Pre-clinical drug tests in the mdx mouse as a model of dystrophinopathies: an overview

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Duchenne muscular dystrophy is a lethal X-linked muscle disease affecting 1/3500 live male birth. It results from defects in the sub-sarcolemmal protein dystrophin, a component of the dystrophin-glycoprotein complex (DGC) which links the intracellular cytoskeleton to the extracellular matrix. The absence of dystrophin leads to muscle membrane fragility, muscle necrosis and gradual replacement of skeletal muscle by fat and connective tissue, through a complex and still unclear cascade of interconnecting events. No cure is currently available, with glucocorticoids being the sole drugs in clinical use in spite of their remarkable side effects. A great effort is devoted at performing pre-clinical tests on the mdx mouse, the mostly used homologous animal model for DMD, with the final aim to identify drugs safer than steroids and able to target the pathogenic mechanisms so to delay pathology progression. This review updates the efforts on this topic, focusing on the open issues about the animal model and highlighting the classes of pharmaceuticals that are more promising as disease-modifiers, while awaiting for more corrective therapies. Although caution is necessary in data transfer from mdx model to DMD patients, the implementation of standard operating procedures and the growing understanding of the pathology may allow a more accurate evaluation of therapeutics, alone or in combination, in pre-clinical settings. A continuous cross-talk with clinicians and patients associations are also crucial points for proper translation of data from mouse to bedside.

Key words: Duchenne muscular dystrophy, mdx mouse model, pharmaceuticals, pre-clinical studies, translational research

Dystrophinopathies

Dystrophinopathies are due to defects in the dystrophin gene on the X chromosome, with Duchenne muscular dystrophy (DMD) being the most common and severe form, affecting approximately 1/3500 male birth (1). Dystrophin is a subsarcolemmal protein linking the intracellular cytoskeleton to the extracellular matrix

via the interaction with glycoproteins, in the so called dystrophin-glycoprotein complex (DGC). The absence of dystrophin, as in DMD, leads to destruction of the DGC, with the loss of mechanical stability and of proper mechano-transduction signalling. In fact dystrophin-deficient myofibres are more susceptible to contraction injury with consequent myofibre necrosis and ultimately the replacement of myofibres by fibrous and fat tissue; a progressive failure of regeneration efficiency also occurs (1). No cure is currently available, and current patient's standard care includes different approaches with a mean life expectancy around 30s (2). In this frame, glucocorticoids are the sole drugs clinically used to delay pathology progression, in spite of their remarkable side effects (1, 2). Efforts are devoted toward strategies to restore the expression of full-length or short-form of dystrophin via exon-skipping, stem cells or small molecules able to force read-through premature stop-codon mutations (see 3 for review). Other promising approaches include small molecules able to enhance the dystrophin surrogate utrophin, and stabilize or reduce degradation of DGC (3-5). Although these approaches have been validated by extensive pre-clinical investigation, they will not be covered by the present review, that instead is focused on a parallel valuable strategy, i.e. the pre-clinical studies to identify pharmaceutical compounds, novel or repurposed, with a better safety profile with respect to corticosteroids and having a similar or greater efficacy as disease modifiers. This approach is hindered by the complex cascade of pathological events whose causal and temporal occurrence is still unclear. The extensive pre-clinical studies on the mdx mouse have then the dual aim to identify candidate key events that may be targeted by drugs and to evaluate potential efficacy of pharmaceuticals

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upon sub-chronic and chronic treatments. A large plethora of data have been obtained so far with identification of promising, yet difficult to prioritarize, strategies. Key issues and results are described below.

The mdx mouse: standard operating procedures for pre-clinical tests

The mdx mouse has a premature stop codon mutation on exon 23 of the dystrophin gene, leading to a lack of the mature protein. The absence of dystrophin results in an acute onset of skeletal muscle necrosis around 3 weeks of post-natal life, followed by an extensive period of degeneration and regeneration until necrosis gradually decreases and a relatively low level is reached in adult mice (3-4 months) with pathology stabilization. The pathology is far more benign than in DMD, and cardiomyopathy and fibrosis appear only in very late stage of the disease. The benign phenotype of the mdx mouse raises the main concerns about its appropriateness for pre-clinical studies; in fact drug effects can be hardly estimated while no clear consensus exists about the readout parameters that are more predictable for the human disease. In addition, a large variability exists between the experimental approaches used by various research groups and this, together with the high inter- and intra-individual variability of pathology, makes difficult to compare results obtained in different laboratories. A detailed discussion about this topic is out of the scope of the present review. More specific reviews are available describing the effort of focused experts panels to find a consensus on the most reliable approach to enhance data predictability in mdx mouse (www.treat-nmd.eu/research/pre-clinical/ SOPs [6-8]). Accordingly, standardized protocols for the assessment of various endpoints resulted from specialized working groups of experts and are available on www. treat-nmd.eu/research/pre-clinical/SOPs.   Importantly to mention is the consensus raised around the protocol of forced exercise on horizontal treadmill, as the one in use in the laboratory of this review's author, as a strategy to prolong the degenerative phase and then to enhance patient's like alterations useful for pre-clinical evaluation of therapeutics (6, 9). This is based on the hypothesis of contractile susceptibility of dystrophic muscle, and is supported by the more severe signs in the mdx diaphragm which undergoes a continuous respiratory activity (6).

It is important to remind the need to distinguish between primary and secondary readout parameters: primary readouts are more related to the pathology progression, and are main endpoints for evaluation of therapeutics: these include basal physiological/functional tests in vivo, among which mouse strength, capacity for exercise and spontaneous locomotor activity, and terminal tests including histological analyses of muscle sections (properly stained to quantify the area of damage and regeneration, fiber size and/or fibrosis) blood parameters (creatine kinase, CK) and muscle force/function.

Assessment of additional in vivo and/or ex vivo parameters, as secondary readouts, are useful for gaining more insight into drug's mechanism of action or of drug impact on specific signalling pathways. For instance the potential ability of a drug to act on channels involved in abnormal calcium entry, may require patch clamp experiments and/or specific imaging approaches, while biochemistry and molecular biology are necessary to evaluate drug impact on specific protein expression (8, 10).

Pharmaceutical testing in mdx mice: an overview

In spite of the variety of approaches used, the extensive research carried out in independent laboratories concentrates on few drug categories, which include the following: (i) anti-inflammatory and immuno-modulators, (ii) anti-oxidants, iii) anabolic compounds, iv) anti-fibrotic drugs. A brief rationale for focusing on drugs acting with the above mechanism of action is given in each of the following paragraphs. Importantly, a high level of cross-talk exists between various pathways; then a certain level of overlap or multiple actions can be found for some pharmaceutical interventions. An additional paragraph v) miscellaneous, briefly mentions pharmaceuticals that act through different, yet potentially relevant, mechanism of action (i.e. drug acting as anti-ischemic, on altered calcium homeostasis, etc.).

Food supplements and aminoacids will not be specifically discussed herein, unless their action is relevant for a clear "pharmacological" rather than pure "nutraceutical" effect. Due to the extreme large field, the present review cannot be exhaustive; some of the unmentioned approaches can be found in previous reviews on the topic (11, 12).

Anti-inflammatory and immunosuppressive drugs

Inflammation is a clear hallmark of dystrophic muscle and contributes to myofiber necrosis (2). Early inflammation is important to remove dead myofibers and to activate muscle repair program; however a chronic inflammatory state is established in dystrophic muscle with overactivity of Nuclear-factor-kB (NF-kB), overproduction of cytokines, such as Tumour Necrosis Factor (TNF)α and deregulation of M1/M2 macrophages population (13, 14). Interestingly, partial increase in dystrophin expression markedly reduces inflammatory

markers (15), thus further corroborating the view that the inflammatory cascade represents an important drug target, mostly at early stages of the disease. In this frame, corticosteroids, whose clinically efficacy is the result of many actions, also exert an important anti-inflammatory and immunosuppressive effect that may in part account for their efficacy, in both mdx mice and humans (1, 2). According to this view, drugs with an anti-cytokine action, such as those contrasting TNFα, have been proven independently by our and Grounds' group to protect mdx muscle against early necrosis. TNFα is a key pro-inflammatory cytokine that stimulates the inflammatory response, and pharmacological blockade of TNFα activity with the neutralising antibody infliximab (Remicade) or with a chimera compound bearing its soluble receptor, such as etanercept (Enbrel) is clinically effective at reducing symptoms of chronic inflammatory diseases. In mdx mice, infliximab delays and reduces the necrosis of dystrophic muscle in young mdx mice (16). A protective effect of TNFα blockade is reinforced by further studies using etanercept or the specific antibody against murine TNFα; in exercised adult mdx mice additional physiological benefits on mouse strength, chloride channel function (a cellular biomarker muscle state) and CK levels have been observed (17-19). The profile of the anti-TNFα drugs in mdx mice overlap that observed with cyclosporine, suggesting the importance to modulate immune response (20). The controversial outcome and/or impact of these drugs in clinical settings on DMD may derive from their toxicity (enhanced risks of serious infections) and/or difficulty in finding proper human doses in young patients; however these drugs may also be adjuvant for future gene/cell therapies. Other compounds, acting as inhibitors of NF-kB or as wide anti-inflammatories, such as flavocoxid, have been found beneficial in mdx mice, with a wide modulation in function and in expression of various pro-inflammatory pathways (21, 22). Some of these actions also overlap a possible anti-oxidant effect, due to the expected cross-talk between the two pathways (see below) (21). Similarly, classical non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and flurbiporfen have been found to ameliorate function and disease course in mdx, especially if combined with or linked to a nitric oxide (NO)-donating moiety which is claimed to enhance satellite cells activation and myogenic program (23, 24). By contrast, we could not confirm in the exercised mdx mouse model, a similar efficacy of meloxicam, a NSAID with higher affinity for the inducible-form of cyclo-oxygenase 2 (COX-2). This could be related to the role of COX-2 derived eicosanoids in promoting muscle regeneration (18, 25). Clinical trials in DMD boys with various anti-inflammatories are under considerations. However, the choice of the best drug is still unclear and must carefully consider the risk/benefit ratio also in relation to patient's age, the drug-specific toxicological profile and the delicate role of inflammation in muscle repair.

Anti-oxidants

High level or unbalanced production of reactive oxygen species (ROS) can damage tissues, including skeletal muscle (26). Enhanced signs of oxidative stress and ROS are present in mdx mouse muscle before the onset of pathology signs (27). The high ROS production might be related to inflammatory cell infiltrations, to an inability of dystrophic muscle to properly respond to oxidative injury, or to mitochondrial sufferance. However, recent evidences suggest that a cytoskeleton-dependent stretch-activation of NADPH oxidase (NOX-2) accounts for an unbalanced production of the highly reactive superoxide anion (O_2^-) specie and oxidative stress (X-ROS) in dystrophic muscle and heart (28). According to this view, we have described that O_2^- production is higher in exercised versus non-exercised mdx muscles (29). Interestingly, a chronic and early treatment of exercised mdx mice with enalapril, a drug blunting angiotensin-II production by inhibiting angiotensin-converting enzyme (ACE), leads to a dose-dependent reduction of O_2^- in muscles, along with a reduction of activated NF-kB (30). Angiotensin-II is the main endogenous regulator of NOX-2, supporting the interest of this latter as specific target. Interestingly, Angiotensin-II is also involved in fibrotic process in skeletal muscle and heart (see dedicated paragraph), and ACE-inhibitors and antagonists of the AT1 receptors, such as losartan, are already used in DMD patients to control cardiomyopathy (2, 31, 32). The early anti-oxidant effect of enalapril may help the design of proper anti-oxidant strategies, as most of the approaches used had limited translational impact for the wide and aspecific action of the scavenger used or the limited knowledge about the ROS targets in the dystrophic muscles. In this sense, important findings have been obtained in mdx mouse phenotype with N-acetyl-cysteine (NAC), a well known anti-oxidant compound. NAC has been described to protect dystrophic myofibers against eccentric muscle damage and to contrast abnormal calcium influx, then linking oxidative stress to key pathological features (33). A more recent study showed the direct ability of NAC to contrast the enhanced thiol oxidation in mdx muscles providing additional evidences about ROS targets and their impact in signalling in skeletal muscle (34). NAC is a rather inexpensive and safe drug that may deserve a more detailed clinical study in DMD patients. Other anti-oxidants such as idebenone, acts as Coenzyme Q, and has been mainly tested for its effects at heart level (35),

while green tea extracts ([-]-epigallocatechin gallate) are subject of an intense research by Ruegg's group and other laboratories. Early addition of green tea extracts to diet significantly reduced muscle damage in the EDL muscle of 4 week old mice and improved muscle function in 8 week old mice after 5 weeks of treatment; a synergic effect with low-intensity exercise has also been described (36-38). The diet enriched by epigallocatechin gallate seems to be more effective, although slower, than the systemic subcutaneous one; however higher doses that those normally used for food supplements are required (39, 40). The mechanism of action is also rather complex, including increased level of glutathione and inhibition of the transcription of pro-inflammatory and pro-oxidative pathways.

Importantly, ROS also act by activating NF-kB. Green tea extracts and other natural anti-oxidant, such as curcumin and genistein have been reported to reduce NF-kB activation; this has been claimed to play an important role in the potential benefit in mdx mice, although controversial results are present in the literature (41-43). Resveratrol has been also tested for its potential anti-oxidant effects. Hori et al., described the ability of this sirtuin 1 activator to reduce the markers of oxidative stress and the expression of NOX subunits (44); in parallel we found that resveratrol can reduce the O_2^- in muscles of exercised mdx mice, while enhancing exercise performance and decreasing histological and biochemical markers of damage (unpublished observation).

The ability of BN82270, a dual compound with anti-oxidant and anti-calpain activity, to contrast some pathology signs in the mdx mice, such as exercise-induced weakness and the high plasma CK, can be likely due to the anti-oxidant moiety, also in relation to the less relevant role of calpain proteases in the pathology (45).

Anabolic drugs

The possibility of increasing muscle mass and consequently muscle strength by anabolic drugs seems a reasonable approach for a muscle wasting disorder such as DMD. Nonetheless this is one of the most widely trialed therapeutic strategies and include drugs acting via different mechanisms, such as anabolic steroids, myostatin-blocking antibodies and β_2-adrenoceptor agonists (β_2-agonists). However, controversial results have been obtained, leading to possible concern that enlargement of muscle fiber size may in fact lead to make fibers more susceptible to contraction-induced injury, since larger type II fibers are more preferentially affected in dystrophic muscles.

This hypothesis has been recently rejected by Lynch's group using the muscle specific β_2-agonist formeterol. Its anabolic action is associated with an enhanced protein synthesis and decreased calpain activity and in mdx mice it increases muscle mass and fiber size as well as force (46-48). This drug can be of value due to the less cardiac side effects with respect to classical β_2-agonists, which however gave controversial results in both dystrophic patients and mice (see 12).

Anabolic steroids, including both testosterone and nandrolone, also gave controversial results and a tendency to increase muscle fiber degeneration has been observed (49). No clear benefit or mechanism of action have been described in mdx mice treated with anabolic steroids and this may in part account for the controversial results in DMD patients (50). Part of the controversy may be related to the side effects of anabolic steroids on off-target tissues, raising interest toward selective-androgen receptor modulators under current development.

Similar interest has been devoted to IGF-1, a somatomedin which is involved in muscle regeneration and mediates part of the anabolic action of growth hormone in skeletal muscle. Administration of IGF-1 enhances mouse force and ameliorates some IGF-1 sensitive parameters such as chloride channel conductance in EDL and diaphragm muscles of exercised mdx mice; a recent IGF-1-pegylated formulation also protected muscles against contraction-induced damage. In parallel transgenic mdx with a muscle specific over-expression of IGF-1 are protected by necrosis; the actions of IGF-1 are more important during the growing phase (9, 51-53). However, the remarkable pro-tumour and metabolic actions of IGF-1 are major concerns for its chronic use.

More recently a great attention concentrated to strategies aimed at contrasting myostatin, a negative regulator of muscle mass. Myostatin antibodies or the stimulation of its natural antagonist follistatin resulted in increase in body and muscle mass and muscle size, along with an improved performance and reduced signs of muscle degeneration in mdx mice (54-57). Although the great enthusiasm toward these compounds their real benefit in clinical settings is unclear yet.

Anti-fibrotic drugs

The progressive inefficiency of regeneration program and the unbalanced pro-fibrotic signalling lead to a gradual re-placement of muscular tissue with fibrotic one. Fibrosis is a rather late phenomenon; however, it is generally accepted that contrasting pro-fibrotic signals would ultimately result in an improved muscle regeneration and in an increase in muscle mass and strength. TGF-β1 and its signalling pathways (i.e. phosphorylated SMADs) are overactive in mdx muscle. Then pro-fibrotic cytokines and the pro-fibrotic signals have been targeted in the mdx mice. A recent study with

a neutralizing antibody against all the three isoforms of TGF-β markedly reduced hydroxyproline levels and plasma creatine kinase, ameliorated respiratory function and grip strength in 9 month old mice, being more effective than losartan on many parameters (58). However, an early treatment with TGF-β1 antibodies showed the ability to reduce the development of fibrosis, although inflammation markers were increased (59), in line with the delicate balance between anti- and pro-inflammatory signals. Halofunginone is an anti-fibrotic drug tested in mdx mice with a wide action on many fibrotic-markers: in fact it reduced collagen expression and the non-muscle area, meanwhile improving respiratory and heart function. Halofunginone has been suggested to inhibit Smad 3 phosphorylation downstream TGF-β1 and it is at the moment under further development.

As anticipated above, other important and clinically relevant anti-fibrotic interventions are the ACE inhibitors and the antagonists of type 1 receptor for angiotensin-II. Cohn et al., first demonstrated the anti-fibrotic action of losartan in models of muscular dystrophy, among which the mdx mouse; this action adds to the early anti-inflammatory action described by us, corroborating the interest of this class of drugs in muscular dystrophy.

Miscellaneous

The lack of dystrophin and the destruction of the DGC leads to a delocalization of the nitric oxide sinthase in myofibers (nNOS). This is believed to have a great impact in contracting muscles, where the proper formation of NO triggers vasodilatation and increases blood flow. Then a functional ischemia can be recurrent in dystrophin-less muscles and can strongly contribute to myofiber damage for hypoxia-reperfusion injury as well as metabolic distress. According to this view, drugs able to mimic NO action, either by enhancing its formation (L-arginine, NO-donors) or supporting its downstream signalling, exerts positive effect in mdx mice. The restoration of NO signalling also has additional advantages in increasing utrophin expression and in stimulating myogenic program, since follistatin is also under the control of NO (60, 61). Phosphodiesterase (PDE) inhibitors able to enhance the level of cyclic nucleotides also act through similar mechanisms, since NO signals via cGMP. Inhibitors of PDE5, other than ameliorating heart function in mdx mice, have also been shown to enhance exercise performance, that has been ascribed to an enhanced vasodilatation (62, 63). These potential beneficial effect at muscle level should also be considered in DMD patients undergoing trials with sildenafil. Similar results have been obtained in our laboratories by the use of pentoxifylline, an aspecific inhibitor of phosphodiesterase. The action of this drug is

likely the result of both the increase in cGMP and cAMP, exerting then a wide action on many different parameters. In fact we found the ability of this drug to enhance exercise resistance and regeneration signs, as expected by a NO-like signalling, meanwhile reducing calcium influx and markers of oxidative stress and inflammation, likely via enhanced cAMP (10, 29). A trial with pentoxifylline in DMD patients was little successful due to relevant side effects of this aspecific drug.

Stabilization of sarcolemma has also been attempted in the mdx mouse model. The use of aspecific compounds, such as poloxamer P188, or molecules able to enhance binding to and expression of integrins, such as laminin 111, although promising, raised concerns in relation to possible deregulation of the extracellular matrix and the unclear outcome in terms of fibrosis and muscle stiffness (64-66).

Blocks of channels involved in calcium entry can be another reasonable approach; however the molecular identity of these channels, necessary to develop specific modulators, is still unclear. Mechanosenstive channels or various members of the TRP channel family, such as TRPC1 or TRPV2, have been indicated as possible candidates but a real evidence is still lacking (10, 67-68). Taurine, a safe aminoacid, has an interesting wide action in dystrophic muscle. Supplementation of taurine exerts a marked increase in fore limb strength of exercised mdx mouse and ameliorates chloride conductance and calcium homeostasis in isolated muscles (9). In addition it has been recently proven to exert synergistic effects with prednisolone in the mdx mouse (69). A more detailed assessment of its mechanism of action in dystrophic is currently ongoing in our laboratory.

The possibility to sustain muscle metabolism via drugs able to mimic the beneficial effects of exercise, without the mechanical stress, are also providing interesting results (70, 71). However, a possible pathology-related alteration of these pathways can in fact contribute to the damaging action of exercise in dystrophic muscle; this needs to be clarified for validating these pathways as drug targets.

As anticipated in previous paragraph, stimulation of regeneration is another important mechanism, although must be paralleled by reduction of degeneration to avoid rapid exhaustion of satellite cells. Evaluation of regeneration efficiency requires a detailed evaluation of the proportion of centronucleated fibers and the detection of specific markers of myogenesis. Due to the cross-talk between damage and regenerative pathways, such an estimation in muscle of mdx mice that already have a high level of regeneration is not always a simple task (6).

Conclusion

The mdx mouse is extensively used for pre-clinical evaluation of therapeutics in dystrophinopathies. The great efforts devoted to standardize the approaches may help to enhance translation of data from mouse to humans, which remains however a delicate task. Pre-clinical scientists should be aware of the great expectation of novel therapeutic for these severe diseases and caution should be used when concluding about potential efficacy in mdx mice as a proof of therapeutic outcome in patients, especially when related to drugs of easy access. Assessment of interest to move a potential candidate towards clinical trials requires strictly controlled studies, dedicated head-to-head evaluation of similar drugs, and the multi-disciplinary evaluation of data package by expert panels, such as the TREAT-NMD Advisory Committee for Therapeutics. Nonetheless, the mdx mouse is a valuable tool, when properly used, and largely contributed to enhance our understanding and approach to this rare pathology.

Acknowledgments

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47