

# Clinical Use of Immunosuppressants in Duchenne Muscular Dystrophy

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## Abstract

Duchenne muscular dystrophy (DMD) is a degenerative disease primarily affecting voluntary muscles with secondary consequences on heart and breathing muscles. DMD is an X-linked recessive disease that results in the loss of dystrophin, a key muscle protein. Inflammation can play different roles in DMD; it can be a secondary response to muscle degeneration, a primary cause of degeneration, or can contribute to the disease progression. Several immunosuppressants have been used with the aim to reduce the inflammation associated with DMD. Most recently, myoblast transplantation has shown the possibility to restore the dystrophin lack in the DMD patient's muscle fibers and this evidence has emphasized the importance of the use of immunosuppressants and the necessity of studying them and their secondary effects. The aim of this review is to analyze the main immunosuppressants drugs starting from the mdx mice experiments and concluding with the most recent human clinical studies.

**Key Words:** Duchenne, dystrophy, immunosuppressants, dystrophin

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## INTRODUCTION

Duchenne muscular dystrophy (DMD) is characterized by a progressive loss of muscle function. Inflammatory pathways mediated by neutrophils, macrophages, and associated to cytokines have been suggested to have a possible role in the damage of dystrophic muscles. (Reactive oxygen species may be important in both the activation of and the damage caused by this inflammatory pathway in mdx muscle.<sup>1</sup>) Gosselin et al reported that a persistent inflammatory response has been observed in dystrophic skeletal muscle leading to an alteration in extracellular environment,

including an increased presence of inflammatory cells such as macrophages and elevated levels of various inflammatory cytokines such as tumor necrosis factor alpha (TNF $\alpha$ ) and tumor necrosis factor beta.<sup>2</sup> Moreover, the proinflammatory cytokine TNF was shown to increase necrosis of skeletal muscle. Studies conducted on the DMD mdx mouse model support this fact reporting that the depletion of inflammatory cells such as neutrophils, cromolyn blockade of mast cell degranulation, or pharmacological blockade of TNF reduces necrosis of dystrophic myofibers.<sup>3</sup> Patients affected by DMD show problems climbing stairs, rising up from the floor, and are unable to run and in a variable way the most of them lose ambulation by 7 to 12 years. Other DMD complications are the progressive loss of respiratory function that can lead to respiratory failure, scoliosis, weight loss, cardiomyopathy, and finally death as a result of respiratory and cardiac complications. Unfortunately, there is no cure for this disease. Corticosteroids slow its progression, although their mechanism of action is not well known. Two corticosteroids, prednisone and deflazacort, have been used extensively because of their ability to improve skeletal muscle function. Recently, the interest on the suppressing drugs acting against TNF level and suppressing calcineurin signals has increased. Beyond the anti-inflammatory chemical compounds, growing interest either in mice or in humans was focused on immunosuppressant drugs that potentially might give clinical benefit during the DMD course. The interest in immunosuppressants is also growing

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because of the host transplant, potential immunosuppressant schedule that should be suitable to increase myoblast or mesangioblast graft survival supporting, in the meantime, the autologous cripple mass function.

### AIM

The aim of this review is to revisit the main important DMD clinical trials starting from the experimental studies in mice.

### MDX MICE AND IMMUNOSUPPRESSANTS

MD is caused by loss of expression of dystrophin, a protein of 427 kDa that links the cytoskeleton to a complex of proteins localized on the surface of the membrane of muscle fibers and is able to interact with the extracellular matrix. The literature evidences that the most commonly used DMD model is the mdx mouse because of his genetic mutation resulting in the loss of dystrophin.

### CALCINEURIN INHIBITORS

Calcineurin (Fig. 1) is a serine/threonine phosphatase controlled by cellular calcium, initially identified in extracts of mammalian brain.

Neural recruitment results in sarcolemmal membrane depolarization followed by the increase in intracellular  $Ca^{++}$  levels.

This increase in intracellular  $Ca^{++}$  activates  $Ca^{++}$ /calmodulin-dependent phosphatase calcineurin and  $Ca^{++}$ /calmodulin-dependent kinase pathways.

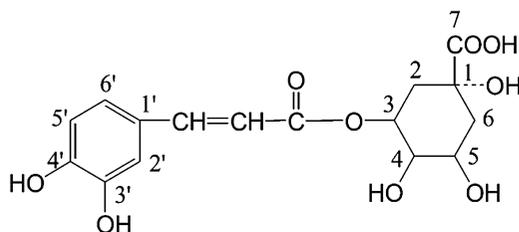


FIGURE 1. Calcineurin chemical structure.

It phosphorylates nuclear factor of activated T-cells (NFAT) that are important in the transcription of interleukin-2 genes.

Calcineurin and NFAT play an important role in the activation of Type I and IIA myosin heavy chain (MHC), oxidative enzyme, and utrophin A genes; it also acts through myocyte-enhancing factor 2-dependent transcription.

Calcineurin inhibitors are orally administered for the treatment of atopic dermatitis<sup>4</sup> and seborrheic dermatitis.<sup>5</sup> Calcineurin inhibition has been observed using cyclosporine A block activation of lymphocyte T, causing an immunosuppressant effect.

Parsons et al<sup>6</sup> showed that inhibition of calcineurin may benefit some types of muscular dystrophy. They examined the effect of altered calcineurin activation in a delta-sarcoglycan-null (*scgd*<sup>-/-</sup>) mouse model of limb-girdle muscular dystrophy (LGMD; delta sarcoglycan is a model of LGMD2F). The authors showed that genetic deletion of a loxP-targeted calcineurin B1 gene using a skeletal muscle-specific Cre allele in the *scgd*<sup>-/-</sup> background substantially reduced skeletal muscle degeneration and histopathology compared with the *scgd*<sup>-/-</sup> genotype alone.

A similar regression in *scgd*-dependent disease manifestation has also been observed in calcineurin A(beta) gene-targeted mice in both skeletal muscle and heart, whereas increased calcineurin expression, using a muscle-specific transgene, is able to promote the increase of cardiac fibrosis and the decrease of cardiac ventricular shortening. An increased calcineurin expression is also correlated with an increase of muscle fiber loss in the quadriceps.

### Debio 025

In the last 2 years, several studies and experiments involving the use of mdx mice have been performed using a cyclophilin inhibitor named Debio 025 ( $C_{63}H_{113}N_{11}O_{12}$ ; Fig. 2). This drug, developed by the Debiopharma Group<sup>TM</sup>, (Lausanne, Switzerland) was first used as a treatment for hepatitis C.<sup>7</sup>

Debio-025 is a synthetic cyclosporine without immunosuppressive properties but

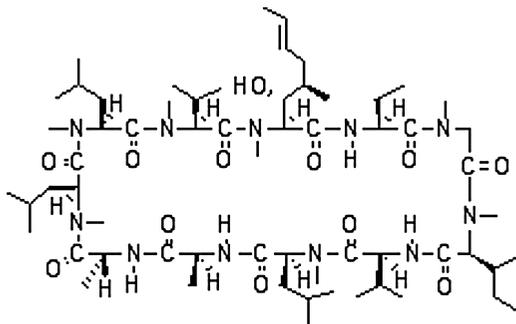


FIGURE 2. Debio 025 chemical structure.

a high inhibitory effect against peptidyl prolyl *cis-trans* isomerase activity of cyclophilin A (CypA). The lack of immunosuppressive effects compared with that of cyclosporine was demonstrated both *in vitro* and *in vivo*. Debio-025 is able to selectively inhibit the replication of HIV-1 in a CD4<sup>+</sup> cell line and in peripheral blood mononuclear cells. Its strong activity has been demonstrated against various isolated HIV-1 subtypes, including the isolated ones with multidrug resistance to reverse transcriptase and protease inhibitors. Debiopharm has not started Debio 025 human experimentation yet. Ptak et al<sup>8</sup> demonstrated that Debio-025 seems to interfere with the function of CypA during the progression/completion of HIV-1 reverse transcription.

Reutenauer et al<sup>9</sup> measured the effects of Debio 025 on muscle necrosis and function in mdx mice. Mice models of DMD were treated daily by means of a tube passed through the mouth down to the stomach (gavage) for 2 weeks with Debio 025 (10, 30, or 100 mg/kg<sup>-1</sup>), cyclosporine A (CsA) (10 mg/kg<sup>-1</sup>), or placebo. The authors observed a protective effect of low concentrations of Debio 025 against cell death. Histology demonstrated that Debio 025 partially protected the diaphragm and soleus muscles against necrosis. Hindlimb muscles from mice receiving Debio 025 at 10 mg/kg<sup>-1</sup> relaxed faster, showed alteration in the stimulation frequency-dependent recruitment of muscle fibers, and displayed a higher resistance to mechanical stress. The authors concluded that Debio 025 improved the structure and the function of the dystrophic mouse muscle,

suggesting that therapies targeting the mPTP may be helpful to patients with DMD.

In several muscular dystrophies, there is a compromise of the support network that connects myofilament proteins within the cell to the basal lamina outside the cell, making the sarcolemma more permeable or leaky. Millay et al<sup>10</sup> showed that the deletion of the gene encoding cyclophilin D (Ppif) is responsible for the mitochondria insensitivity to the calcium overload-induced swelling associated with a defective sarcolemma leading to the myofiber necrosis in two distinct models of muscular dystrophy. The authors evidenced that mice lacking delta-sarcoglycan (Scgd<sup>-/-</sup> mice) displayed markedly less dystrophic disease in both skeletal muscle and heart in the absence of Ppif. Moreover, the premature lethality associated with deletion of Lama2, encoding the alpha-2 chain of laminin-2, was rescued, as other indices of dystrophic disease were. Treatment with the cyclophilin inhibitor Debio-025 was able to reduce mitochondrial swelling and necrotic disease manifestations in mdx mice and in Scgd<sup>-/-</sup> mice. Based on the previously described evidence, the authors concluded that mitochondrial-dependent necrosis represents a prominent disease mechanism in muscular dystrophy, suggesting that inhibition of Ppif could provide a new pharmacologic treatment strategy for these diseases.

### Cyclosporine A

CsA (C<sub>62</sub>H<sub>111</sub>N<sub>11</sub>O<sub>12</sub>; Fig. 3) is a fungal metabolite derived by *Tolypocladium inflatum*. CsA was discovered in 1971 and has potent immunosuppressive properties. Cyclosporine inhibits calcineurin by binding to the protein and inhibiting its ability to dephosphorylate substrates such as NFATc family members, thus preventing their nuclear localization. This drug is able to prevent graft rejection inhibiting the T-cell receptor signal transduction pathway through the formation of the CsA–cyclophilin complex that inhibits calcineurin (protein phosphatase 2B). CsA also inhibits nitric oxide synthesis induced by interleukin 1α, lipopolysaccharides, and TNFα and can block cytochrome C

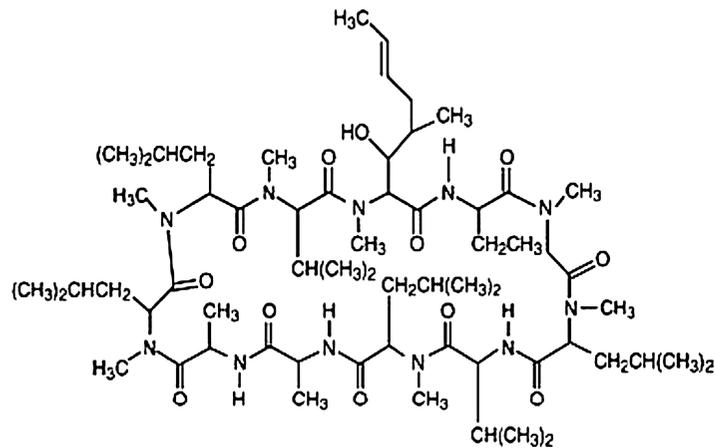


FIGURE 3. Cyclosporine A chemical structure.

release from mitochondria. Gonçalves et al<sup>11</sup> evidenced some CsA important side effects such as liver and kidney damage, high blood pressure, hirsutism, nausea and emesis, and gingival overgrowth.

CsA use in organ transplantation was approved in 2001 to prevent graft rejection in kidney, liver, heart, lungs, and combined heart-lung transplants. CsA is able to prevent the rejection after bone marrow transplantation and during prophylaxis of host-versus-graft disease. CsA has also been widely and successfully used in allogeneic hematopoietic cell transplantation (HCT). Hogan et al<sup>12</sup> reported the use of CsA in several clinical trials involving human patients undergoing HCT. This drug is also used for the treatment of psoriasis, atopic dermatitis, rheumatoid arthritis, and nephrotic syndrome despite of its time- and dose-dependent toxicity against kidneys. This drug is also widely used in postallogeneic organ transplant to reduce the activity of the patient's immune system with risks of organ rejection.

CsA is metabolized into a vast spectrum of metabolites and exerts its immunosuppressive action by inhibiting the enzyme calcineurin phosphatase.

Marx et al<sup>13</sup> conducted a study to investigate possible additive effects of calcium antagonists on the CsA-induced inhibition of cellular immunity. Human T-cells were isolated using standard methods and stimulated with phytohemagglutinin (n = 8), the

monoclonal antibody OKT3 (n = 6), or mixed lymphocyte reaction (n = 5). Verapamil, nifedipine, nimodipine, or diltiazem was added ( $5 \times 10^{-7}$  to  $5 \times 10^{-5}$  M) to the cultures either alone or in combination with CsA (62.5, 125, and 250 ng/mL). 3H-thymidine uptake was measured to estimate the proliferative responses and dose-response curves were constructed for the Ca antagonists and their combinations with CsA. A 50% inhibition of T-cell proliferation in the different stimulation assays was achieved with  $3.2 \times 10^{-5}$  to  $5.3 \times 10^{-5}$  M verapamil,  $2.5 \times 10^{-5}$  to  $4.3 \times 10^{-5}$  M nifedipine,  $3.7 \times 10^{-6}$  to  $5 \times 10^{-6}$  M nimodipine, and greater than  $5 \times 10^{-5}$  M diltiazem. Thus, in combination with CsA, a dose-dependent additive inhibitory effect of the Ca antagonists on T-cell proliferation was observed. This effect was less pronounced in the OKT3 assay, intermediate after phytohemagglutinin stimulation, and most pronounced in mixed lymphocyte reaction. Even in low concentrations, which correspond to therapeutic serum concentrations, Ca antagonists have an additive inhibitory effect in mixed lymphocyte reaction. The authors concluded that Ca antagonists exert a dose-dependent inhibitory effect on T-cell proliferation. A combination of CsA with verapamil, nifedipine, nimodipine, or diltiazem is more effective than each drug given alone. This additive effect of Ca antagonists and CsA may possibly contribute to better graft survival in clinical transplantation.

De Luca et al<sup>14</sup> tested CsA in dystrophic mdx mice to analyze its effects on a dystrophic mouse model. The study involved 22 mdx and 10 wild-type male mice aged 4 to 5 weeks. Mdx mice were treated with 10 mg/kg of CsA for 4 to 8 weeks throughout a period of exercise on treadmill, a protocol that worsens the dystrophic condition. The authors observed that CsA prevented the 60% drop of forelimb strength induced by exercise. A significant amelioration was observed in the histologic profile of CsA-treated gastrocnemius muscle with reductions of nonmuscle area (20%), centronucleated fibers (12%), and degenerating area (50%) compared with untreated exercised mdx mice. Consequently, the percentage of normal fibers increased from 26% to 35% in CsA-treated mice. Decreases in creatine kinase and markers of fibrosis were also observed. Using electrophysiological recordings *ex vivo*, the authors found that CsA counteracted the decrease in chloride conductance, a functional index of degeneration in diaphragm and extensor digitorum longus muscle fibers. However, electrophysiology and fura-2 calcium imaging did not show any amelioration of calcium homeostasis in extensor digitorum longus muscle fibers. No significant effect was observed on utrophin levels in diaphragm muscle. The previously described data show that the CsA treatment is able to significantly normalize many functional, histologic, and biochemical end points by acting on events that are independent or downstream of calcium homeostasis. The beneficial effect of CsA may involve different targets, reinforcing the importance of immunosuppressant drugs in muscular dystrophy.

Stupka et al<sup>15,16</sup> tested the hypothesis that the calcineurin signal transduction pathway is essential for the successful regeneration after severe degeneration (observed in the limb muscles of young mdx mice aged 2–4 weeks) and that inhibition of this pathway using CsA would exacerbate the dystrophic pathology. The authors treated 18-day-old mdx mice and C57BL/10 mice with CsA for 16 days. CsA administration severely

disrupted muscle regeneration in mdx mice but had a minimal effect in C57BL/10 mice. Muscles from CsA-treated mdx mice had fewer centrally nucleated fibers and extensive collagen, connective tissue, and mononuclear cell infiltration than muscles from vehicle-treated littermates. The deleterious effects of CsA on muscle morphology were accompanied by a 30% to 35% decrease in maximal force-producing capacity. These observations indicate that the calcineurin signal transduction pathway is a significant determinant of successful skeletal muscle regeneration in young mdx mice. The authors demonstrated that calcineurin activation ameliorates the dystrophic pathology of hindlimb muscles in mdx mice and decreases their susceptibility to contraction damage. They tested how muscle morphology and function would be improved by overexpression of calcineurin. An alpha transgene in skeletal muscle of mdx mice observed that hindlimb muscles from mdx mice, which overexpressed calcineurin had a prolonged twitch time course and were more resistant to fatigue if compared with control mdx mice. Moreover, the proportion of centrally nucleated fibers was reduced, indicating improvement of myofiber viability. The previously described findings brought the authors to the conclusion that the calcineurin activation is able to increase the expression of the markers of regeneration, in particular developmental myosin heavy chain isoform and myocyte enhancer factor 2A, and is able to ameliorate the mdx pathophysiology through its effects on muscle degeneration and regeneration and endurance capacity.

### Tacrolimus

Another immunosuppressive drug tested for therapy in muscular dystrophy mice is tacrolimus (FK-506; PROGRAF; Astellas Pharma Inc.; C<sub>44</sub>H<sub>69</sub>NO<sub>12</sub>; Fig. 4), a metabolite isolated from *Streptomyces Tsukubaensis* in 1984.

FK-506 exerts a potent inhibitory effect on T-lymphocyte activation. It binds to immunophilins FK-506 binding proteins (FKBP-12) leading to the development of a complex of

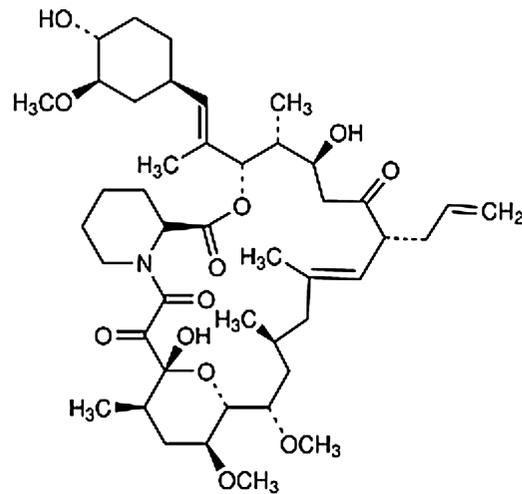


FIGURE 4. Tacrolimus chemical structure.

FKBP-12, calcium, calmodulin, and calcineurin-inhibiting phosphatase activity of calcineurin. This prevents the dephosphorylation and the translocation of the activated T-cells (NFAT) nuclear factor and inhibits transcription of early T-cell activation gene, interleukin (IL)-2, TNF $\alpha$ , and proto-oncogenes suppressing the expression of IL-2 and IL-7 receptor.

This results in the inhibition of T-lymphocyte activation. FK-506 is also able to inhibit the mixed lymphocyte reaction, generation of cytotoxic T-cells, and T-cell dependent B-cell activation.<sup>17</sup> FK-506 is used for the treatment of severe atopic dermatitis, severe refractory uveitis after bone marrow transplants, vitiligo, atopic dermatitis, and to suppress the inflammation associated with ulcerative colitis, a form of inflammatory bowel disease.

### TUMOR NECROSIS FACTOR BINDING PROTEINS

#### Etanercept

Etanercept (Enbrel, Immunex, Seattle, WA) (C<sub>2224</sub>H<sub>3475</sub>N<sub>621</sub>O<sub>698</sub>S<sub>36</sub>; Fig. 5) was developed by Immunex and was released in late 1998. Etanercept is a large molecule of 150 kDa made from the combination of two naturally occurring soluble human 75-kD TNF receptors linked to an Fc portion of an IgG1. The effect is an artificially engineered dimeric fusion protein. This molecule binds to

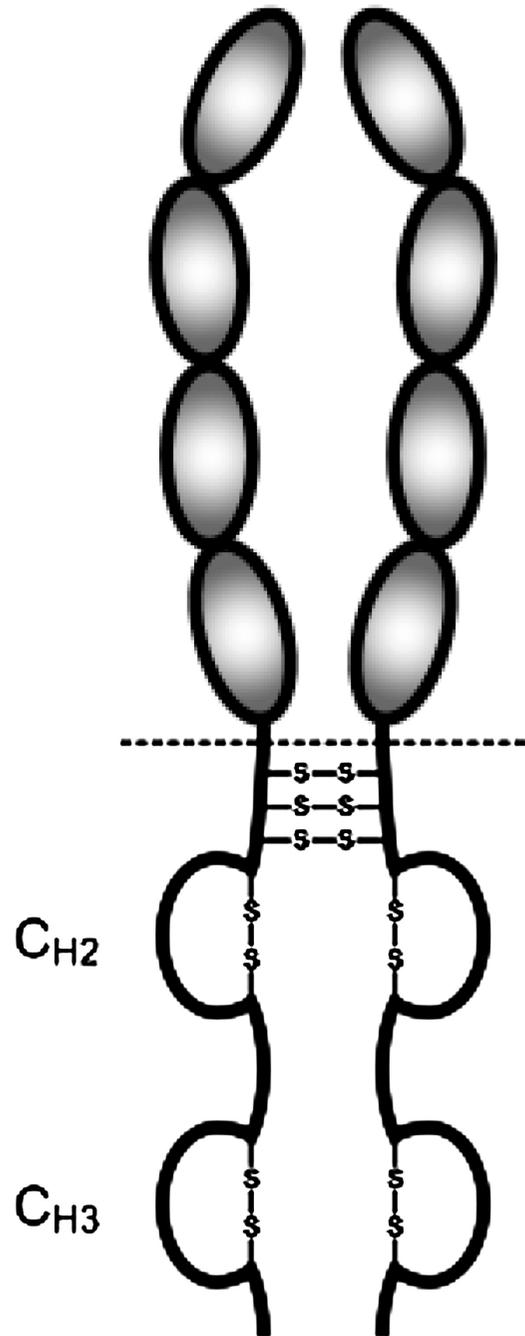


FIGURE 5. Etanercept structure.

TNF $\alpha$  and decreases its role in disorders involving excess inflammation in humans and other animals, including autoimmune diseases such as ankylosing spondylitis, juvenile rheumatoid arthritis, psoriasis, psoriatic arthritis, rheumatoid arthritis, and, potentially, in a variety of other disorders mediated by excess TNF $\alpha$ .

Pierno et al<sup>18</sup> evaluated the role of TNF $\alpha$  or cyclo-oxygenase-2 eicosanoids in dystrophinopathies.

The authors treated adult dystrophic mdx mice with the anti-TNF $\alpha$  etanercept (0.5 mg/kg) or the cyclo-oxygenase-2 inhibitor meloxicam (0.2 mg/kg) for 4 to 8 weeks.

Throughout the treatment period, the mdx mice underwent a protocol of exercise on a treadmill worsening the progression of pathology; gastrocnemius muscles from exercised mdx mice showed an intense staining for TNF $\alpha$  by immunohistochemistry. In vivo etanercept, but not meloxicam, improved the exercise-induced forelimb force drop. Electrophysiological recordings *ex vivo* showed that etanercept was able to counteract the decrease in chloride conductance, a functional index of myofiber damage, in both diaphragm and extensor digitorum longus muscle. Instead, meloxicam is effective only in extensor digitorum longus muscle. None of the drugs ameliorate calcium homeostasis detected by electrophysiology and/or spectrofluorimetry. Etanercept more than meloxicam reduced plasma creatine kinase (CK) and etanercept-treated muscles showed a reduction of connective tissue area and of profibrotic cytokine transforming growth factor- $\beta$ 1 versus untreated ones. The histology profile of gastrocnemius was significantly improved with a reduction of degenerating area and CK levels were only slightly lower. The previously described findings suggest that TNF $\alpha$ , but not cyclo-oxygenase-2, plays a key role in different phases of dystrophic progression and anti-TNF $\alpha$  drugs can be used in combination therapies in DMD.

Hodgetts et al<sup>19</sup> tested, in dystrophin-deficient mice, the hypothesis that the initial sarcolemmal breakdown resulting from dystrophin deficiency is exacerbated by inflammatory cells, specifically neutrophils, and that cytokines, specifically TNF $\alpha$ , is able to contribute to myofiber necrosis. Antibody depletion of host neutrophils resulted in a delayed and significantly reduced amount of skeletal muscle breakdown in young dystrophic mdx mice. A more striking and prolonged

protective effect was seen after pharmacologic blockade of TNF $\alpha$  bioactivity using etanercept. The extent of exercise induced myofiber necrosis in adult mdx mice after voluntary wheel exercise was also reduced after etanercept administration. The previously reported data show a clear role for neutrophils and TNF $\alpha$  in necrosis of dystrophic mdx muscle *in vivo*. Etanercept is a highly specific anti-inflammatory drug, widely used clinically, and its potential application to muscular dystrophies is suggested by this reduced breakdown of mdx skeletal muscle. Etanercept caused the following side effects: redness, itching, pain, or swelling at the injection site; colds; cough; headache; and nausea.

### Infliximab

Infliximab (Remicade; Centocor Ortho Biotech Inc.; Malvern, PA) (C<sub>6428</sub>H<sub>9912</sub>N<sub>1694</sub>O<sub>1987</sub>S<sub>46</sub>; Fig. 6) is a chimeric monoclonal antibody targeted against TNF $\alpha$  and approved by the U.S. Food and Drug Administration in 1998 to treat children (age 6 years or older) and adults with Crohn disease who do not respond to traditional therapies. There is evidence that the overstimulation of TNF $\alpha$  is implicated in causing psoriasis and other autoimmune disorders because rheumatoid arthritis and infliximab can prevent TNF $\alpha$  from triggering inflammation in the body by blocking the activities of cell surface receptors.

Grounds et al<sup>20</sup> tested infliximab in young dystrophic mdx mice to confirm the

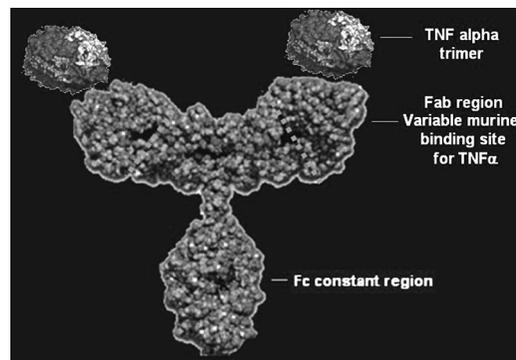


FIGURE 6. Infliximab structure.

hypothesis previously described by Hodgetts et al. Mdx mice aged 7 days were injected intraperitoneally weekly with 10 g infliximab before the onset of muscle necrosis and dystropathology that normally occurs at 21 days postnatally. Infliximab-treated and control mdx mice were also compared with untreated mdx/ $\text{TNF}\alpha^{-/-}$  mice. After the mice were killed, inflammatory cell infiltration, muscle necrosis, and myotube formation were evaluated by histologic analysis from 18 to 28 days. Muscle damage was also visualized by penetration of Evans blue dye into myofibers. The authors concluded that infliximab greatly reduced the breakdown of dystrophic muscle in contrast to the situation in mdx and mdx/ $\text{TNF}\alpha^{-/-}$  mice. Necrosis and the dystropathology were reduced and had no adverse effect on new muscle formation. Infliximab caused the following side effects: upper respiratory tract infections, urinary tract infections, cough, rash, back pain, nausea, vomiting, abdominal pain, headache, weakness, fever, and low or high blood pressure.

## STEROID-BASED DRUGS

### Prednisone and Deflazacort

Glucocorticoids are routinely and effectively used to treat chronic inflammatory diseases. There is evidence in literature of their use both in mice and human clinical trials with beneficial effects in the treatment of DMD. The two main steroids used are prednisone (Fig. 7) and deflazacort (Fig. 8).

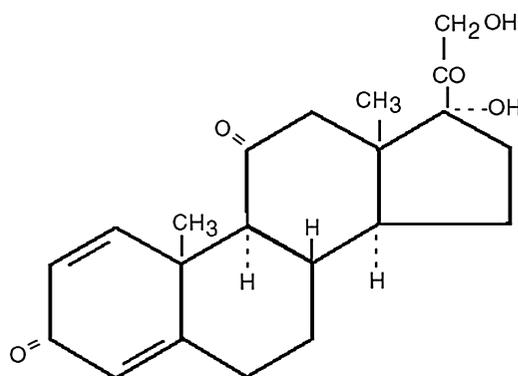


FIGURE 7. Prednisone chemical structure.

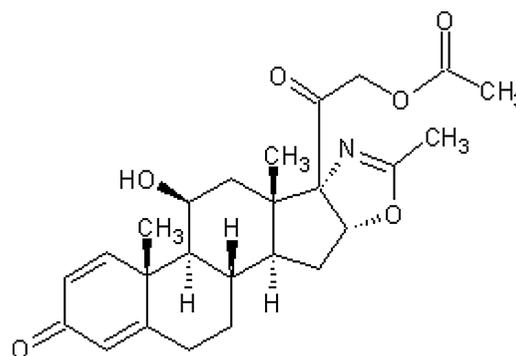


FIGURE 8. Deflazacort chemical structure.

These are probably equally effective in stabilizing muscle strength but may have different side effect profiles (for instance, deflazacort causes less weight gain).

Prednisone is used in autoimmune diseases, severe asthma, severe allergies, rheumatoid arthritis, Bell's palsy, Crohn disease, pemphigus and sarcoidosis, uveitis, and other inflammatory disease.

It is also used in various kidney diseases such as nephrotic syndrome, mononucleosis, and to prevent and treat rejection in organ transplantation. Deflazacort ( $\text{C}_{25}\text{H}_{31}\text{NO}_6$ ), an oxazoline derivative of prednisone ( $\text{C}_{21}\text{H}_{26}\text{O}_5$ ), with high immunosuppressant capacity, is a synthetic glucocorticoid that has a crucial role in the treatment of patients with autoimmune disorders associated with central nervous system or metabolic manifestations.

Anderson et al<sup>21</sup> studied the effects of deflazacort and prednisone on muscle regeneration in mdx mice during a period of 4 to 5 weeks. They tested and compared these immunosuppressant drugs evaluating their power to decrease dystrophy through inflammatory effect suppression and increasing new muscle formation after crush injuries. Deflazacort but not prednisone increased the centronucleation index of accumulated damage and repair and myotube growth over the long term. In crush-injured left tibialis anterior muscle, the fusion of proliferative muscle precursors to myotubes was increased only after deflazacort and the diaphragm muscle was much less inflamed, and fiber diameter was greater after deflazacort. The authors observed that only

deflazacort but not prednisone promoted myogenic repair over short and longer terms in addition to stimulating fiber growth.

Archer et al<sup>22</sup> treated dystrophic mdx mice for 3 weeks with placebo, deflazacort, or deflazacort plus either L-arginine or N(G)-nitro-L-arginine methyl ester (a nitric oxide synthase inhibitor). Experiments were designed to test whether treatment with deflazacort and L-arginine (a substrate for nitric oxide synthase) would change the extent of fiber injury induced by 24 hours of voluntary exercise. Deflazacort, especially combined with L-arginine, spared quadriceps muscle from injury-induced regeneration compared with placebo treatment despite an increase in membrane permeability immediately after exercise. Deflazacort alone prevented the typical progressive loss of function (measured as voluntary distance run over 24 hours) that was observed 3 months later in placebo-treated mice. Therefore, combined deflazacort plus L-arginine treatment spared mdx dystrophic limb muscle from exercise-induced damage and the need for regeneration and induced a persistent functional improvement in distance run.

St-Pierre et al<sup>23</sup> reported that activation of a JNK1 (c-Jun-N-terminal kinase 1)-mediated signal transduction cascade contributes to the progression of the DMD phenotype, in part by phosphorylation and inhibition of a calcineurin sensitive NFATc1 transcription factor. The authors, in this study, observed that 1) deflazacort treatment restored myocyte viability in muscle cells with constitutive activation of JNK1 and in dystrophic mdx mice; 2) deflazacort treatment did not alter JNK1 activity itself, but rather led to an increase in the activity of the calcineurin phosphatase and an upregulation of NF-ATc1-dependent gene expression; 3) the prophylactic effect of deflazacort treatment was associated with increased expression of NFATc1 target genes such as the dystrophin homologue utrophin; 4) the muscle-sparing effects of deflazacort were completely abolished when used in conjunction with the calcineurin inhibitor cyclosporine. The

authors conclude that deflazacort attenuates loss of dystrophic myofiber integrity by upregulating the activity of the phosphatase calcineurin, which in turn negates JNK1 inhibition of NFATc1-mediated phosphorylation and nuclear exclusion of NFATc1. The potential to increase precursor specification, strength, and possible membrane stability may be useful in directing long-term benefits for patients with DMD and short-term amplification of precursors before myoblast transfer.

Recently, Marques et al<sup>24</sup> studied deflazacort (1.2 mg/kg) in 6-month-old mdx mice for 15 months. The histomorphometric analysis demonstrated reduction of myocardial fibrosis in treated mice.

The authors concluded that long-term therapy with deflazacort is effective in slowing down the progression of fibrosis in the dystrophin-deficient heart.

## CYTOSTATICS

### Azathioprine

Azathioprine (C<sub>9</sub>H<sub>7</sub>N<sub>7</sub>O<sub>2</sub>S; Fig. 9) is a purine synthesis inhibitor used in organ transplantation, rheumatoid arthritis, pemphigus, or Crohn disease and ulcerative colitis. It is able to inhibit the proliferation of cells, in particular leukocytes, but patients will be more susceptible to infections.

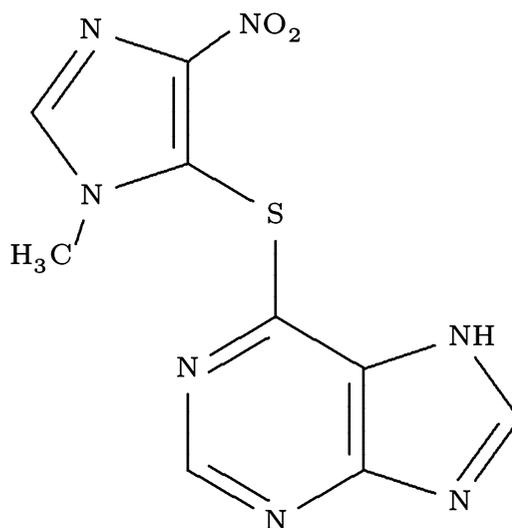


FIGURE 9. Chemical structure of azathioprine.

Weller et al<sup>25</sup> administered therapeutic doses of methylprednisolone, azathioprine, CsA, and cyclophosphamide to mdx mice aged 15 to 45 days. These doses failed to significantly influence the time course and prevalence of necrosis and regeneration or serum CK activity.

### OTHER IMMUNOSUPPRESSIVE DRUGS

#### Mycophenolate Mofetil

Mycophenolate mofetil (MMF; CellCept, Roche, Switzerland; Fig. 10) is a salt form of the immunosuppressive drug mycophenolic acid. The salt form is much better tolerated and allows good and rapid absorption by the body before it is converted to the active agent, mycophenolic acid.

Mycophenolic acid is a selective inhibitor of inosine monophosphate dehydrogenase, thereby preventing the synthesis of guanosine nucleotide and resulting in cytostatic effect on T and B lymphocytes, inhibiting proliferation, and antibody production. It is used primarily in immunosuppressive regimens to prevent rejection of allogeneic cardiac, hepatic, and renal transplants.

More recently, it has been used to treat various nontransplant-related conditions, including autoimmune skin disorders: psoriasis, atopic dermatitis, sarcoidosis, cutaneous vasculitis, and lupus erythematosus.

MMF is available in both oral and intravenous preparations.

Strober et al<sup>26</sup> tested MMF in mdx mice. Mdx mice were treated through intraperitoneal injection daily with 80 mg/kg MMF, 1 mg/kg prednisone, or vehicle. Injections were started on day of life 10 and mice were killed at 3, 4, and 5 weeks of age. The

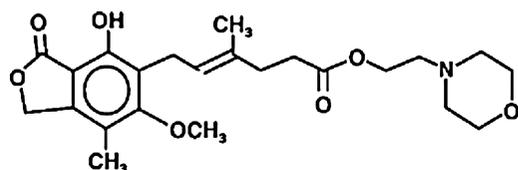


FIGURE 10. Chemical structure of mycophenolate mofetil.

diaphragm, tibialis anterior, and quadriceps muscles were removed and the sections were evaluated by an observer blinded to treatment type for necrosis, central nuclei, and inflammatory infiltrate. This study brought the following results: the MMF group showed a significantly smaller percentage of central nuclei than the control group and the prednisone-treated group for the quadriceps at 4 weeks and the tibialis anterior at 4 and 5 weeks; MMF treatment inhibited muscle degeneration in mdx mice better than steroids; a trend toward improvement in necrosis and degeneration in the quadriceps and tibialis anterior was seen but for greater significance, more samples have to be analyzed; MMF reduced the percentage of centrally located nuclei in the quadriceps and tibialis anterior muscles of mdx mice compared with mice treated with prednisone. The authors concluded that MMF, a drug with already excellent safety data in transplant patients, is a good candidate for treatment of DMD.

### IMMUNOSUPPRESSANTS IN CLINICAL TRIALS

Here are summarized the most important human clinical trials in DMD we found in the literature involving the use of immunosuppressant drugs such as deflazacort, azathioprine, prednisone, oxandrolone, tacrolimus, and CsA.

Biggar et al<sup>27</sup> compared the long-term effects of the deflazacort treatment using two treatment protocols from Naples (N) and Toronto (T). The study involved boys, aged between 8 and 15 years, with DMD who had 4 or more years of deflazacort treatment. Diagnostic criteria were proximal muscle weakness evident before 5 years and increased serum CK and genetic testing and/or a muscle biopsy consistent with DMD. Thirty-seven boys were treated with protocol-N using deflazacort at a dose of 0.6 mg/kg per day for the first 20 days of the month and no deflazacort for the remainder of the month. Boys with osteoporosis received daily vitamin D and calcium. Deflazacort treatment started between 4 and 8 years of age. Thirty-two were

treated with protocol-T using deflazacort at a dose of 0.9 mg/kg per day plus daily vitamin D and calcium. Treatment started between 6 and 8 years of age. All boys were monitored every 4 to 6 months. The results were compared with age-matched control subjects in the two groups (19 for protocol-N and 30 for protocol-T). For the boys treated with protocol-N, the authors observed that 97% were ambulatory at 9 years (control, 22%), 35% at 12 years (control, 0%), and 25% at 15 years (control, 0%). For the 32 boys treated with protocol-T, the authors reported that 100% were ambulatory at 9 years (control, 48%), 83% at 12 years (control, 0%), and 77% at 15 years (control, 0%). In boys aged 13 and older, a scoliosis of greater than 20° developed in 30% of the boys on protocol-N, 16% on protocol-T, and 90% of control subjects. For protocol-N, no cataracts were observed, whereas in protocol-T, 30% of boys had asymptomatic cataracts that required no treatment. Fractures occurred in 19% (control, 16%) of boys in protocol-N and 16% (control, 20%) of boys on protocol-T. The authors conclude that: 1) collaborative studies are important to develop treatment protocols in DMD; 2) deflazacort treatment long term has beneficial effects in both protocols; 3) the protocol-T seems to be more effective and frequently is associated with asymptomatic cataracts; and 4) alternate-day administration seems less effective than daily treatment and the long-term beneficial effects of steroid treatment in both protocols have a dose-dependent response for deflazacort.

Biggar et al<sup>28</sup> designed a study to report deflazacort long-term effects on muscle strength and side effects in DMD. The study involved 54 boys (30 treated with deflazacort), aged between 7 and 15 years with DMD, who were reviewed retrospectively. The authors observed that: 1) the boys not treated with deflazacort stopped walking at  $9.8 \pm 1.8$  years; 2) seven of 30 treated boys had stopped walking at  $12.3 \pm 2.7$  years ( $P < 0.05$ ), and of the 23 boys who were still walking, 21 were older than 10 years; 3) pulmonary function (percent predicted functional vital capacity)

was significantly greater in treated boys at 15 years ( $88\% \pm 18\%$ ) than in boys not treated ( $39\% \pm 20\%$ ) ( $P < 0.001$ ); 4) between 9 and 15 years, treated boys were shorter; 5) between 9 and 13 years, treated boys weighed less; 6) after 13 years, the treated boys maintained their weight, whereas boys not treated lost weight; 7) asymptomatic cataracts developed in 10 of 30 boys who received deflazacort; and 8) hypertension, glucosuria, acne, infection, and bruising were not more common. The authors conclude that deflazacort can preserve gross motor and pulmonary function in boys with DMD with limited side effects. Deflazacort, as this study shows, seems to have a very significant impact on health, quality of life, and healthcare costs for boys with DMD and their families and it is associated with few side effects, but it must be considered only a starting point for a future and more complete solution.

Biggar et al<sup>29</sup> compared the clinical course of 74 boys 10 to 18 years of age with DMD treated ( $n = 40$ ) and not treated ( $n = 34$ ) with deflazacort. Treated boys were able to rise from supine to standing, climb stairs, and walk 10 m without aids 3 to 5 years longer than boys not treated. After 10 years of age, treated boys had significantly better pulmonary function than boys not treated and after 15 years of age, eight of 17 boys not treated required nocturnal ventilation compared with none of the 40 treated boys. For boys older than 15 years of age, 11 of 17 boys not treated required assistance with feeding compared with none of the treated boys. By 18 years, 30 of 34 boys not treated had a spinal curve greater than 20° compared with four of 40 treated boys. By 18 years, seven of 34 boys not treated had lost 25% or more of their body weight (treated zero of 40) and four of those seven boys required a gastric feeding tube. By 18 years, 20 of 34 boys not treated had cardiac left ventricular ejection fractions less than 45% compared with four of 40 treated boys and 12 of 34 died in their second decade (mean,  $17.6 \pm 1.7$  years), primarily of cardio-respiratory complications. Two of 40 boys treated with deflazacort died at 13 and 18

years of age from cardiac failure. The treated boys were significantly shorter, did not have excessive weight gain, and 22 of 40 had asymptomatic cataracts. Long bone fractures occurred in 25% of boys in both the treated and not treated groups. The authors conclude that these long-term observations are most encouraging. The major benefits of daily deflazacort appear to be the prolonging ambulation, improved cardiac and pulmonary function, delaying the need for spinal instrumentation, and greater independence for self-feeding. Deflazacort has a very significant impact on health, quality of life, and health-care costs for boys with DMD and their families and is associated with few side effects.

Houde et al<sup>30</sup> collected data over an 8-year period for 79 patients with DMD, 37 of whom were treated with deflazacort. Deflazacort (dose of 0.9 mg/kg adjusted to a maximum of 1 mg/kg according to the side effects) was started when boys showed functional decline resulting in difficulties to ambulate. The mean length of treatment was 66 months.

Treated boys stopped walking at  $11.5 \pm 1.9$  years, whereas nontreated boys stopped walking at  $9.6 \pm 1.4$  years. Cardiac function, assessed by echocardiography every 6 to 12 months, was better preserved as shown by a normal shortening fraction in treated ( $30.8\% \pm 4.5\%$ ) versus untreated boys ( $26.6\% \pm 5.7\%$ ,  $P < 0.05$ ), a higher ejection fraction ( $52.9\% \pm 6.3\%$  treated versus  $46\% \pm 10\%$  untreated), and lower frequency of dilated cardiomyopathy (32% treated versus 58% untreated). No change was observed in blood pressure, left ventricle end-diastolic diameter, or cardiac mass. Scoliosis was much less severe in treated ( $14^\circ \pm -2.5^\circ$ ) than in untreated boys ( $46^\circ \pm -24^\circ$ ) and no spinal surgery was necessary in treated boys. Limb fractures occurred in 24% of treated and in 26% of untreated boys, whereas vertebral fractures occurred only in the treated group (seven of 37 compared with zero for the untreated group). In both groups, weight excess was observed at 8 years of age, and its frequency tripled between the ages of 8 and

12 years. More patients had weight excess in the treated group (13 of 21 [62%]) than in the untreated group (six of 11 [55%]) at 12 years of age. Cataracts developed in 49% of the treated patients and in almost all of these patients developed after at least 5 years of treatment. The authors confirmed that deflazacort use in DMD prolongs walking by at least 2 years, slows the decline of vital capacity, and postpones the need for mechanical ventilation.

Quality of life seemed improved in terms of prolonged independence in transfers and rolling over in bed as well as sitting comfortably without having to resort to surgery.

Manzur et al<sup>31</sup> realized a study to assess whether glucocorticoid corticosteroids stabilize or improve muscle strength and walking in boys with DMD. The authors collected all the randomized or quasirandomized trials involving patients with a definite diagnosis of DMD who were treated with glucocorticoids such as prednisone, prednisolone, deflazacort, or others with a minimum treatment period of 3 months. The primary observed outcome measure was the prolongation of walking (independent walking without long leg calipers). The secondary observed outcome measures were strength outcome measures, manual muscle strength testing using Medical Research Council strength scores, functional outcome measures, and adverse events. The authors identified six randomized controlled trials that met the inclusion criteria. The data from one small study used prolongation of walking as an outcome measure and did not show significant benefit. The meta-analysis of the results from four randomized controlled trials with 249 participants showed that glucocorticoid corticosteroids improved muscle strength and function over 6 months. Improvements were seen in time taken to rise from the floor (Gowers' time), 9 m walking time, four-stair climbing time, ability to lift weights, leg function grade, and forced vital capacity. One randomized controlled trial with 28 participants showed that glucocorticoid corticosteroids stabilize muscle strength and function for up to 2 years. The most effective

prednisolone regime appears to be 0.75 mg/kg per day given in a daily dose regime. Not enough data were available to compare efficacy of prednisone with deflazacort. The following adverse effects had been seen: excessive weight gain, behavioral abnormalities, cushingoid appearance, and excessive hair growth were all more common with glucocorticoid corticosteroids than placebo. Long-term adverse effects of glucocorticoid therapy could not be evaluated because of the short-term duration of the randomized studies. A number of nonrandomized studies with important efficacy and adverse effects data were tabulated and discussed. The authors concluded that there is evidence from randomized controlled studies that glucocorticoid corticosteroid therapy in DMD improves muscle strength and function in the short term (6 months to 2 years); the most effective prednisolone regime appears to be 0.75 mg/kg per day given daily; in the short term, adverse effects were significantly more common but not clinically severe; long-term benefits and hazards of glucocorticoid treatment cannot be evaluated from the currently published randomized studies; nonrandomized studies support the conclusions of functional benefits, but also identify clinically significant adverse effects of long-term treatment; these benefits and adverse effects have implications for future research studies and clinical practice.

Balaban et al<sup>32</sup> realized a study to determine and compare the long-term effects of prednisone and deflazacort on 49 boys aged 12 to 15 years with DMD over a 7-year follow-up period. Eighteen had been treated with prednisone, 12 with deflazacort, and 19 had no drug treatment. Analyzing lower and upper limb motor functions, pulmonary function, prevalence of surgery for scoliosis, and side effects, they reach these results: boys in the steroid groups were significantly more functional and performed better on all tests than boys not treated ( $P < 0.05$ ); there was no significant difference between the deflazacort- and prednisone-treated groups ( $P > 0.05$ ); the number of boys having scoliosis

surgery among the treated groups was significantly less than nontreated boys ( $P < 0.05$ ); the control group's pulmonary capacity was decreasing and significantly less than both prednisone- and deflazacort-treated boys; both deflazacort and prednisone had beneficial effects on pulmonary function and scoliosis; cataracts, hypertension, behavioral changes, excessive weight gain, and vertebral fracture were noted as serious side effects. The results of this long-term study are very encouraging and both prednisone and deflazacort seem to have a significant beneficial effect on slowing the disease progress. Their use in DMD may prolong ambulation and upper limb function with similar potency. Both steroids are also able to improve pulmonary function, in addition to delay the need for spinal interventions, with similar therapeutic profiles.

Dubowitz et al<sup>33</sup> reported a 5-year follow up of two 4-year-old boys with classic Duchenne dystrophy with an out-of-frame deletion in the Duchenne gene and absence of dystrophin in their muscle, who had a quite remarkable response to an intermittent, low-dosage regime of prednisolone (0.75 mg/kg per day for 10 days each month or alternating 10 days on and 10 days off). The authors reported that: 1) in the first case, there was complete remission of all clinical signs of dystrophy, sustained almost fully up to the present time; and 2) in the second, the initial response was almost as marked, sustained for almost 5 years before showing a fairly rapid decline over the ensuing year that resulted in loss of independent ambulation at the age of 10. Both boys remained around the 50th percentile for height and weight and showed no evidence of demineralization of bone on consecutive dual x-ray absorptiometry scanning of the spine nor any signs of chronic prednisolone toxicity. Although this study involved a limited number of patients, it showed that there may be an optimal window for treatment in the early stages of the disease and further larger-scale controlled studies should be targeted more selectively at this stage of the disease. This report also showed

that a regime of low-dosage, intermittent prednisolone, with cycles of 10 days of treatment, either per month or alternating with 10 days off treatment, is well tolerated in children affected by DMD.

Markham et al<sup>34</sup> demonstrated that treatment, with either prednisone or deflazacort, appears to have an impact on the decline in cardiac function seen with DMD and are equally effective at preserving cardiac function. Shortening fraction was significantly lower in the untreated group than in the steroid-treated group. Despite the theoretical adverse effects of steroid use to cardiac function such as obesity, ventricular hypertrophy, hypertension, and lipid abnormalities, the benefits appear to outweigh these risks. The beneficial impact of steroid treatment on cardiac function may be sustained beyond the duration of treatment prolonging survival in patients with this disease.

Griggs et al<sup>35</sup> reports a randomized controlled trial of prednisone and azathioprine with the aim to assess the longer-term effects of prednisone and to determine whether azathioprine alone or in combination with prednisone is able to improve strength. The study involved 99 boys aged 5 to 15 years affected by DMD. They were divided into three groups: placebo; 0.3 mg/kg prednisone per day; and 0.75 mg/kg prednisone per day. After 6 months, 2 to 2.5 mg/kg azathioprine per day was added into the first two groups and placebo added to the third group. The study showed that the beneficial effect of prednisone (0.75 mg/kg per day) is maintained for at least 18 months and is associated with a 36% increase in muscle mass. Weight gain, growth retardation, and other side effects were associated with prednisone and azathioprine did not have a beneficial effect. The authors concluded that prednisone's beneficial effect is not the result of immunosuppression.

Markham et al<sup>36</sup> studied the effect of steroids in cardiac function of patients with DMD. They evaluated left ventricular systolic function and cardiac geometry of those subjects through transthoracic echocardiogram; 111 patients 21 years of age or younger

affected by DMD were selected. They were divided into two groups: untreated (never exposed or treated for less than 6 months) and steroid-treated (steroids were administered longer than 6 months); subjects did not differ in age, height, weight, body mass index, systolic and diastolic blood pressure, or left ventricular mass. Among the treated patients, 29 received prednisone and 19 received deflazacort. There was no difference in the shortening fraction between the two treated subgroups. Treated subjects not receiving steroids still had a normal shortening fraction, which was no different from the shortening fraction of those still receiving treatment. The authors concluded that in DMD, cardiac function, as measured by shortening fraction, remains normal until approximately the age of 10 years in the majority of patients.

Kirschner et al<sup>37</sup> conducted a randomized, multicenter, double-blind placebo-controlled trial. One hundred fifty-three patients were randomized to receive either placebo or 4 mg/kg CsA. After 3 months, both groups received additional treatment with intermittent prednisone (0.75 mg/kg, 10 days on/10 days off) for another 12 months. In each group, 73 patients were available for intention-to-treat analysis. Baseline characteristics were comparable in both groups. There was no significant difference between the two groups concerning primary (manual muscle strength according to Medical Research Council) and secondary (myometry, loss of ambulation, side effects) outcome measures. Peak CsA values were measured blindly and ranged from 12 to 658 ng/mL (mean, 210 ng/mL) in the verum group. The authors concluded that CsA does not improve muscle strength as a monotherapy and does not improve the efficacy of intermittent prednisone in DMD.

Calcineurin inhibitors induced chronic nephrotoxicity as described by Naesens et al.<sup>38</sup> They observed that calcineurin inhibitors after renal and nonrenal transplantation alter all kidney compartments (glomeruli, arterioles, tubule interstitium).

Sharma et al<sup>39</sup> tested CsA in 15 patients affected by DMD and observed an increase in

the muscular force generation, measuring titanic force and maximum voluntary contraction (MVC) of both anterior tibial muscles. Normally tetanic force and MVC declined during 4 months in patients with DMD. During 8 weeks of CsA treatment (5 mg/kg per day), tetanic force significantly increased ( $25.8\% \pm 6.6\%$ ) and MVC ( $13.6\% \pm 4.0\%$ ) occurred within 2 weeks. Side effects from CsA, gastrointestinal and flu-like symptoms, were transient and self-limiting.

Straathof et al<sup>40</sup> retrospectively analyzed 35 DMD patients' data who were treated with 0.75 mg/kg prednisone per day intermittently 10 days on/10 days off. Prednisone was started during the ambulant phase at age 3.5 to 9.7 years (median, 6.5 years). The median period of treatment was 27 months (range, 3–123 months). The authors reported the following results: the median age at which ambulation was lost was 10.8 years (mean, 10.9 years; 95% confidence interval, 10.0–11.8 years); nine patients (26%) had excessive weight gain; eight boys (21%) had a bone fracture, which was when four of these eight children lost the ability to walk. The treatment was stopped in two obese patients, two hyperactive boys, and one patient after a fracture. Based on the previously described data, the authors conclude that prednisone 10 days on/10 days off has relatively few side effects and extends the ambulant phase by 1 year compared with historical controls.

### OXANDROLONE IN CLINICAL TRIALS

Oxandrolone (Fig. 11) is an anabolic steroid and corresponds to the chemical name of the active ingredient in oxandrin and ANAVAR. Oxandrin is a registered trademark of (Bio-Technology General Corp.; NASDAQ:BTGC) in the United States and/or other countries. ANAVAR was originally the registered trademark of Searle Laboratories. This androgenic steroid with potential neuroprotective properties is widely used to prevent muscle loss and is not immunosuppressive. Oxandrolone increased locomotor recovery concomitant with reduced

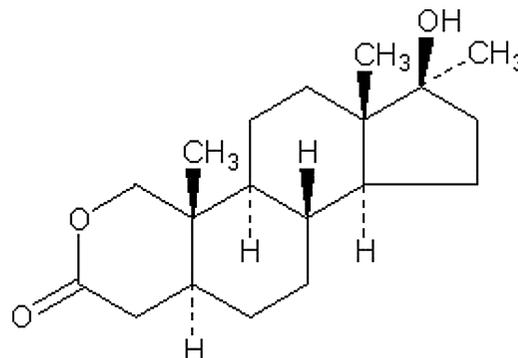


FIGURE 11. Oxandrolone chemical structure.

loss of cord tissue in a standard weight drop model of spinal cord contusion injury.<sup>41</sup> As a result of its ability to enhance skeletal muscle myosin synthesis, there is evidence in literature of some clinical trials involving its use for the treatment of DMD.

Balagopal et al<sup>42</sup> studied the mechanism of action of oxandrolone to understand if it may be beneficial in DMD altering global gene expression profile in the affected patients. The authors combined isotope studies and gene expression analysis to measure the fractional synthesis rate of MHC, the key muscle contractile protein, the transcript levels of the isoforms of MHC, and global gene expression profiles in four children (ages: 8.3, 10.4, 12.8, and 16.7 years); one of the subjects (age, 16.7 years) was wheelchair-dependent. The others were ambulatory. Gastrocnemius muscle biopsies and blood samples were collected during the course of a primed 6-hour continuous infusion of L-[U-<sup>13</sup>C]leucine on two separate occasions, before and after the 3-month treatment with oxandrolone ( $0.1 \text{ mg/kg}^{-1} \times \text{day}^{-1}$ ). Microarrays and reverse transcriptase-quantitative polymerase chain reaction were used to perform gene expression analysis. The following results were reported: MHC synthesis rate increased 42%, and this rise was accounted for, at least in part, by an upregulation of the transcript for MHC8 (perinatal MHC); gene expression data suggested a decrease in muscle regeneration as a consequence of oxandrolone therapy, presumably because of a decrease in muscle degeneration. Despite

the small sample size, this study demonstrated that the anabolic effect of oxandrolone on muscle in DMD may be mediated by a stimulation of the synthesis rate of MHC, a key contractile protein in skeletal muscle. They also observed significant augmentation of the fractional synthesis rate (FSR) of MHC in response to oxandrolone in children and changes in gene expression profile and transcript levels of isoforms of MHC. Oxandrolone showed to have an anabolic effect on DMD muscle and decrease muscle degeneration, easing the demands for muscle regeneration. A long-term therapy study with oxandrolone may prove if this steroid is able to prolong muscle function in patients affected by DMD.

Fenichel et al<sup>43</sup> treated 10 boys with DMD with oxandrolone for 3 months. They observed an improvement of  $0.315 \pm 0.097$  (mean of the changes in the average muscle score). The expected mean change in muscle score after 3 months from natural history data is a loss of 0.1. The difference of 0.415 between the actual and expected values is significant at  $P < 0.01$ . These data, although derived from a short-numbered study, suggest that oxandrolone treatment may provide functional benefit for DMD-affected children.

Following the encouraging results from a preliminary study, Fenichel et al<sup>44</sup> realized in 1997 (reported in the previous paragraph) a 6-month, randomized, double-blind, placebo-controlled study of oxandrolone in boys with an established diagnosis of DMD performed in 2001. The authors used a change from baseline to 6 months in the average muscle strength score as the primary efficacy measure. The authors reported: 1) the mean change from baseline for the oxandrolone group was +0.035; and 2) the mean change from baseline for the placebo group was -0.140. Although the oxandrolone group did not get worse and the patients taking the placebo showed some deterioration in strength, the difference was not significant ( $P = 0.13$ ). The average of the four quantitative muscle tests (QMTs) showed a significant improvement in the oxandrolone-treated boys

as compared with placebo. No adverse reactions attributable to oxandrolone were recorded. Oxandrolone did not produce a significant change in the average manual muscle strength score as compared with placebo, but the mean change in QMT was significant. The authors concluded that oxandrolone may be useful before initiating corticosteroid therapy because it is safe, accelerates linear growth, and may have some beneficial effect in slowing the progress of weakness.

## SUMMARY

The Quality Standards Subcommittee of the American Academy of Neurology and the Practice Committee of the Child Neurology Society<sup>45</sup> have developed practice parameters as strategies for the management of corticosteroid treatment in boys with DMD. The guideline authors reviewed all available research concluding that prednisone has a beneficial effect on muscle strength and function in boys with DMD and should be offered (at a dose of 0.75 mg/kg per day) as treatment. The side effects may require a decrease in prednisone to dosages of 0.3 mg/kg per day giving less robust but significant improvement. Deflazacort (0.9 mg/kg per day) can also be used for the treatment of DMD in countries where it is available. Benefits and side effects of corticosteroid therapy need to be monitored. The guideline authors emphasize that an offer of treatment with corticosteroids should include a balanced discussion on potential benefits and risks. Possible side effects of corticosteroid therapy should be closely monitored by a physician. A nutrition and exercise program may prevent some of these side effects.

Unfortunately, great concern has risen about the multiple side effects of immunosuppression with possible infections and induction of lymphomas and cancers in the long run, and multi-, low-dosage, immunosuppressive compounds might be the right answer to counteract the risk of complications.

Ideally, the best choice should enclose a mix of drugs able to preserve the existing

muscle mass function, guaranteeing homologous graft survival, and integration in the contracting network.

In the future, as a result of the great toxicities, risks, and serious side effects associated with the use of immunosuppressant drugs, it could be important to consider also hematopoietic cell transplantation as a serious alternative for the treatment of DMD. In fact, Parker et al<sup>46</sup> designed a study to determine whether allogeneic muscle progenitor cells can be successfully transplanted in an immune-tolerant recipient. The authors induced immune tolerance in two DMD-affected dogs (xmd) through HCT. Xmd dogs are a random-bred, large animal model of DMD for preclinical myogenic stem cell transplantation. Studies that are characterized by a point mutation in the consensus splice acceptor site in intron 6 of the dystrophin gene, introduced a stop codon within the modified reading frame, resulting in a near complete absence of dystrophin protein and making it an ideal model to investigate potential therapies. The authors investigated if myeloablative and nonmyeloablative HCT in xmd canines would permit donor-derived myogenic stem cells to stably engraft and restore dystrophin expression. This study showed that: 1) injection of freshly isolated muscle-derived cells from the HCT donor into either fully or partially chimeric xmd recipients restored dystrophin expression up to 6.72% of wild-type levels, reduced the number of centrally located nuclei, and improved muscle structure; and 2) dystrophin expression was maintained for at least 24 weeks. Moreover, the authors demonstrated that HCT established immune tolerance in xmd canines, permitting stable engraftment of donor muscle-derived cells in the absence of pharmacologic immunosuppression. So HCT could be a good starting point to be able in the future to restore dystrophin expression in patients with DMD and the xmd canine model could be ideal to optimize donor muscle-derived cell isolation methods and test molecules that stimulate donor cell proliferation or reprogram recipient muscle to enhance fusion.

The immunosuppressant drugs characteristics are summarized in Table 1 and Table 2.

## CONCLUSIONS

These studies and clinical trials update the immunosuppressant drugs use in DMD. Summarizing, we reached the following conclusions. Debio 025 is able to improve the structure and the function of the dystrophic mouse muscle. Those findings are encouraging for the future use of immunosuppressant drugs in the treatment of DMD. CsA is able to improve many functional, histologic, and biochemical end points associated with DMD and in the future it may contribute to a better graft survival in clinical transplantation. Calcineurin increases the expression of the markers of regeneration, in particular developmental myosin heavy chain isoform and myocyte enhancer factor 2A, and is able to significantly improve the mdx pathophysiology through its effects on muscle degeneration and regeneration and endurance capacity; etanercept use evidences a reduced breakdown of mdx skeletal muscle leading to a potential application to muscular dystrophies in the near future. Infliximab seems to greatly reduce the breakdown of dystrophic muscle and the necrosis and the dystropathology without adverse effect on new muscle formation. Deflazacort, but not prednisone, promotes myogenic repair over short and longer terms and is able to stimulate fiber growth. Some studies evidence that both seem to have a significant beneficial effect on slowing DMD progress. Their use may prolong ambulation and upper limb function. Both steroids are also able to improve pulmonary function in addition to delay the need for spinal interventions with similar therapeutic profiles. Deflazacort shows very important improvements in walking by at least 2 years, slows the decline of vital capacity, and postpones the need for mechanical ventilation in patients with DMD. Deflazacort has also got a very significant

TABLE 1. Immunosuppressant Therapeutic and Side Effects in Duchenne Muscular Dystrophy (DMD) Studies

Immunosuppressant Drugs	Therapeutic Effect	Side Effects on DMD Studies
Debio 025	Reduces necrotic disease manifestation; partially protected the diaphragm and soleus muscles against necrosis	Not investigated
Cyclosporine A (CsA)	Significant reduction of degenerating areas and a reduced percentage of nonmuscle tissue replacing muscle fibers with a greater preservation of viable contractile material	Liver damage, nephrotoxicity, high blood pressure, hirsutism, emesis, gingival overgrowth
Etanercept	Reduced amount of skeletal muscle breakdown	Redness, itching, pain, or swelling at the injection site; colds; cough; headache; nausea
Infliximab	Reduced breakdown of dystrophic muscle, necrosis and the dystropathology; it has no adverse effect on new muscle formation	Upper respiratory tract infections, urinary tract infections, cough, rash, back pain, nausea, vomiting, abdominal pain, headache, weakness and fever, low or high blood pressure
Prednisone	Improves strength and function; preserves cardiac function	Cataracts, hypertension, behavioral changes, excessive weight gain, vertebral fracture, growth retardation
Deflazacort	Increases myotube growth over the long term, promotes myogenic repair over short and longer terms, prevents the typical progressive loss of function; preserves cardiac function; prolongs walking by at least 2 years, slows the decline of vital capacity, and postpones the need for mechanical ventilation	Cataracts, hypertension, behavioral changes, excessive weight gain, vertebral fracture, behavioral abnormalities, cushingoid appearance and excessive hair growth, respiratory insufficiency
Azathioprine Mycophenolate mofetil (MMF)	No sufficient therapeutic effect Inhibits muscle degeneration	Weight gain, growth retardation Not investigated
Tacrolimus (FK-506)	Administered after transfer of dystrophin gene AV-mediated, it reduces immune response	Not investigated

**TABLE 2.** Immunosuppressant Drug Target and Clinical Trial Evidence

Immunosuppressant Drugs	Drug Target	Test in mdx Mice	Human Clinical TRIALS
Debio 025	High inhibitory potency against cyclophilin A	+	–
Cyclosporine A (CsA)	Inhibits calcineurin	+	+
Calcineurin	Activates of Type I and IIa myosin heavy chain (Mhc), oxidative enzyme and utrophin A genes; also acts through myocyte-enhancing factor 2 (MEF2) dependent transcription		
Etanercept	Blocks TNF $\alpha$	+	–
Infliximab	Blocks TNF $\alpha$	+	–
Prednisone	Glucocorticoid receptor	+	+
Deflazacort	Glucocorticoid receptor	+	+
Azathioprine	Purine synthesis inhibitor; inhibits proliferation of leukocytes	+	+
Mycophenolate mofetil (MMF)	Inhibits inosine monophosphate dehydrogenase, preventing synthesis of guanosine nucleotide and inhibiting proliferation of T and B lymphocytes	+	–
Tacrolimus (FK-506)	Inhibits transcription of early T-cell activation gene, IL-2, TNF $\alpha$ and proto-oncogenes; suppressing expression of IL-2 and IL-7 receptor	+	+

TNF $\alpha$ , tumor necrosis factor alpha; IL, interleukin.

impact on health, quality of life (prolonged independence in transfers and rolling over in bed as well as sitting comfortably without having to resort to surgery), and healthcare costs for patients with DMD and their families, but it has to be considered only a start point to a future and more complete solution. MMF treatment inhibits muscle degeneration in mdx mice better than steroids. MMF shows improvements in necrosis and degeneration in the quadriceps and tibialis anterior muscles of mdx mice better than steroids. MMF is already used safely in transplant patients and it could be one of the best candidates for DMD treatment.

Lately, it has been hypothesized that treatment of mdx mice with inhibitors of the proteasomal pathway could potentially rescue the expression level and localization pattern of dystrophin and dystrophin-associated proteins. In fact, there is evidence that the loss of dystrophin in muscle cells derived from both mdx mice and DMD patients may render the dystrophin-glycoprotein complex

(DGC) more susceptible to proteolytic degradation<sup>47</sup> and that the proteasomal pathway is involved in the pathogenesis of various muscle diseases.<sup>48,49</sup> An experimental study<sup>50</sup> shows that in vivo administration of MG-132, an inhibitor of the proteasomal pathway, effectively restores the expression levels and the localization pattern of dystrophin and of dystrophin-associated proteins, normally absent or greatly reduced in mdx mice skeletal muscles. These findings have been later confirmed by the same research group<sup>51</sup> showing that treatment with MG-132 is able to rescue the expression of dystrophin,  $\beta$ -dystroglycan, and  $\alpha$ -sarcoglycan in skeletal muscle explants from patients with Duchenne or Becker muscular dystrophy. The important results obtained from mdx mice and from DMD patients led this research group to test 2 new dipeptide boronic acid inhibitors blocking the proteasomal-dependent degradation pathway, that is, Velcade (bortezomib or PS-341; Food and Drug Administration approved for treatment of multiple myeloma) and MLN273 (PS-273). They injected Velcade and MLN273 into the mdx mice gastrocnemius muscle,

observing the rescue of the expression of  $\alpha$ -dystroglycan,  $\beta$ -dystroglycan,  $\alpha$ -sarcoglycan, and dystrophin. Moreover, localized treatment with Velcade and MLN273 reduced the activation of nuclear factor-kappaB (NF $\kappa$ B), which is involved in a pathway linked to inflammation responses in myopathies and DMD.<sup>52</sup> A recent study<sup>53</sup> has shown that Velcade, a drug that selectively blocks the ubiquitin-proteasome pathway, when systemically administered in mdx mice over a 2-week period, restores the membrane expression of dystrophin and DGC members and improves the dystrophic phenotype. Moreover, the same study has shown that treating with the same compound explants from muscle biopsies of DMD or BMD patients, dystrophin,  $\alpha$ -sarcoglycan, and  $\beta$ -dystroglycan protein levels are upregulated in explants from BMD patients, whereas the proteins of the dystrophin glycoprotein complex, in DMD cases, have increased. The ubiquitin-proteasome pathway blocker Velcade may have, in the future, important clinical implications for the pharmacological treatment of muscular dystrophy in humans, although further studies are required.

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