Perspective

GLPG0492, a novel selective androgen receptor modulator, improves muscle performance in the exercised-mdx mouse model of muscular dystrophy

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A B S T R A C T

Anabolic drugs may counteract muscle wasting and dysfunction in Duchenne muscular dystrophy (DMD); however, steroids have unwanted side effects. We focused on GLPG0492, a new non-steroidal selective androgen receptor modulator that is currently under development for musculo-skeletal diseases such as sarcopenia and cachexia. GLPG0492 was tested in the exercised mdx mouse model of DMD in a 4-week trial at a single high dose (30 mg/kg, 6 day/week s.c.), and the results were compared with those from the administration of α-methylprednisolone (PDN; 1 mg/kg, i.p.) and nandrolone (NAND, 5 mg/kg, s.c.). This assessment was followed by a 12-week dose-dependence study (0.3–30 mg/kg s.c.). The outcomes were evaluated in vivo and ex vivo on functional, histological and biochemical parameters. Similar to PDN and NAND, GLPG0492 significantly increased mouse strength. In acute exhaustion tests, a surrogate of the 6-min walking test used in DMD patients, GLPG0492 preserved running performance, whereas vehicle- or comparator-treated animals showed a significant increase in fatigue (30–50%). Ex vivo, all drugs resulted in a modest but significant increase of diaphragm force. In parallel, a decrease in the non-muscle area and markers of fibrosis was observed in GLPG0492- and NAND-treated mice. The drugs exerted minor effects on limb muscles; however, electrophysiological biomarkers were ameliorated in extensor digitorum longus muscle. The longer dose-dependence study confirmed the effect on mdx mouse strength and resistance to fatigue and demonstrated the efficacy of lower drug doses on in vivo and ex vivo functional parameters. These results support the interest of further studies of GLPG0492 as a potential treatment for DMD.

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1. Introduction

Duchenne muscular dystrophy (DMD) and the homologue dystrophic condition in the mdx mouse model, are caused by X-chromosome gene mutations that lead to the absence of the protein dystrophin [1,2]. Dystrophin is a subsarcolemmal component of a multi-molecular network (the dystrophin–glycoprotein complex, DGC) that ensures a physical linkage between the intracellular cytoskeleton and the extracellular matrix. The widely accepted theory is that dystrophin provides mechanical stability to myofibre during contraction. The absence of dystrophin triggers a complex and still unclear sequence of events that eventually leads to progressive myofibre degeneration, failing regeneration and fibrosis, thus resulting in muscle weakness and wasting [1]. Various key events in the process have been identified, and many of them are related to muscle activity, such as recurrent states of ischemia due to impaired nitric-oxide induced vasodilatation, alterations in calcium homeostasis via mechano-sensitive pathways, an early
and self-sustained inflammatory response paralleled by oxidative stress, and an impaired balance between protein synthesis and degradation [3–10]. The complexity of the causal and temporal sequence of the pathological events justify the effort expended towards therapeutic strategies aimed at replacing or correcting the gene defect, although many obstacles have to be overcome before the clinical use of such innovative strategies [11]. Until then, pharmacological approaches are compelling for the enhancement of muscle function and reduction of wasting to improve the quality of life of DMD patients [12]. Among the various strategies explored, there is a particular interest in anabolic agents, based on their ability to exert a stimulatory action on muscle mass and strength while promoting protein synthesis. In this context, various drugs have been tested with a variable level of success, including myostatin-blocking agents, β2-adrenoceptor agonists and anabolic steroids [13–17]. The latter have produced controversial results in both pre-clinical and clinical studies. In fact, nandrolone has been reported to worsen the pathological progression in mdx mice, although the androgen-sensitive muscle levator ani was shown to present a milder pathological progression compared to the diaphragm [18,19]. Oxandrolone has been observed to exert differential protection in relation to the duration of the treatment [20,21]. In addition, the reduction of the expression of several genes related to the muscle regeneration program has been described, implying that oxandrolone has the ability to decrease regeneration and metabolic muscle demands [22]. Some of the controversial effects observed with anabolic steroids may be related to their relevant effects on androgen-sensitive tissues other than skeletal muscle, with the possibility that beneficial effects are masked by the action of the steroids on off-target sites. Selective androgen receptor modulators (SARM) are non-steroidal compounds synthesised for more specific actions on bone and skeletal muscle [23,24]. One such compound is GLPG0492, a small molecule with remarkably low activity on the prostate compared to muscle and bones. Because of its profile, we decided to assess the potential benefits of GLPG0492 in the treadmill-exercised mdx mice, as a model of DMD. The first phase of the study was a 4-week treatment in which 30 mg/kg GLPG0492 was compared with nandrolone (5 mg/kg), the classic anabolic steroid, and α-methylprednisolone (PDN; 1 mg/kg), the clinically used gold-standard glucocorticoid [12]. In the second phase, a treatment with multiple doses of GLPG0492 (0.3, 3 and 30 mg/kg) was performed up to 12 weeks, in order to assess the dose- and time-dependent effects. A multidisciplinary in vivo and ex vivo approach was used to evaluate the outcome of the treatment on disease-sensitive indices and clinically relevant parameters and to elucidate the drug mechanism of action on dystrophic muscle. The results suggest that GLPG0492 is highly promising as an adjuvant compound for improving performance in dystrophic patients, although its complex mechanism of action deserves further investigation to better assess its therapeutic potential.

2. Methods

The animals housing, all the in vivo experiments and most of the ex vivo studies were done at the Unit of Pharmacology, Department of Pharmacy-Drug Sciences, University of Bari (Italy), legally authorised to these experimental procedures by the Italian Minister of Health (Authorisation n. 219/95 – A 19/5/1995). The animal research protocol was designed in conformity with the Italian Law for Guidelines for Care and Use of Laboratory Animals (D.L. 116/92), and European Directive (2010/63/UE). The experimental procedures used respected the standard operating procedures for pre-clinical tests in mdx mice available on http://www.treat-nmd.eu/research/preclinical/SOPs/.

2.1. In vivo experiments

2.1.1. Animal groups, treadmill running and drug treatment

A total of 56 mdx male mice of 4–5 weeks of age (C57BL/10ScSn-Dmdmdx/J; Jackson Laboratories, USA), and homogeneous for body weight, fore limb force and exercise resistance were used. The mice, either vehicle or drug treated, underwent a 30 min running on an horizontal treadmill (Columbus Instruments, USA) at 12 m/min, twice a week (keeping a constant interval of 2–3 days between each trial), for 4–6 weeks or 12 weeks. The exercise protocol facilitates the estimation of drug efficacy on pathology-related parameters [25,26] (http://www.treat-nmd.eu/resources/research-resources/dmd-sops). For the 1st study the following groups were used: 13 vehicle-treated mice (7 on corn oil + ethanol and 6 on sterile water), 6 mice treated with GLPG0492 at 30 mg/kg s.c.; 5 mice treated with nandrolone at 5 mg/kg s.c. and 6 mice treated with PDN i.p. (1 mg/kg). A dose of GLPG0492 in the high-effective range on other animal models was chosen to avoid false negative data in this first test in dystrophic animals. The treatment started one day before the beginning of the exercise protocol, and continued until the day of sacrifice. The dose was formulated so to inject 0.1 mL/10 g body weight. Vehicle for both GLPG0492 and nandrolone was 10% corn oil in 90% ethanol. Particular care in animal handling and environment was used to avoid any animal discomfort and stress during daily injection. However, due to the oily nature of the vehicle, skin bruises of various entities occurred. These did not appear to influence animal well being and muscle force vs. those without skin reactions; however for the second study (12-week treatment) the drug doses were formulated so to inject sub-cutaneously 0.05 mL/10 g body weight, in order to minimise the risk. No skin bruises were observed in the 12-week treatment. This second study included the following groups: 7 vehicle-treated mice and three groups treated with GLPG0492: 5 mice at 0.3 mg/kg; 8 mice at 3 mg/kg and 6 mice at 30 mg/kg. The dose scaling was chosen based on the result of the first study and to evaluate dose- and time-dependency of drug effects. In both studies, drugs were administered once a day, 6 days a week (Monday to Saturday). The one day-off protocol, suitable for chronic treatment regimen, has been decided for internal organisation. Animals for both the 1st and the 2nd study were randomly assigned to each group. Non-exercised (sedentary) mdx and wild-type (wt; C57BL/10ScSn) mice, treated or not with vehicle were left free to move in the cage, without additional exercise and monitored at the same time points of exercised counterparts, if needed. Every week all mice were monitored for body weight and forelimb force by means of a grip strength meter (Columbus Instruments, USA); the end of the 4th–5th week was considered for statistical analysis [25,26]. For the second study the additional time points were the 8th and the 12th weeks. At these time-points, as well as at the beginning of the protocol (time 0), an exercise resistance test on treadmill was also performed. All mice were made running on a horizontal treadmill for 5 min at 5 m/min, then increasing the speed of 1 m/min each minute. For each mouse, the total distance run until exhaustion was measured. At the end of the 4th and of the 12th week of exercise/treatment the ex vivo experiments were started [27]. Evaluation of in vivo parameters was performed in a blind fashion. Due to the time-consuming nature of some of the ex vivo experiments no more than one-two animals could be sacrificed per day. The animals continued to be exercised/treated until the day of sacrifice.

2.2. In vitro studies

2.2.1. Muscle preparations and organ biopsies

Animals of 8–10 and 16–18 weeks of age belonging to the different groups were anaesthetised with 1.2 g/kg urethane i.p. Extensor digitorum longus (EDL) muscle of one hind limb was removed and
rapidly placed in the recording chamber for isometric recordings. The contralateral EDL muscle was used for the electrophysiological recordings. Gastrocnemius (GC) muscle from one side was removed and processed for histology, while the contralateral one was snap frozen in liquid nitrogen and stored at -80 °C until use for biochemical and real-time PCR analysis. The same procedure was used for various parts of the left half-side of Diaphragms (DIA), while strips of the right half-side were used for isometric tension measurements.

Soon after mouse death, different organs were removed and rapidly weighed in order to evaluate potential effect of drug treatment on other tissues. Hind limb bones were removed for determination of morphometry by microCT, while plasma was collected for determination of creatine kinase, lactate dehydrogenase as well as testosterone and GLPG0492 level.

2.2.2. Isometric contraction

EDL muscle and strips of diaphragm were securely tight at tendon insertion and placed in a muscle chamber containing the normal physiological solution (in mM): NaCl 148; KCl 4.5; CaCl2 2.0; MgCl2 1.0; NaHCO3 12.0; NaH2PO4 0.44 and glucose 5.55, continuously gassed with 95% O2 and 5% CO2 (pH=7.2–7.4). For the diaphragm, the ribbon side was fixed to a 25 g isometric force transducer (FORT25, WPI, Inc., FL, USA) connected to a TCI 102 transducer interface and an MP 100 acquisition unit (Biopac Systems, Santa Barbara, CA, USA), while the opposite end was fixed to a chamber hook. The EDL muscle was connected by one tendon at the dual-mode muscle lever–system transducer (300C dual mode transducer, Aurora Scientific, ON, Canada). An electrical stimulation field was obtained with two axial platinum wires connected to a stimulator (LE 12406, 2Biological Instruments, VA, Italy). After an equilibration period (30 min), the preparation was stretched to its optimal length (Lo); measured with an external calliper, i.e. the length producing the maximal twitch to a 0.2 ms 40 V pulse. Then the stimulation protocol was initiated. Maximal twitch tension and contraction kinetic (time to peak and half relaxation time) were evaluated as the mean values from 5 single twitches elicited by pulses of 40 V and 0.2 ms (every 30s); Maximal tetanic tension and frequency for half-maximal activation (Hz/50) have been calculated from the force–frequency curve constructed with trains of 0.2 ms 40 V pulses from 10 to 140 Hz; train duration was 450 ms for diaphragm and 350 ms for EDL muscle. A protocol of 10 tetani at 100 Hz (450 ms and 350 ms for diaphragm and EDL, respectively) spaced by 5 s intervals allowed to calculate the muscle fatigue as % drop of tension at the 10th pulse vs. the first tetani. The eccentric contraction has been measured in EDL muscle with a 120 Hz tetanic stimulation of 500 ms having a gradual stretch up to 10% during the last 200 ms. The protocol consisted of 10 of such tetani spaced by 15 s; the stretch-induced damage was estimated as the % drop of tension between 1st and 10th tetani. Data was collected and analysed using AcqKnowledge software vs. 3.8 (Biopac System). At the end of experiments, samples were removed, cleaned from tendons, dried and weighted. Diaphragm strips had variable mass (ranging from 7 to 10 mg) due to the preparation procedure. Absolute values of tension were normalised by cross sectional area according to the equation SPl=P/(Mass/L0 × D) where P is absolute tension; Mass is the muscle mass from tendon to tendon; D is the density of skeletal muscle assumed to be 1.06 g/cm³; L0 is determined by multiplying L0 by previously determined muscle length to fibre length ratios (0.44 for the EDL and 1 for the diaphragm) [27,28].

2.2.3. Electrophysiological recordings by intracellular microelectrodes

EDL muscles were bathed at 30±1 °C in the normal physiological solution (see composition above) continuously gassed with 95% O2 and 5% CO2 (pH=7.2–7.4; 27±1 °C). Standard two intracellular microelectrode current clamp method was used to measure the membrane electrical properties of muscle fibres, among which membrane resistance (Rm), according to the cable equation (fibre input resistance of 140 Ω cm²) [25,26]. The total membrane conductance (gm) was calculated as 1/Rm in normal physiological solution.

The mechanical threshold (MT) was determined in the presence of tetrodotoxin (3 µM) using a two microelectrode “point” voltage clamp method [25,26]. In brief, the two microelectrodes (spaced about 50 µm) were inserted into the central region of a superficial fibre, continuously viewed using a stereomicroscope (100× magnification). Depolarising command pulses of duration ranging from 500 to 5 ms (0.3 Hz) were progressively increased in amplitude from the holding potential (H) of ~90 mV until visible contraction. The threshold membrane potential (V, in mV) was read on a digital sample-and-hold millivoltmeter at the various pulse durations (t in ms) for each fibre; mean values at each t allowed the construction of a “strength-duration” curve. Rheobase voltage (R, in mV) and the time constant (τ) to reach the rheobase was obtained by a non-linear least square algorithm using the following equation: V = (H – R exp(t/τ))/((1 – exp(t/τ))) [25,26].

2.2.4. Muscle histology and bone micro CT

Gastrocnemius (GC) muscles and hemidiaphragm were dissected by surrounding tissue, fixed in paraﬃn, routinely processed in descending alcohol and then paraﬃn wax embedded. Six µm transversally cut sections were stained with haematoxylin–eosin to calculate the percentage of both healthy myoﬁbres with peripheral nuclei (peripherally nucleated ﬁbres) and regenerating/regenerated myoﬁbres, showing central nuclei (centrally nucleated ﬁbres). Morphometric analysis was performed by using an Image J analysis software on the entire muscle section, selecting about 5 ﬁelds/section. For each muscle two sequential cross-sections were used; at least three animals per experimental group were assessed [26,29]. MicroCT scans of the metaphyseal region of tibias were performed at an isotropic resolution of 8 µm, using a threshold of 275 to obtain trabecular bone structural parameter (BV/TV) using the Scanco Medical µCT scanner CT (µCT 20; Scanco Medical AG, Bassersdorf, Switzerland). The mean tissue of the scanned area was between 0.5 and 1.25 mm from the growth plate in the trabecular bone of the tibial proximal metaphysis. The morphometric measurements were performed blinded to the treatment for each mouse and validated by a second independent experimenter, always in a blind fashion.

2.2.5. Determination of transforming growth factor-β1 level

Total and active TGF-β1 protein was measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer’s instructions (RandD System, Minneapolis, MN, USA). Briefly, 10–20 µg of frozen muscle tissue was homogenised in 500 µL of a solution containing 1% Triton X-100, 20 mM Tris pH 8.0, 137 mM sodium chloride, 10% glyceral, 5 mM ethylenediaminetetraacetic acid and 1 mM phenylmethylsulphonyl fluoride [26,27,29]. TGF-β1 levels were expressed as pg of TGF-β1/µg of total protein.

2.2.6. Plasma levels of creatine kinase, lactate dehydrogenase, testosterone and GLPG0492

Blood was collected from ventricular camera soon after animal death in heparin rinsed tubes. The blood was centrifuged at 3000 × g for 10 min and plasma was separated. Creatine kinase (CK) and lactate dehydrogenase (LDH) determination was performed by standard spectrophotometric analysis by using diagnostic kits (Sentinel, Farmalab – Italy). CK and LDH were determined on the same day of plasma preparation.

Serum testosterone levels were evaluated at day 7 in each group using a RIA kit (Orion Diagnostica, Finland). In addition, circulating
levels of GLPG0492 were quantified at steady-state at 3 or 5 h post-dose by LC–MS/MS in Galapagos bio-analytical department.

2.2.7. Real-time PCR

For each Dia and GC muscle sample, total RNA was isolated with RNasy Fibrous Tissue Mini Kit (Qiagen) and quantified by using a spectrophotometer (ND–1000 NanoDrop, Thermo Scientific). Total RNA (400 ng) was used for reverse transcription. Synthesis of cDNA was performed using random hexamers ( annealed 10 min, 25 °C) and Superscript II reverse transcriptase (Invitrogen–Life Technologies) incubated at 42 °C for 50 min.

Real-time PCR was performed in triplicate using the Applied Biosystems Real-time PCR 7300 Fast System. TaqMan hydrolysis primer and probe gene expression assays were ordered by Applied Biosystems with the following assay IDs: Insulin-like growth factor 1 (encoded by igf1 gene) assay ID: Mm_00439560_m1; Follistatin (encoded by Fst gene) assay ID: Mm_00514982_m1, myogenin (encoded by myog gene) assay ID: Mm_00446194_m1 and Peroxisome proliferator activated receptor γ coactivator-1α (PGC1α; encoded by ppargc1a gene), assay ID: Mm_01208835_m1. Each reaction ran on a singleplex reaction. The setup of reactions consisted 8 ng of cDNA, 0.5 μL of primer and probe set, 5 μL of TaqMan Fast Universal PCR master mix No AmpErase UNG (2x) (Applied Biosystems) and H2O Nucleotide free for a final volume of 10 μL, under the following PCR conditions: step 1, 95 °C for 20 s; step 2, 95 °C for 3 s; and step 3, 60 °C for 30 s; steps 2 and 3 were repeated 45 times. The results were compared with a standard curve and normalised to expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (gpdh) (assay ID Mm_00868315_g1) and hypoxanthine guanine phosphoribosyl transferase (hprt1) (assay ID Mm_00446968_m1).

2.3. Statistics

All data is expressed as mean± standard error of the mean (S.E.M.). The S.E. estimate for the fitted rheobase (R) values (and relative statistical analysis) were obtained as previously described [25,26]. Statistical analysis for direct comparison between two means was performed by unpaired Student’s t test. Multiple statistical comparisons between groups were performed by one-way ANOVA, with Bonferroni t post test hoc correction for allowing a better evaluation of intra- and inter-group variability and avoiding false positive. The recovery score by the drug treatment has been evaluated according to the Standard Operating Procedures described in the Treat-NMD web site (http://www.treat-nmd.eu/research/pediatric/DMD_SOPs) using the following equation:

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\text{Recovery score} = \frac{[\text{mdx treated}] - [\text{mdx untreated}]}{[\text{wildtype}] - [\text{mdx untreated}]} \times 100.
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3. Results

3.1. Effect of GLPG0492 on forelimb strength and resistance to treadmill exercise

A time-dependent increase in body weight was observed in all groups in both studies (Figs. 1 and 2). The increase was significantly lower in the PDN-treated mice, agreeing with previous observations [25,30]. In the second study, the group of mdx mice treated with GLPG0492 at 3 mg/kg showed lower body weight increases compared with the other treatment groups (Fig. 2A).

The mouse strength increased age-dependently in all groups. At time 0 (i.e., 4 weeks of age), all of the mdx mice groups were weaker than wild-type (wt) mice group. The weakness was exacerbated by the exercise protocol in the groups of vehicle-treated mdx animals in both studies (Figs. 1 and 2). In agreement with previous studies, this weakness was less evident in non-exercised mdx mice [data not shown; [26,30]]. Significant protection against the 4-week exercise-induced weakness was observed in the group of mice treated with GLPG0492, nandrolone and PDN (Fig. 1B). To avoid any possible bias due to individual differences in body weight, the normalised strength was calculated. This analysis showed significant protection from GLPG0492 and PDN, whereas slight protection was observed with nandrolone (Fig. 1C). The dose- and time-dependent study confirmed the ability of the SARM to significantly protect mouse strength, even at lower doses, and to maintain efficacy over time (Fig. 2B). In agreement with the first study phase, significant protection against exercise-induced weakness was observed in the mice treated with GLPG0492 at 3 and 30 mg/kg after 4 weeks of treatment. After 8 weeks of treatment, all three doses tested led to a significant increase in the absolute forelimb strength, and this protection was maintained for up to 12 weeks. At this stage, the highest value of forelimb strength was found in the 30 mg/kg-treated mice. A similar time-dependent trend was observed when the strength was normalised to body weight (Fig. 2C).

Another index of neuromuscular function is the resistance to treadmill exercise, which is representative of the 6-minute walk tested clinically used for DMD patients, and is measured as the maximal distance that each mouse is able to run in an acute exhaustion test. The test was performed at the beginning of experimental section (T0) and at T4, T8 and T12 according to the duration of the treatment. A significant decrease in resistance was observed in the untreated exercised group, whereas a different degree of protection was observed in the GLPG0492-treated mice (Figs. 1D and 2D). In fact, after 4 weeks of treatment, 30 mg/kg GLPG0492 was the most effective drug to protect against fatigue when comparing treated mice running a longer distance than vehicle-treated mice (+62.5%). The recovery score was 26%. In contrast, PDN- and nandrolone-treated mice did not show any improvement (Fig. 1D).

In the dose- and time-dependent experiments, we confirmed that after 4 weeks of treatment, GLPG0492-treated mice could run for a greater distance than the untreated group. Lower doses (0.3 and 3 mg/kg) were more effective than 30 mg/kg. This latter dose, however, significantly increased the resistance to exercise with respect to vehicle-treated counterparts, with a comparable, although greater, efficacy with respect to the first study. The slightly different improvement from 30 mg/kg dose between the two studies was attributed to the expected intergroup variability. After 12 weeks of treatment, GLPG0492, at all three doses, significantly increased the total distance run. No clear dose relationship could be observed on this parameter; the maximal activity was constantly maintained over 12 weeks with the 3 mg/kg GLPG0492 dose (Fig. 2D).

3.2. Effect of GLPG0492 on mass of muscles and androgen-sensitive tissues

Muscle performance can be a direct consequence of the hypertrophic effect exerted by anabolic drugs. Thus, we first evaluated the outcome of drug treatment on the masses of the EDL, soleus and levator ani muscles, taken as normalised values over body weight, to evaluate the hypertrophic action of the drug on fast- and slow-twitch and androgen-sensitive muscle phenotypes, respectively. The mass of other androgen-sensitive tissues, such as heart and prostate, was also evaluated. The results of both the 1st and 2nd studies are shown in Figs. 3 and 4, respectively. As shown, no significant changes by GLPG0492 were observed in any of the muscles sampled, which exclude a phenotype-dependent hypertrophic drug effect in the mdx mice. In contrast, a significant increase in

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the levator ani muscle weight was observed in nandrolone-treated mice. No changes were observed in the mass of the prostate and heart in both the 1st and the 2nd studies. However, a significant decrease in the normalised prostate weight after 12 weeks of treatment suggested potential anti-androgen activity at the highest GLPG0492 dose (30 mg/kg). As potential target organs, the masses of the liver, spleen, and kidney were measured, and few changes were observed in both studies (Figs. 3B and 4B). In the 1st study, a significant increase in liver weight was observed with all drugs, and significant increases in the kidney and spleen weights were detected in the nandrolone and GLPG0492 groups, respectively. However, after 12 weeks of treatment, a significant increase in kidney weight was observed for all three doses of GLPG0492, whereas a tendency towards a lower spleen weight was observed at the 3 and 30 mg/kg doses. The differences observed in the 2nd study could be related to the longer treatment duration and adaptation (i.e., for the liver and spleen) or exacerbation (kidney) of tissue-specific drug activity.

3.3. Effect of GLPG0492 on the functional parameters of isolated muscles

3.3.1. Diaphragm Resistance to exercise may be dependent on the contractile performance of respiratory muscles. Therefore, the decreased exercise performance of mdx mice may be due to the serious impairment in the diaphragms of mdx mice. We tested whether the enhanced resistance to running observed with GLPG0492 correlated with an improvement of diaphragm contractile efficiency. The maximal twitch and tetanic forces of diaphragm (DIA) strips were measured by isometric contraction. A significant increase in twitch tension was observed in the nandrolone-treated group, while a similar trend was found with GLPG0492; however, all the three drugs tested in the 1st study significantly increased the DIA tetanic tension after 4 weeks of treatment (Fig. 5A and B). The recovery scores for tetanic tension were 35% and 37% for GLPG0492 and nandrolone, respectively.
After 12 weeks of treatment with scaling doses of GLPG0492, a significant increase in both twitch and tetanic tension was observed only in 0.3 mg/kg GLPG0492-treated mice, with recovery scores of 41% and 50%, respectively; a slight increase was observed at the higher doses (Fig. 5C and D). No significant changes were observed in the contraction kinetics or in other calcium-dependent parameters (twitch/tetanus ratio and Hz50) after 4 or 12 weeks of exercise and/or treatment (data not shown). A trend of reducing muscle fatigue was observed after 4 weeks in the drug-treated groups primarily with GLPG0492 and PDN treatment, but this trend was not confirmed after 12 weeks of any of the GLPG0492 doses.

3.3.2. Extensor digitorum longus muscle

The contractile profile of the EDL muscle was also monitored to evaluate the muscle-specific action of GLPG0492 as well as the contribution of limb muscles to the general increase in mouse force observed in vivo. No significant amelioration was observed on twitch or tetanic forces in the drug-treated animal groups in the first study. In addition, no significant differences were observed between experimental groups in the contraction and relaxation times, the calcium-dependent parameters and fatigue, although a partial recovery from fatigue was observed with nandrolone and PDN (data not shown). We then evaluated whether lower doses and/or prolongation of treatment may produce a greater effect on the limb muscles. The prolonged treatment with GLPG0492 exerted a significant increase in EDL muscle twitch force at 3 and 30 mg/kg, whereas the increase was not significant at the lowest dose (Fig. 6A). An increase in tetanic tension ranging between 10% and 20% for the different doses was also observed; however, the increase was not significant (Fig. 6B). We then evaluated the ability of GLPG0492 to protect dystrophic EDL muscle against fatigue and eccentric contraction force drop. As shown in Fig. 6C, a wt-like resistance to fatigue was observed in EDL muscles treated with GLPG0492 at 3 and 30 mg/kg, although the values did not reach the...
statistical significance vs. untreated mdx mice, most likely due to
the high inter-individual variability. In addition, partial protection
was observed for the force drop induced by eccentric contraction
when using GLPG0492 at 0.3 and 3 mg/kg, but not at the highest
dose tested (Fig 6D). The analysis of the calcium-dependent param-
eters indicated no significant alteration at any dosage, suggesting
no effect of the drug on calcium homeostasis.

This issue was addressed at the myofibre level by measuring the
voltage threshold for contraction (mechanical threshold; MT) using
electrophysiological experiments. A shift of MT and rheobase volt-
age towards more negative potentials is a typical hallmark of mdx
EDL muscle fibres and is indicative of calcium homeostasis distress,
resulting from greater release, slower reuptake or higher basal
cytosolic levels [4,25]. The 4-week treatment with GLPG0492 at

Fig. 3. Effect of 4-week treatment with GLPG0492 and comparators on the weight of androgen-sensitive tissues and other potential target tissues. Each bar represents the mean ± S.E.M. from 5 to 10 animals and shows the tissue mass normalised with respect to the individual body weight of mdx mice treated with either vehicle (corn oil and water; Mdx + Vctrl) or with 30 mg/kg GLPG0492 (Mdx + GLPG0492), 5 mg/kg nandrolone (Mdx + NAND) or 1 mg/kg α-methylprednisolone (Mdx + PDN). The drugs were given 6 days per week. In (A), the figure shows the weight of androgen-sensitive tissues, i.e., the heart, prostate, levator ani, EDL and soleus muscles. The normalised values for the levator ani have been scaled by a factor of ten for graphical reasons. The ANOVA analysis and Bonferroni t test indicated significant differences only for the levator ani weight (F > 4; p < 0.015). Significantly different vs. mdx vehicle-treated (p < 0.05). In (B), the figure shows the weights of the spleen, liver and kidneys. The normalised values for the liver have been scaled by a factor of ten for graphical reasons. An ANOVA analysis and the Bonferroni t test indicated significant differences only for liver weight (F > 3; p < 0.04); significantly different vs. mdx vehicle-treated (p < 0.02).

Fig. 4. Dose- and time-dependent effect of GLPG0492 on the weight of androgen-sensitive tissues and other potential target tissues. Each bar represents the mean ± S.E.M. from 5 to 8 animals and show the tissue mass normalised with respect to the individual body weight of mdx mice treated with either corn oil (Mdx + Vctrl) or with GLPG0492 at 0.3, 3 or 30 mg/kg (Mdx + GLPG0492). The drugs were given 6 days per week. In (A), the figure shows the weights of androgen-sensitive tissues, i.e., heart, prostate, levator ani, EDL and soleus muscles. The normalised values for the levator ani have been scaled by a factor of ten for graphical reasons. An ANOVA analysis and the Bonferroni t test indicated significant differences only for levator weight (F > 12; p < 5.4 × 10−3); significantly different vs. mdx vehicle-treated (p < 1.4 × 10−1). In (B), the figure shows the weight of the spleen, liver and kidneys. The normalised values for the liver have been scaled by a factor of ten for graphical reasons. An ANOVA analysis and Bonferroni t test indicated significant differences only for kidney weight (F > 19; p < 1.9 × 10−6); significantly different vs. mdx vehicle-treated (p < 0.03).
30 mg/kg led to a significant shift in the potential for fibre contraction towards WT values at all durations of depolarising pulses. Both nandrolone and PDN were less effective than GLPG0492 (Fig. 7A). The fit of the data points indicated that the rheobase voltage of GLPG0492-treated EDL myofibres was similar to that of the WT and more positive than the values obtained with nandrolone and PDN. This latter compound exerted the same effect observed in previous studies [25,30]. An improvement was also observed in the kinetics for reaching the rheobase. In fact, the time constant for the GLPG0492-treated EDL myofibres was remarkably shorter than that of the untreated myofibres (Fig. 7B and C).

The results from increasing the duration of the treatment on MT are shown in Fig. 8A–C. The strength-duration curves for the three doses of GLPG0492 were all shifted to more positive potentials when compared with the untreated groups, suggesting that the effect was maintained over time. However, a dose-dependent relationship was not observed, suggesting a saturating process or rather the occurrence, at higher drug doses and longer treatment duration, of other effects on calcium-dependent handling mechanisms that mask the beneficial drug effect on this parameter. Such a mechanism could partially account for the absence of effects on the calcium-dependent contractile parameters described above.

A similar trend was observed for the effect of the drug treatment on the decrease of $g_m$ (mostly due to a decrease in chloride channel conductance, $gCl$), a typical cellular hallmark of myofibre sufficiency in diaphragm and exercised EDL muscle of mdx mice. In the first study, all of the drug treatments resulted in a significant increase in $g_m$, and the effect was particularly evident with GLPG0492, which produced effects comparable to those of PDN [25] (Fig. 7D).

In the dose- and time-dependent study, all of the doses of GLPG0492, after 12 weeks, significantly counteracted the exercise-induced decrease of $g_m$ in EDL muscle; however, the effect was not greater than the effect found after 4 weeks, and again, a dose-dependent relationship was not observed (Fig. 8D).

3.4. Plasma level of creatine kinase (CK) and lactate dehydrogenase (LDH)

A marked elevation of plasma creatine kinase is a typical diagnostic marker of muscular dystrophy, along with the functional impairment and the altered histological profile. Similarly, an increase in lactate dehydrogenase is observed and is a sign of metabolic distress. As shown in Table 1, no significant amelioration was observed with any of the drugs on CK or LDH. A slight, but not significant reduction of LDH was observed in GLPG0492-treated mdx mice in the 4-week study. The absence of an effect of PDN on CK and LDH has been already observed in previous studies [25,30].
Fig. 6. Effect of GLPG0492 on isometric and eccentric contraction of extensor digitorum (EDL) muscle. The figure shows the contractile parameters of the isolated EDL muscles from wt and mdx mice treated with either corn oil (Mdx + V1) or with GLPG0492 at 0.3, 3 or 30 mg/kg (Mdx + GLPG0492). The drugs were given 6 days per week. In (A), the normalised values for maximal isometric twitch (sPhw measured in kN/m²) are shown. ANOVA test indicated significant differences with F=4 and p < 0.05. The post hoc Bonferroni t-test results are indicated as follows: *significant difference vs. wt (p < 0.05) and **vs Mdx + V1 (0.05 < p < 0.025). In (B), the normalised values of maximal isometric tetanic tension (sPht measured in kN/m²) are shown. ANOVA test indicated significant differences with F=4 and p < 0.03. The post hoc Bonferroni t-test results are indicated as follows: *significant difference vs. wt (p < 0.01) and **vs MDX (0.01 < p < 0.02). In (C), the muscle fatigue, defined as the percentage drop of force at the 10th pulse with respect to the first contraction, is shown. No significant difference was observed as evaluated with ANOVA. A Bonferroni t-test indicated significant differences, and the results are indicated as follows: *significant difference vs wt (p < 0.005). In (D), the percentage of tension reduction during eccentric contraction (calculated as the drop at the 10th pulse vs the tension at the first eccentric stimulus) is shown. ANOVA test indicated significant differences with F=4 and p < 0.02. The post hoc Bonferroni t-test results are indicated as follows: *significant difference vs wt (p < 0.05)Each bar represents the mean S.E.M. for 4–7 animals per group.

Table 1
Effect of GLPG0492 on the plasma biomarkers of mdx mice.

<table>
<thead>
<tr>
<th></th>
<th>CK (U/L)</th>
<th>LDH (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1st study</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1448 ± 144 (5)</td>
<td>580 ± 67 (5)</td>
</tr>
<tr>
<td>Mdx + V1</td>
<td>13,805 ± 4129 (10)</td>
<td>5866 ± 1947 (10)</td>
</tr>
<tr>
<td>Mdx + GLPG0492 30 mg/kg</td>
<td>11,556 ± 1514 (5)</td>
<td>3857 ± 777 (5)</td>
</tr>
<tr>
<td>Mdx + NAND 5 mg/kg</td>
<td>16,434 ± 2751 (5)</td>
<td>4817 ± 1075 (5)</td>
</tr>
<tr>
<td>Mdx + PDN 1 mg/kg</td>
<td>17,739 ± 1840 (6)</td>
<td>7124 ± 2275 (6)</td>
</tr>
<tr>
<td><strong>2nd study</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1634 ± 291 (5)</td>
<td>716 ± 376 (5)</td>
</tr>
<tr>
<td>Mdx + V1</td>
<td>11,438 ± 2059 (7)</td>
<td>3696 ± 559 (7)</td>
</tr>
<tr>
<td>Mdx + GLPG0492 0.3 mg/kg</td>
<td>15,435 ± 2845 (5)</td>
<td>4977 ± 957 (5)</td>
</tr>
<tr>
<td>Mdx + GLPG0492 3 mg/kg</td>
<td>16,199 ± 1902 (8)</td>
<td>3666 ± 354 (8)</td>
</tr>
<tr>
<td>Mdx + GLPG0492 30 mg/kg</td>
<td>18,847 ± 1766 (6)</td>
<td>6962 ± 1495 (6)</td>
</tr>
</tbody>
</table>

Columns are as follows: groups of mice used: wild-type C57BL/10 mice (WT) and mdx mice treated with vehicle (water or corn oil; Mdx + V1) or only corn oil; Mdx + V1) (1st and 2nd studies); Mdx mice treated with GLPG0492 30 mg/kg (Mdx + GLPG0492), nandrolone 5 mg/kg (Mdx + NAND) or α-methylprednisolone 1 mg/kg (Mdx + PDN) (1st study); Mdx mice treated with GLPG0492 0.3 mg/kg (Mdx + GLPG0492 0.3 mg/kg), 3 mg/kg (Mdx + GLPG0492 3 mg/kg) and 30 mg/kg (Mdx + GLPG0492 30 mg/kg) (2nd study). The table shows the plasma levels of creatine kinase (CK) and lactate dehydrogenase (LDH) measured by standard spectrophotometric methods. The values are the means ± SEM. The number of animals is indicated in brackets. For each parameter, the significant differences between groups were evaluated using the ANOVA test for multiple comparisons (F-values) and the Bonferroni t-test post hoc correction. No significant difference was observed by ANOVA. The post hoc Bonferroni t-test results are indicated as follows: *significantly different with respect to mdx exercised mice with 4.9 x 10⁻⁹ < p < 0.01.

3.5. Effect of GLPG0492 on muscle histology, markers of fibrosis and bone morphology

In line with these observations, no effect on both CK and LDH was observed at the end of 12 weeks of treatment with any of the GLPG0492 doses (Table 1).

Representative pictures of the histology profile of the mdx diaphragm and GC muscles for the various experimental conditions are shown in Fig. 9. Both muscles clearly presented the typical dystrophic features, such as the alteration of the muscle architecture, with the presence of areas of necrosis, infiltrates and a large non-necrose area, likely due to the deposition of fibrotic and adipose tissue. A large variability in fibre size and the presence of centronucleated fibres (CNF), isolated or in clusters near necrotic fibres, were also present along with other infiltrates, such as mononuclear inflammatory cells. The alterations were still present in the groups of treated muscles, showing a very high inter-individual variability (see as example Fig. 9), which barely allowed for the estimation of the qualitative signs of amelioration.

However, with respect to the first study, the morphometric analysis indicated a significant decrease in the total area of muscle damage (necrosis + non muscle tissue) in the diaphragm of GLPG0492- and nandrolone-treated mice but not in PDN-treated ones. Interestingly, this result was entirely due to a reduction in the non-muscle area (Fig. 10A). In contrast, minor effects of the treatments were observed on GC, including a slight reduction of necrosis by nandrolone and PDN (from 9.0 ± 5% of vehicle treated
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**Fig. 7.** Effect of 4-week treatment with GLPG0492 and comparators on functional cellular parameters in extensor digitorum longus (EDL) muscle fibres of mdx mice. In (A), the data, expressed as the means ± S.E.M. from 14 to 30 values from 2 to 5 preparations, show the voltages for the contraction of EDL myofibres (mechanical threshold) at increasing pulse duration in wild-type mice (WT, black circles) and in mdx mice treated either vehicle (corn oil and water; Mdx + V0.2%, white circles), 30 mg/kg GLPG0492 (white triangles), 5 mg/kg nandrolone (upside-down black triangles) or 1 mg/kg PDN (white rhombus). The drugs were given 6 days per week. The voltage threshold values of myofibres of mdx mice treated with 30 mg/kg GLPG0492, 5 mg/kg nandrolone or 1 mg/kg PDN were significantly more positive with respect to those of mdx mice treated with vehicle (p < 0.03 or less by Student’s t test) at each pulse duration. For some data points, the standard error bar is not visible because it is smaller than the symbol size. In (B) and (C), the rheobase voltage, in mV and the time constant, in ms, with relative standard errors, have been calculated from the fit of data points of the voltage–duration curves in A, using the equation described in Section 2. In (D), the total resting membrane ionic conductances (gms) in μS/cm² of EDL muscle fibres of the same experimental groups described in A are shown. The bars represent the means ± SEM from the number of 3–5 prep/25–37 fibres. For each parameter, the significant differences between groups were evaluated using ANOVA for multiple comparisons (F values) and the Bonferroni t-test post hoc correction. Significant differences were found for rheobase voltage (F > 4; p < 0.003) and gms (F > 7; p < 0.0002). The post hoc Bonferroni t-test results are indicated as follows: *significantly different with respect to wt mice with p < 0.05 and **significantly different with respect to mdx exercised mice with p < 0.02.

Due to the reduction in non-muscle area observed in the diaphragm after the 1st treatment, the level of the profibrotic TGF-β1 protein were measured using an enzyme-linked immunosorbent assay (ELISA) in parallel with the total protein content in diaphragm muscle.

The classic Bradford assays indicated an increase of total protein content in the treated muscles from both the 1st and the 2nd study. For the latter, the 0.3 mg/kg treatment showed the greatest increase (total protein content of 9487 ± 2787 μg/mL; n = 5; vs. 3940 ± 251 μg/mL; n = 5, corn-oil treated mice) followed by 3 mg/kg with total protein content of 6048 ± 785 μg/mL (n = 5); 30 mg/kg showed only a modest increase (5085 ± 342 μg/mL; n = 4). These results corroborate the anabolic action of GLPG0492 on dystrophic muscle, with lower doses already exerting a ceiling effect. **Fig. 10B** shows the effect of drug treatment on the TGF-β1
levels. After 4 weeks of treatment, both GLPG0492 and nandrolone significantly reduced the total and active TGF-β1 levels. This effect was maintained over 12 weeks, and an inverse dose-dependent relationship was observed, with 0.3 mg/kg being the most effective dose. A similar trend was observed for the active TGF-β1 level (Fig. 10C), again corroborating the ceiling effect observed for some parameters with low doses of GLPG0492.

The potential outcome of drug treatment on the trabecular bone structural parameter (BV/TV) values was assessed after 12 weeks of treatment. The exercised mdx mice presented a 30% decrease of BV/TV vs. wt; however, no significant effect of GLPG0492 was observed (data not shown).

3.6. Plasma level of drug and testosterone

Due to the limited dose-dependent effect observed on various parameters, the plasma levels of GLPG0492 were determined during the long-term treatment. As shown in Fig. 11, the plasma levels of GLPG0492 were in the range expected from a single acute dose. Interestingly, a dose-dependent relationship was observed.

We also evaluated the possible impact of the treatment on testosterone levels. First, we estimated the basal differences between wt and mdx mice at 8 weeks of age and the impact of 4 weeks of exercise on those differences. As shown in Fig. 12A, a slightly higher value of testosterone was observed in mdx vs. wt mice and in the exercised groups vs. the non-exercised groups. These differences were not significant. The 12-week treatment with GLPG0492 did not lead to significant changes in the testosterone plasma levels at doses of 0.3 or 3 mg/kg. A slight reduction was observed at 30 mg/kg, but the reduction did not reach significance, likely because of the great inter-individual variability (Fig. 12B).

3.7. Effect of GLPG0492 on target-gene expression

To elucidate the mechanism of action of GLPG0492 on dystrophic muscle, gene expression analysis was performed. A few genes were selected as potential targets of SARM action based on the available evidence. In particular, insulin-like growth factor-1 (IGF-1) and follistatin, genes involved in control of muscle mass; myogenin, a marker of muscle
regeneration; and peroxisome-proliferator-receptor γ-coactivator (PGC)-1α (ppargc1a), a modulator of muscle metabolism and of mechano-transduction signalling were chosen.

The analysis focused on the two doses, 0.3 mg/kg and 3 mg/kg, for which the effects on other parameters eliminated the occurrence of non-specific/toxic effects. The values were normalised to the levels of the GADPH and HPRT1 housekeeping genes; however, because no differences were observed with the two housekeeping genes, only the effects normalised for GADPH are shown in Fig. 13. No significant changes were observed in both gastrocnemius and diaphragm muscles, although slight increases in IGF-1, myogenin and PGC-1α were observed in the 0.3 mg/kg-treated group in gastrocnemius muscle.

4. Discussion

The present study is the first in vivo and ex vivo pre-clinical evaluation of GLPG0492, a novel selective androgen receptor modulator (SARM), on a mouse model of Duchenne muscular dystrophy. The rationale for the study was the expected benefit of anabolic compounds to counteract the muscle wasting that occurs in dystrophic conditions without the unwanted side effects typical of anabolic steroids [18–22]. Our studies show a clear improvement in the in vivo performance of GLPG0492–treated mdx mice in terms of both increases in animal strength and resistance to exercise fatigue, which predict a potential benefit for dystrophic patients. In fact, the test used to evaluate fatigability to exercise performance is considered representative of the 6-min walk test used for DMD patients.

The in vivo effects observed after short-term treatment with the high dose of 30 mg/kg were greater than those observed with the natural comparator, the steroid nandrolone, as well as those of prednisolone, the “gold standard” for pharmacological management of Duchenne patients [12]. Importantly, the beneficial effects observed for the in vivo parameters was maintained over time and was evident at 10- and 100-fold lower doses, emphasising the remarkable sensitivity of dystrophic animals towards this compound. Accordingly, the lack of a dose-dependent relationship observed with many parameters might be because the low doses are near the maximal effective ones. In fact, the plasma levels of GLPG0492 in chronically treated mice were clearly dose-dependent and comparable to those after acute dosing, ruling out potential saturation in the pharmacokinetic of GLPG0492.
Surprisingly, no significant drug effect was observed on gross muscle mass and histologically on fibre diameter (data not shown), suggesting a lack of hypertrophic action. These findings are quite different from the results observed with other approaches, such as myostatin-inhibitors or β2 agonists, for which hypertrophic action appears to be the mechanism responsible for the functional benefit in dystrophic mice [13,14,16]. Whether hypertrophy is a real desired effect in enhancing performance is still controversial. In addition, drug-mediated hypertrophy may be more evident during the growing phase of dystrophic animals [15,17,31]. We actually tested the SARM in growing young adult mdx mice, showing that the absence of hypertrophy is not an age-related issue.

Importantly, the effect of androgens and SARM on muscle mass has often been observed under conditions of experimentally induced atrophy, including those from corticosteroids, hypogonadism, or ageing sarcopenia [32–34]. In mdx mice, the basal levels of testosterone were slightly higher than in wt, whereas the
Fig. 12. Plasma testosterone levels in wt and mdx mice. In (A), the bars show the serum testosterone levels of 8-week-old wild type and mdx mice either exercised for 4 weeks (WT EXER; MDX EXER) or not (WT SED; MDX SED). Each bar is the mean ± S.E.M. from 5 to 6 animals. Significant differences between groups were evaluated by Student’s t test. *Significantly different with respect to wt mice with \( p < 0.05 \). In (B), the bars show the effect of GLPG0492 on plasma testosterone levels in mdx mice. Each bar is the mean ± S.E.M. from 5 to 7 animals.

exercise-induced increase was slightly lower, suggesting a possible but not failure of the axis muscle activity-testosterone [35–38]. GLPG0492, as with many developed SARMs, acts as a partial agonist of androgen receptors [23,24]. Thus, the absence of hypertrophy in our experimental settings may be related to the normal hormonal conditions of the animals used and the occurrence of antagonism of testosterone actions. This hypothesis is supported by the slight decrease in the testosterone level and the parallel decrease in prostate weight observed in the mdx mice after prolonged administration with the highest dose of GLPG0492. However, the treatment of the non-exercised mdx mice with GLPG0492 at 3 mg/kg for 4 weeks produced minor, if any, amelioration of the in vivo performance compared to that observed in exercised mice (data not shown), which is likely related to the lower phenotype severity.

Importantly, DMD patients experience delayed puberty with reduced testosterone levels, often related to the use of corticosteroids [39], which predicts a greater sensitivity to SARMs with respect to the mdx animals.

From a functional perspective, an improvement in contraction strength was observed with the isolated muscles of mdx mice, and different sensitivities for the diaphragm and hind limb EDL muscle were observed. These results imply that there are muscle-specific actions of the drug possibly due to differences in either drug distribution or androgen-receptor expression. The slight but significant increase in strength recorded in the diaphragm is important from a therapeutic perspective, as this muscle presents impairment more
similar to that in patients [2,40]. Importantly, the EDL muscle was also partially protected by GLPG0492, with respect to fatigue and damaging eccentric contraction, during the longer treatment duration. In parallel, the cellular indices of myofibre distress such as the mechanical threshold and resting sarcotensional tonic conductance were ameliorated in treated EDL myofibres, although this amelioration was neither time- nor dose-dependent. The functional improvement in both respiratory and limb muscle may, at least partially, account for the observed in vivo effects; however, the exact correlation between the in vivo and ex vivo performances is currently under further evaluation.

No marked signs of amelioration, such as reduced degeneration or enhanced regeneration, were evident in the histological analysis of the GLPG0492-treated muscles, despite the increase in protein synthesis, which suggests anabolic activity. In addition, the biochemical plasma markers of muscle sufferance, such as CK and LDH, were not improved. Also, no changes were found in the expression of genes that can be the target of androgen-like drugs and are involved in regeneration and growth, such as IGF-1, follistatin and myogenin or in muscle metabolism, such as PGC-1α. These results raise questions about the mechanism of action of the SARM in the skeletal muscle of dystrophic animals. However, it is important to emphasise that GLPG0492-treated diaphragms showed a significant decrease in TGF-β1, a marker of fibrosis, which paralleled, in the first study, a decrease in non-muscle area. Although not investigated herein, the reduction of TGF-β1 could be related to the ability of androgens to interfere with the WNT-β catenin pathway, which eventually results in the inhibition of TGF-β1 production and signalling [23]. This action is important, due to the key role of fibrosis in dystrophinopathies and the potential usefulness of a less fibrotic respiratory muscle to contract and sustain training performance. Additionally, part of the amelioration of the alterations in the in vivo performance may result from the action of GLPG0492 on other systems, such as the peripheral and central nervous systems. In fact, androgens have been claimed to enhance motor nerve function and increase the number of synaptic contacts, the density of acetylcholine receptors and the level of neurotrophins such as BDNF [38,41]. These potential effects in dystrophic animals need to be addressed in future studies.

Despite the tissue-selective action, the mechanism of action of both steroid hormones and SARMs on skeletal muscle is not yet clear, and our data indicate that this is particularly true for dystrophic animals. Androgen receptors (AR) are classical intracellular receptors that modulate the transcription of specific genes once activated. Several tissue specific co-activators and co-repressors play a key role in the action of testosterone [42]. SARMs have also been claimed to interfere with AR and modulate a different set of genes [23]. In skeletal muscle, some of these co-activators belong to the supervillin family, a little known family of proteins with relevant actions as cytoskeletal components [43,44]. Some of the supervillin-related proteins, such as arhcrillin co-segregate with dystrophin at the costamere level [44]. Then, the absence of dystrophin may affect the localisation and function of co-activators of AR, leading to a difference in androgen-mediated physiological signalling. These aspects deserve additional specific studies.

5. Conclusions

The results of this pre-clinical study suggest that GLPG0492 may improve the quality of life of DMD patients due to its ability to enhance functional performance. Further studies are necessary to clarify its mechanism of action and its potential role as a primary agent to counteract muscle wasting or as an important adjuvant with other therapies, such as corticosteroids. Pre-clinical studies with low doses of GLPG0492 in combination with PDN will be essential to demonstrate the potential advantages and clear therapeutic applications in DMD.

Conflicts of interest

None.

Acknowledgments

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References

drolone enhances skeletal muscle myosin synthesis and alters global gene expres
sion profile in Duchenne muscular dystrophy. American Journal of Physi

[23] Bhasin S, Jasuja R. Selective androgen receptor modulators as function pro
moting therapies. Current Opinion in Clinical Nutrition and Metabolic Care

receptor modulators in preclinical and clinical development. Nuclear Receptor
Signaling 2008;6:e010.

Enhanced dystrophic progression in mdx mice by exercise and beneficial effects
of tauine and insulin-like growth factor-1. Journal of Pharmacology and Exper

disciplinary evaluation of the effectiveness of cyclosporine a in dystrophic mdx

pathological events in exercised dystrophic mdx mice are targeted by pentoxi
fylline: outcome of a large array of in vivo and ex vivo tests. Journal of Applied

[28] Brooks SV, Faulkner JA. Contractile properties of skeletal muscles from young,

Enalapril treatment discloses an early role of angiotensin II in inflammation-
and oxidative stress-related muscle damage in dystrophic mdx mice. Pharma

[30] Cozzoli A, Rolland JF, Capogrosso RF, Sblendorio VT, Longo V, Simonetti S,
et al. Evaluation of potential synergistic action of a combined treatment with
alpha-methyl-prednisolone and tauine on the mdx mouse model of Duchenne
muscular dystrophy. Neuropathology and Applied Neurobiology 2011;37(3),
243–56.b.

stimulus is needed for IGF-1 to induce skeletal muscle hypertrophy in vivo.

tive androgen receptor modulator on dexamethasone-induced and hypogonadism-

[33] Cao W, Reiser PJ, Coss CC, Phelps MA, Kearbey JD, Miller DD, et al. Selective
androgen receptor modulator treatment improves muscle strength and body
composition and prevents bone loss in orchidectomized rats. Endocrinology

stand on androgen and human growth hormone use. Journal of Strength and

[35] De Luca A. Pre-clinical drug tests in the mdx mouse as a model of dys

Testosterone physiology in resistance exercise and training: the up-stream

[37] West DW, Phillips SM. Anabolic processes in human skeletal muscle: restor
ing the identities of growth hormone and testosterone. Physician and

[38] Verhovshek T, Cai Y, Osborne MC, Sengelaub DR. Androgen regulates
brain-derived neurotrophic factor in spinal motoneurons and their target mus


[40] Willmann R, Possekel S, Dubach-Powell J, Meier T, Ruegg MA. Mammalian
animal models for Duchenne muscular dystrophy. Neuromuscular Disorders

[41] Bleisch WV, Harrelson A. Androgens modulate endplate size and ACh recep

[42] Heemers HV, Tindall DJ. Androgen receptor (AR) coregulators: a diversity of
functions converging on and regulating the AR transcriptional complex.

[43] Ting HJ, Yeh S, Nishimura K, Chang C. Supervillin associates with andro
gen receptor and modulates its transcriptional activity. Proceedings of the
National Academy of Sciences of the United States of America 2002;99(2):
661–6.

a muscle-specific isoform of supervillin, is an early expressed component
of the costameric membrane skeleton. Journal of Cell Science 2003;116(Pt
11):2261–75.