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ABSTRACT

Blockade of TGF-β Signaling Through the Activin Type IIB Receptor
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Antagonism of the activin receptor signaling pathway represents a promising potential therapy for the muscular dystrophies and other muscle wasting disorders (i.e., cachexia or sarcopenia). Previous research has shown that antagonism of activin signaling promotes muscle growth, attenuates muscle wasting, and restores function in both wild type and diseased animals. Our laboratory has recently developed a novel small molecule (SGI-1252) that inhibits activin downstream (i.e., Smad2/3 phosphorylation) signaling.

Purpose: In this study we determined how eight weeks of orally administered SGI-1252 affected TGF-β signaling, whole body mass, individual limb muscle mass, and muscle fiber cross sectional area (CSA).

Methods: Wild-type (WT) mice were treated with SGI-1252 or a vehicle control (VC) via oral gavage (400 mg/kg 3 times per week) for 8 weeks. Body mass was measured twice per week during the 8-week treatment period. At the end of the treatment period, gastrocnemius and tibialis anterior (TA) muscles were excised, weighed, and prepared for histological and biochemical analyses.

Results: Following 8 weeks of treatment, there was no difference in weight gain between SGI-1252 (24.8 ± 1.8g) and VC treated mice (23.2 ± 1.5g) ($p = 0.06$). Gastrocnemius whole muscle mass was significantly greater in the SGI-1252 treated group relative to the VC treated mice (139.6 ± 12.8 mg vs 128.8 ± 14.9 mg) ($p = 0.04$), although when normalized with body mass there was no difference in gastrocnemius mass. For the TA muscle, there were no significant differences in whole muscle mass between SGI-1252 and VC groups, yet TA muscles in the SGI-1252 treated group had a reduced muscle fiber CSA compared to controls (621 ± 44 μm^2 vs 749 ± 36 μm^2) ($p = 0.0005$). There was a statistical trend of decreasing Smad2 phosphorylation in the SGI-1252 treated TA muscles (mean SGI-1252 = 0.668 vs VC = 0.848) ($p = 0.06$), and no significant differences in Smad2 phosphorylation in the gastrocnemius.

Conclusions: Contrary to our hypothesis, 8 weeks of orally administered SGI-1252 was not effective in promoting increases in whole body mass, limb whole muscle mass, or myofiber cross sectional area. This may be due to the inability of SGI-1252, at the administered dose, to effectively decrease signaling downstream of the activin receptor. Clearly, studies using a wider range of doses and delivery methods will be needed to ascertain the efficacy of SGI-1252 as a potential therapeutic.

Keywords: TGF-β, myostatin, activin, SGI-1252, Smad, phosphorylation, ALK, JAK/STAT

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INTRODUCTION

Approximately 40% of human body mass is skeletal muscle [1]. Muscle mass is correlated with strength, and studies that have looked at older individuals have shown that with an increase in muscle mass comes an associated improvement in function/movement, seemingly from the correlated strength gains [2, 3]. The preservation of skeletal muscle mass and strength through the lifespan is essential to maintain health and function and also has potential to decrease the negative side effects associated with aging and muscle wasting diseases [4-6]. Aging and certain muscle wasting diseases including cancer (cachexia) and muscular dystrophy are associated with skeletal muscle atrophy and a concomitant loss of function [2, 3, 7]. Members of the transforming growth factor beta (TGF-β) family of proteins have been associated with the loss of muscle seen in these muscle wasting diseases [8, 9].

The transforming growth factor beta (TGF-β) superfamily is a family of ligands/cytokines that play essential roles in the development and growth of different cell types and are required during the developing stages of many tissues [10, 11]. The TGF-β superfamily consists of a diverse group of cytokine proteins including the TGF-β proteins, growth differentiation factors (GDFs), activins, bone morphogenic proteins (BMPs), and others [8]. Many of these cytokines have profound effects on cellular proliferation, differentiation, and growth [12-17]. TGF-β ligands signal through transmembrane receptor complexes consisting of type I and type II receptor subunits [18-20]. For example, the active form of TGF-β1 binds to the TGF-β type II receptor and either activing-like kinase 1 (ALK1) or TGF-β type I/ALK5 receptors, while the active form of myostatin (another TGF- β family member) binds either the activin type II A or B receptor (ActRIIA/B) and either TGF-β type I/ALK4 or ALK5 type I receptors [8, 18, 19, 21, 22]. Upon binding and activating their respective activin receptors, both

signaling pathways lead to the phosphorylation/activation of intracellular transcription factor signaling proteins called Smads, particularly Smad2 and Smad3 [8, 18, 23-25]. When Smad2 and Smad3 are phosphorylated, they form a complex with Smad4 and translocate into the nucleus where they regulate expression of genes associated with differentiation and muscle protein synthesis [8, 23, 25].

Skeletal muscle is potently influenced by activation of the activin receptor complexes by the TGF-β family of cytokines. This is clearly illustrated by the exaggerated growth of muscles found in animals deficient of myostatin (GDF-8) [26]. Studies in myostatin null mice have shown that there is a profound increase in skeletal muscle mass [26]. Other studies investigating heterozygous or partial blockade of myostatin in mice have also shown significant increases in skeletal muscle mass [27], demonstrating that myostatin is an important regulator of skeletal muscle growth. Inhibition of activin signaling not only leads to an increase in muscle growth but also an attenuation of muscle wasting [28-31]. Other TGF-β ligands also influence growth and differentiation of skeletal muscle and, when inhibited, lead to a reduction of muscle wasting. For example, the activins are potent negative regulators of skeletal muscle mass and lead to decreases in protein synthesis under muscle wasting conditions [32, 33]. Rodent studies have shown that elevated levels of activin A promote skeletal muscle wasting and fibrosis. However, when activin levels are attenuated, muscle wasting and fibrotic tissue buildup is reversed, even in advanced stages of muscle wasting [33]. Likewise, TGF-β suppresses gene expression in skeletal muscle and has been reported to alter proliferation of satellite cells [16, 34, 35]. More specifically, TGF-β1 has been reported to inhibit transcription of the MyoD gene in myoblasts and transcriptional activity of the MyoD protein, leading to decreased myogenesis [36-38]. These studies demonstrate the role of the activin pathway in skeletal muscle. Given the role of activin

signaling activity on skeletal muscle mass, antagonism of the activin receptor complexes may represent a potential therapeutic avenue to attenuate the negative side effects seen in muscle wasting disorders.

Small molecule inhibitors have proven extremely useful for investigating signal transduction pathways and have the potential for development into therapeutics for inhibiting signal transduction pathways whose activities contribute to human disease. We have recently developed and characterized a small molecule, SGI-1252, which shows antagonistic activity against the activin type I receptors ALK4 and ALK5. Data collected recently in our laboratory have demonstrated that SGI-1252 effectively inhibits activin signaling by decreasing ALK4/5 kinase activity. Furthermore, treatment of primary muscle cells with SGI-1252 decreases Smad2/3 phosphorylation in a dose dependent manner (unpublished data). Previously published data indicate that SGI-1252 is an orally bioavailable molecule with good tolerability, making it an ideal pharmacological candidate for muscle wasting disorders [39]. However, it is currently unknown whether SGI-1252 is effective at inhibiting activin signaling and promoting muscle mass gains in vivo. Therefore the purpose of this study was to determine the effects of SGI-1252 administration on activin signaling (i.e., Smad2/3 phosphorylation) and hind limb skeletal muscle mass in mice. We hypothesized that administration of SGI-1252 would result in reduced signaling through the activin receptor in mouse skeletal muscle, resulting in reduced Smad phosphorylation thus leading to an increase in overall mouse weight, hind limb muscle mass, and myofiber size.

METHODS

Research Design

Wild-type mice were randomly assigned and treated with SGI-1252 or a vehicle control (VC) orally, via a modified gavage (400 mg/kg 3 times per week) for 8 weeks. The treatment

group was administered SGI-1252 (in 5% dextrose at 400 mg/kg) while the control group received a sham treatment (5% dextrose in water). Mice were administered the drug or vehicle control solution through a modified/partial gavage technique. Specifically, mice were secured by scruffing, and administration of the solution was performed by inserting a pipet tip to the back of the throat and slowly ejecting the liquid, allowing the mice to swallow. Differences in Smad2 phosphorylation, wet muscle weight, and muscle fiber cross sectional area were analyzed after 8 weeks of treatment, while overall body mass was measured twice per week. The 8-week treatment period was used to align with future experimentation that will be done with mdx mice, who demonstrate the dystrophic phenotype from 4 to 12 weeks of age (a total of 8 weeks).

Animals

Twelve male wild-type C57BL/10ScSnJ mice (n = 6 for each treatment group), 4–6 weeks of age were used in this study. Sample size was estimated based on previously published studies and computed assuming a power (1 - β) of 0.90 and α of 0.05 [29, 40, 41]. Animals were housed 3 to a cage at a Brigham Young University animal housing facility. They were kept on a 12-hour light-dark cycle (6 am to 6 pm). Mice were fed standard chow and water ad libitum. All Institutional Animal Care and Use Committee procedures and protocols were followed in the treatment and handling of the mice.

Animal Mass

Animal mass was determined for each mouse on a digital scale twice each week and before being sacrificed.

Tissue Dissection and Processing

The gastrocnemius and tibialis anterior were dissected from each hind limb after euthanization. Muscles were cleaned of excess connective tissue and blood and weighed.

Muscles from the right hind limb were prepared for immunohistochemical analysis, while muscles from the left hind limb were used for protein analysis. Once dissected, right hind limb muscles were mounted in gum tragacanth and frozen in isopentane cooled by liquid nitrogen. Left hind limb muscles were flash frozen in liquid nitrogen. Harvested skeletal muscle tissue was stored at -80° C until analysis.

Muscle Fiber Cross Sectional Area

The frozen, mounted tissue samples were serially sectioned at 8 μm using a cryostat microtome (Microm HM 525, Thermo Fisher Scientific, Waltham, MA), and mounted on microscope slides for analysis with a light microscope. Tissue cross sections were bathed in Curtis stain (9 parts saturated aqueous Picric acid, 1 part 1% Ponceau S, and 1 part Glacial acetic acid) for two minutes, rinsed two times in 100% ethanol, and allowed to air dry. After drying, samples were mounted with a cover slip with Canada balsam. The Canada balsam was allowed to dry before images were taken using a ZEISS Axiovert 135 light microscope. At least 10 random images at 200X magnification were taken of individual muscles, equating to ~160 fibers for each muscle in each condition. Muscle fiber area was measured using Olympus cellSens microscope imaging software.

Tissue Homogenization

Tissue samples were homogenized in lysis buffer at a ratio of 9 μl per mg tissue. Cell signaling lysis buffer (cat# 43-040 from Millipore, Darmstads, Germany) was used as the lysis buffer, Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) (cat# 78440 from Thermo Fischer Scientific, Waltham, MA) was also added to the homogenate. Samples were homogenized on ice using a glass-on-glass spear. Homogenates were centrifuged at 10,000 g at 4° C for 10 minutes. An aliquot of the resulting supernatant was analyzed for total protein

concentrations with the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The remaining supernatant was stored at -80° C for later analysis.

Western Blot

Samples were prepared for Western blot analysis by combining a fixed amount of total protein (100 µg) with an equal volume of loading buffer (0.2% SDS, 20% glycerol, 25% 4x buffer, 5% Beta-Mercaptoethanol and 0.0025% bromophenol blue). Samples were boiled, vortexed, and quickly centrifuged. A molecular weight ladder, negative and positive control, and each prepared homogenized sample were loaded into the wells of a 10% polyacrylamide mini gel which was then submerged in running buffer and run in an electrophoresis unit (Mini Protean II; Bio-Rad Laboratories, Hercules, CA) at 200 volts for 40 minutes, after which proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Protein transfer occurred through a transfer cell sandwich that was submerged in chilled transfer buffer at 100 volts for 60 minutes, while maintaining constant cooling. Following protein transfer, the membrane was rinsed with dH_2O, submerged in Ponceau Red Staining Solution, and rinsed again with dH_2O. Images were taken to ensure for proper protein transfer to membrane and to serve as a loading control. The membrane was then placed in a 5% blocking solution (5% milk powder in TBST) at room temperature for one hour with gentle rotation. It was then rinsed in 1x TBST (Tris-Buffer Saline + 0.1% Tween-20) at room temperature. The membrane was then exposed to primary antibodies probing for each specific protein in a 5% blocking solution (5% BSA in TBST): (Phospho-Smad2 (Ser465/467) (138D4) Rabbit mAB (#3108)) 1:500 and (Smad2 (D43B4) XP® Rabbit mAB (#5339)) 1:1000 and incubated overnight at 4° C with gentle rocking. All antibodies were obtained from Cell Signaling Technology® (CST). Following incubation, the membrane was rinsed in 1x TBST at room temperature and then incubated for 1

hour in recommended secondary antibody (Anti-rabbit IgG, HRP-linked Antibody (#7074, CST)) in 5% blocking solution (5% milk powder in TBST). A chemiluminescent solution (Clarity™ Western ECL Substrate, Bio-Rad Laboratories Inc., Berkeley, CA) was applied to each blot according to the manufacturer's instructions and each blot was imaged using a Bio-Rad imager (Universal Hood II, Chemiluminescent Imaging with Chemi Doc™ XRS, Quantity One Software, Bio-Rad Laboratories, Hercules, CA) to quantify total protein amount.

Statistical Analysis

Analysis for body weight over time was made using t-tests to compare each time point for treated vs. control, with a Bonferroni correction to account for multiple comparisons. Analyses for muscle mass, muscle fiber CSA, and Smad2 phosphorylation were made using t-tests. Analysis for individual muscle mass while accounting for body weight were made by dividing muscle mass by body weight and then using t-tests to compare treated vs. control. Statistics were run with JMP software. Data are presented as mean ± SD and significance was set at $p < 0.05$.

RESULTS

Body Mass

There were no significant differences in body mass between SGI-1252 (24.8 ± 1.8 g) and VC (23.2 ± 1.5 g) treated mice at the end of the 8-week treatment period due to our statistical parameters (Figure 1). However, pre and post body weights were different at a p-value of 0.06.

Muscle Mass

Gastrocnemius wet muscle mass increased significantly following 8 weeks of SGI-1252 treatment (139.6 ± 12.8 mg) relative to the VC treated group (128.8 ± 14.9 mg) ($p = 0.04$) (Figure 2). We suspected a measuring or recording error with one of the data points for the

gastrocnemius muscle of the VC mice. To identify whether this data point was an outlier, a linear regression equation was produced to predict gastrocnemius mass relative to individual muscle weights (TA, soleus, and plantaris) and body mass for each mouse. We found that the data point of concern was far outside the 95% confidence interval that was generated from the linear regression equation, while all of the other data points fell within or close to the 95% confidence intervals for their respective measurements. Therefore we excluded the data point and re-analyzed the data. The new model showed that gastrocnemius wet muscle mass still increased in the SGI-1252 treated mice (139.6 ± 12.8 mg vs 125.2 ± 8.7 mg) ($p = 0.004$). However, when normalized to body weight, there were no significant differences in gastrocnemius muscle mass between SGI-1252 treated (142.9 ± 18.6mg) and VC treated (131.3 ± 22.0mg) mice (Figure 3). There were no significant differences in TA muscle mass between the SGI-1252 (36.5 ± 5.2 mg) and VC (37.7 ± 5.3 mg) treated mice ($p = 0.18$) (Figure 3).

Muscle Fiber Cross Sectional Area

Following 8 weeks of treatment, there were no significant differences in gastrocnemius muscle fiber CSA between SGI-1252 treated (2416 ± 195 μm^2) and VC treated (2494 ± 257 μm^2) mice. However, contrary to our hypothesis, 8 weeks of SGI-1252 treatment reduced TA muscle fiber CSA (621 ± 44 μm^2) relative to the VC treatment (749 ± 36 μm^2) ($p = 0.0005$) (Figure 4).

Smad2 Phosphorylation

Western blot analysis revealed that 8 weeks of oral SGI-1252 administration did not affect Smad2 phosphorylation in the gastrocnemius muscle relative to the VC treated mice, (SGI-1252 = 0.75 ± 0.3 vs VC = 0.84 ± 0.4) ($p = 0.37$) (Figure 5). Likewise, Smad2 phosphorylation in the TA showed no significant difference with SGI-1252 treatment (SGI-1252 = 0.67 ± 0.1 vs

VC = 0.85 ± 0.2) ($p = 0.06$), though we did note a trend towards statistical significance (Figure 5). Smad2 phosphorylation was measured in intensity units.

DISCUSSION

The primary purpose of this study was to determine the *in vivo* effects of the small molecule SGI-1252, when administered orally, on whole body mass, individual limb muscle mass, muscle fiber CSA, and activin signaling in wild-type mice. As antagonism of activin signaling via a variety of peptide-based molecules has been widely shown to induce marked gains in muscle size in wild-type mice [8, 32, 42, 43], we hypothesized that SGI-1252 treatment would result in decreased skeletal muscle activin signaling (decreased Smad phosphorylation), leading to an increase in overall mouse weight, hind limb muscle mass, and myofiber size. Contrary to our hypothesis, we found that 8 weeks of orally administered SGI-1252 did not induce body mass increases relative to a control group and had variable effects on muscle fiber CSA, even decreasing TA muscle fiber CSA. Whereas previous studies have used genetic approaches or peptide-based molecules (i.e., antibodies or soluble receptors) [8, 26, 29, 42, 44] to antagonize activin signaling, this is the first study to our knowledge to use a small molecule inhibitor. Reasons that SGI-1252 did not induce consistent increases in whole body and limb muscle mass are not known, but could possibly be attributed to a number of factors, including: low bioavailability of the drug, insufficient dose, poor drug specificity, and potential off-target drug interactions.

In this study we did not assess blood levels of SGI-1252, making it difficult to directly determine whether oral administration resulted in increased serum SGI-1252 concentration. Nevertheless, oral administration of SGI-1252 has been previously evaluated in mice, and it was shown that SGI-1252 was highly bioavailable at the same dose used in the current study [39].

Furthermore, unpublished pharmacokinetic data provided by SuperGen (primary developer of the compound) indicate that a 300 mg/kg oral dose of SGI-1252 results in a peak serum concentration of 4.0 ± 1 μg/mL 6h following administration. SGI-1252 levels are then reduced to preadministration levels after 24h. Studies in muscle cell culture performed in our laboratory have demonstrated that SGI-1252 effectively reduces signaling downstream of the activin receptor (Smad2/3 phosphorylation) at an IC_{50} of 236 ng/mL (unpublished data). Collectively, these data suggest that the peak serum concentration of SGI-1252 following oral delivery would be sufficient to decrease activin/Smad signaling, but that a 3-times-per-week dosing schedule may not have been sufficient to keep serum levels chronically high enough to see a biological effect. In support of this notion, we found suggestive, but nonsignificant decreases in Smad2 phosphorylation in the TA. Interestingly, despite the large number of published studies that have administered a variety of activin inhibitor compounds for 2–12 weeks [29, 42, 45-47], none have reported on changes in Smad phosphorylation in the muscles of the treated mice. The lack of Smad signaling data in these repeat dose studies makes it difficult to determine whether decreased Smad phosphorylation is necessary for muscle mass gains in mice using antagonists of activin signaling. However, studies that have inhibited activin signaling show an increase in Smad phosphorylation in cell culture [28, 48]. Alternatively, our inability to measure changes in Smad2 phosphorylation may be related to the timing of mice being sacrificed after administration of the final SGI-1252 dose (typically 24 to 48 hours). As phosphorylation events are transient, we may have missed the effect of the drug dose by sampling the muscle 24-48 hours following the last dose. For example, Wang et al. [48] demonstrated that increased levels of phosphorylated Smad3, through TGF-β induced phosphorylation, can be detected as early as ~10–15 minutes and peaks at 1 h after treatment and subsequently drops off thereafter.

In support of our hypothesis, we did find a significant increase in body mass (7% increase) at a p-value of 0.06 in mice treated with SGI-1252 compared to controls. Three previous studies have shown that inhibition of activin signaling leads to an increase in body weight in mice. One study that inhibited signaling with a soluble receptor for 28 days found a 16% increase in body mass relative to controls [45], while two other studies that inhibited signaling with two different antibodies, one for 15 weeks and another for 12 weeks, showed a 10% [46], and 12.4% [29] respective increase in mass compared to their corresponding controls. Notably, the growth slope of the SGI-1252 treated and control mice begins to diverge after 5 weeks of treatment, with a steeper slope in the SGI-1252 treated group, suggesting that the drug treated mice may have gained significantly more weight had the treatment period been extended. This finding supports the aforementioned notion that either the absolute dose (400 mg/kg) and/or the frequency of doses (3X per week) were insufficient to increase muscle mass over the 8-week treatment period. We also found that SGI-1252 treatment significantly increased gastrocnemius mass by 7.7% relative to controls when not normalizing to body mass. Two previous studies have shown a 13.8 to 28.2% [42] and 44% [45] increase in gastrocnemius mass, each with potent inhibition of activin signaling through soluble receptor antagonism in wild-type mice, with concomitant increases in muscle fiber hypertrophy. However, contrary to the findings of these studies, we found that SGI-1252 treatment had no effect on gastrocnemius muscle fiber CSA. Our data do not provide any clear explanation for this discrepancy, but other studies have shown that genetic mutation of myostatin can produce muscle gains resulting from hyperplasia [26, 49] rather than hypertrophy. Furthermore, one study showed that injection of follistatin, an inhibitor of activin signaling [24], in genetically mutated mice produced muscle gains resulting from hyperplasia [50]. An analysis of total muscle fiber quantity would help determine whether

hyperplasia can explain the increase in gastrocnemius mass of SGI-1252 treated mice. However, the most plausible explanation regarding the gastrocnemius muscle mass discrepancy is simply that SGI-1252 likely did not induce meaningful muscle fiber hypertrophy and had no significant or specific effect on muscle mass. Due to the normalization of muscle mass to body mass taking away any significant effect, it is likely that any changes seen in muscle mass can be attributed to normal growth patterns. Also, seeing the almost identical increases in gastrocnemius mass (7.7%) and body mass (7%) relative to controls, this further supports that this is the most likely explanation.

One of the more robust and unexpected findings of the present study was a decrease in TA muscle fiber CSA with SGI-1252 treatment. Two previous studies have shown that administration of an activin antagonist did not protect the muscle from experimentally induced myofiber atrophy [51], and in one case worsened it [52]. However, there is no other indication that activin antagonism, under non atrophy conditions, results in atrophy of myofibers in any muscle. The atrophy response in the TA of SGI-1252 treated mice could potentially be explained by off-target drug effects. SGI-1252 has been shown previously to be a potent inhibitor of the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway [39]. Recently, the JAK/STAT signaling pathway has been shown to regulate skeletal muscle myogenesis and satellite cell activity [53, 54]. Specifically, transient inhibition of JAK/STAT signaling promotes expansion of the muscle satellite cell pool by limiting terminal differentiation [53, 54]. In a recent study Tierney et al. [53] showed that chronic inhibition of STAT3 led to a decrease in muscle regeneration, attributed to decreased satellite cell differentiation and potentially decreased fusion. With increasing age, satellite cells decrease in function and number [55, 56]. The decrease in function and number of satellite cells have been linked with the

decreases seen in muscle mass that occur with aging and disease [55, 57, 58]. In the same manner that muscle atrophy in aging and disease are related to decreased satellite cell number and function, the decrease in muscle fiber CSA of the TA in our study could possibly be explained by the extended (8-week) chronic inhibition of JAK/STAT signaling by the administration of SGI-1252. Chronic inhibition of JAK/STAT signaling results in decreased satellite cell differentiation [53, 54] and possibly fusion. Therefore 8 weeks of chronic inhibition of JAK/STAT signaling could result in prolonged decreases of satellite cell fusion. Interleukin-6 (IL-6), a JAK/STAT pathway ligand, is a cytokine that has been linked closely to STAT3 activity. For example, Tierney et al. showed that IL-6-induced STAT3 signaling plays a regulatory role in Myod1 transcription, suggesting that STAT3 may also play a role in satellite cell-mediated hypertrophy and muscle wasting [53]. Serrano et al. [59] found, in support of this idea, that genetic loss of IL-6 blunted muscle hypertrophy *in vivo*. Xiao [60], also found that the loss of STAT3 leads to inhibition of satellite cell-mediated compensatory muscle hypertrophy and a decrease in MyoD expression. These data demonstrate the possible role of JAK/STAT signaling in satellite cell-mediated hypertrophy and muscle wasting, which would potentially explain the decreases in TA muscle fiber CSA. For example, if JAK/STAT signaling was being chronically inhibited for 8 weeks as expected, we could expect to see a decrease in muscle fiber CSA with young (4-week) growing mice, strictly arising from an inability to properly activate the hypertrophy signaling pathways in skeletal muscle as the mice grow and mature or from an inability to maintain proper myonuclear domain due to decreased satellite cell contribution, and therefore decreasing the ability to synthesize new contractile proteins to maintain growing muscle [61-63]. Resulting in either actual muscle fiber atrophy or more likely just an inhibition of fiber hypertrophy that is associated with normal growth. Due to chronic inhibition of

JAK/STAT signaling, decreased satellite cell fusion, decreased satellite cell-mediated hypertrophy, and altered hypertrophy signaling could possibly explain the decreases seen in muscle fiber CSA in TA muscles. Addressing the issue that TA muscles decreased in muscle fiber CSA while gastrocnemius muscle did not could be explained by the difference in fiber type, as TA muscles are richer in type IIb fibers compared to the more mixed-fiber gastrocnemius. The TA muscle would likely atrophy more because type IIb fibers tend to be larger [64], more prone to atrophy [65], and more dependent on fusion of satellite cells [66]. Further research would need to be done to investigate the exact interaction of inhibited JAK/STAT signaling and the proposed methods of decreasing muscle fiber CSA in skeletal muscle. Specifically, inhibited JAK/STAT signaling effects on satellite cell-mediated hypertrophy, satellite cell fusion, and hypertrophy signaling and how these effect muscle during developing stages, especially regarding the specificity in type IIb fibers.

CONCLUSION

In conclusion, oral administration of the activin receptor antagonist SGI-1252 to wild-type mice did not induce widespread increases in body mass or individual limb muscle mass as we had expected. The drug was well tolerated and did not induce any indications of toxicity when administered at 400 mg/kg, 3 times per week. In fact, treated mice appeared to be on a trajectory towards greater body mass relative to control mice had the treatment period been extended. It is not entirely clear why SGI-1252 did not improve muscle mass in the wild-type mice, but the most likely explanations include insufficient dose and dose frequency, and/or off-target effects involving the drug's inhibitory activity on JAK/STAT signaling. Based on the results of the current study, it is still unclear whether SGI-1252 has the potential to be developed into a successful therapeutic for muscle wasting disorders. Clearly, more studies need to be done

using a wider range of doses and modes of administration. Studies to assess the effectiveness of SGI-1252 in preventing muscle wasting during experimentally induced atrophy (i.e., disuse or cachexia) will also be important in further defining its therapeutic potential. Studies have also suggested recently that inhibitors of the JAK/STAT signaling pathway may have utility to counteract the functional exhaustion of satellite cells in pathological conditions [53]. In that light, given its excellent tolerability and oral bioavailability, SGI-1252 should be further investigated for its regulation of satellite cell activity and skeletal muscle myogenesis.

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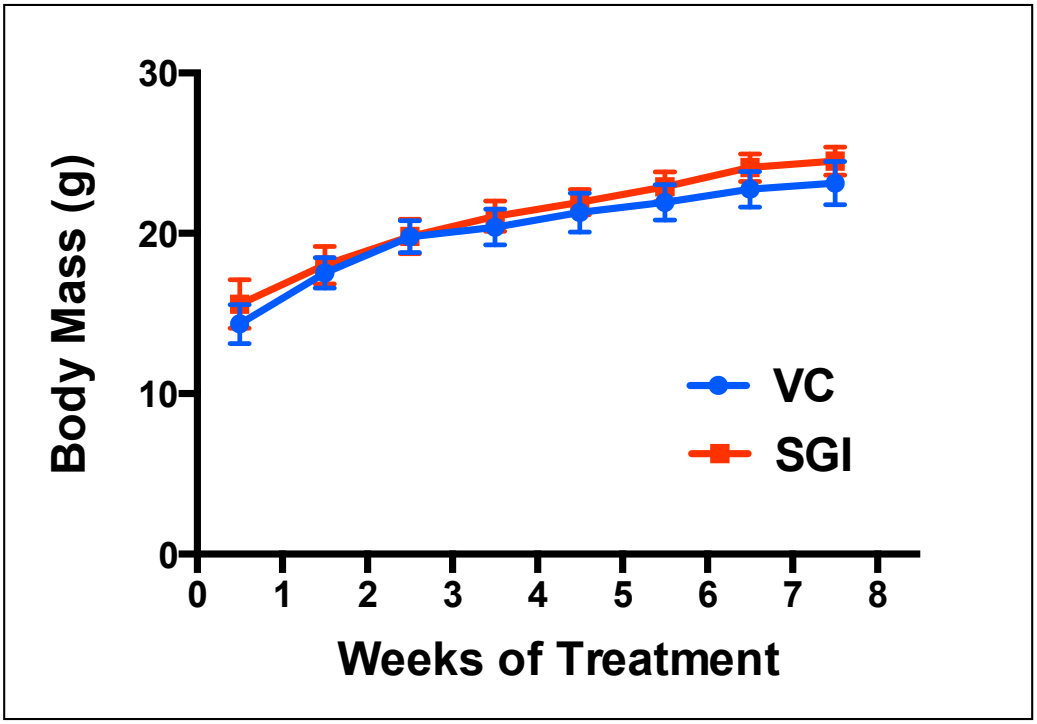

Figure 1: Body mass over 8 weeks of treatment. No differences were observed with body mass over the 8-week treatment period between SGI-1252-treated mice and control mice.

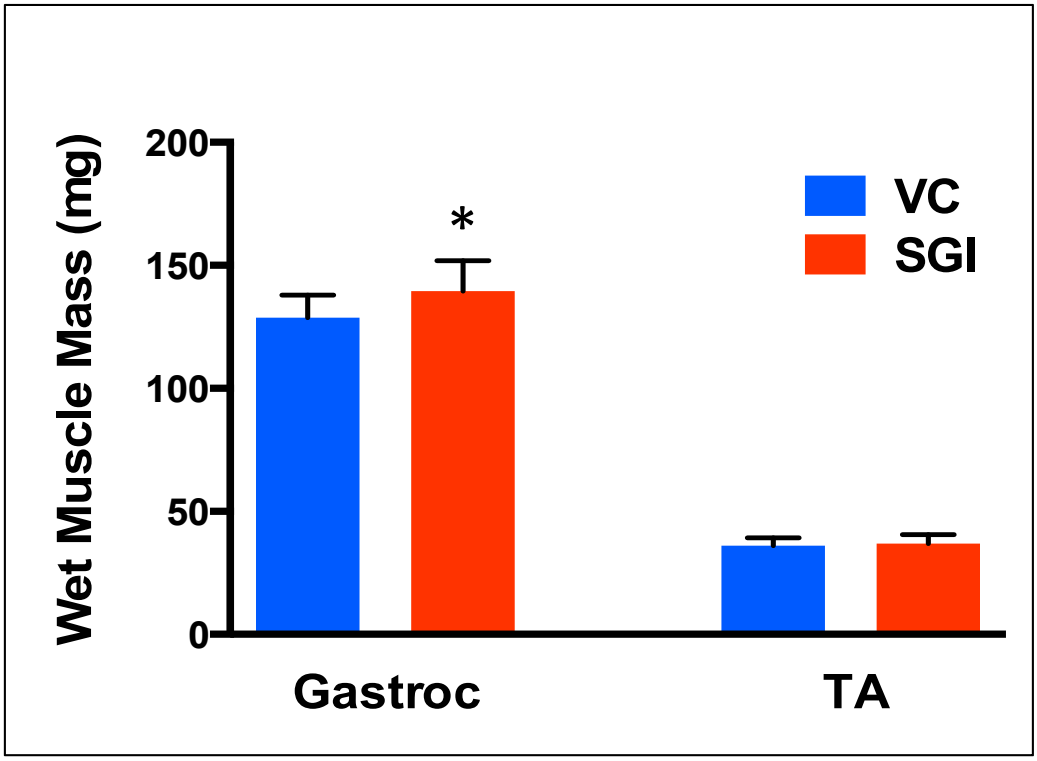

Figure 2: Gastrocnemius and TA wet muscle mass after 8-week treatment. Gastrocnemius wet muscle mass increased significantly in SGI-1252-treated mice compared to controls. Data are means ± SD. * indicates significant difference ($p < 0.05$) from vehicle control (VC).

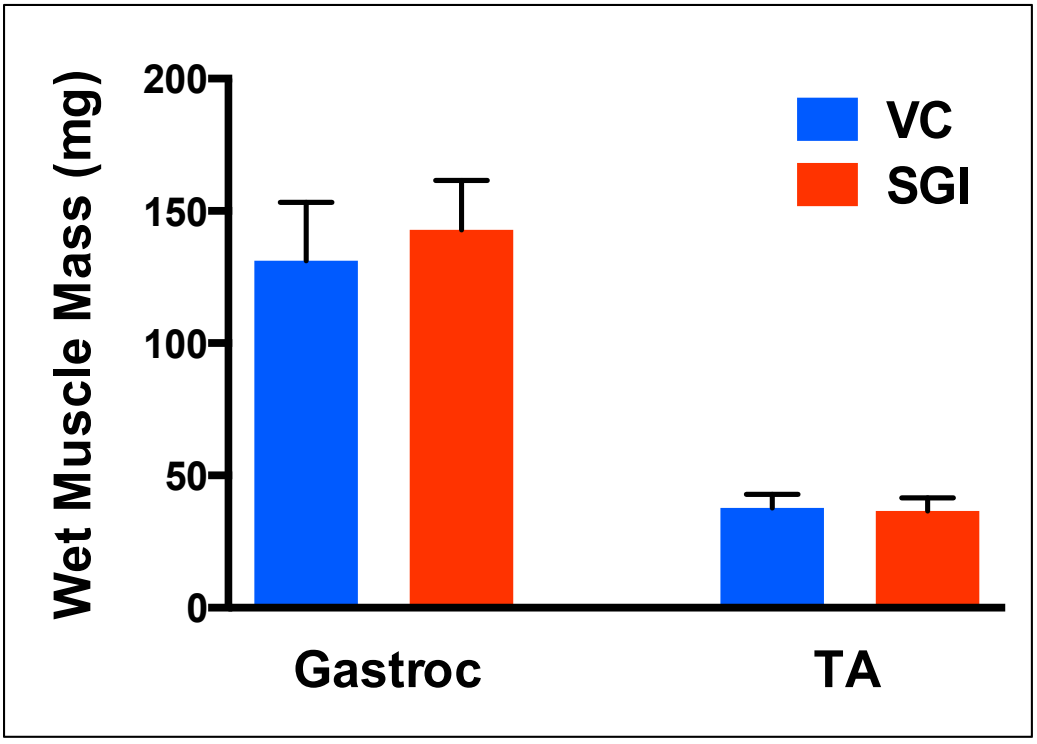

Figure 3: Gastrocnemius and TA wet muscle mass after normalizing for body mass after 8-week treatment. There were no changes in gastrocnemius or TA mass after the 8-week treatment when accounting for body mass.

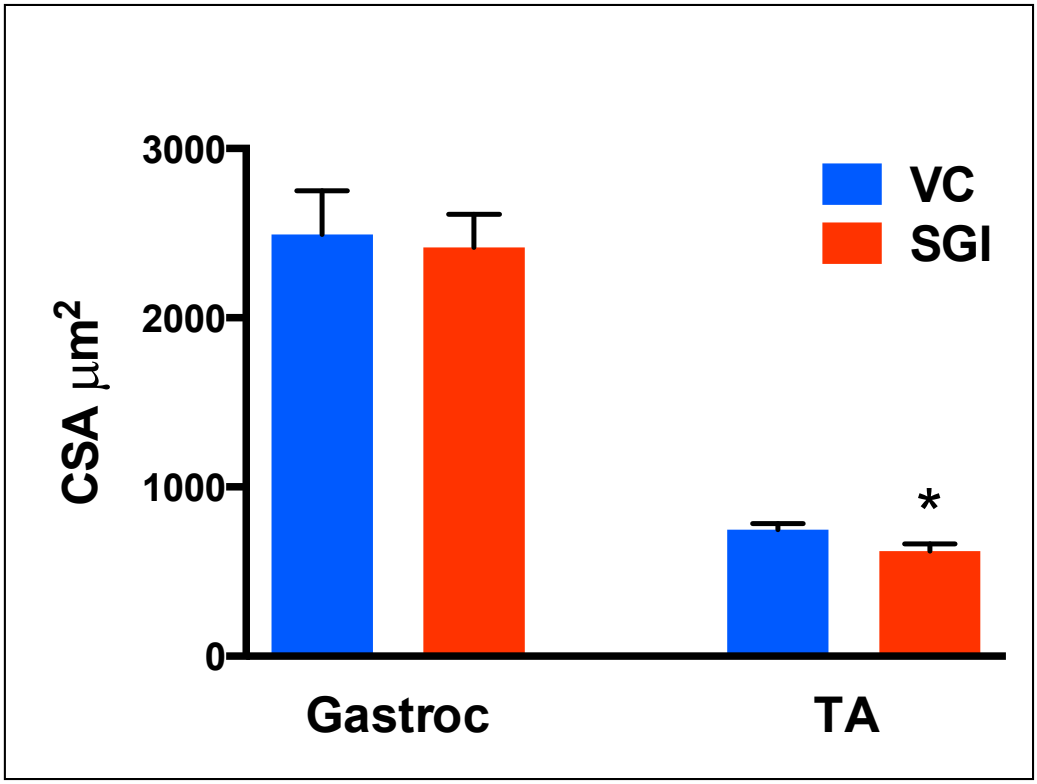

Figure 4: Cross sectional area (CSA)/fiber size after 8-week treatment. CSA/fiber size shown in µm² values are not comparable between muscles (Gastrocnemius and TA). There were no significant differences in muscle fiber CSA between SGI-1252-treated mice and control mice for gastrocnemius muscles, however there was a significant difference with TA muscles, with SGI-1252-treated mice having a smaller muscle fiber CSA compared to controls. Data are means ± SD. * indicates significant difference ($p < 0.05$) from vehicle control (VC).

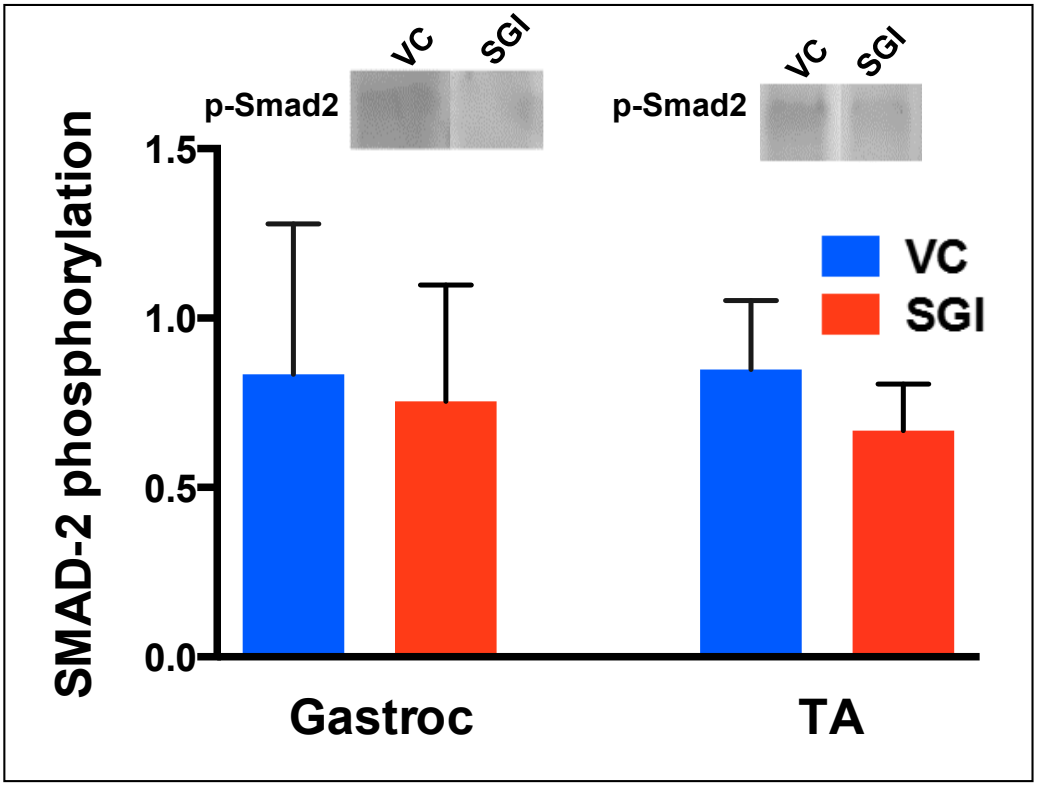

Figure 5: Phosphorylation of Smad2 in gastrocnemius and TA muscles, as measured by Western blot. Smad2 Phosphorylation shown as intensity units. Although there is suggestive evidence of SGI-1252-treated TA muscles having reduced phosphorylation/activity compared to VC, there were no significant differences for either muscle.