysis. See text for details.

associated proteins at the plasma membrane of skeletal muscle. These results may have important clinical implications for promising new avenues in the treatment of patients with DMD or BMD.

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