Omecamtiv mecarbil lowers the contractile deficit in a mouse model of nebulin-based nemaline myopathy

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Abstract

Nemaline myopathy (NEM) is a congenital neuromuscular disorder primarily caused by nebulin gene (NEB) mutations. NEM is characterized by muscle weakness for which currently no treatments exist. In NEM patients a predominance of type I fibers has been found. Thus, therapeutic options targeting type I fibers could be highly beneficial for NEM patients. Because type I muscle fibers express the same myosin isoform as cardiac muscle (Myh7), the effect of omecamtiv mecarbil (OM), a small molecule activator of Myh7, was studied in a nebulin-based NEM mouse model (*Neb* cKO). Skinned single fibers were activated by exogenous calcium and force was measured at a wide range of calcium concentrations. Maximal specific force of type I fibers was much less in fibers from *Neb* cKO animals and calcium sensitivity of permeabilized single fibers was reduced (pCa_{50} 6.12 ±0.08 (cKO) vs 6.36 ±0.08 (CON)). OM increased the calcium sensitivity of type I single muscle fibers. The greatest effect occurred in type I fibers from *Neb* cKO muscle where OM restored the calcium sensitivity to that of the control type I fibers. Forces at submaximal activation levels (pCa 6.0–6.5) were significantly increased in *Neb* cKO fibers (~50%) but remained below that of control fibers. OM also increased isometric force and power during isotonic shortening of intact whole soleus muscle of *Neb* cKO mice, with the largest effects at physiological stimulation frequencies. We conclude that OM has the potential to improve the quality of life of NEM patients by increasing the force of type I fibers at submaximal activation levels.

Introduction

Nebulin is a giant filamentous protein, located in the skeletal muscle sarcomere[1] where it winds around the actin filament, from the Z-disk to near the pointed end of the actin filament[2]. Nebulin is believed to play an important role in muscle contraction[3–5], it maintains myofibrillar alignment[6] and functions as a thin filament stabilizer that regulates thin filament length[7]. Nebulin deficiency due to mutations in the nebulin gene (NEB) is an important cause of Nemaline Myopathy (NEM)[8–11]. Although the phenotypes of nebulin-related NEM patients are variable, a common functional feature is skeletal muscle weakness [12–17]. No treatments for NEM exist to improve muscle function. In NEM patients a predominance of type I fibers has been found [13, 16]. Thus, therapeutic options targeting type I fibers would be highly beneficial for NEM patients.

Omecamtiv mecarbil (OM) is a selective small-molecule activator of cardiac myosin (Myh7 or β-MHC) that was developed as a treatment for heart failure [18, 19]. Single molecule studies have shown recently that OM suppresses the size of the working stroke of myosin and prolongs the duration of its attachment to actin [20]. It has been proposed that OM-inhibited myosin heads cooperatively activate the thin filament at submaximal activation levels, recruiting OM-free myosin heads and increasing force [20].

Although OM has been developed for heart failure patients, cardiac myosin (Myh7 or β-Myh) is identical to the type I myosin expressed by slow skeletal muscles[21] suggesting that OM might also be effective in skeletal muscle. Furthermore, type I fibers are the dominant fiber type in most human muscles [22, 23]. The percent type I fibers in healthy humans is >60% for a wide range of muscle types [23] and examples of type I rich muscles in the mouse are the piriformis (~60%), the quadratus femoris (~70%)[24], and the soleus (~50%, Fig 1). Furthermore, studies have shown that in both mouse models of NEM and in NEM patients there is an additional shift towards type I fibers [12–15, 25]. Thus, OM might be effective in increasing skeletal muscle force, particularly in NEM patients. We investigated whether OM has a beneficial effect on slow skeletal muscle and used a conditional nebulin knockout mouse model (*Neb* cKO) which expresses low levels of nebulin and has severe weakness in peripheral and respiratory muscles[25], phenocopying NEM[9, 16, 25, 26]. Additionally, there is a fiber type I predominance [25], as in NEM patients. Hence, we tested the hypothesis that OM ameliorates the force deficit in *Neb* cKO mice.

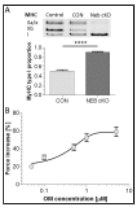

Fig 1

Fiber type composition of *Neb* cKO muscle and dose-response curve of OM.

Materials and methods

The conditional nebulin knockout (*Neb* cKO) mouse was previously described [25]. Mice that were positive for MCK-Cre and homozygous for the floxed nebulin allele were nebulin deficient and are referred to as *Neb* cKO. Littermate controls (CON) were WT for nebulin[25]. Studies were performed on membrane-permeabilized soleus muscle fibers and intact whole soleus muscles. Six months old *Neb* cKO (189±3 days old) and CON (186±1 days old) mice were used for this study. In this work we focused on the fiber type I rich soleus muscle. The weight of the soleus muscle was 7.7 ± 0.5 mg (n = 20) in CON and 11.6 ± 0.7 mg (n = 25) in Neb cKO mice and the physiological cross-sectional area at mid-belly was 0.58 ± 0.03 mm^2 (CON) and 0.91 ± 0.05 mm^2 (*Neb* cKO). This is similar to our previous study [25] in which it was found that most muscles in the *Neb* cKO mice are atrophied, with as exception the soleus muscle that hypertrophies. Furthermore, it is known from previous work that the fiber cross-sectional area of type I fibers in the soleus is reduced significantly in the *Neb* cKO while the number of type I fibers is greatly increased [25]. All experiments were in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals and approved by the University of Arizona Institutional Animal Care and Use Committee.

Omecamtiv mecarbil

Omecamtiv mecarbil (OM) was purchased from Selleckchem (Houston, TX) and was dissolved in dimethylsulfoxide (DMSO) to make a stock solution as instructed by the manufacturer. OM stock solution was then added to the experimental solution to prepare the final desired concentration of OM. In most studies, we used a low OM dose (0.1 μM) to limit effects on cardiac muscle that occur mainly at OM concentration > 0.3 μM [18, 19, 27–30].

Skinned muscle fiber mechanics

Experimental procedure The procedures for skinned muscle mechanics were as described previously[31]. Briefly, soleus muscles were skinned overnight at ~4°C in relaxing solution (in mM: 40 BES, 10 EGTA, 6.56 $MgCl_2$, 5.88 NaATP, 1 DTT, 46.35 K-propionate, 15 creatine phosphate, Ionic strength 180 mM, pH 7.0 at 20°C) containing 1% (w/v) Triton X-100 and protease inhibitors (in mM: 0.01 E64, 0.04 leupeptin and 0.5 PMSF). Muscles were then washed thoroughly with relaxing solution and stored in 50% glycerol/relaxing solution at −20°C[32]. Either fiber bundles (Fig 2) or single fibers (Figs 3 and 4) were dissected and mounted using aluminum T clips between a length motor (ASI 322C, Aurora Scientific Inc.), and a force transducer element (ASI 403A, Aurora Scientific Inc.) in a skinned fiber apparatus (ASI 802D, Aurora Scientific Inc.). Sarcomere length was set in passive fibers to 2.4 μm using a high-speed camera and video-based sarcomere length software (ASI 900B, Aurora Scientific Inc.); in a small subset of control fibers we measured sarcomere length and found no difference in sarcomere shortening between OM treatment vs. vehicle (DMSO). Muscle bundles/fibers were activated in pCa (pCa = –log($[Ca^{2+}]$) 4.0 activating solution (in mM: 40 BES, 10 $CaCO_3$ EGTA, 6.29 $MgCl_2$, 6.12 Na-ATP, 1 DTT, 45.3 potassium-propionate, 15 creatine phosphate, Ionic strength 180 mM, pH 7.0 at 20°C) and protease inhibitors. Fiber width and depth (built-in prisms allow for side view of fibers and measurement of depth) were measured at three points along the fiber, and the cross-sectional area (CSA) was calculated assuming an elliptical cross-section. The CSA of type I fibers was on average 1300 μm^2 for CON and 1183 μm^2 for *Neb* cKO fibers. Specific force was expressed as force per CSA (mN/mm^2) and used for comparison of force between groups. In a separate series of experiments we also used skinned cardiac muscle strips (LV papillary)[33] and performed OM dose-response experiments (pCa 6.0) as explained above for skeletal muscle, except that the sarcomere length to which the passive cardiac muscle was set prior to activation was 2.0 μm.

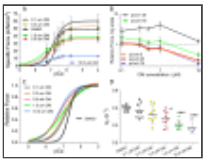

Fig 2

Effect of OM on force-pCa relations of permeabilized fiber bundles from Neb cKO mice.

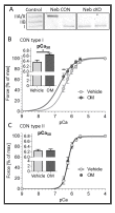

Fig 3

Effect of OM on active force of single soleus fibers from CON mice.

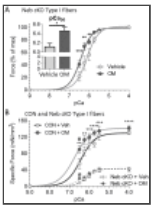

Fig 4

Effect of OM on active force of single type I fibers of *Neb* cKO and comparison with CON type I fibers.

Protocols

Preparations were in relaxing solution and then immersed in pre-activating solution (relaxing solution with a 10-fold lower EGTA concentration), followed by activation with incrementally increased pCa (pCa = –log([Ca^{2+}]), ranging from 6.5 to 4.0. Solutions with different pCa were created by mixing varying volumes of relaxing and activation solutions. The obtained force–pCa relation was fit with a Hill equation, providing pCa_{50} (pCa giving 50% of maximal active force) and the Hill coefficient, n_H, an index of myofilament cooperativity. Experiments were performed at 20°C. Separate groups of muscle bundles/fibers were used to test the effect of vehicle (DMSO) and OM (in DMSO). Fibers from a total of five CON and five *Neb* cKO mice, with multiple fibers (4–6) per animal were used in fiber experiments. Five to seven soleus bundles from two *Neb* cKO were used for dose-response studies. k_{tr}-measurements: The rate of tension redevelopment (ktr) was measured at steady-state force by rapidly shortening (1 ms) the fiber at one end of the fiber resulting in unloaded shortening of the fiber for 20 ms. Remaining bound cross-bridges were detached by rapidly restretching the fiber to initial length and the tension redeveloped [34]. ktr was determined by fitting the rise of force to the following equation (one-phase association curve): F = Fss*(1−ektr*t)+c, where F is force at time t, Fss is steady-state force. After mechanical experiments, the bundles/fibers were stored in SDS-sample buffer for gel electrophoresis and myosin isotyping (see below).

Intact muscle mechanics

Muscle experiments Mice were anesthetized with isoflurane and sacrificed by cervical dislocation. The soleus muscle was dissected and mounted to the isolated muscle test system (1200A, Aurora Scientific Inc.) that has been described previously[35]. In brief, both tendons were tied with 5–0 silk sutures, and the muscle was attached between the lever arm of dual servomotor-force transducer (300C, Aurora Scientific Inc.) and a fixed hook. The experimental bath was filled with Krebs-Ringer solution containing (in mM) 137 NaCl, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 1 NaH_2PO_4, 24 $NaHCO_3$, 11 Glucose with 95% O_2 and 5% CO_2 supply (pH = 7.4). The temperature during the experiments was 30°C. The muscle was placed between platinum electrodes connected to an electrical stimulator (701C, Aurora Scientific Inc.) for muscle activation. The optimal muscle length (L_0) was determined by adjusting overall muscle length until maximal force was generated with a 400ms 20Hz stimulation frequency (pulse duration of 200μs with biphasic polarity). The muscle length at L_0 was measured using a digital caliper. At L_0, an initial tetanic contraction at 150Hz stimulation frequency was imposed. Measured forces were normalized to the physiological cross-sectional area (PCSA) to obtain specific force (mN/mm^2). PCSA (in mm^2) was calculated using muscle mass (in g), fiber length (in mm), and muscle density (1.056 g/mm^3);PCSA = Muscle mass / (Fiber length × Muscle density). Since muscle fibers of soleus align to their tendon with a pennation angle and do not span the full muscle length, fiber length is estimated from muscle length and pennation angle. As reported in Burkholder et al.[36], the ratio of fiber and muscle length for soleus is 0.72. We used this ratio to calculate the fiber length from the measured muscle length.

Force-frequency protocol To establish the force-frequency relation, active forces at various stimulation frequencies were measured. 14 CON and 14 *Neb* cKO mice were used for the force-frequency protocol. The muscle was stimulated at 1, 5, 10, 20, 30, 40, 60, 100 and 150 Hz and waiting for 30, 30, 60, 90, 90, 120, 120, 180 and 180 s, respectively, in between stimulations. The first force-frequency protocol was performed in the vehicle solution (DMSO), and the experimental solution was then switched to OM solution. The muscle was incubated in OM solution for 15 min with twitch stimulation every minute to monitor changes in force. When the increase in force was stable, the force-frequency protocol was measured.

Force-velocity protocol To determine the force-velocity relation, the load-clamp technique was used as previously described[37]. Shortening velocities were measured during isotonic contraction against loads 85, 70, 55, 40, 25, 15, and 5% of maximal force at 25 Hz of stimulation frequency. Each contraction started out with a 200 ms isometric phase (to reach the plateau force), and then a 150 ms load-clamp was applied. A series of load-clamps was performed in solution with vehicle and the same protocol was then repeated in OM solution. The muscle was incubated in OM solution for 15 min with twitch stimulation every minute before the protocol with OM solution was performed. After completion of each experiment, the sutures and tendons were carefully removed, and the muscle mass was then measured. The muscle was quick-frozen in liquid nitrogen and stored at −80°C for later analysis of fiber type composition.

Analysis The force-frequency curve was fit with a Hill equation to calculate the half-maximal frequency (frequency giving 50% of maximal force increase beyond twitch force) and the Hill coefficient (n_H), an index of the steepness of the curve. Shortening velocity (in mm/sec) was measured as a slope of linear portion of the shortening period (~40 ms duration) after force was stable. For each preparation, shortening velocity was normalized by

muscle fiber length. Muscle power production was calculated by multiplying the shortening velocity and the applied load. Force-power curves were fitted by 2nd order polynomial non-linear regression, and the maximal power production was determined from the apex of the force-power curve.

Myosin heavy chain isoform determination and distribution

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to determine the myosin isoform composition of the muscle lysates and single fibers as previously described [35]. The stacking gel contained a 4% acrylamide concentration (pH 6.7), and the separating gel contained 8% acrylamide (pH 8.7) with 30% glycerol (v/v). The gels were run for 24 h at 15 °C and a constant voltage of 275 V. Gels for whole muscle lysates were stained with Coomassie blue and single fiber gels were silver-stained. Gels were scanned and analyzed with ImageJ (v1.49, NIH, USA).

Statistical analyses

Data are presented as mean ± SEM. GraphPad Prism 6 was used to calculate statistics. For statistical analysis one-way ANOVA, two-way ANOVA and the t-test with multiple testing corrections were used, as appropriate. For permeabilized single fibers study, Student's t-tests were performed on force-pCa curve (each pCa) and pCa_{50} of each fiber type and genotype (OM vs. Vehicle; Figs 3 & 4). For the force-frequency curve of the intact whole muscle experiment, paired t-tests were used at each stimulation frequency (OM vs. Vehicle). Student's t-tests were also used for comparison of genotype (CON vs. cKO) in MHC type I proportion (Fig 1A) and relative force increase (Fig 5A) in intact whole muscle experiment. Repeated measure 2-way ANOVA and multiple post-hoc t-test using Sidak method were performed for half-frequency, Hill-coefficient (Fig 5B and 5C), and maximal power (Fig 6B) analysis. $p < 0.05$ was considered to be statistically significant.

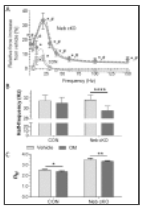

Fig 5

Effect of OM on force of intact soleus muscle.

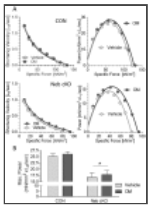

Fig 6

Effect of OM on power production in intact soleus muscle.

Results

Fiber type composition of *Neb* cKO muscle and Dose-response of OM

It has been previously reported that nebulin deficiency leads to a shift in myosin heavy chain isoform distribution from fast type II to slow type I MHC[25]. In order to test this in the mice studied here, SDS-PAGE was performed on lysates obtained from muscles used in the mechanical studies. Representative MHC gels and analyzed results are shown in Fig 1A. The average proportion of type I MHC isoform in soleus muscle was 51±3% in control (CON) muscle and 91±1% in *Neb* cKO muscle. Thus the soleus muscle of *Neb* cKO muscle expresses predominately type I myosin. We also performed studies to establish the OM dosage for our experiments and measured the OM effect in intact *Neb* cKO soleus muscle activated with 20 Hz stimulation. As shown in Fig 1B, OM significantly increased force; this effect was seen already at 0.05 μM OM and followed a dose-response curve with an EC_{50} of 0.6 μM.

The OM dose-response curve was also established for skinned fiber bundles dissected from soleus muscle of *Neb* cKO mice. (Note that these fiber bundles are fiber-type mixed and that studies with fiber-typed single fibers are described later in the Results section). Fiber bundles were sub-maximally activated by exogenous calcium at a range of OM concentrations (S1A Fig). All tested OM concentrations increased force production. Interestingly, the force increase reaches a plateau between 0.5 μM and 1.0 μM, and declined at a higher dose (S1A Fig). The half-maximal effect occurred at ~0.1 μM (the force decline at 10 μM precluded fitting the data to a standard dose-response curve). For a comparison we also performed experiments on submaximally-activated permeabilized left ventricular papillary muscle and found that force increased more gradually and did not decline at high OM doses (S1B Fig, see also Discussion).

We also determined the OM effect on the full force-pCa relationship of soleus fiber bundles. At 0.1 and 0.3 μM OM, force was increased, particularly at submaximal activation levels (Fig 2A and 2B). At 0.5 μM and higher the force increase at submaximal activation was small and at maximal activation, force was depressed (Fig 2B). Note that force depression was absent in the intact muscle experiments (Fig 1), most likely because these experiments were performed at a frequency (20 Hz) that produces submaximal force levels (see also Discussion). Normalized force-pCa curves of soleus fiber bundles, with force normalized to the maximal force (pCa 4.0), are left shifted in OM (increased pCa_{50}) and less steep (reduced Hill coefficient), Fig 2C. We also investigated cross-bridge cycling kinetics by measuring the rate constant of tension redevelopment (k_{tr}) in *Neb* cKO bundles at a range of OM-concentrations. Starting at 0.5 μM OM, k_{tr} is decreased (Fig 2D). As k_{tr} correlates with myosin ATPase activity [34, 38], these experiments suggest that high OM doses slow ATPase activity. Based on these results and the desire to limit the likelihood of OM effects on cardiac muscle [18, 27, 28, 30] (see also Discussion), 0.1 μM OM was used in a follow-up study focused on single fibers that have been fiber-typed.

Force measurements on fiber-typed single fibers

The effect of OM on contractility was first studied by measuring the Ca^{2+} dependence of force in membrane-permeabilized soleus slow type I and fast type II single fibers. This comparative study was performed only on CON fibers (that express nebulin) because of the limited number of type II fibers in *Neb* cKO soleus muscle (out of the 50 studied fibers only four fibers (one treated with vehicle and three treated with OM) were type II, which precluded statistical testing). Examples of single fiber gels are shown in Fig 3A. Single fibers were activated with incrementally increasing concentrations of Ca^{2+} with or without 0.1 μM OM. Treatment of fibers with OM resulted in a robust increase in submaximal force production in type I fibers (Fig 3B). We did not observe any effect of OM on type II fibers (Fig 3C). The results in type I fibers show an OM-induced increase in pCa_{50} (the pCa for half-maximal force) of 0.29 pCa-units (insets of Fig 3B and 3C). Thus, at a submaximal activation level, OM significantly affects force production of type I fibers.

Soleus type I fibers from *Neb* cKO mice were studied next. Compared to type I fibers from CON muscle these fibers had significantly lower maximal specific force and significantly lower calcium sensitivity (Table 1), as shown by their reduced pCa_{50} (6.12 in cKO and 6.36 in CON). The low specific-force levels of the *Neb* cKO fibers are likely due to the absence of nebulin but the role of other adaptive changes cannot be excluded. It is known that at the whole muscle level profound changes in expression occur in MHC, Troponin-T, Troponin-I and Tropomyosin isoforms [25]. This is likely due to the known fiber-type switch from fast to slow, but determining whether differences exist when comparing the same fiber type in different genotypes will require future work focused on single fiber proteomics. OM had no effect on the maximal active force, but at submaximal Ca^{2+} levels, forces were significantly increased (Fig 4A). The greatest increase was at low Ca^{2+} levels, the effect became less as Ca^{2+} was increased and was absent at maximal activation (Fig 4A). At intermediate calcium levels the increase was large, for example at pCa 6.5, OM increased specific force from 8.4±2.6 mN/mm^2 to 19.2±3.5 mN/mm^2. However, it is important to point out that although specific force was more than double; it still was much below that of untreated control fibers (55 mN/ mm^2 at pCa 6.5).

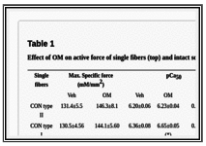

Table 1

Effect of OM on active force of single fibers (top) and intact soleus muscle (bottom).

The OM-induced increase in force at sub-maximal activations in type I *Neb* cKO fibers resulted in a leftward shift of the force-pCa curves (Fig 4A). The Hill coefficient (n_H), a measurement of the filament cooperativity, was also determined. Although mean n_H values were reduced by OM, the differences were not significant (Table 1). The OM-induced leftward shift of the force- pCa relation increased the pCa_{50} from 6.12±0.10 to 6.46±0.08 in *Neb* cKO fibers (Fig 4A) which is close to that of type I CON fibers in vehicle (pCa 6.36, Table 1).

Effect of OM on intact muscle

Intact soleus muscles were electrically stimulated at a range of frequencies to determine their force-frequency relation. The maximal specific force of *Neb* cKO soleus was ~1/3 of the maximal specific force of CON soleus, consistent with the maximally activated single fiber data (Table 1) and highlighting that the force deficit of intact *Neb* cKO muscle has a myofilament origin. OM has a small but significant effect on force at all stimulation frequencies. The increase was maximal at 20Hz (32±5%) and gradually decreased at higher stimulation frequencies (Fig 5A, dark symbols). However, in CON muscle the effect was less (Fig 5A, light gray symbols) and only reached significance at 10 Hz (increase 5±2%) and 20Hz (increase 7±2%). From the force-frequency curves, the frequency for half-maximal force increase beyond twitch force (defined as half-frequency) and the Hill coefficient (n_H) were calculated (Fig 5B and 5C). The average half-frequency was decreased by OM but this effect was only significant in *Neb* cKO muscle (Fig 5B and Table 1). Hill coefficients were significantly decreased by OM in both genotypes, and the effect was largest in cKO muscle (Table 1).

The shortening velocity at a range of load levels was also measured. The shortening velocity was normalized by muscle length, and the force-velocity curves were converted to force-power curves and the effects of OM were studied. Fig 6A show example experiments and 6B show the mean results of all experiments. OM had no significant effect on CON muscles but it enhanced the maximal power in *Neb* cKO muscles, the effect was small but significant (Fig 6B).

Discussion

Nemaline myopathy (NEM) is the most common non-dystrophic congenital myopathies, with features that include muscle weakness, muscle atrophy, presence of nemaline rod-bodies and type I fiber predominance[8, 9, 12–17]. It impacts both peripheral and respiratory muscles, and many NEM patients have difficulties in locomotion and respiratory function[17]. However, targeted treatment options for NEM patients are not available. Although OM has been developed as a heart failure drug, the expression of cardiac myosin (Myh7 or β-MHC) in both heart and slow skeletal muscle[21], predicts that OM is effective in slow skeletal muscle as well, and the findings of the present study clearly bear this out. OM greatly increases the force of single type I fibers activated at submaximal activation levels and significantly increases the calcium sensitivity, as reflected by the increased pCa_{50} (Fig 4). Considering the large number of type I fibers in humans [22–24] and the additional shift in fiber type distribution of nebulin-deficient muscle and NEM patients toward type I fibers [12–15], OM might be a therapeutic option to ameliorate the muscle weakness in NEM patients. That OM is well-tolerated and is currently in phase-3 clinical trials for treatment of heart failure makes it an attractive therapeutic option. Our findings suggest that OM is far from a cure. However, it has a good potential to bring relief in nebulin-based nemaline myopathy patients, not only because of their type I predominance but also because nebulin deficiency appears to increase the effectiveness of OM. Below we discuss our findings in detail.

OM has been developed to increase the contractile force of cardiac muscle in heart failure patients[18]. Unlike various existing calcium sensitizers[39] OM does not affect calcium transients, which limits possible side effects (e.g., altering calcium-dependent signaling processes) [18, 40]. Recent single molecule studies [20] provide evidence that OM binds myosin, causing long-lived strongly bound myosin heads that do not generate force. From these studies, a model was proposed in which these OM-bound heads cooperatively activate the thin filament at submaximal activation levels, recruiting OM-free myosin heads and thereby increasing force [20]. This mechanism naturally explains the sarcomere length dependence of the magnitude of the OM-effect that has been found in sub-maximally activated cardiac muscle[41], with larger OM effects at short sarcomere length where the baseline activation level of the thin filament is lower and more OM-free heads can be recruited to the force generating pool than at longer sarcomere length. It can also explain the reduced OM effects at high OM levels reported by Nagy et al. for both cardiac myocytes and diaphragm fibers[42], and seen here in soleus fiber bundles (S1A Fig): at high OM levels the OM-bound heads that do not generate force outnumber the additionally recruited OM-free force generating heads. The dose-response curve that we measured on cardiac muscle does not decline at high OM levels (S1B Fig) a finding similar to that of Gollapudi et al (their Fig 2)[41]. This can be explained by the short sarcomere length that was used in these studies (2.0 μm in our study and 1.9 μm in [41]), where baseline activation is low, and the long sarcomere length used by Nagy et al in their cardiac myocyte study (2.3 μm) where the baseline activation level is high. Thus a mechanism based on an increased attachment lifetime of OM bound non-force producing myosin heads that enhances thin filament activation can explain the various findings. An additional mechanism recently proposed by Kampourakis et al. [43] to explain the OM effect involves the ON and OFF states of the thick filament [44, 45]. In the OFF state, many of the myosin heads in the thick filament are bent back towards the center of the sarcomere and are unable to interact with actin and in the ON state they are in a more perpendicular position and available for actin binding and force generation. By promoting the ON state of the thick filament and increasing the thin filament activation level, OM might facilitate actomyosin interaction and enhance force development at low to intermediate calcium levels [43, 45, 46]. The extent to which both thick filament and thin filament based mechanisms are responsible for OM-induced force increase requires future research.

The effects that were found in type I fibers (increased pCa_{50}-values and no effect on the maximal force) are in general similar to the OM effects reported in cardiac muscle. In cardiac muscle of mouse[47], rat[42, 43], guinea pig[41] and human[28], OM increases calcium sensitivity (higher pCa_{50}), and typically has no effect on the maximal active force. Our findings are also consistent with the work of Nagy et al who recently used OM on rat diaphragm muscle fibers[42]. Although the myosin isoform was not directly determined, Nagy et al did measure the kinetics of force generation (K_{tr}) which also provides insights in the fiber type (slow kinetics for type I fibers and fast kinetics for type II fibers). In both their and our study, OM clearly increases the calcium sensitivity of slow type I fibers (fibers with low K_{tr} values in Nagy et al[42] and Myh7 in our study). The Nagy et al study also found a small but significant effect of OM on type II fibers (identified by Nagy et al as having fast kinetics[42]), which we did not observe. This issue warrants follow-up. If future studies were to show that OM also affects type II MHC isoforms, it would extend OM's usefulness for treating skeletal muscle diseases with as it would increase the proportion of fibers being affected by OM. It would also represent a treatment option for myopathies with fiber type II dominance.

Most cardiac studies were performed at OM levels higher than ours (0.3–1.0 μM), except for studies on guinea pig myocardium[41] and human myocardium[28] (both of which found no effect at 0.1 μM) and a study on rat myocytes [45]that did find an effect at 0.1 μM but that was smaller than in the present study (e.g., ΔpCa_{50} ~0.1, our study ~0.3). Thus it appears that the sensitivity to OM is higher in our study on type I skeletal muscle fibers than in published studies on cardiac muscle. The explanation for this is not immediately clear but could reside in the distinct protein isoform composition of thin and thick filament proteins involved in activation in slow skeletal muscle compared to cardiac muscle. It is also possible that the

tissue-specific expression of nebulin (present in skeletal muscle but absent in cardiac muscle[48]) is part of the explanation, or that some other putative adaptation in the proteome of nebulin-free skeletal muscle fibers play a role. Future work is required to fully understand the details of the OM effect on both cardiac and skeletal muscle. The higher OM sensitivity of skeletal muscle type I fibers, compared to cardiac muscle, is beneficial as it provides a therapeutic window for a sole skeletal muscle effect. For OM to be useful for increasing skeletal muscle force in nebulin-based nemaline myopathy patients, undesirable effects in the heart have to be avoided. Slowing of force relaxation in the heart is of a particular concern since this can impair diastolic filling and cause ischemia, especially at high heart rates. However, clinical trials have shown that OM is well tolerated without adverse effects up to ~10 μM in healthy individuals, and in heart failure patients at plasma concentrations of up to ~1.0 μM (reviewed in[39]). Thus it is possible that the doses established in the present study will be able to increase skeletal muscle force in Nemaline Myopathy patients without adverse effects on the heart. Finally, it is also worth pointing out that when using OM in heart failure patients, undesirable effects on skeletal muscle have to be avoided. This could occur, for example, if high OM doses required for cardiac benefits in heart failure patients were to suppress force production or crossbridge cycling kinetics of type I fibers of the diaphragm. The possible clinical application of OM requires study of both the cardiac and skeletal muscle systems.

The effect of OM on intact muscle reached a maximum at 20Hz and then gradually decreased at higher stimulation frequencies (Fig 5A). This is similar to the findings in single fibers and, thus, in both preparations, the greatest effect of OM occurs at submaximal activation levels. Because skeletal muscles operate at submaximal activation levels during normal activity [22, 49, 50], OM is expected to enhance force development under clinically meaningful conditions. OM does not nearly recover the large functional deficit of nebulin deficient muscle and, thus, OM is far from 'a cure.' Considering that our work was performed at a low OM concentration (0.1 μM), which is below the EC_{50} of 0.6 μM, it is likely that the effect of OM can be augmented by increasing the OM dose. From our present study it is possible to conclude OM is likely to provide relief to NEM patients as increases in force of ~20% are clearly attainable, which is likely to improve quality of life for patients with muscle weakness.

It is also worth noting that OM has a small effect on intact muscle of *Neb* cKO mice at simulation frequencies that result in maximal tetanic force (Fig 5A), and that this is not the case in single fibers that are maximally activated (pCa 4.0) (Fig 4B, Table 1). This suggests that unlike in maximally activated skinned fibers, in intact muscle of *Neb* cKO mice, the number of myosin molecules participating in maximal tetanic contraction is not at its maximal level. This conclusion is consistent with an X-ray study on intact muscle that revealed that, compared to CON muscle, *Neb* cKO muscle has thick filaments that partially remain in the OFF state during a maximal tetanus [51]. Our findings indicate that OM corrects this effect by more completely switching the thick filament to its ON state. This mechanism (a deficit in the ON state of the thick filament in the absence of nebulin) can also explain the larger effect of OM on type I fibers of *Neb* cKO muscle compared to CON type I fibers. Finally, it also provides an explanation for the only study in which OM was found to increase the force of maximally activate skinned cardiac muscle (pCa ~4.0)[41]. This finding was obtained at a short sarcomere length (1.9 μm) where the thick filament might not be fully turned ON with calcium[52], and, thus, OM is able to augment the ON state of the thick filament and increase maximal force.

In summary, this study shows for the first time that at submaximal activation levels OM increases force and power of slow skeletal muscles. This is expected to be beneficial for nemaline myopathy patients where type I fibers dominate, with predicted benefits for respiratory and peripheral muscle function. OM currently is in a Phase 3 Heart Failure clinical trial (GALACTIC-HF). The previous phase I and phase II clinical trials have shown that OM is well tolerated to plasma levels of ~1.0 μM [39], a level beyond the level used in the present study. We conclude that OM has the potential to improve skeletal muscle function in NEM patients and bring much needed relief. Finally, it is also worth considering that although type II fibers do not appear to benefit from OM, fast skeletal muscle troponin activators (FASTA) have been developed that selectively activate type II fibers by increasing their sensitivity to calcium [53]. Indeed it has been shown that FASTAs increase force at submaximal activation levels in type II skeletal muscle fibers from nebulin-deficient mice and nemaline myopathy patients [54, 55]. Thus, by using a combination of both OM and FASTA, an increase in force of all fiber types might be achievable.

Supporting information

S1 Fig

A) Dose-response of OM effect on force production by soleus fiber bundles from Neb cKO mice (n = 6). Specific force was measured at pCa 6.75. OM increases specific force at low OM doses, the effect is maximal between 0.5 and 1.0 μM OM, and is much less at 10 μM OM. B) OM Dose-response using LV permeabilized papillary muscle from control (n = 7) and Neb cKO mice (n = 7). Specific force was measured at pCa 6.0. Specific force follows a dose-response curve with EC50 of 0.62±0.04 μM OM (control) and 0.79±0.08 μM OM (Neb cKO). No significant difference in EC50 (See text for details).

(TIF)

Click here for additional data file.(1.0M, tif)

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Data Availability

All data are contained within the paper and its Supporting Information files.

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Decision Letter 0

Agustín Guerrero-Hernandez, Academic Editor

6 Aug 2019

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Omecamtiv mecarbil lowers the contractile deficit in a mouse model of nebulin-based nemaline myopathy.

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Please include the following items when submitting your revised manuscript:

- A rebuttal letter that responds to each point raised by the academic editor and reviewer(s). This letter should be uploaded as separate file and labeled 'Response to Reviewers'.
- A marked-up copy of your manuscript that highlights changes made to the original version. This file should be uploaded as separate file and labeled 'Revised Manuscript with Track Changes'.
- An unmarked version of your revised paper without tracked changes. This file should be uploaded as separate file and labeled 'Manuscript'.

Please note while forming your response, if your article is accepted, you may have the opportunity to make the peer review history publicly available. The record will include editor decision letters (with reviews) and your responses to reviewer comments. If eligible, we will contact you to opt in or out.

We look forward to receiving your revised manuscript.

Kind regards,

Agustín Guerrero-Hernandez

Academic Editor

PLOS ONE

Journal requirements:

When submitting your revision, we need you to address these additional requirements.

1. Please ensure that your manuscript meets PLOS ONE's style requirements, including those for file naming. The PLOS ONE style templates can be found at

http://www.journals.plos.org/plosone/s/file?id=wjVg/PLOSOne_formatting_sample_main_body.pdf and http://www.journals.plos.org/plosone/s/file?id=ba62/PLOSOne_formatting_sample_title_authors_affiliations.pdf

2. To comply with PLOS ONE submissions requirements, in your Methods section, please provide additional information on the animal research and ensure you have included details on (1) methods of sacrifice for all parts of your study (including the study described in section 2.3), (2) methods of anesthesia used prior to cervical dislocation.

[Note: HTML markup is below. Please do not edit.]

Reviewers' comments:

Reviewer's Responses to Questions

Comments to the Author

1. Is the manuscript technically sound, and do the data support the conclusions?

The manuscript must describe a technically sound piece of scientific research with data that supports the conclusions. Experiments must have been conducted rigorously, with appropriate controls, replication, and sample sizes. The conclusions must be drawn appropriately based on the data presented.

Reviewer #1: Yes

Reviewer #2: Yes

****

2. Has the statistical analysis been performed appropriately and rigorously?

Reviewer #1: I Don't Know

Reviewer #2: Yes

****

3. Have the authors made all data underlying the findings in their manuscript fully available?

The PLOS Data policy requires authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception (please refer to the Data Availability Statement in the manuscript PDF file). The data should be provided as part of the manuscript or its supporting information, or deposited to a public repository. For example, in addition to summary statistics, the data points behind means, medians and variance measures should be available. If there are restrictions on publicly sharing data—e.g. participant privacy or use of data from a third party—those must be specified.

Reviewer #1: No

Reviewer #2: Yes

****

4. Is the manuscript presented in an intelligible fashion and written in standard English?

PLOS ONE does not copyedit accepted manuscripts, so the language in submitted articles must be clear, correct, and unambiguous. Any typographical or grammatical errors should be corrected at revision, so please note any specific errors here.

Reviewer #1: Yes

Reviewer #2: Yes

****

5. Review Comments to the Author

Please use the space provided to explain your answers to the questions above. You may also include additional comments for the author, including concerns about dual publication, research ethics, or publication ethics. (Please upload your review as an attachment if it exceeds 20,000 characters)

Reviewer #1: This paper describes the effects OM has on a nebulin KO model. The premise is that since type 1 fibres are mainly affected and predominate in nemaline myopathy due to nebulin mutations, it may have a positive effect on contractility. Whilst 0.1μM OM has limited effects that are greater in the nebula KO, there is a considerable issue about the chosen dosage.

1 In this system EC50 appears to be about 0.6μM (no sem is given), so 0.1μM is a rather low level of dosage. It is necessary to explore this system in greater depth, using-

1a Effects on contractility and pCa50 at a range of doses- is there a maximum with decline at higher concentrations as Nagy et al. found?

1b The low dose is chosen to avoid cardiac effects. Since none of the experiments involves cardiac muscle this is not relevant to the study. However it would be important to check the dose-response curve of cardiac muscle in this system and also of the nebulin KO, which may be different from wt.

1c There should be some discussion as to whether a combined cardiac and type 1 fibre effect might actually be advantageous given that nebulin is also present in heart muscle.

2 The authors seem to have misunderstood the mechanism of OM action. (line 46) no mention of the work of Woody et al (ref 36) which clearly explains the mechanism- in fact refs 21 and 22 seem to be wholly unsuitable, being clinically al trials.

In the discussion lines 310-317 are essentially wrong- Woody shows that OM causes non-moving attached cross bridges that are inhibitory but which activate the THIN FILAMENT cooperatively. SRX has nothing to do with this- you are thinking of Mavacamten, perhaps.

3 in the discussion the authors should compare OM with the fast skeletal-specific troponin activators as treatments for Nemaline myopathy. Some of these were tested with the nebulin KO mouse.

(Collibee et al. 2018; Hwee et al. 2017, 2015,2014; de Winter et al. 2013

4 Figures. You should use a consistent shade for the points on graphs: in figs 2 & 3 OM is light, in Figs 4 & 5 OM is dark !

other points:

Some poorly constructed sentences; the text should be inspected and corrected by an English expert. line 15 is an obvious example.

line 37 nebulin is BELIEVED to play.....

line 41 Few or none? If a few state what they are.

line 53 Can you specify which are the main type 1 muscles (in mouse and in human)

Figure 5- Please also show the original force-velocity curves which were used to create the force-power plots.

Reviewer #2: This MS by Lee et al reports a study of the effect of omecamtiv mecarbil (OM), a small molecule activator of cardiac beta/skeletal muscle type I myosin, on correcting the contractile abnormality of mouse skeletal muscle lacking nebulin for potential use in the treatment of NEB nemaline myopathy. Skinned single fiber and intact muscle studies were performed. The results suggest beneficial effect of OM treatment. The studies used comprehensive quantitative approaches which the authors have expertise and extensive past experiences. The experiments are carefully performed and the data are clearly presented. While this work is considered as potentially interesting to the field by providing valuable information on whether and how manipulate myosin ATPase function and kinetics could therapeutically correct or mitigate the functional defect of a thin filament function due to the loss of nebulin, a skeletal muscle specific protein. There are some issues that need the authors' attention:

1. For the rationale and significance of this study, the authors should emphasize the effect of OM on increasing myosin activity to treat myopathies, since this drug is unlikely to be directly useful in the treatment of Neb myopathy for its cardiac effect in humans.

2. Muscle weight and fiber size information should be presented and discussed for whether and how atrophy has effect on the contractile function of whole muscle or single fibers.

3. The whole muscle functional parameters should be normalized to the contents/% of type I myosin.

4. Since there was no anticipated effect of OM on maximum force whereas it increased calcium sensitivity (pCa50), the effect on intrinsic myosin ATPase activity should evaluated. The authors referenced previous Ktr study, which should also be examined here.

5. In addition to Force-Power curve, the primary contractile velocity data should be shown and discussed.

6. Examples of the SDS-gel confirmation of single fibber types should be shown. This will also help to evaluate the myofilament protein contents of the Neb KO and WT muscle fibers studied. If there are any adaptive changes in the KO muscle, they can be discussed for potential significance in altering contractility.

7. The difference between normal cardiac and skeletal muscles in their nebulin contents may contribute to their contractile properties and the responses to OM treatment, a worthwhile point for discussion.

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Do you want your identity to be public for this peer review? For information about this choice, including consent withdrawal, please see our Privacy Policy.

Reviewer #1: No

Reviewer #2: No

[NOTE: If reviewer comments were submitted as an attachment file, they will be attached to this email and accessible via the submission site. Please log into your account, locate the manuscript record, and check for the action link "View Attachments". If this link does not appear, there are no attachment files to be viewed.]

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2019; 14(11): e0224467.
Published online 2019 Nov 13. doi: 10.1371/journal.pone.0224467.r002

Author response to Decision Letter 0

1 Oct 2019

Reviewer #1:

Comment: “This paper describes the effects OM has on a nebulin KO model. The premise is that since type 1 fibres are mainly affected and predominate in nemaline myopathy due to nebulin mutations, it may have a positive effect on contractility. Whilst 0.1μM OM has limited effects that are greater in the nebula KO, there is a considerable issue about the chosen dosage.

1a Effects on contractility and pCa50 at a range of doses- is there a maximum with decline at higher concentrations as Nagy et al. found?

1b The low dose is chosen to avoid cardiac effects. Since none of the experiments involves cardiac muscle this is not relevant to the study. However it would be important to check the dose-response curve of cardiac muscle in this system and also of the nebulin KO, which may be different from wt.

1c There should be some discussion as to whether a combined cardiac and type 1 fibre effect might actually be advantageous given that nebulin is also present in heart muscle.”

Response: First of all, thank you reviewer for the positive overall evaluation and the constructive comments that allowed us to strengthen our manuscript. We have taken your comments to heart and performed the following experiments.

To answer the issue about the maximum with decline at higher concentrations (question 1a) we performed a new series of experiments on Neb cKO fiber bundles that were activated at a range of pCa values and in the presence of 0, 0.1, 0.3, 0.5, 1.0 and 10 μM OM (new Figure 2, text on page 12, lines 215-222). At submaximal activation OM increased force and the effect peaked at 0.3 μM OM and declined at OM >0.5 μM OM. Similar findings are reported by Nagy et al. who studied permeabilized rat diaphragm fiber bundles activated to ~25% of maximal. They found that OM increased force with a maximum at ~0.3 μM OM and a reduction in the effect at OM >~1.0 μM OM. These new studies support the earlier conclusion that Neb cKO fibers 0.1 μM OM is sufficient to see robust effects on force at submaximal activation. Although 0.3 μM OM has a slightly larger effect, the benefit beyond 0.1 μM is small and to limit the potential for cardiac effects we elected to use in our single fiber experiments 0.1μM OM.

In response to question 1b we measured the dose-response curve of cardiac muscle in both WT and Neb cKO LV papillary muscle (See Supplemental Figure 1B). We find EC50 values of ~0.7 μM, similar to Nagy et al for rat cardiac myocytes, but we find no drop-off at OM concertation >1.0 μm, unlike in the work of Nagy et al (their Fig. 1). Please note that other studies (e.g., Gollapudi et al, Biophys J, 2017) also found no drop-off. We believe that this is likely due to a sarcomere length difference (we worked at 2.0 μm SL as did Gollapudi et al, whereas Nagy et al, used 2.3 μm, a length at which the baseline activation level is already high due to length-dependent activation); future follow-up work is required to test this and other possible explanations. In our revised manuscript we discuss the issue of ‘drop-off ‘that that is sometimes present but not always. See page 20, line 368-376.

In response to question 1c, we would like to first highlight that nebulin is not expressed in adult cardiac muscle (Kolb et al., J Mol Cell Cardiol. 2016;97:286-94.). As for the possible utility of omecamtiv in patients, this has to be evaluated using an organismal-level perspective. For omecamtiv to be useful for increasing force of skeletal muscle in nebulin-based nemaline myopathy patients, undesirable effects in the heart have to be avoided. It is known that at high OM concentrations, the relaxation rate of the heart is reduced and that this can impair diastolic filling and cause ischemia, especially at high heart rates. This has to be avoided when treating skeletal muscle myopathies with OM. Fortunately, these cardiac effects occur at high OM concentrations (>~ 1 μM), or ~10-fold higher concentrations than those that are effective in increasing skeletal muscle force. We now add this as a discussion item, see page 22 lines 423-35.

Comment: “2 The authors seem to have misunderstood the mechanism of OM action. (line 46) no mention of the work of Woody et al (ref 36) which clearly explains the mechanism- in fact refs 21 and 22 seem to be wholly unsuitable, being clinically al trials.”

Response: We have updated our discussion of the OM mechanism. See page 3, lines 45-50.

Comment: “In the discussion lines 310-317 are essentially wrong- Woody shows that OM causes non-moving attached cross bridges that are inhibitory but which activate the THIN FILAMENT cooperatively. SRX has nothing to do with this- you are thinking of Mavacamten, perhaps.)”

Response: We had referred to the work by Kampourakis et al (J. Physiol, 2018) who used omecamtiv (not mavacamten) to investigate the thick filament ‘on’ and ‘off’ states. These authors provide evidence that in passive muscle OM promotes the thick filament ON state. Although the Woody et al study deserves most of the focus in our Discussion, we don’t think that this study excludes that in addition to enhancing thin filament activation, OM might also increase the thick filament ON state. In response to your comments we have revised our Discussion. See page 20, lines 386-395

Comment: “3 in the discussion the authors should compare OM with the fast skeletal-specific troponin activators as treatments for Nemaline myopathy. Some of these were tested with the nebulin KO mouse. (Collibee et al. 2018; Hwee et al. 2017, 2015,2014; de Winter et al. 2013)”

Response: We have added this suggested discussion item. See page 24, lines 469-475.

Comment: “4 Figures. You should use a consistent shade for the points on graphs: in figs 2 & 3 OM is light, in Figs 4 & 5 OM is dark !)

Response: You are right and we have made the suggested change. See revised Figures 3 and 4.

Comment: “Some poorly constructed sentences; the text should be inspected and corrected by an English expert. line 15 is an obvious example.

Responses: Done. See revised ms. and throughout.

Comment: line 37 nebulin is BELIEVED to play.....

Responses: Indeed this is better and we made the suggested change. See line 37 of revised ms.

Comment: line 41 Few or none? If a few state what they are.

Responses: Done. See line 42 of revised ms.

Comment: line 53 Can you specify which are the main type 1 muscles (in mouse and in human)

Responses: Done. See top of page 3, lines 54-56.

Comment: Figure 5- Please also show the original force-velocity curves which were used to create the force-power plots.”

Responses: Done. See left panels of Fig. 6A

Reviewer #2

“This MS by Lee et al reports a study of the effect of omecamtiv mecarbil (OM), a small molecule activator of cardiac beta/skeletal muscle type I myosin, on correcting the contractile abnormality of mouse skeletal muscle lacking nebulin for potential use in the treatment of NEB nemaline myopathy. Skinned single fiber and intact muscle studies were performed. The results suggest beneficial effect of OM treatment. The studies used comprehensive quantitative approaches which the authors have expertise and extensive past experiences. The experiments are carefully performed and the data are clearly presented. While this work is considered as potentially interesting to the field by providing valuable information on whether and how manipulate myosin ATPase function and kinetics could therapeutically correct or mitigate the functional defect of a thin filament function due to the loss of nebulin, a skeletal muscle specific protein.”

Responses: Thank you for your positive evaluation and for your comments that further helped us to improve our manuscript.

There are some issues that need the authors’ attention:

Comment: “1. For the rationale and significance of this study, the authors should emphasize the effect of OM on increasing myosin activity to treat myopathies, since this drug is unlikely to be directly useful in the treatment of Neb myopathy for its cardiac effect in humans.”

Responses: It is possible that it will turn out that OM will not be useful for nebulin-based nemaline myopathy patients but, in our opinion, the jury is still out and more future work will be required. Based on clinical studies ~1 μM OM is well tolerated in patients (see manuscript for details). Thus the 10-fold lower dose that is effective in type-I skeletal muscle fibers should be well-tolerated. Even if it turns out that OM will not be useful for treating skeletal muscle myopathies, the effect of OM on type-I skeletal muscle fibers still needs to be carefully considered, because skeletal muscle effects will have to be taken into account when treating heart failure patients with OM (e.g. depressing force in type-I diaphragm fibers has to be avoided). We added the suggested discussion, see page 22, lines 423-435.

Comment: “2. Muscle weight and fiber size information should be presented and discussed for whether and how atrophy has effect on the contractile function of whole muscle or single fibers.”

Responses: We have added this information, see page 5, lines 71-76 and page 6, lines 104-105. Please note that when forces are compared between genotypes, specific force is shown (force normalized to the cross-sectional area of the fibers/muscles), and muscle hypertrophy will not affect these comparisons.

Comment: “3. The whole muscle functional parameters should be normalized to the contents/% of type I myosin.”

Responses: If effects were to occur at maximal activation, they could easily be normalized because all fiber types will be maximally activated under those conditions and their forces are additive. However, the effects of OM are mainly seen at submaximal activation and under those conditions the different fiber types will contribute to overall muscle force at different levels (because of their different force-frequency relations) adding uncertainty as to how to normalize by MHC content. Nevertheless, assuming simple addition at submaximal activation we have normalized for type-I MHC content (See Figure 5A of revised ms. and text on page 17, lines 323-325).

Comment: “4. Since there was no anticipated effect of OM on maximum force whereas it increased calcium sensitivity (pCa50), the effect on intrinsic myosin ATPase activity should evaluated. The authors referenced previous Ktr study, which should also be examined here.”

Responses: Unfortunately we are currently unable to perform ATPase measurements in loaded fibers since we lost this expertise in our group and these measurements have to wait until some future opportunity arises to carry out this work. However, we have performed Ktr experiments and found that Kts is reduced at 0.5 μM OM and higher concentrations indicating decreased myosin ATPase activity, see new Figure 2C and page 13, line 233-237.

Comment: “5. In addition to Force-Power curve, the primary contractile velocity data should be shown and discussed.”

Responses: Done. See Figure 6, left panels.

Comment: “6. Examples of the SDS-gel confirmation of single fibber types should be shown. This will also help to evaluate the myofilament protein contents of the Neb KO and WT muscle fibers studied. If there are any adaptive changes in the KO muscle, they can be discussed for potential significance in altering contractility.”

Responses: As you suggest, we now show gel examples of single fibers, see new Fig. 3A. As far as evaluating the protein composition of muscle fibers, this is easily doable for MHC because of the abundance of this protein, but for more typical proteins with lower abundance, this is challenging. Perhaps you meant studies at the whole muscle level. If so, we would like to point out that they have been done previously. We have studied the thin filament regulatory proteins (fast and slow skeletal muscle isoforms of Tm, TnI, TnT, and TnC) in soleus muscle and found the isoform composition to have shifted away from fast towards slow isoforms of skeletal muscle, when comparing Neb cKO and WT mice. This work has been published (Kiss et al., PNAS, 2018.). Performing this type of study at the single fiber level is challenging and time-consuming, in part due to the small size of mouse muscle fibers and especially in the Neb cKO mouse (see our previous work, Li et al, 2015, Human Molecular Genetics). For now, we revised our text highlighting our previous study at the whole muscle level and indicating that future work should focus on single fiber proteomics to reveal possible adaptive changes in the Neb cKO fibers (comparing the same fibers type in Neb cKO and WT mice). The difference between control and Neb cKO fibers is likely largely due to nebulin but adaptive changes (in addition to the fiber type switch) cannot be excluded and your point is well taken. See page 15, lines 275-281.

Comment: “7. The difference between normal cardiac and skeletal muscles in their nebulin contents may contribute to their contractile properties and the responses to OM treatment, a worthwhile point for discussion.”

Responses: We agree and now include this in the discussion. See page 22, lines 417-420.

A final thank you to both reviewers for their time spent reviewing and for their excellent comments. We have taken these comments to heart, performed multiple additional experiments, and revised our manuscript throughout. We hope that you agree that our manuscript has been much improved and that our manuscript is now ready for acceptance. Thank you again.

Attachment

Submitted filename: *Omecamtiv revision Resp to Reviewers Comments oct 01FINAL.docx*

Click here for additional data file.(33K, docx)

2019; 14(11): e0224467.

Published online 2019 Nov 13. doi: 10.1371/journal.pone.0224467.r003

Decision Letter 1

Agustín Guerrero-Hernandez, Academic Editor

15 Oct 2019

Omecamtiv mecarbil lowers the contractile deficit in a mouse model of nebulin-based nemaline myopathy.

PONE-D-19-14908R1

Dear Dr. Granzier,

We are pleased to inform you that your manuscript has been judged scientifically suitable for publication and will be formally accepted for publication once it complies with all outstanding technical requirements.

Within one week, you will receive an e-mail containing information on the amendments required prior to publication. When all required modifications have been addressed, you will receive a formal acceptance letter and your manuscript will proceed to our production department and be scheduled for publication.

Shortly after the formal acceptance letter is sent, an invoice for payment will follow. To ensure an efficient production and billing process, please log into Editorial Manager at https://www.editorialmanager.com/pone/, click the "Update My Information" link at the top of the page, and update your user information. If you have any billing related questions, please contact our Author Billing department directly at authorbilling@plos.org.

If your institution or institutions have a press office, please notify them about your upcoming paper to enable them to help maximize its impact. If they will be preparing press materials for this manuscript, you must inform our press team as soon as possible and no later than 48 hours after receiving the formal acceptance. Your manuscript will remain under strict press embargo until 2 pm Eastern Time on the date of publication. For more information, please contact onepress@plos.org.

With kind regards,

Agustín Guerrero-Hernandez

Academic Editor

PLOS ONE

Additional Editor Comments (optional):

Reviewers' comments:

Reviewer's Responses to Questions

Comments to the Author

1. If the authors have adequately addressed your comments raised in a previous round of review and you feel that this manuscript is now acceptable for publication, you may indicate that here to bypass the “Comments to the Author” section, enter your conflict of interest statement in the “Confidential to Editor” section, and submit your "Accept" recommendation.

Reviewer #1: All comments have been addressed

Reviewer #2: All comments have been addressed

****

2. Is the manuscript technically sound, and do the data support the conclusions?

The manuscript must describe a technically sound piece of scientific research with data that supports the conclusions. Experiments must have been conducted rigorously, with appropriate controls, replication, and sample sizes. The conclusions must be drawn appropriately based on the data presented.

Reviewer #1: Yes

Reviewer #2: Yes

****

3. Has the statistical analysis been performed appropriately and rigorously?

Reviewer #1: Yes

Reviewer #2: Yes

****

4. Have the authors made all data underlying the findings in their manuscript fully available?

The PLOS Data policy requires authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception (please refer to the Data Availability Statement in the manuscript PDF file). The data should be provided as part of the manuscript or its supporting information, or deposited to a public repository. For example, in addition to summary statistics, the data points behind means, medians and variance measures should be available. If there are restrictions on publicly sharing data—e.g. participant privacy or use of data from a third party—those must be specified.

Reviewer #1: Yes

Reviewer #2: Yes

****

5. Is the manuscript presented in an intelligible fashion and written in standard English?

PLOS ONE does not copyedit accepted manuscripts, so the language in submitted articles must be clear, correct, and unambiguous. Any typographical or grammatical errors should be corrected at revision, so please note any specific errors here.

Reviewer #1: Yes

Reviewer #2: Yes

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6. Review Comments to the Author

Please use the space provided to explain your answers to the questions above. You may also include additional comments for the author, including concerns about dual publication, research ethics, or publication ethics. (Please upload your review as an attachment if it exceeds 20,000 characters)

Reviewer #1: OK OK K OKK OKK OKK OKK OKK OKK OKK OKK OKK OKK OKK OKK OKK OKK OKK OKK OKK OKK OKK OKK OK

Reviewer #2: My comments have been clearly addressed. I have no further concerns. This work should be published to make a valuable contribution to the field.

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7. PLOS authors have the option to publish the peer review history of their article (what does this mean?). If published, this will include your full peer review and any attached files.

If you choose “no”, your identity will remain anonymous but your review may still be made public.

Do you want your identity to be public for this peer review? For information about this choice, including consent withdrawal, please see our Privacy Policy.

Reviewer #1: Yes: Steen Marston

Reviewer #2: Yes: J.-P. Jin

2019; 14(11): e0224467.
Published online 2019 Nov 13. doi: 10.1371/journal.pone.0224467.r004

Acceptance letter

Agustín Guerrero-Hernandez, Academic Editor

5 Nov 2019

PONE-D-19-14908R1

Omecamtiv mecarbil lowers the contractile deficit in a mouse model of nebulin-based nemaline myopathy.

Dear Dr. Granzier:

I am pleased to inform you that your manuscript has been deemed suitable for publication in PLOS ONE. Congratulations! Your manuscript is now with our production department.

If your institution or institutions have a press office, please notify them about your upcoming paper at this point, to enable them to help maximize its impact. If they will be preparing press materials for this manuscript, please inform our press team within the next 48 hours. Your manuscript will remain under strict press embargo until 2 pm Eastern Time on the date of publication. For more information please contact onepress@plos.org.

For any other questions or concerns, please email plosone@plos.org.

Thank you for submitting your work to PLOS ONE.

With kind regards,

PLOS ONE Editorial Office Staff

on behalf of

Dr. Agustín Guerrero-Hernandez

Academic Editor

PLOS ONE