From Basic Research to Clinical Trials: Preclinical Trial Evaluation in Mouse Models

Sasha Bogdanovich[1,3] and Emidio E. Pistilli[2]
[1]University of Pennsylvania School of Medicine and Pennsylvania Muscle Institute,
Department of Physiology, Philadelphia, Pennsylvania
[2]West Virginia University School of Medicine,
Division of Exercise Physiology, Morgantown, West Virginia
[3]Current address: University of Kentucky College of Medicine,
Department of Ophthalmology and Visual Sciences, Lexington, Kentucky
USA

1. Introduction

Duchenne Muscular Dystrophy (DMD) is a fatal, X chromosome-inherited disease which affects the whole world population equally and has an incidence of 1 in 3500 boys. The disease is progressive in nature with the first signs of muscle wasting appearing as early as age 3 (Dubowitz 1975; Jennekens et al. 1991). The disease slowly weakens the skeletal muscles of the arms and legs (mostly muscles of shoulder and pelvic girdles) and abdomen. By the early teens, heart and respiratory muscles may also become affected. Nearly all children with DMD lose the ability to walk between the age of 7 and the early teen years, with activities involving the arms, legs or trunk requiring assistance or mechanical support. Patients are typically confined to a wheelchair and they rarely survive the fourth decade of their life. Most DMD patients die due to respiratory or cardiac failure because of the progressive damage to the diaphragm or cardiac myopathy. A milder form of muscular dystrophy, termed Becker Muscular Dystrophy (BMD), has been characterized with a milder clinical presentation (Becker 1955). Children with this variation remain physically active and independent later in life compared to DMD patients. Symptoms begin to appear after 20 years of age and patients live longer compared to DMD patients.

Histologically, muscles from DMD patients are characterized by increased variation in muscle fiber size, necrosis of individual muscle fibers and replacement of necrotic fibers by fibrofatty tissue (Emery 1995; Engel 1994). In addition, an increase in serum Creatine Kinase (CK), derived from degenerating muscle fibers, has been recognized as one of the main diagnostic characteristics of the disease (Engel 1994; Guyton 1995). In 1983, Kay Davies of London, England found linkage between a DNA marker and the DMD gene located on the short arm of the X chromosome (Xp21) (Davies et al. 1983). This discovery finally confirmed the long time theory that DMD inheritance is through the X chromosome and explained Duchenne's notes of affected boys while girls remained without symptoms.

The culmination of DMD research occurred in 1986 when Louis Kunkel of Boston, United States of America (USA) isolated and cloned the gene which caused DMD / BMD (Kunkel et al. 1986). One year later (1987) Eric Hoffman from the same laboratory identified the protein product of the DMD / BMD gene (Hoffman et al. 1987). This protein was called dystrophin. The discovery of the dystrophin gene mutation as the cause of DMD opened the door for diagnostic development and therapeutic strategies for this disease (Bogdanovich et al. 2004).

1.1 Dystrophin

Disease symptoms are progressive and characterized by quantitative and qualitative changes in dystrophin protein (Brown 1997; Khurana et al. 1990). The dystrophin gene is located on the short arm of chromosome Xp21 and is a large gene comprised of 79 exons. With a total size of 3Mb, the gene represents about 0.1% of the entire human genome. The dystrophin protein consists of 3645 amino acids (AA) and has a molecular mass of 426 kDa (Coffey et al. 1992; Monaco et al. 1986). The molecule can be divided into 4 parts: an N-terminal actin binding domain, a central rod, a cysteine rich segment and a C-terminal end (Einbond & Sudol 1996; Koenig et al. 1988; Ponting et al. 1996; Roberts et al. 1996;). Dystrophin forms a link between the extra and intra-cellular cytoskeleton, and it is hypothesized that dystrophin acts to neutralize stressful events that come from outside the cell toward the intracellular matrix, although the full function of dystrophin is still unknown. With a lack of dystrophin protein, the sarcolemma is susceptible to contraction-induced ruptures and permanent cell damage is inevitable (Wrogemann & Pena 1976). Previous experiments have demonstrated an influx of extracellular Ca^{2+} or sarcoplasmatic reticulum leakage due to membrane disruption, which can result in proteolitic activities by Ca^{2+} dependent proteases. This cycle leads to necrosis, inflammatory cell infiltration, and phagocytosis. Additionally, the presence of mast cells can stimulate the release of basic fibroblast growth factor (bFGF) which contributes to fibrotic changes in dystrophic muscle.

The clinical presentation of DMD depends on the nature of the *dystrophin* gene mutation mutation. Nonsense mutations in the *DMD* gene lead to a premature stop codon that blocks dystrophin translation (Hoffman et al. 1987). In the case of a frame shift mutation, there is an exchange of one nucleotide base with another, resulting in the synthesis of shorter or longer dystrophin variants. These mutations result in a milder clinical presentation, namely BMD, where expression of dystrophin protein is present but reduced (Monaco et al. 1988). Dystrophin mutations can be localized in any of its 4 parts and the mutation localization is in direct correlation with the severity of clinical disease presentation. The most severe cases of DMD are described with mutations in the cystein rich component because of its multiple functions (Beggs et al. 1991; Koenig et al. 1989), while mutations in the other 3 parts result in milder clinical presentation.

Disease diagnosis starts with taking a careful history of the disease as well as laboratory testing. The level of CK in the blood is significantly higher in DMD patients, especially at the beginning of the disease, where it can often be 10 times higher compared to normal (Engel 1994). Many advantages are achieved by genetic analysis of DMD mutations. It is possible to discover mutations in the DMD gene using polymerase chain reaction (Flanigan et al. 2003; White et al. 2002). These methods are used routinely in prenatal diagnosis of male fetuses. Typically, DMD patients become wheelchair bound before 13 years of age, while BMD patients can remain physically active after 16 years of age. More invasive methods such as

electrodiagnostics and muscle biopsy are used in cases of negative genetic analysis, for the differential diagnosis between DMD and BMD, and especially in autosomal disorders such as Limb-Girdle Muscular Dystrophy, where big differences between clinical presentations and laboratory testing do not exist (Laval & Bushby 2004). In such cases, muscle biopsy samples are used for histopathology and immunohistochemistry examination.

2. Mouse models of DMD

The identification and utilization of animal models in biomedical research is a necessary step in evaluating disease pathology and designing effective therapies for that disease (Table 1). In 1984, a mouse model of DMD was identified and has been termed the *mdx* mouse (*mdx* = muscular disease x-chromosome). The identification of a mouse model for DMD proved to be useful for further understanding of both the normal function of dystrophin and the pathology of the disease (Petrof et al. 1993; Stedman et al. 1991), and the *mdx* mouse currently remains the most widely used mouse model of DMD (Brockdorff et al. 1987; Cavanna et al. 1988). *Mdx* mice have a natural mutation in the dystrophin gene, caused by a point mutation. Compared to human DMD patients, *mdx* mice have a relatively mild disease presentation, characterized by periods of muscle fiber degeneration and regeneration starting approximately 2 weeks after birth although not all muscles are similarly affected. Despite the milder disease phenotype in these mice, the characteristic cycles of muscle fiber degeneration and regeneration provide a variable to evaluate therapies in this mouse model. In addition, biochemical analyses have demonstrated that there is a consistent increase in serum CK in the *mdx* mouse, mirroring an important biomarker in human patients (Bulfield et al. 1984; Moens et al. 1993). Inasmuch as there are recognized limitations of this mouse model, the *mdx* mouse remains a widely used animal in biomedical research.

The *mdx* mouse is very suitable for experiments designed to elucidate function and causality of the disease, as well as for gene and pharmaceutical therapy. It is in fact proving to be useful for furthering our understanding on both the normal function of dystrophin and the pathology of the disease. Experiments using *mdx* mice have provided us with invaluable information regarding the function of the gene product involved in DMD as well as the dystrophin-associated proteins (DAP) and the dystroglycan/sarcoglycan complex (DGC/SGC).

Additional allelic variants of the original *mdx* mouse mutation were created by treating mice with the mutagen, N-ethylnitrosourea (Rafael et al. 2000). This resulted in the formation of 4 new mouse models with specific mutations in the dystrophin gene and relevant increases in circulating CK levels in the blood. These mouse models have been described previously (Chapman et al. 1989; Cox et al. 1993; Im et al. 1996). Briefly, the *mdx*[2cv] mouse has a mutation in intro 42; the *mdx*[3cv] mouse has a mutant splice acceptor site in nintron 65; the *mdx*[4cv] mouse has a "C" to "T" substitution in exon 53; and *mdx*[5cv] has an "A" to "T" transition in exon 10. Due to the different sites of these point mutations, different dystrophin isoforms can be expressed in the form of revertant fibers. It has been proposed that these strains of *mdx* mice may be usefule to elucidate the role of these various dystrophin isoforms, although the original *mdx* variant remains the mouse model most utilized (Banks & Chamberlain 2008).

The mild phenotype of *mdx* mice is a recognized limitation of this mouse model. For example, *mdx* mice maintain cage activity and do not have significant exercise limitations (De Luca et al. 2003). Indeed, forced treadmill exercise has been used as a way to increase the degree of muscle pathology present in these mice (Fraysse et al. 2004). Also, there is only

approximately a 20% difference in the lifespan of the *mdx* mouse compared to wild-type, thus limiting the ability to detect a therapy-based improvement on lifespan (Chamberlain et al. 2007). For these reasons, a more severe mouse model was developed that introduced the knockout of the utrophin gene into the *mdx* mouse; thus creating the dystrophin:utrophin double knockout mouse (DKO) (Deconinck et al. 1997, 1998; Grady et al. 1997; Huang et al. 2011). These mice have a severely limited lifespan coupled with severe impairments in muscle function (Chamberlain et al. 2007).

Most commonly used mouse models in muscular dystrophies		
Muscular Dystrophy	**Gene product**	**Mouse model**
Duchenne/Becker MD	Dystrophin	*mdx*, *mdx²⁻⁵ᶜᵛ*, *mdx:utr⁻ᐟ⁻*
Limb-Girdle MD		
Type 1C	Caveolin 3	*cav3⁻ᐟ⁻*
Type 2A	Calpain 3	*capn3⁻ᐟ⁻*
Type 2B (Miyoshi myopathy)	Dysferlin	SJL, A/J
Type 2C	*γ*-Sarcoglycan	*Sgcg⁻ᐟ⁻*
Type 2D	*a*-Sarcoglycan (Adhalin)	*sgca⁻ᐟ⁻*
Type 2E	*β*-Sarcoglycan	*sgcb⁻ᐟ⁻*
Type 2F	*δ*-Sarcoglycan	*sgcd⁻ᐟ⁻*
Congenital MD (CMD)		*dy*, *dy²ʲ*, *dy³ᵏ*, *dyʷ*, *dyᴾᴬˢ*

Table 1. Mouse models in MDs

3. Current progress and evaluation

It has recently been proposed that a set of standard operating procedures be established for evaluating pre-clinical testing data in *mdx* mice (Grounds et al. 2008; Nagaraju & Willmann 2009; Spurney et al. 2009; Willmann et al. 2011). Through the universal adoption of standardized laboratory assays, the results of multiple pre-clinical trials performed in independent laboratories could be evaulated. The laboratory assays that we have employed in designing our new scaling system have been identified as robust tests for evaluating endpoints in the *mdx* mouse (Spurney et al. 2009). Before introducing the new scaling methodology, we will review the results of a number of preclinical trials performed in the *mdx* mouse. The data from these trials will then be utilized in presenting and evaulating the new scaling system, we call the Multiparametric Muscle Improvement Score (MMIS). These preclinical trials use a variety of parameters to establish the beneficial effect of the administered compound, and can be catogorized as either functional, morphological, or biochemical. Functional evaluation was done using a specially designed system to quantify *ex vivo* isometric and eccentric contractions (ECC) in freshly dissected muscle. Morphologic measures were quantified from hematoxylin and eosin (H&E) or immuno-stained muscle sections, and included counting of total muscle fibers, single fiber cross-sectional area, and the percentage of centrally nucleated fibers. Determination of serum creatine kinase and up-regulation of utrophin constituted the biochemical evaluation. Improvement in any of these individual parameters would suggest a therapy-based improvement in the dystrophic phenotype in *mdx* mice. However, some parameters are suggestive of more significant clinical improvement (i.e. greater muscle force, lower serum CK) and are therefore given more weight in our scaling system.

Preclinical trials were performed to determine the extent that small molecule therapies could up-regulate utrophin, and therefore functionally compensate for the loss of dystrophin in the muscles of *mdx* mice. These small moleculaes included heregulin (Krag et al. 2004) and biglycan (Amenta et al. 2011). In general, these utrophin-based up-regulation strategies resulted in very similar benefits in the *mdx* mouse. Parameters that were improved included isometric force following ECC, increased regenerative capacities of *mdx* muscle (i.e. greater number of regenerative fibers), and less necrotic areas in the diaphragm muscle. However, these strategies did not show improvements isometric force producing capacity of limb muscles or the levels of serum creatine kinase. The improvements in the ECC force coupled with the up-regulation of utrophin demonstrate that utrophin can functionally substitute for the loss of dystrophin. However, the incomplete amelioration of the dystrophic phenotype suggests that these therapies have limitations when administered individually.

A number of preclinical studies were performed to analyze the effects of blocking the activity of myostatin, a negative regulator of muscle growth and member of the TGF-β family (McPherron et al. 1997; McPherron & Lee 1997). Strategies included using antibodies directed against myostatin and administration of the myostatin propeptide, to sequester circulating myostatin and neutralize its activity. Administration of these compounds to *mdx* mice resulted in significant improvements in overall body and skeletal muscle mass. Muscle mass increases were greater in response to the myostatin propeptide, and may be due to a higher binding affinity for myostatin compared to the myostatin antibody (Bogdanovich et al. 2002, 2005). In addition, muscle function was improved as evidenced by greater absolute forces in muscles from treated mice in both trials. However, the propeptide-based strategy also resulted in improvements in specific force (i.e. force normalized to muscle cross-sectional area). Both strategies demonstrated improvements in overall muscle histopathology and the levels of serum creatine kinase, suggesting an improvement in the sarcolemma by a utrophin independent mechanism. Collectively, these myostatin blockade strategies were effective in stimulating increases in muscle mass and muscle function, along with measures of muscle histopathology and serum CK. However, complete amelioration of the dystrophic phenotype was not accomplished due to the fact that no improvements were noted in the force loss following eccentric lengthening contractions.

Furthermore, we wanted to determine whether myostatin blockade would be beneficial to additional mouse models of muscular dystrophy. We utilized the myostatin-antibody strategy in gamma sarcoglycan (*Sgcg$^{-/-}$*) knockout mice, a model of Limb-Girdle 2C muscular dystrophy (LGMD 2C) (Bogdanovich et al. 2008). Interestingly, myostatin antibody blockade did not show desired improvement in this mouse model. Improvement was minimal and evident in body and muscle weight increases. Physiological improvement was notable only in one parameter, absolute force improvement. Histopathological and biochemical parameters were unchaged when compared to control mice. Therefore, unique disease characteristic of LGMD 2C compared to DMD in mouse models may have led to these differing results and a preferential benefit in the *mdx* mouse.

Recently, we tested the efficacy of a novel myostatin blockade strategy using a soluble form of the activin type IIB receptor (ActRIIB) (Pistilli et al. 2011). The ActRIIB is the receptor for myostain as well as other member of the TGF-β superfamily. The solubilized form of the receptor (sActRIIB) would be able to bind to and sequester multiple TGF-β superfamily members, thereby potentially providing a greater therapeutic effect. In this preclinical trial, two doses of sActRIIB were utilized, a low dose of 1,0 mg/kg bodyweight and a high dose 10,0 mg/kg bodyweight. Notable differences were observed when comparing these two

dosing strategies. The high dose of sActRIIB resulted in dramatic increases in body weight and lean muscle mass, while minimal changes in overall body mass were noted in the response to the low dose. Both doses of sActRIIB improved absolute forces produced by limb muscles. However, the low dose significantly improved specific force, indicating an improvement in force producing capacity independent of muscle size. Serum CK levels were also lower in sActRIB treated mice. Unfortunately, force loss follwing eccentric lengthening contractions and muscle histopathology were not significantly improved in these trials. Therefore, as with the myostatin antibody and the myostatin propeptide, complete amelioration of the dystrophic phenotype was not observed with sActRIIB therapy.

The results of these trials demonstrate that small molecule-based therapies have the potential to improve a number of paremeters related to the dystrophic phenotype in *mdx* mice. However, as noted, no therapy has been able to completely ameliorate the phenotype and rescue the *mdx* mouse. Also, evaluating the therapeutic efficacy of drugs identified through preclinical trials is inefficient, due to the large number of preclinical trials published

Multiparametric Muscle Improvement Score -MMIS	
Bogdanovich / Khurana points	
Body weight (g)	1
Muscle weights (mg)	2
Absolute force, twitch (mN)	1
Specific force, twitch (mN/mm^2)	3
Absolute force, tetanus (mN)	4
Specific force, tetanus (mN/mm^2)	5
Eccentric contractions improvement	5
Centrally nucleated fibers	5
Loss of fibrotic changes	3
Decreased CK value	5
Total score	**34**

Total umeric score: 34
01-08 no improvement
09-22 intermediate
23-34 optimal

Table 2. Multiparametric Muscle Improvement Score

in animal models, and the differential methodology used to evaluate the data. Therefore, a scaling system that can evaluate the therapeutic efficacy of independent preclinical trials would be useful to identify those compounds with the most therapeutic promise. The purpose of this research was to formulate a single, objective scoring system to evaluate preclinical trial data arising from multiple laboratories. More detailed and precise quantification of different therapies can be obtained by utilizing the Multiparametric Muscle Improvement Score (MMIS) system (Bogdanovich 2009).

4. Methods

The authors have complied preclinical trial data acquired during the last 10 years, and evaluated the data using the MMIS scoring system (Bogdanovich 2009). This system consists of ten of the most important anatomical, physiological and biochemical elements directly related to improvement of the dystrophic phenotype in mouse models of muscular dystrophy. These include: body and muscle weight changes, muscle force production during isometric and lengthening contractions, evidence of histological improvement, and reductions in circulating creatine kinase (see Table 2). These elements are weighted with one single numerical value from 1 (least important) to 5 (most important). Only statistically significant improvements in measured parameters can be scored. Scoring is done in such a way that every parameter receives maximal value if there is improvement. Scored elements are summarized at the end and the final number is the improvement score.

Comparison of possible therapeutic strategies for muscular dystrophies (before MMIS system)							
Mice	mdx	mdx	Sgcg^{-/-}	mdx		mdx	mdx
Treatment	**Myostatin blockade or inhibition**					**Utrophin upregulation**	
	Antibody	Propeptide	Antibody	sActRIIB		Heregulin	Biglycan
				1mg/kg	10mg/kg		
	Bogdanovich et al. 2002 (Nature)	Bogdanovich et al. 2005 (FASEB J)	Bogdanovich et al. 2007 (Muscle&Nerve)	Pistilli et al. 2011 (Am. J. Pathol.)		Krag et al. 2004 (PNAS)	Amenta et al. 2010 (PNAS)
Body weight (g)	+	+	+	-	+	-	-
Muscle weights (mg)	+	+	+	+	+	-	-
Absolute force, twitch (mN)	+	+	+	+	+	-	-
Specific force, twitch (mN/mm^2)	-	-	-	+	-	-	-
Absolute force, tetanus (mN)	+	+	+	+	+	-	-
Specific force, tetanus (mN/mm^2)	-	+	-	+	-	-	-
Eccentric contractions	-	-	-	-	-	+	+
Centrally nucleated fibers	+	-	-	-	-	+	+
Loss of fibrotic changes	+	+	-	-	-	+	+
Decreased CK value	+	+	-	+	+	-	-

Legend: Sgcg$^{-/-}$, gamma sarcoglycan (LGMD 2C mouse model); -, no improvement; +, positive improvement

Table 3. Evaluation of possible therapeutic stratagies for muscular dystrophy (before MMIS system)

5. Results

The scoring system is objective and excludes human error. It is possible to review and rank therapies based on a final single numeric score using the MMIS scale. With the MMIS scale, we have objectively assessed the therapeutic efficacy of multiple drug therapies in mouse models of muscular dystrophy (*mdx* mouse, *Sgcg-/-* mouse) (Table 3, 4). However, the limitations of these promising methods were identified objectively using the MMIS scale. Despite significant effects on muscle mass and muscle force production, neither strategy completely ameliorated the dystrophic phenotype with regards to eccentric lenghtening contractions or histological improvement as identified using the MMIS scale.

Comparison of possible therapeutic strategies for muscular dystrophies (MMIS system)						
Mice	*mdx*	*mdx*	*Sgcg-/-*	*mdx*		*mdx*	*mdx*
	M y o s t a t I n b l o c k a d e o r i n h i b i t i o n					**Utrophin upregulation**	
	Antibody	*Propeptide*	*Antibody*	*sActRIIB* 1mg/kg 10mg/kg		Heregulin	*Biglycan*
Treatment	Bogdanovich *et al.* 2002 (Nature)	Bogdanovich *et al.* 2005 (FASEB J)	Bogdanovich *et al.* 2007 (Muscle&Nerve)	Pistilli *et al.* 2011 (Am. J. Pathol.)		Krag *et al.* 2004 (PNAS)	Amenta *et al.* 2010 (PNAS)
Body weight (g)	1	1	1	0	1	0	0
Muscle weights (mg)	2	2	2	2	2	0	0
Absolute force, twitch (mN)	1	1	1	1	1	0	0
Specific force, twitch (mN/mm²)	0	0	0	3	0	0	0
Absolute force, tetanus (mN)	4	4	4	4	4	0	0
Specific force, tetanus (mN/mm²)	0	5	0	5	0	0	0
Eccentric contractions	0	0	0	0	0	5	5
Centrally nucleated fibers	5	0	0	0	0	5	5
Loss of fibrotic changes	3	3	0	0	0	3	3
Decreased CK value	5	5	0	5	5	0	0
Total score	**21**	**21**	**8**	**20**	**13**	**13**	**13**

Legend: *Sgcg-/-*, gamma sarcoglycan (LGMD 2C mouse model) ; Total numeric score: 34; 01-08 no improvement; 09-22 intermediate; 23-34 optimal

Table 4. Possible therapeutic stratagies for muscular dystrophy (MMIS system)

6. Conclusion

Currently, there is a need for standardization of measurement and objective evaluation of different preclinical studies of the muscular dystrophies, which would allow for a better understanding of the disease and its response to potential therapies. We suggest that the MMIS provides a single numeric value useful for cross-comparing different preclinical studies and prioritizing drug development for muscular dystrophy therapy. We suggest that the use of the MMIS scale will allow for precise and rigorous evaluation of functional improvements of therapeutic interventions performed in preclinical trials.

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8. References

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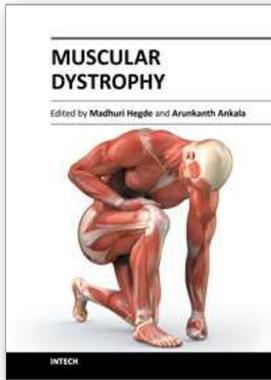
Muscular Dystrophy

Edited by Dr. Madhuri Hegde

With more than 30 different types and subtypes known and many more yet to be classified and characterized, muscular dystrophy is a highly heterogeneous group of inherited neuromuscular disorders. This book provides a comprehensive overview of the various types of muscular dystrophies, genes associated with each subtype, disease diagnosis, management as well as available treatment options. Though each different type and subtype of muscular dystrophy is associated with a different causative gene, the majority of them have overlapping clinical presentations, making molecular diagnosis inevitable for both disease diagnosis as well as patient management. This book discusses the currently available diagnostic approaches that have revolutionized clinical research. Pathophysiology of the different muscular dystrophies, multifaceted functions of the involved genes as well as efforts towards diagnosis and effective patient management, are also discussed. Adding value to the book are the included reports on ongoing studies that show a promise for future therapeutic strategies.

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