Author’s response to reviews

Title: Characterization of GLPG0492, a selective androgen receptor modulator, in a mouse model of hind limb immobilization

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Author’s response to reviews: see over
Dear Editor,

Please find enclosed a copy of our revised manuscript entitled “Characterization of GLPG0492, a selective androgen receptor modulator, in a mouse model of hind limb immobilization” (Authors: R. Blanqué, L. Lepescheux, M. Auberval, D. Merciris, D. Minet, C. Cottereaux, P. Clément-Lacroix, P. Delerive & F. Namour) we would like to resubmit for publication in *BMC Musculoskeletal Disorders*.

All authors have read and approved the contents of this manuscript. We have not submitted and are not going to consider for submitting this manuscript to elsewhere in whole or in part in any language except as an abstract.

In order to address the different concerns pointed out by the reviewers, we added new results (table 1, figure 3A & 3B) and some comments and corrections to the revised manuscript as suggested by the reviewers.

We believe to have satisfactorily responded to the reviewers’ comments and hope our manuscript is now acceptable for publication in *BMC*.

Looking forward to your response in due course,

Sincerely yours,

Philippe Clément-Lacroix, Ph.D.
Answer to reviewer 1 (Mark Tarnopolsky)

1) We agree that PGC-1α4 must be discussed and investigated deeper in the paper. For that, we have added one paragraph in the discussion and in the result (page 10, line 7 to 21). Even modulation of gene expression both by immobilization and by treatment is light in our model at day 7. The two treatment inversely regulated PGC-1α (up-regulated) and PGC-1α4 (down-regulated).

2) The primary objective of the metabolomic data was to identify a signature of GLPG0492 efficacy related to muscle function. This metabolomic signature might be considered as a bona fide biomarker of target engagement in future clinical studies. Even though the measurement of the muscle fractional synthetic rate remains a gold standard as endpoint for muscle anabolic agent, additional biomarkers are needed to establish target engagement in early clinical development.

This approach requires very little amount of blood and is considered as non-invasive. Moreover, these “targeted” approaches (1H-NMR, LC-MS/MS, GC-MS/MS) are quite sensitive. Finally, kinetic analysis can easily be performed on the same patients (Phase 2) or healthy volunteers (phase 1) which makes the approach attractive from an experimental point of view.

Testosterone metabolomic signature was not determined under these conditions because testosterone is a full androgen receptor agonist with no tissue selectivity in contrast to GLPG0492 which demonstrated clear SARM activity. So, the signature that we would have obtained with testosterone would have been very complex to compare with the GLPG0492 profile since testosterone is known to significantly affect directly or indirectly the physiology of many tissues. Non-steroidal SARMs such as GLPG0492 differ from testosterone in several respects. Unlike testosterone, which is converted to active metabolites (estradiol and DHT), non-steroidal SARMs do not undergo aromatization or 5-α-reduction, and act as agonists in muscle and bone and as partial agonists in prostate and seminal vesicles. In conclusion, GLPG0492 metabolomic profile was mainly studied to identify putative biomarkers in the plasma compartment that might be related to muscle function and potentially translated into the clinical space. Testosterone profile was therefore not determined as control due to the very different biological and safety profile of this drug compared to the SARM from a drug development point of view.
**Answer to reviewer 2 (Paula Tavares)**

1) Our aim was to confirm the activity of SARM molecule (GLPG0492) on an immobilization muscle atrophy mouse model and to identify putative biomarkers in the plasma compartment that might be related to muscle function and potentially translated into the clinical space. (Included in abstract page 2) For that we
   a. evaluated selective muscle and prostate weight after seven days of immobilization, to confirm previous publication demonstrating that GLPG0492 is a SARM (Fig 1)
   b. confirmed anabolic muscle properties of GLPG0492 on muscle fibers atrophy by histology analysis of FCSA complemented with the distribution of fast and slow fibers (Fig 2)
   c. checked that key gene expression clearly linked to muscle atrophy, are increased by immobilization, and are down regulated by GLPG similarly to testosterone (Fig 3a & 3b)
   d. realized on this model a 1H-NMR analysis on the plasma compartment to identify biomarkers related to muscle function and potentially translated into clinical study phase. (Fig 4, Table 3)

2) For this experiment, at sacrifices, muscle samples were collected and immediately snap frozen before cryo-sectioning. As described in the main text of the paper, we have performed a double IHC for laminin and myosin light chain, allowing to visualize all fibers border and to discriminate slow-twitch fibers from the fast-twitch ones. Both fibers type FCSA were then measured by image analysis. We agree that a staining with myosin ATPase would be informative on fibers sub-typing. Nevertheless, we can’t perform this additional evaluation because today no more frozen muscle samples are available.

3) The sentence has been modified to be on line with previous report.

4) Mice were sacrificed using CO2 followed by decapitation in accordance to ethical guidelines edited by Animal Institutional Care and Use Committee of Galapagos controlled by French Authorities (agreement n° B 93 063 06, DDPP, Seine Saint Denis).

5) Blood collection was performed at sacrifice by decapitation

6) GLPG0492 has been previously tested in a standard castrated male rodent model, in which it demonstrated robust anabolic properties on levator ani (LA) muscle comparable to testosterone propionate (TP) but dissociated from the androgenic activity on ventral prostate (VP), after oral dosing at 30 mg/kg. The dose displaying 50% activity on LA was 0.75 mg/kg/day, while at the maximum 30% activity can be achieved on VP at the highest dose tested (30 mg/kg/day). Normalizing sham-operated rat at 100% and ORX (orchidectomized) rat at 0%, we observed in the treated group that the LA weight was fully restored (117%), at a level similar to that of the sham-operated group. Conversely, the increase in prostate weight in rats treated with GLPG0492 was much lowers (25%), with the prostate weight closer to that of the ORX group than the sham-operated group. This indicates that dissociation observed in the short-term model is maintained after long-term treatment with 10 mg/kg/day of GLPG0492 by the oral route (Nique et al., 2012a & Nique et al. 2012b). Therefore, the doses of both GLPG0492 and TP were selected based on these dissociation studies conducted in the rat model.
For FCSA measurement, an average of 2000 to 5000 fibers (fast+slow fibers) per animal was quantified. Then each fiber type was calculated as = (fast or slow fibers number) divided by (total fibers number) x 100 and expressed in %. As suggested by the reviewer, the distribution frequency of muscle fibers was added (fast and low) as a novel figure 2C.

**Results:**

The whole statistical analysis has been reviewed and significance levels were updated in the revised manuscript. Legends have been check to be consistent through all the graphs and figures.

There is no discrepancy. GLPG0492 in contrast to testosterone propionate did not significantly increase prostate weight as compared to the group CTL (immobilized) and are in line with our previous studies (Nique et al., 2012a & Nique et al. 2012b). Only reported significance (#) is versus the intact animal (non-immobilized). Due to the immobilization, we observed a decreasing trend of prostate weight/BW. That has been also clarifying in the legend of figure 1. So as compared to CTL (immobilized), all the immobilized groups treated with GLPG0492 didn’t exhibit prostate weight increasing in opposite to TP.

We apologize for the confusion with figures and texts. The whole statistical analysis has been reviewed and significance levels were updated in the revised manuscript. Please see new figure 2B

X-axis is Fiber distribution (%) That has been added on the two graphs. Then each fiber type was calculated as = (fast or slow fibers number) divided by (total fibers number) x 100 and expressed in %.

Done, please see new figures

In the 5th paragraph, line 9-11, we have modified the sentence. The decrease of BCAAs post immobilization is notable but not significant.

No these analysis has not been performed. Liver have not been collected. At time of study, we didn’t have access to X-ray device, such as Piximus, to analyze the fat/lean mass ratio. So, we agree that fatty acid oxidation is just an assumption. We have adapted consequently the comments inside the text.

Body weight data at day 0 and day 7 have been including in the Table 1. No significant difference exists a day 0. The whole of immobilized groups exhibit a significant body weight loss as compared to the intact group, at day 7. Nor TP, neither GLPG0492 succeed to significantly counteract this loss.

Text has been corrected according this point.

Gene expression de IGFI and LC3 are significantly increased by the immobilization. Nevertheless, these increases are not down regulated by TP and GLPG0492 after 7 days of treatment. The two graphs are now added in Figure 3B.
18) We have included a paragraph to discuss this point: “Increased of atrogin-1 and MuRF1 proteins have been reported in hind limb suspension rat model. L-leucine and the leucine metabolite β-hydroxy-β-methylbutyrate (Ca-HMB) have been reported to attenuate the increase in atrogin-1 and MuRF1 proteins in hind limb suspension rat model. [Maki, 2012]. Another recent paper suggest that leucine, could down regulated the MurF1 protein in mouse cachexia model [Mirza, 2014]. In our study, modulation of BCAAs, especially leucine, by GLPG0492 is not strong enough to explain, to it alone, the significant gene regulation of MurF1.”

19) Table 3 is now modified as requested.

**Conclusion**

20) We take your points. According these comments we have modulate the discussion and especially don’t include reference to possible treatment of sarcopenia and cachexia with our compounds. Formally, this current paper didn’t strictly report supportive data on these two musculoskeletal diseases. For information, another paper is currently in preparation with oncology models of cachexia. It is clear that our data need to be considered for this model and this timing. As we mentioned in our discussion, lot of other studies are still needed to fully understand the role of androgen and SARM in muscle