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Investigating the Effects of Lestaurtinib on Skeletal Muscle Regeneration

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THE EFFECTS OF LESTAURTINIB ON SKELETAL MUSCLE REGENERATION

by

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ABSTRACT

Repurposing drugs to enhance skeletal muscle repair mechanisms may unveil novel treatments for certain muscular pathophysiological states while maintaining high safety and efficacy standards at lower costs. The anti-cancer drug lestaurtinib appears to induce fusion and differentiation of myoblasts *in vitro*, inviting an attempt in the present study to determine if these results are replicable *in vivo*. To initiate skeletal muscle regeneration, tibialis anterior muscles were injured in both legs of C57BL/6J mice (n = 30) using the myotoxin, cardiotoxin (CTX). At 3 days post-CTX, the affected area on the right leg was treated with lestaurtinib at a concentration of either 40 nM or 200 nM; the left leg was used as a vehicle-treated control. Muscles were excised at either 4, 7, or 14 days post-CTX and then histologically examined to determine the effect of lestaurtinib on muscle regeneration. Fibre cross-sectional area, collagen-positive area (%), embryonic myosin heavy chain (emb-MyHC) positive area (%), and number of emb-MyHC-positive cells were assessed. The regeneration process appeared to be mostly unaffected following lestaurtinib treatment as indicated by no difference in fibre cross-sectional area, number of newly regenerated myofibres, and emb-MyHC-positive area. However, significantly reduced collagen-positive area was observed in lestaurtinib-treated muscle at 7 and 14 days post-CTX. Divergent effects on collagen content were observed between lestaurtinib-treated muscles, suggesting that each drug level exhibited opposing effects on collagen synthesis and/or degradation. These data suggest that single dose treatment of 40 or 200 nM of lestaurtinib at 3 days post-injury does not augment muscle repair, although the 200 nM dose may reduce collagen accumulation. Further investigation of lestaurtinib treatment on muscle regeneration in myopathic states may reveal utility in suppressing muscle fibrosis; however, further study is needed to understand the mechanisms by which lestaurtinib exerts its effects on muscle *in vivo*.

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AML	Acute Myeloid Leukemia
CEP-701	Codename for lestaurtinib
CSA	Cross-sectional area
CTX	Cardiotoxin
DMD	Duchenne Muscular Dystrophy
DMSO	Dimethylsulfoxide
ERK	Extracellular signal-regulated kinase
FLT3	Fms-like tyrosine kinase 3
HGF	Hepatocyte growth factor
huSMPs	Human skeletal muscle precursors
IC_{50}	Half maximal inhibitory concentration
JAK2	Janus kinase 2
MPD	Myeloproliferative disorders
MHC	Myosin heavy chain
MRF	Myogenic regulatory factor
Mrf4	Myogenic regulatory factor 4
Myf5	Myogenic factor 5
MyoD	Myoblast determination protein
NGS	Normal goat serum
PBS	Phosphate buffered saline
PRK1	Serine/threonine-protein kinase N1
TA	Tibialis anterior
NTRK1	High affinity nerve growth factor receptor
RTK	Receptor tyrosine kinase
V617F	Mutation encoding a valine to phenylalanine substitution at position 617

1. INTRODUCTION

Skeletal muscle is a multinucleated contractile tissue under voluntary control of the somatic nervous system and thus responsible for producing movement of the skeleton. Acute skeletal muscle damage is frequently sustained through exercise, injury, and daily mechanical stress (Proske & Morgan, 2001; Tidball, 2005). To maintain functional capacity under duress of regular tissue damage, skeletal muscle is adept at regenerating following damage, a process tightly regulated through coordinated interplay between various cellular and molecular systems. This process is critically dependent on the activation and differentiation capacity of satellite cells – myonuclei-like stem cells that reside within skeletal muscle between the basement membrane and the basal lamina (Tedesco et al., 2010). These cells remain in a quiescent state at the periphery of the muscle fibre until damage is sustained, at which point they activate, proliferate as muscle precursor cells (myoblasts), differentiate into muscle-specific cells (myocytes), and fuse to damage sites or to each other to form new muscle fibres (Chargé & Rudnicki, 2004; Lieber, 2002; Zammit, 2008). A small portion of proliferated myoblasts de-differentiate back into satellite cells and return to quiescence, repopulating the cell pool (Lieber, 2002; Zammit, 2008).

The impact of satellite cells on the regenerative capacity of skeletal muscle is most clearly evident in aging (Shefer et al., 2006; Snow, 1977) and various muscle diseases (Shi, 2006); both are pathophysiological states typified by slow and sometimes incomplete muscle tissue regeneration. In these states, the capacity for regeneration in response to damage is diminished over time due, in part, to impaired satellite cell self-renewal mechanisms, leading to a less potent cell population. As such, research that seeks to further the understanding of the mechanisms that underlie skeletal muscle regeneration is imperative in unveiling new treatments that may ameliorate symptoms associated with pathophysiology. Specifically, potential drug therapies that

encourage satellite cell renewal, activation, proliferation, and/or differentiation could be the key to realizing new medical treatments for individuals with impaired regenerative capacity.

Recently, a drug repurposing strategy was developed to isolate existing drugs with the potential to augment skeletal muscle regeneration in pathophysiological states. Identifying candidate compounds was the first step. Employment of a high-throughput screen revealed several compounds capable of altering the phenotype of satellite cells (see Appendix A for description of screening methods). The screen revealed a cancer IND called lestaurtinib (CEP-701) that stood out for its ability to produce a desirable satellite cell phenotype while minimizing cell death (Krause et al., 2015). Given these results, the effect of lestaurtinib was assessed in a preclinical model. When applied to activated satellite cells in differentiation media, lestaurtinib conserved these desirable phenotypic effects by inducing differentiation of myoblasts and encouraging their subsequent fusion to form myotubes (see Appendix B for details of preliminary cell culture experiments).

It is presently unknown if lestaurtinib treatment could improve muscle regeneration *in vivo*, possibly by accelerating fusion and/or differentiation of myoblasts. Thus, given these data from the pilot studies, the present study seeks to assess the drug's effects in an *in vivo* preclinical model. Specifically, the results of this study will determine if lestaurtinib could reduce the time required to regenerate damaged skeletal muscle, and/or contribute to greater skeletal muscle fibre size, by accelerating fusion and/or differentiation of myoblasts *in vivo*. Proof of concept in a live model could provide support for the advancement of lestaurtinib to Phase II clinical trials for this novel indication and invite further research into the drug's mechanism of action in this unique treatment setting.

To explore the circumstances in which lestaurtinib could potentially augment skeletal muscle regeneration, it is important to first understand the function of satellite cells in greater detail, and how both their activity and capacity for self-renewal is regulated during regeneration. To clarify the context in which satellite cells operate, the following review first outlines the skeletal muscle regeneration process and expands on the morphology, activity, and self-renewal ability of satellite cells. Next, the effects of specific pathophysiological states on skeletal muscles and satellite cells are reviewed. Finally, lestaurtinib's role in the clinical field is explored.

1.1 Overview of Adult Skeletal Muscle Regeneration

Damage to muscle fibres can be sustained in several ways: near constant micro-damage from daily mechanical stress; acute trauma from exercise (Proske & Morgan, 2001; Sorichter et al., 1999; Tidball, 2005); or directly through puncture or laceration. Damage can also be induced purposely for scientific study through freezing, crushing, or treatment with chemicals or myotoxins (Gutiérrez et al., 1990). Despite the potential for frequent damage, and regardless of severity, skeletal muscle has considerable regenerative capability.

Muscle repair follows a well-defined, three-phase process consisting of degeneration, inflammation, and regeneration (Chargé & Rudnicki, 2004). This process is similar between humans and mice, with a few minor differences in timing of key elements (reviewed in Mierzejewski et al., 2020). Directly following injury, the beginning of the degenerative phase is marked by necrosis – progressive destruction of the damaged muscle fibre. If damage to the muscle cell is great enough, the entire fibre induces a self-destructive program and ultimately dies (Chargé & Rudnicki, 2004). Chemotactic factors released from the damaged/dying muscle fibre activate local inflammatory cells which, in turn, signal to circulating inflammatory cells, such as neutrophils and macrophages, and induce their migration to the damage site on the muscle fibre

(Bartocci et al., 1987; Deuel et al., 1981). Neutrophils are the first to infiltrate the damage site and are thought to play a role in both early phagocytosis and the breaking down of cellular debris left over after muscle damage is sustained (Lowe et al., 1995; Tiidus, 1998). In mouse muscle, neutrophil numbers peak between 6 to 24 hours following damage and decrease after 72 hours (Tidball & Villalta, 2010). In human muscles, neutrophil numbers peak much more rapidly, about 45 to 120 minutes following damage (Fielding et al., 1993; Fortunato et al., 2018).

Following neutrophil infiltration, macrophages overtake the inflammatory process by clearing cellular debris through phagocytosis (Arnold et al., 2007; Tidball, 1995; Tidball & Villalta, 2010). Macrophages are present within the regeneration muscle in two distinct populations. The first population, M1, is comprised of pro-inflammatory macrophages that remove debris and necrotic tissue through phagocytosis and amplify the inflammatory response by expressing and secreting high levels of signalling cytokines (Tidball & Villalta, 2010). In mouse muscle, M1 macrophages peak between 24 and 48 hours following damage. In human muscle, this population is observed later, by about 48 hours following injury. Infiltration of M2 macrophages follow M1, with some overlap occurring in timing. M2 macrophages are anti-inflammatory and nonphagocytic – their role is to mitigate inflammation and contribute to the activation of satellite cells, so the rest of the regeneration process can proceed (Arnold et al., 2007; Tidball & Villalta, 2010). In mouse muscle, M2 macrophage numbers peak around 48 hours following damage. As with M1 macrophages, this occurs later in human muscles, with M2 macrophage numbers peaking between 4 and 6 days following damage (Tidball & Villalta, 2010). Overall, this process is critical in both the inflammatory phase and during new fibre formation. When macrophage populations are disrupted, the myofibre fails to regenerate (Lu et al., 2011).

Following inflammation, regeneration commences when satellite cells activate. In mouse muscle, the increase in activated satellite cell number occurs 72 to 96 hours following damage (Wosczyna & Rando, 2018). This timing is similar in human muscle – the peak of activated satellite cells is typically observed at 72 hours following damage (Crameri et al., 2004; Mackey et al., 2009). Following activation, satellite cells proliferate, differentiate, then fuse to damaged fibres or to each other to form new fibres. New (or partially new) fibres display several distinct characteristics, including reduced size, centrally located nuclei, evidence of elevated protein synthesis, and presence of embryonic myosin heavy chain (emb-MyHC), a marker of newly formed myotubes (Whalen et al., 1990). Eventually, new fibres become larger and their nuclei migrate to the periphery of the fibre, indicating completion of regeneration (Hall-Craggs, 1974). Remodeling of the extracellular matrix (ECM) also occurs while new muscle fibres are established. Fibroblasts contribute heavily to this process. With damage, they activate and subsequently migrate to damage sites, where they temporarily stabilize the ECM by releasing proteins such as collagen, elastin, fibronectin, laminin, and proteoglycans. These proteins act as a scaffold for new muscle fibres (Mann et al., 2011).

The formation of new muscle fibres proceeds quickly in healthy skeletal muscle. Typically, between two and three days post-injury, new myotubes have formed, indicated by emb-MyHC expression (Ciciliot & Schiaffino, 2010; Krause et al., 2013). Around this time, nerve terminals re-establish contact with the newly formed myotubes (Jirmanová & Thesleff, 1972), and re-innervation is complete by about day four or five (Grubb et al., 1991). By five or six days post-injury, force production and the muscle's overall structure is restored, though newly regenerated fibres are smaller and still feature centrally located nuclei (Kalhovde et al., 2005). Generally, after four weeks post-injury, newly formed muscle fibres are functionally indistinguishable from non-

injured fibres, though nuclei may remain centrally located for upwards of 50+ days (Fig. 1; Table 1; D'albis et al., 1998; Paoni et al., 2002).

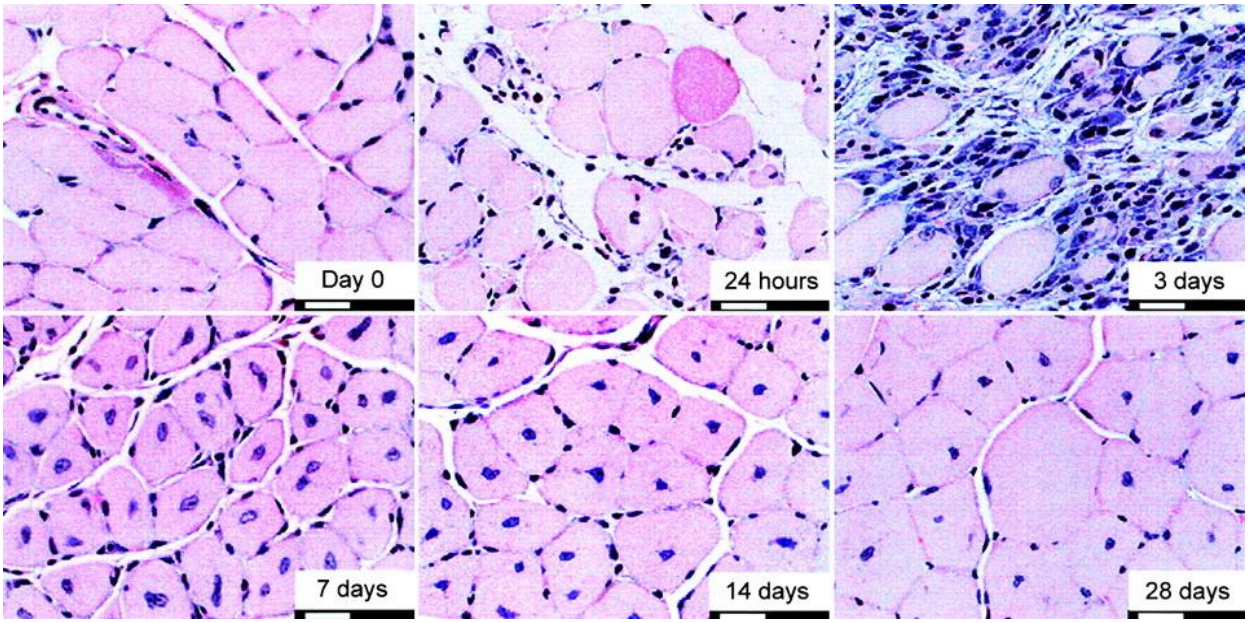


Figure 1. *Time course of muscle fibre regeneration from onset of damage to 28 days post injury (Paoni et al., 2002).*

Regeneration Time Course	
Time Post-Damage	**Activity/Characteristic**
1 - 6 hours	Neutrophil numbers increase and invade tissue
24 - 48 hours	Inflammatory response peaks as macrophages invade
1 - 4 days	Inflammatory response and swelling decreases Satellite cell proliferation most active
3 days	Satellite cells differentiate into myoblasts New myotubes evident through emb-MyHC expression
4 - 6 days	Force production has returned to approximately normal levels Overall architecture of muscle restored Regenerated fibres are smaller and display centrally located nuclei
3 - 4 weeks	Fibre size is similar to that of undamaged muscle Some new fibres may still feature centrally located nuclei
> 4 weeks	Muscle is functionally restored but newly formed fibres may still feature centrally located nuclei

Table 1. *Regeneration time course from onset of muscle damage to fully restored muscle fibres.*

1.2 The Role of Satellite Cells in Skeletal Muscle Regeneration

1.2.1 Regulation of Satellite Cell Activity Following Muscle Fibre Damage

Regeneration of skeletal muscle is a complex process involving many extracellular growth factors, intracellular signalling pathways, and transcription factors. Of these, the myogenic regulatory factors (MRFs) myogenin, MRF4, MyoD, and Myf5 and the transcription factor Pax7 play the greatest role in the determination and differentiation of satellite cells (Fig. 2; Braun et al., 1994; Buckingham et al., 2003; Rudnicki et al., 1992, 1993; Zhao & Hoffman, 2008).

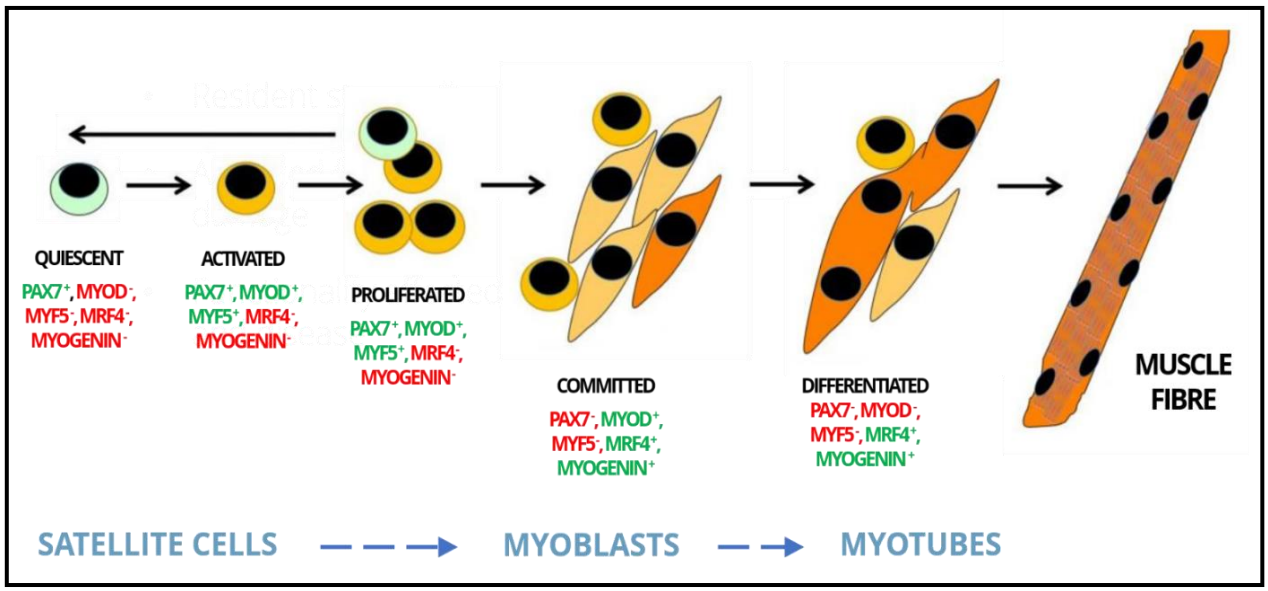


Figure 2. *An illustrated representation of differences in expression in each of the MRFs and Pax7, depending on satellite cell activity and where the cell is in the myogenic program.*

Activation. Hepatocyte growth factor (HGF) is mainly responsible for inducing satellite cell activation following damage to the extracellular matrix of skeletal muscle fibres. When HGF is released from the extracellular matrix post-damage, it binds to its receptors (Cmet) on satellite cells, stimulating their activation (Tatsumi & Allen, 2004). At this point, satellite cells begin to express *MyoD* and *Myf5*, and are already expressing *Pax7* (Fig. 2). Myf5 is crucial for ensuring

commitment of satellite cells to the full cell cycle before they differentiate. This is evidenced in *Myf5*-null mice, where absence of expression in late fetal stages leads to premature differentiation causing growth defects and pre-natal death (Montarras et al., 2000).

Proliferation. Satellite cell proliferation is regulated through interactions between several mitogens – including HGF – and intracellular protein kinase signalling pathways. Activated satellite cells relay the mitogenic signal to initiate proliferation via multiple signalling pathways, one of which includes extracellular signal-regulated kinase (ERK; Shefer et al., 2001). HGF binding to satellite cell Cmet receptors leads to ERK phosphorylation which, in turn, induces satellite cell proliferation (Volonte et al., 2005). Through proliferation to cell commitment, continued expression of *MyoD* and concurrent downregulation of *Pax7* and *Myf5* indicate satellite cells that have committed to the myogenic program (Fig. 2; Megeney et al., 1996).

Differentiation. MyoD plays a crucial role in ensuring timely regeneration by establishing timely satellite cell differentiation. Mice with mutant *MyoD* are viable, but satellite cells take longer to proliferate and differentiate (Sabourin et al., 1999) and so the formation of new muscle fibres is delayed (Megeney et al., 1996). In this case, satellite cells tend to undergo premature senescence, exhausting the cell pool more rapidly than in mice with normal expression of *MyoD* (Montarras et al., 2000).

Myogenin and Mrf4 are present together in both committed and differentiated satellite cells. *Myogenin* is a transcriptional activator of genes whose expression helps to drive post-natal differentiation of satellite cells by ensuring that these cells cease proliferation (Fig. 2; Buckingham & Montarras, 2008; Cornelison & Wold, 1997). Though expression of *myogenin* is downregulated in newly fused myotubes, *Mrf4* remains detectable and is the only MRF present in new muscle fibres as well (Fig. 2). Mrf4 is thought to be especially important in facilitating normal contraction,

as *Mrf4*-mutant mice tend to experience a downregulation of specific sodium channels at the neuromuscular junction and along the muscle fibre membrane (Zhou & Bornemann, 2001).

1.2.2 Maintenance of Cell Pool

If satellite cells were unable to repopulate following incorporation into new muscle tissue, the number of quiescent satellite cells available to commit to the myogenic program would decrease over time and eventually deplete. Therefore, renewal of the quiescent satellite cell pool is crucial to ensure an adequate reserve for future regeneration events. The timing of expression of *Pax7* and specific MRFs regulates the self-renewal process. As proliferation begins, satellite cells co-express *MyoD* and *Pax7* (Pax7-positive/MyoD-positive; Fig. 2). About 48 hours following the first division, most satellite cells down-regulate the expression of *Pax7*, retain *MyoD* expression, and begin to express *myogenin* (Fig. 2). The co-occurrence of these three events induces differentiation and the new myoblast is then incorporated into the growing myotube.

However, a small portion (~20%) of satellite cells follows a different route – *MyoD* is down-regulated while *Pax7* expression is retained (Pax7-positive/MyoD-negative; Halevy et al., 2004; Schultz, 1996; Zammit et al., 2004). In these cells, *myogenin* is never expressed at all. It is assumed that maintaining expression of *Pax7* signals these cells to return to a quiescent state in lieu of differentiating. In this state, they remain transcriptionally active and can later proliferate and express *myogenin* to differentiate or proliferate and return to the quiescent cell pool once again (Zammit et al., 2004). This process is called asymmetric division – one daughter cell differentiates and commits to myogenesis while the other returns to a quiescent state (Baroffio et al., 1995; Schultz, 1996; Beauchamp et al., 1999). In contrast, satellite cells can also repopulate the cell pool through symmetric division. This occurs when neither daughter cells differentiate; instead, both return to a quiescent state (Price et al. 2014).

1.3 Pathophysiological Dysregulation of Regeneration

Disruption of any phase of muscle repair can result in unsuccessful regeneration, typically characterized by myofibre degeneration, inflammation, and fibrosis. Because satellite cells are highly dependent on the niche within which they exist, pathophysiological states such as aging, disease, obesity, and cancer cachexia can disrupt both their activity and functionality and, by extension, the key events necessary for successful regeneration of skeletal muscle. This section reviews several mechanisms by which pathophysiological states contribute to impaired satellite cell function in order to highlight relevant areas that could potentially benefit from therapeutic countermeasures.

1.3.1 Aging

Aging skeletal muscle is associated with a condition called sarcopenia, characterized by a progressive loss of functional characteristics and an impaired ability to regenerate following damage. With sarcopenia, the inflammatory phase following damage is prolonged, fibrosis increases, and new fibres replace damaged ones less frequently, leading to diminished muscle mass and an associated loss of strength (Ali & Garcia, 2014; Muscaritoli et al., 2010). The cause of sarcopenia is multi-factorial – contributing factors include age-related changes in biology such as neurological decline (Chai et al., 2011; Verdijk et al., 2012), hormonal shifts (Sakuma & Yamaguchi, 2012), and increased inflammation (Jo et al., 2012). Environmental factors include an increasingly sedentary lifestyle and inadequate nutritional intake, both of which contribute to weight gain/obesity (Koster et al., 2011; Robinson et al., 2012).

The aging-related loss of regenerative capacity is associated with a compromised satellite cell population, including discontinuation of activation following damage and reduction of the quiescent satellite cell pool. Several contributing mechanisms have been proposed, including

disruption of asymmetric division, decline in proliferation, and failure to return to a quiescent state following regeneration (Shefer et al., 2006). It is suggested that these mechanisms contribute to a continually growing committed cell pool and a concurrently diminishing reserve pool with each iteration of regeneration.

Several intrinsic and extrinsic factors are associated with the underlying cause of impaired satellite cell function with age. One such intrinsic factor is the increased exposure to oxidative stress that occurs with aging quiescent satellite cells. Damage from oxidative stress is inefficiently repaired within these cells, leading to increased DNA damage over time (Bohr, 2002), which ultimately modulates their self-renewal, proliferation, and differentiation capabilities (reviewed in Kozakowska et al., 2015). Another intrinsic factor associated with the inability of satellite cells to return to a quiescent state following damage is aberrant cell-autonomous signalling. Fibroblast growth factor receptor (FGFR) tyrosine kinase helps to coordinate satellite cell regulatory factors with extracellular signals (Hannon et al., 1996; Kudla et al., 1998). For example, satellite cells express FGFR1, which activates the intracellular p38α/β mitogen-activated protein kinase (MAPK) pathway. This pathway prevents terminal differentiation and regulates both asymmetric division and the exit of satellite cells from quiescence (Jones et al., 2005; Kudla et al., 1998; Troy et al., 2005). However, these regulatory mechanisms tend to be defective in the skeletal muscles of aged mice due to an insensitivity to FGF ligands and increased p38α/β MAPK activity (Bernet et al., 2014). This suggests that while FGF–p38α/β MAPK signalling is important in ensuring a robust reserve satellite cell pool, aging-related changes to signalling activity contribute to the quiescent population's decline and resulting regenerative impairments.

Contributing extrinsic factors include reduced expression of *Notch* and *TGF-β*, as well as age-dependent increases in Wnt signalling. Notch signalling helps regulate the activation of

quiescent satellite cells and their subsequent proliferation. Because Notch signalling decreases with age, satellite cell activation is correspondingly reduced following muscle damage (Conboy & Rando, 2002; Conboy et al., 2005). Additionally, aging muscles tend to express elevated levels of *TFG-β*, which contribute to elevated levels of phosphorylated Smad3 within satellite cells. In the regulation of satellite cell proliferation, phosphorylated Smad3 and endogenous Notch are antagonistic of one another. As a result, the proliferative capacity of the satellite cell is reduced (Carlson et al., 2008). Together with compromised satellite cell activation, this effect further compromises the muscle's regenerative capacity. An additional extrinsic factor altered by aging is the Wnt signalling pathway. Following the transition from proliferation-promoting Notch signalling, Wnt signalling plays a role in satellite cell differentiation. In aged muscle, Wnt ligands are chronically elevated, contributing to increased signalling activity associated with enhanced fibrosis and reduced myogenesis (Brack et al., 2007, 2008).

1.3.2 Myopathy

Myopathies are diseases of, or disease complications that affect, skeletal muscle in which some collection of structural and functional impairments lead to a loss of contractile function. Dystrophies are a specific group of myopathies caused by a disconnection of the actin protein in the cytoskeleton of the muscle fibre and dystroglycan proteins in the sarcolemma. Without this link, regular contractions persistently injure the muscle, resulting in constant damage to the fibres (Lapidos, 2004; Wallace & McNally, 2009). Satellite cells attempt to regenerate fibres at the damage sites, but they cannot repair the dysfunctional proteins that result in the separation of the cytoskeleton from the plasma membrane. As damage accumulates at a higher-than-normal rate, the regenerative process is continually active, resulting in perpetual recruitment of satellite cells. Because satellite cells cannot sufficiently compensate for the increasing degeneration, their self-renewal mechanisms become exhausted and their finite reserve cell pools eventually deplete (Blau

et al., 1983; Sacco et al., 2008. As regeneration lags, more damage accumulates, leading to advanced wasting of the muscle and a progressive replacement of muscle tissue with fibrotic tissue (Rahimov & Kunkel, 2013; Wallace & McNally, 2009).

The impaired regenerative capacity of satellite cells in dystrophic muscle is assumed to be caused by premature senescence due to repetitive cycles of replication (Decary et al. 2000; Sacco et al., 2010). This contributes to a loss of many functional characteristics earlier than would normally be expected (i.e. with aging; Kudryashova et al., 2012). Several studies have suggested additional contributing factors. For instance, early studies have shown that satellite cells in dystrophic muscle undergo delayed or inadequate differentiation (Delaporte et al., 1984; Jasmin et al., 1984). Alterations in Notch signalling could be another contributing factor. Blocking Notch signalling in mouse satellite cells results in a dystrophic phenotype and reduces satellite cell activation and proliferation (Lin et al., 2013). Similarly, Notch signalling is attenuated in satellite cells of mice lacking dystrophin expression (Dmd^{mdx}) and is associated with a reduced capacity for self-renewal (Jiang et al., 2014). Lastly, the dystrophic stem-cell niche may also contribute to reduced myogenic potential in satellite cells. One study compared the proliferative capacity of satellite cells isolated from a mouse model of congenital muscular dystrophy ($Large^{myd}$). Those cells that remained attached to single isolated muscle fibres showed a reduced ability to proliferate; however, when fully isolated, they proliferated and differentiated at levels comparable to cells from control mice (Ross et al., 2012). This suggests that the dystrophic microenvironment may play a role in reducing the satellite cell's capacity for regeneration.

1.3.3 Obesity

Obesity is defined as excess fat accumulation that significantly increases certain health risks (Purnell, 2018), and is characterized by increased lipid deposition in both adipose and non-

adipose tissue (such as skeletal muscle; van Herpen & Schrauwen-Hinderling, 2008). Satellite cell function and muscle regeneration is thought to be impaired in obese individuals, contributing to a loss of muscle mass and function. For instance, accumulation of lipid metabolites in skeletal muscles of obese humans has been shown to result in lipotoxicity, which negatively affects cell signalling and metabolism (Adams et al., 2004; Hulver et al., 2003; Lelliott & Vidal-Puig, 2004). However, other skeletal muscle processes that may be affected by lipid metabolites remain elusive. Though the mechanisms that contribute to impaired muscle regeneration in humans with obesity are currently unclear, studies of obese mice have suggested several contributing mechanisms that may affect the regenerative capacity of satellite cells.

One such mechanism is leptin resistance, a condition commonly observed in obese humans (reviewed in Pan et al., 2014). In leptin signalling-deficient mice, both satellite cell proliferation and *MyoD* expression are reduced (Nguyen et al., 2011; Peterson et al., 2008). Similarly, myoblasts from leptin-receptor deficient mice express reduced levels of *MyoD* and *myogenin* and exhibit attenuated fusion during differentiation (Arounleut et al., 2013). These findings suggest that impaired regeneration may be attributed in part to reduced proliferative capacity and inadequate differentiation, caused by a decreased response to leptin signalling in obese individuals.

Inflammation is another mechanism that may affect satellite cell activity in individuals with obesity. Obese humans tend to experience chronic low-grade systemic inflammation (reviewed in Gregor & Hotamisligil, 2011 and Monteiro et al., 2010). Inflammation signals the release of HGF in skeletal muscle, which stimulates satellite cell activation in response to damage (Tatsumi & Allen, 2004). This suggests that chronic inflammation stimulates continual satellite cell activation, leading to premature senescence and impairment of regenerative capacity over time similar to that observed in aging skeletal muscle (Blau et al., 2015). Additionally, circulating pro-inflammatory

cytokines (such as TNF-α, IL-1β and IL-6) have also been shown to attenuate myoblast proliferation and differentiation (Langen et al., 2006), and may prevent protein synthesis (Frost et al., 1997) and thus growth of muscle fibres during regeneration.

Lastly, defects in skeletal muscle metabolism may also contribute to attenuated regeneration in obese individuals. In skeletal muscle fibres isolated from obese patients with type 2 diabetes, AMP-activated protein kinase (AMPK) functioning is impaired due decreased diacylglycerol kinase-delta (DGKδ), one of its key enzymes (Jiang et al., 2016). In healthy muscle, AMPK plays a role in ensuring the proper functioning of satellite cells by upregulating transcription of Pax7, Myf5, myogenin, and MyoD (Joe et al., 2010). It also increases *GLUT4* expression (McGee et al., 2008), whose translocation to the plasma membrane is associated with increased satellite cell proliferation and differentiation (Mokbel et al., 2014). Thus, decreased AMPK activity associated with obesity could be a contributing mechanism to impaired regeneration observed in obese individuals.

Together, these mechanisms show that satellite cell function and muscle regeneration is vulnerable to dysregulation in obese individuals, which can result in a loss of muscle mass and function. This can decrease quality of life for these individuals and may contribute to pain and weakness in the muscle. As such, therapies with the potential to ameliorate symptoms associated with dysregulation of skeletal muscle regeneration in other pathophysiological states (such as aging or muscle disease), may also be of benefit for individuals experiencing attenuated regeneration due to obesity.

1.3.4 Cancer Cachexia

Cachexia is a syndrome experienced by most cancer patients and is associated with weight loss due, in part, to progressive muscle wasting. Muscle wasting is involuntary and is not

successfully reversed through nutritional support or supplementation (Bennani-Baiti & Walsh, 2009; Fearon et al., 2011). Cancer patients experiencing cachexia also tend to experience cancer-induced anorexia – a loss of appetite unrelated to chemotherapy or other forms of treatment. Cancer-induced anorexia is irreversible by increased caloric intake and contributes to further body wasting (Fearon et al., 2011). Cachexia is common – over half of all individuals with cancer experience it to some degree (Dodson et al., 2011). The syndrome progresses in stages, with little weight loss occurring in early-stage or mild cachexia. Conversely, severe cachexia is fatal and is responsible for 20% of all cancer-related deaths (Dodson et al., 2011). Severe cachexia is classified by the percentage of weight loss experienced – around 25-30% of total body weight. Loss of muscle mass contributes significantly to the overall loss of body weight, and the extent of total weight lost at any stage is related to length of survival time (Fearon et al., 2006). Therefore, therapies seeking to reverse loss of muscle mass may be beneficial in increasing survival rates in some cancer patients.

However, there is ongoing debate as to whether muscle loss is caused by a reduction in protein synthesis, an increase in degradation, or a combination of both (reviewed in Johns et al., 2013). Though there has been considerable study of cachexia in a variety of animal models, the activated pathways and key mediators involved in the syndrome differ between each model. For example, both tumour growth and development of cachexia in murine models tend to advance rapidly (i.e. within a matter of days; Bennani-Baiti & Walsh, 2011), which does not best represent the chronic tumor growth and body wasting typically observed in humans with the condition. Thus, it is important that those seeking to treat human cancer cachexia attempt to target validated biomarkers of the condition in humans, rather than based on other models of the syndrome. As such, the following sections provide an overview of the current systemic mediators and myogenic-

associated mechanisms involved in human cancer cachexia. Note that because there are no licensed treatments for the disorder in humans, these mediators and mechanisms are based on observation only.

1.3.4.1 Systemic Mediators. ***Acute Phase Response.*** Increased resting energy expenditure (REE) in patients of certain cancers may contribute to cachexia by compounding loss of muscle mass and total body weight. Thermogenesis, the process of producing heat, is one factor that contributes to total energy expenditure in humans. This process is increased in some cancer patients due to an increased ratio of brown adipose tissue (BAT) as white adipose tissue (WAT) atrophies in the early stages of cachexia. Specifically, there is a phenotypic switch from WAT to BAT – a process called WAT browning. This is significant because increased BAT is associated with an increased expression of uncoupling protein 1 (UCP1) in the mitochondria. Here, UCP1 moves cellular respiration away from ATP synthesis toward thermogenesis, ultimately increasing energy expenditure (Petruzzelli et al., 2014, Shellock, Riedinger, & Fishbein, 1986). Furthermore, cancer patients experience an increase in activity of a systemic defense system called the Acute Phase Response (APR; Falconer, Fearon, Plester, Ross, & Carter, 1994). ARP is activated by infection, inflammation, or disease, with the goal of healing the body and/or returning the body to homeostasis (Cray, Zaias, & Altman, 2009). An increase in both thermogenesis and ARP contribute to increased resting energy expenditure, and subsequently body wasting.

Pro-inflammatory cytokines. Patients with cancer have been shown to have high levels of circulating pro-inflammatory cytokines. Cytokine action between host and tumour cells activate peripheral blood mononuclear cells as they pass through the tumour vasculature. When these mononuclear cells are activated, they subsequently release increased levels of pro-inflammatory cytokines. Pro-inflammatory cytokines are involved in the development of cachexia by

contributing to both systemic inflammation and inducing an APR (Wigmore et al., 2002, O'Riordain et al., 1999).

1.3.4.2 Myogenesis- Associated Mechanisms. A decrease in skeletal muscle regeneration may also contribute to the loss of skeletal muscle mass in cancer cachexia. Angiotensin II is increased in cachectic individuals, which suppresses satellite cell function by inhibiting their proliferation (Yoshida et al., 2013). Further evidence suggests that atrophy is also caused by persistent expression of the transcription factor CCAAT/Enhancer Binding Protein beta (C/EBPβ) in the muscle myoblasts of cachectic individuals. Typically, *C/EBPβ* expression is downregulated following myoblast differentiation. However, in a cachectic milieu, *C/EBPβ* expression remains elevated, which inhibits *myogenin* expression (Marchildon et al., 2015). Without myogenin, the myogenic program is compromised, resulting in atrophy.

Though several pathophysiological states may benefit from lestaurtinib's potential as a myotube-promoting compound, this characteristic is particularly applicable in the case of cancer cachexia given the drug's status as an anti-cancer drug. Traditionally, chemotherapy is associated with skeletal muscle atrophy and dysfunction, which compounds loss of muscle mass due to cachexia; as such, a drug that could potentially treat cancer while reversing loss of muscle mass would be especially beneficial. To explore lestaurtinib's potential in the clinical field, a review of the drug's properties and current clinical use as a cancer treatment is summarized below.

1.4 Lestaurtinib (CEP-701)

1.4.1 Background

Lestaurtinib is an orally bioavailable and rapidly absorbed semisynthetic derivative of K-252a, an alkaloid first isolated from *Nocardiopsis* bacteria in 1985 (Kase et al., 1986). Lestaurtinib inhibits several kinases at various half maximal inhibitory concentrations (IC_{50}) including high

affinity nerve growth factor receptor (NTRK1), FLT3, and JAK2, as well as the activation of transcription factors STAT3 and STAT5 (Table 2; "Lestaurtinib: Certificate," 2016). Lestaurtinib is safe for consumption and is generally well tolerated when taken orally – commonly reported mild side effects include nausea (28% of patients), diarrhea (21%), constipation (17%), fatigue (10%), dizziness (7%), and headache (7%; Knapper et al., 2006).

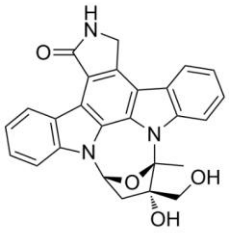
Lestaurtinib – Drug Data	
Codename	CEP-701
Formula	$C_{26}H_{21}N_3O_4$
Molecular Structure	
Description	An orally bioavailable polyaromatic indolocarbazole alkaloid derived from K-252a
Biological Activity	Inhibits: • JAK2 (IC_{50} = 0.9 nM) • FLT3 (IC_{50} = 3 nM) • Trk (A, B, C) (IC_{50} < 25 nM) • PRK1 (IC_{50} = 8.6 nM), • Aurora kinase A (IC_{50} = 8.1 nM) • Aurora kinase B (IC_{50} = 2.3 nM) Prevents STAT3 and STAT5 phosphorylation (IC_{50} = 20 – 30 nM) Exhibits antiproliferative activity *in vitro* (IC_{50} = 30 – 100 nM in HEL92.1.7 cells)
Protein binding	Highly protein bound
Metabolism	Liver – P450 enzyme system
Plasma half-life (oral)	8 – 12 hours
Plasma half-life (IM injection)	Unknown

Table 2. *Information sheet for lestaurtinib (CEP-701; "Lestaurtinib: Certificate," 2016; Kase et al., 1986; Marshall et al., 2005).*

1.4.2 Current Clinical Potential of Lestaurtinib

Though the drug has undergone several clinical trials for the treatment of various cancers over the last two decades (Children's Oncology Group, 2017, 2018; Hexner et al., 2014; M.D. Anderson Cancer Center, 2012; Minturn et al., 2011; Santos et al., 2010; Shabbir & Stuart, 2010), it has shown the greatest promise as a treatment for Acute Myeloid Leukemia (AML). AML is a cancer characterized by increased white blood cells in the bone marrow and an arrest in their maturation resulting in the disruption of the development of normal blood cells (Lowenberg et al., 1999). Currently, AML is a rare cancer; in 2015, only 0.0001% of the world's population (1 million people) were living with the disease. However, from 2005 to 2015, its incidence rose by 34%, and is expected to continue rising as the population ages. Furthermore, most patients with the disease are diagnosed as terminal; very few remit (Jemal et al., 2002).

The gene Fms-like tyrosine kinase 3 (FLT3) codes for a receptor tyrosine kinase and is expressed on bone marrow hematopoietic stem cells. Normally, as these stem cells differentiate, *FLT3* expression is lost. Conversely, almost all patients with AML have *FLT3* expression on their leukemia cells, and in an approximately one-third of these patients FLT3 is mutated resulting in greater proliferation of leukemia blast cells. As such, *FLT3* has been isolated as a potential target for AML drug therapies; inhibition of its deviant receptor tyrosine kinase activity could potentially arrest the overproduction of myeloid cells in the bone marrow (Pemmaraju et al., 2011). lestaurtinib is able to target *FLT3* and treatment with lestaurtinib has been observed to reduce the number of leukemia cells in the bone marrow (Knapper et al., 2006) making it a promising agent in the treatment of acute myeloid leukemia.

Aside from it's success in AML treatment, lestaurtinib has also demonstrated moderate potential as a treatment for prostate cancer, neuroblastoma, and myeloproliferative disorders

(Hexner et al., 2014; Minturn et al., 2011; Santos et al., 2010; Shabbir & Stuart, 2010). Testosterone, the primary androgen in healthy males, is highly related to the development of prostate cancer. While it is critical for the normal formation of male genitalia and secondary sex characteristics, it is suspected of contributing to the development of prostatic tumors (So et al., 2003). Serine/threonine-protein kinase N1 (PRK1) is currently being studied for its role in prostate cancer, as it is involved in the regulation of androgen receptor signalling (Köhler et al., 2012; Sekiya-Kawasaki et al., 2003). Lestaurtinib has been shown to inhibit PRK1 activity both *in vitro* and *in vivo*, highlighting its potential as a treatment for prostate cancer (Köhler et al., 2012).

Neuroblastoma is a pediatric cancer of the sympathetic nervous system where about half of all patients have a survival rate of <40% (George et al., 2006). Trk's have been implicated in the disease. Generally, Trk receptors are critical in the development of the nervous system, playing a role in neuronal survival and differentiation. However, these kinases are expressed differentially in neuroblastoma, suggesting an association with tumorigenesis (Brodeur et al., 2009). At nanomolar concentrations, lestaurtinib has been shown to competitively inhibit ATP binding to the Trk kinase domain (Miknyoczki et al., 1999) suggesting a therapeutic potential against neuroblastoma. Clinical trials have further investigated its efficacy against the disease, showing moderate improvements (Minturn et al., 2011).

Finally, lestaurtinib is able to inhibit certain JAK/STAT signalling pathways, as demonstrated in experiments seeking novel treatments for myeloproliferative disorders (MPDs) – blood diseases characterized by an overproduction of mature red or white blood cells (Santos et al. 2010). Abnormal activation of Janus kinase 2 (JAK2) is implicated in the pathophysiology of MPDs (Levine et al., 2005). In a properly functioning JAK2/STAT5 signalling pathway, a cytokine binds to extracellular transmembrane receptors, activating JAK2. In turn, JAK2 adds a phosphate

group to a tyrosine residue on the receptor, to which STAT5 binds. JAK2 phosphorylates the bound STAT5, causing its dissociation from the receptor. Once the newly phosphorylated STAT5 dimerizes with other STAT proteins, it is ready for translocation into the nucleus where it induces transcription of specific genes (Kisseleva et al., 2002; Rawlings et al., 2004). Most patients with MPDs exhibit constitutive activation of STAT5, caused by a somatic activating mutation in JAK2 that encodes a valine to phenylalanine substitution at position 617 ($JAK2^{V617F}$; Baxter et al., 2005; James et al., 2005; Levine et al., 2005). *In vitro*, lestaurtinib inhibits phosphorylation of $JAK2^{V617F}$ and both STAT5 and STAT3 downstream. Down-regulating JAK/STAT signalling inhibits both STAT5 and the growth of the human erythroleukemia cell line HEL92.1.7 (Hexner et al., 2008). In human trials, lestaurtinib has shown limited efficacy as a treatment for MPDs. While the drug reduces the JAK2 allele burden (the ratio between $JAK2^{V617F}$ and wild type JAK2 in hematopoietic cells) in patients, this effect is only moderate (Hexner et al., 2014; Santos et al., 2010), suggesting a need for further assessment of the role of JAK2 inhibition in treatment of MPDs.

1.5 Clinical Significance of Present Study

The ability of lestaurtinib to produce desirable regenerative effects would be particularly significant given the drug's current role in treating cancer. Traditionally, chemotherapy is associated with skeletal muscle atrophy and dysfunction. Chemotherapeutic drugs generally treat cancers by targeting mitotically active cancer or tumor cells and initiating autologous cell death pathways. As this targeting is non-specific, these drugs can also target highly proliferative healthy cells, causing unwanted side effects in the form of tissue damage or dysfunction. Skeletal muscle contains such healthy cells that maintain proliferative properties throughout their lifespan; therefore, chemotherapeutic drugs often target these cells, causing muscle wasting, fatigue, reduced exercise capacity, and pain (Gilliam & St. Clair, 2011; Greene et al., 1994). These are

persistent, long-term effects that are present long after cessation of chemotherapy (Scheede-Bergdahl & Jagoe, 2013; van Brussel et al., 2006).

Chemotherapy-induced myopathies can compound the loss of body mass that commonly occurs with cancer cachexia, further negatively affecting prognosis for cancer patients. Thus, a drug that could simultaneously treat cancer and reverse cachexia- or chemotherapy-induced myopathy could be instrumental in ensuring greater survival and quality of life among certain cancer patients. As such, research should continue to focus on developing or repurposing candidate cancer drugs that cause little-to-no harm to other tissues, including skeletal muscle. Given the clinical potential for lestaurtinib as a cancer treatment and what is known from pilot data on its effects on myoblasts *in vitro*, the present study aims to determine if lestaurtinib could reduce the time required to regenerate damaged skeletal muscle, and/or contribute to greater skeletal muscle fibre size *in vivo.* This will be achieved by injuring mouse muscles with myotoxin and subsequently treating injured muscles with lestaurtinib to determine if the effects observed from the *in vitro* pilot study can be successfully replicated *in vivo* (Fig. 3).

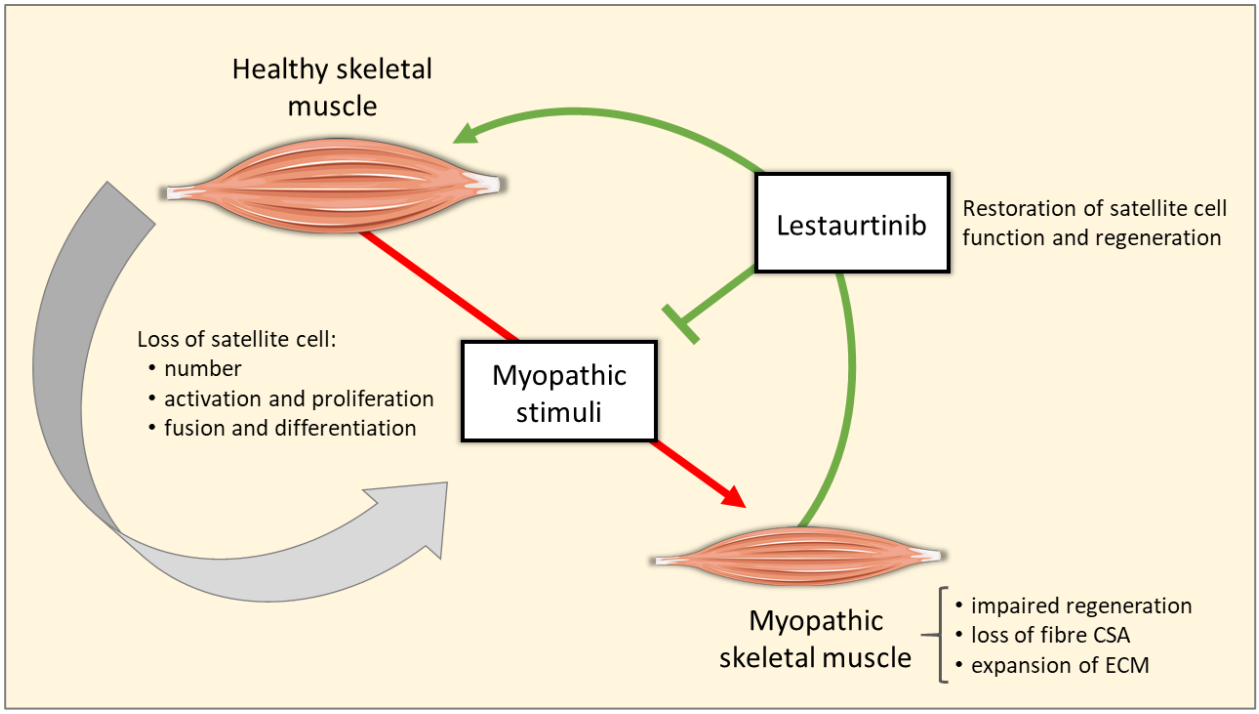


Figure 3. *Summary of potential lestaurtinib effects on satellite cells and skeletal muscle*

2. METHODOLOGY

2.1 Research Questions & Procedures

2.1.1. Effect of Lestaurtinib on Skeletal Muscle Fibre Size

Return to normal fibre size is an indicator of complete healing. In diseased muscle, fibres fail to return to pre-damaged size following repeated bouts of dysfunctional regeneration; thus, a faster return to normal fibre size or overall greater fibre size may be of benefit in certain pathophysiological cases. To determine if lestaurtinib increases skeletal muscle fibre size following damage and/or stimulates an accelerated return to normal fibre size, TA sections were stained for emb-MyHC at 4 days post-CTX injection and subjected to hematoxylin & eosin (H&E) stains at 14 days post-CTX injection. At 4 days post-injury, infiltration of many cell types (e.g.: fibroblasts and inflammatory cells) makes interpreting H&E stains difficult; hence, emb-MyHC stains were used to identify *de novo* regenerating fibres. These fibres will be evaluated for their cross-sectional area (CSA). At 14 days post-injury, regenerating skeletal muscle no longer expresses emb-MyHC but other infiltrating cell types have cleared; hence, H&E stains were used to identify regenerated muscle fibres and their mean fibre CSA. Fibre CSA was quantified for each section, and a mean CSA was calculated for each sample at 4 and 14 days post-CTX injection. If lestaurtinib promotes greater skeletal muscle fibre size following damage, it was hypothesized that mean fibre CSA in regenerating muscle would be greater in lestaurtinib-treated compared to vehicle-treated TAs.

2.1.2. Effect of Lestaurtinib on Collagen Clearing

While the intramuscular space is dominated by collagen, different isoforms exist in varying amounts, both in resting and regenerating skeletal muscle (reviewed in Kjær, 2004). Collagen type I and III dominate all layers of muscular connective tissue, while collagen type IV is mostly present

within the basement membrane. Other collagen types (II, VI, IX, XI-XVI, and XVIII-XIX) are also present within intramuscular connective tissue, but in very small amounts. Of all the collagen isoforms, collagen I makes up most of the intramuscular collagen content in both resting and regenerating muscle. Reports have observed at least 30% and upwards to 97% of intramuscular collagen content being of collagen type I (Light & Champion, 1984). Thus, to characterize the relative amount of collagen within the muscle, collagen I was the chosen isoform for this study.

Collagen I is highly expressed during skeletal muscle regeneration so as to repair the connective tissue component of muscle. In regenerating mouse muscle, the isoform is expressed at similar levels as human muscle, and on par with a similar time course following injury. Excess collagen deposition, as seen in some diseased muscles, leads to formation of fibrotic scar tissue. Typically, muscle fibrosis has been hypothesized to impede inflammatory and myogenic cell migration and function, leading to poor muscle regeneration (Chen & Li, 2009; Fukushima et al., 2001; Krause et al., 2013; Mann et al., 2011; Segawa et al., 2008). Thus, faster collagen clearing may lessen time to full muscle regeneration and restoration of contractile functionality.

To determine if lestaurtinib reduces time to clear collagen post skeletal muscle injury, sections were stained for collagen I content. Collagen-positive area was quantified for each section at 4, 7, and 14 days post-CTX injection. If lestaurtinib reduces time to clear collagen following skeletal muscle damage, it was hypothesized that the overall collagen-positive area observed in the fibre would be less than that of control at 7 days post-CTX injection.

2.1.3. Effect of Lestaurtinib on emb-MyHC Expression

Emb-MyHC is a marker of newly formed myotubes in regenerating skeletal muscle. Therefore, greater emb-MyHC expression may signify increased myotube formation in regenerating muscle. To determine if lestaurtinib promotes increased myotube formation in

regenerating skeletal muscle, sections were subjected to immunohistochemical staining to visualize and quantify emb-MyHC expression. Emb-MyHC-positive area was quantified at 4 and 7 days post-CTX injection. As well, the number of emb-MyHC-positive cells was quantified at 4 days post-CTX injection. If lestaurtinib promotes increased myotube formation in regenerating skeletal muscle, it was hypothesized that emb-MyHC expression would greater in lestaurtinib-treated compared to vehicle-treated TAs.

2.2 Experimental Design

2.2.1 Animal Care

Thirty C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and housed in the Animal Care Facility in the Biology Building at the University of Windsor. Mice were housed four to a cage and had access to water and mice chow. The animal room was maintained at 22°C, 50% humidity and a 12-h/12-h light-dark cycle. All experimental procedures outlined in this proposal were approved by the Animal Care Committee following submission of an Animal Utilization Project Proposal (AUPP #1606).

2.2.2 Injury, Treatment, and Tissue Collection

Mice were divided into three groups to investigate muscle regeneration at different benchmark time points (See Table 1 in Section 1.2): 4, 7, and 14 days post-CTX damage (n = 10 per group). The groups were then divided into subgroups to test for potential differences in regeneration given different treatment concentrations (n = 5 per subgroup): 40 nM and 200 nM, each within a 50 µL vehicle (0.1% dimethylsulfoxide [DMSO]). Skeletal muscle injury was induced in all 30 mice using CTX injections in both TA muscles (in the legs). Doses of lestaurtinib were delivered to affected area in one leg 3 days following CTX injection, while the untreated leg received a vehicle injection (0.1% DMSO) at that time and was used as a control.

To examine the tissues of interest, mice were humanely sacrificed using carbon dioxide inhalation followed by cervical dislocation. TA muscles were excised from both legs, coated in cryosectioning freezing compound, frozen in isopentane cooled by liquid nitrogen, then subsequently transferred to a -80 °C freezer where they were stored until cryosectioning.

2.2.3 Design Rationale

2.2.3.1 Animal Models. An ethical and replicable way to investigate mechanisms involved in skeletal muscle regeneration is by inducing skeletal muscle damage in animal models. Though a wide variety of animals can be used for studying skeletal muscle regeneration, mice and rats are arguably the most popular. This is due to ease of procurement and handling, relatively low cost, availability in several different strains, and overlap in physiological processes with humans. As such, mice were chosen for this present study.

Strain. The C57BL/6J mouse strain is appropriate for general purpose experiments. These mice tend to be healthy and are not susceptible to developing spontaneous conditions that might affect skeletal muscle regeneration. They are susceptible to both obesity and type 2 diabetes, but only when induced through diet (The Jackson Laboratory, 2020). As such, they were chosen for this study so that the effects of lestaurtinib on the regeneration process could be observed in a manually damaged, healthy skeletal muscle without interference from spontaneous pathophysiology that could have potentially confounded the experimental results.

Sex. Because there are no reported sex-based differences in satellite cell populations or activity in healthy human individuals (Kadi et al., 2004; Roth et al., 2000), sex was not a variable investigated in this study. However, varying estrogen levels have been reported to affect muscle regeneration processes (Feng et al., 2004; Tiidus, 2003; reviewed in Velders & Patrick, 2013), including inflammatory responses (Tiidus, 2005) and the activation and proliferation of satellite

cells (Enns & Tiidus, 2010; Larson et al., 2020; Thomas et al., 2010; Seko et al., 2020). Because estrogen levels fluctuate throughout the estrous cycle (Oertelt-Prigione, 2012), male mice were chosen for this study to control for the possibility that the female estrous cycle could transiently affect regeneration.

Age. Additionally, 12-week-old mice were chosen for this study, as this is the earliest age mice are considered developmentally mature (Flurkey et al. 2007). Unlike sex, age has been extensively reported to influence regeneration capacity. Following unimpaired post-natal development of skeletal muscle, age is negatively correlated with regenerative capacity; therefore, adult rather than elderly mice are most representative of the capacity for unimpaired skeletal muscle regeneration. Juvenile mice were not chosen as their muscles are still developing and this would have been an extra variable to consider for this study.

2.2.3.2 Dosage. Initial screening and cell culturing identified lestaurtinib as a compound capable of augmenting the differentiation capacity of activated satellite cells. When treated at concentrations between 40 nM and 1 µM, lestaurtinib appeared to reduce myoblast proliferation compared to untreated controls, while inducing differentiation of myoblasts (Appendix A, Fig. 8). 40 and 200 nM lestaurtinib appeared to have the greatest effect on myotube formation while 1 µM induced some cell death in addition to stimulating myotube formation. Thus, 40 and 200 nM lestaurtinib concentrations were chosen for this study to induce myoblast differentiation into myotubes *in vivo* while minimizing cell death.

2.2.3.3 Timepoints. Satellite cell proliferation and differentiation are most active between 1 to 4 days following injury. However, most of the damaged tissue is still necrotic 24 to 48 hours following injury; on the other hand, by 4 days post-injury, satellite cell fusion and differentiation is largely completed. Therefore, a lestaurtinib treatment time point of 3 days post-CTX was chosen

as this would ensure the highest likelihood of the drug interacting with satellite cells during both proliferation and differentiation.

Excision time points of 4, 7, and 14 days post-CTX were chosen due to their significance in the time course of skeletal muscle regeneration in relation to the variables being measured in this study. For instance, in mouse TAs, collagen-positive area (%) steadily decreases between 5 to 21 days post-injury (Lehto, Duance, & Restall, 1985; Lehto, Sims, & Bailey, 1985; Myllylä et al., 1986). Collagen is secreted in damaged muscle to help repair connective tissue; thus, faster collagen clearing may decrease time to full muscle functionality, promoting faster healing. As such, to determine if collagen is cleared faster with lestaurtinib treatment, these excision time points were chosen to ensure a range of overall collagen area was observed in the muscle, beginning slightly before clearance but not ending before completion.

These time points are also significant in respect to fibre CSA. At 4 days post-injury, regenerating fibres are atrophied and still quite small. By 14 days post-injury, nuclei are still centrally located, but the overall architecture of the myofibre has been re-established (Rosenblatt & Woods, 1992; Krause et al., 2013). On the other hand, at 3-4 weeks post-injury, nuclei begin to migrate back the periphery, making it difficult to confidently identify all regenerated fibres (Paoni et al., 2002). Thus, excision time points of 4 and 14 days post-CTX were chosen to observe fibre-CSA to show the trend of increasing fibre-CSA over time while still being able to easily isolate regenerated fibres due to still-present centrally located nuclei.

Finally, excision time points of 4 and 7 days post-CTX were chosen to examine emb-MyHC-positive area (%) in the muscle. Emb-MyHC is a marker of newly formed myotubes in regenerating skeletal muscle; as such, greater emb-MyHC expression may signify increased myotube formation in regenerating muscles. Expression of emb-MyHC peaks at 4-5 days post-

injury and steadily decreases toward 10 days post-injury (Krause et al., 2013; Rahman et al. 2020). Thus, to measure the difference in emb-MyHC expression between lestaurtinib- and vehicle-treated muscles, excision time points of 4 and 7 days post-CTX were chosen. Though possibly still present at 14 days post-injury, expression of emb-MyHC has decreased significantly in muscle fibres by this time (Schiaffino et al., 2015); as such, this time point was omitted from this analysis

2.3 Data Analysis

2.3.1 Histochemical Analysis

Samples were cross-sectioned with a Leica CM1860 UV cryostat (Leica Microsystems Inc., Concord, ON, Canada) at 10 µm thickness, then fixed to a glass microscope slide. Sections were then analyzed using histological techniques specific to each research question posed above (outlined below).

2.3.1.1 Hematoxylin and eosin. H&E staining was used to identify the basic morphology of the muscle following CTX-induced muscle damage (protocol specifics can be found in Appendix C). This stain was used to identify regions of regenerating muscle that were used to quantity mean fibre CSA at 14 days following CTX damage.

2.3.1.2 Collagen immunofluorescence staining. Collagen staining was performed to determine the area percentage and localization of collagen I present in the muscle fibre (see Appendix D for antibody information). Muscle sections were fixed using 2% paraformaldehyde (PFA) for 5 minutes at 4°C and blocked with 5% normal goat serum (NGS), and 0.1% Triton-X100 in neutral PBS for 40 minutes at room temperature. Primary antibody was applied at 4°C for 16 hours in a humidified chamber. Primary antibody was removed and secondary antibody applied along with 4,6-diamidino-2-phenylindole (DAPI) to identify nuclei. Sections were stored in a dark chamber at room temperature for 1 hour. Following the removal of secondary antibody, a cover

slip was applied using an aqueous mounting medium. Specifics of the protocol can be found in Appendix E.

2.3.1.3 Staining for emb-MyHC expression. Immunostaining was performed to determine the expression pattern and localization of embryonic myosin heavy chain (see Appendix D for antibody information). Mouse IgG block (BMK2202, Vector Laboratories Inc., Burlingame, CA, USA) was applied according to manufacturer's instructions for 1 hour at room temperature. This was followed by a blocking solution of 10% NGS, 1.5% bovine serum albumin (BSA), and 0.2% Triton-X100 in neutral PBS for 1 hour at room temperature. Primary antibody was applied and stored at 4°C overnight in a humidified chamber. Primary antibody was removed and secondary antibody was applied along with 4,6-diamidino-2-phenylindole (DAPI) to identify nuclei. Sections were stored in a dark chamber at room temperature for 1 hour. Following the removal of secondary antibody, a cover slip was applied using an aqueous mounting medium. Specifics of the protocol can be found in Appendix F.

2.3.2 Image Analysis

2.3.2.1 Image Acquisition. Images of stained sections were obtained using a Nikon 90i eclipse microscope equipped with DS-Fi1c colour and DS-Qi1Mc monochromatic cameras (Mississauga, ON, Canada) via Nikon Elements software (version 3.22) and analyzed using ImageJ base software (version 1.52p) with the FIJI plugin suite. Three to five images were captured within the regenerating regions of the muscle at 20x magnification. For the analysis of collagen content, images were auto exposed, and brightness levels adjusted through lookup tables (LUTs) just before or after peak brightness. For the analysis of emb-MyHC content, exposure time remained consistent between slides during the imaging process to allow for comparison of differing brightness values. In all staining procedures, a negative control section (no primary

antibody applied) was used to set the base signal threshold. In all cases, the negative control sections displayed no signal.

2.3.2.1 Fibre CSA. To determine fibre CSA, regenerating fibres were circled in ImageJ using the freehand selection tool. Regenerating fibres were determined as any fibre containing a centrally located nuclei. After each fibre was circled, CSA was measured using the "Analyze > Measure" command. For each image, 50 to 150 fibres were circled; their CSAs were then totaled and averaged to determine a mean CSA for each sample. The scale was set to 3.125 pixels/μm, and the unit of measurement used for CSA was μm^2.

2.3.2.2 Collagen Content. To determine collagen-positive area, signal thresholding was used as a detection method for collagen I using the "Image > Adjust > Color Threshold" command. The brightness level was set at a constant of 38 for each image. Total collagen-positive area was measured with the "Analyze > Analyze Particles" command. No limit was set for circularity or size of particles. Area percentage was recorded for each image, then totaled and averaged for each sample to provide mean collagen area percentage for each sample. Any area within the image that was determined to not be a part of the regenerating section (e.g. area beyond fibre edge) was cropped out of the image and not included in the total area measurement.

2.3.2.3 Emb-MyHC Content. To determine emb-MyHC-positive area, signal thresholding was used as a detection method for emb-MyHC using the "Image > Adjust > Color Threshold" command. The brightness level was set at a constant of 40 for each image. Total emb-MyHC-positive area was measured with the "Analyze > Analyze Particles" command. No limit was set for circularity or size of particles. Area percentage was recorded for each image, then totaled and averaged for each sample to provide mean emb-MyHC area percentage for each sample. Any area within the image that was determined to not be a part of the regenerating section

(e.g. area beyond fibre edge) was cropped out of the image and not included in the total area measurement.

To determine number of emb-MyHC-positive cells, cells were manually counted within regenerating regions using the multi-point tool. The total area of the image was measured using the "Analyze > Measure" command, with the scale set to 3.125 pixels/µm. The total area in μm^2 was converted to mm^2, then number of positive objects was divided by total area (in mm^2) to determine number of positive objects per mm^2. Total number of positive objects per mm^2 was averaged for each sample. Any area within the image that was determined to not be a part of the regenerating section (e.g. area beyond fibre edge) was cropped out of the image and not included in the total area measurement.

2.3.3 Statistical Analysis

A two-way ANOVA (2x3) was conducted to compare outcome measures between lestaurtinib- and vehicle-treated muscle at three different timepoints following induction of skeletal muscle damage. Three treatment conditions were tested: 1) treatment with 40 nM of lestaurtinib, 2) treatment with 200 nM of lestaurtinib, and 3) treatment with vehicle (50 µL of 0.1% DMSO). Lestaurtinib was dissolved in 50 µL of 0.1% DMSO, and each treatment was administered 3 days post-CTX injection. Dependent variables measured included mean fibre cross-sectional area, area percentage of collagen I, and emb-MyHC-positive area (%). For number of emb-MyHC-positive cells, a one-way ANOVA was conducted to compare outcome measures between lestaurtinib- and vehicle-treated muscle at the 4-day recovery time point only. All data passed tests for normality when separated by time point, and Levene's test confirmed homogeneity of variances within each time point. Where the two-way ANOVA revealed any significant interactions between independent variables, the dependent variable was subject to a post-hoc

simple main effects analysis (Bibby, 2010; Rahman et al., 2020), comparing lestaurtinib- and vehicle-treated groups. A Bonferroni adjustment was performed to reveal the time points during the regeneration process (4, 7, or 14 days post-CTX) at which the independent variable affected the dependent variables. All test results were considered significant at an alpha level of .05. All statistical analyses were conducted with the software package SPSS (version 25).

3. RESULTS

3.1 Assessment of Myofibre Cross-Sectional Area

To investigate the acute regenerative capacity of skeletal muscle after treatment with lestaurtinib, damage was induced in both left and right tibialis anterior (TA) muscles via cardiotoxin (CTX) injection and analyzed samples after 4 and 14 days (Fig. 4A; Fig. 4B). The left TA muscle received a vehicle treatment and was used as a control, while the right TA muscle received a lestaurtinib treatment (dissolved in 50 µL of 0.1% DMSO) at a concentration of either 40 nM or 200 nM. Mean TA myofibre cross-sectional area did not differ significantly between vehicle-treated and lestaurtinib-treated mice following damage (main effect of treatment: $p = .503$; Fig. 4C) at any time point, or at either concentration.

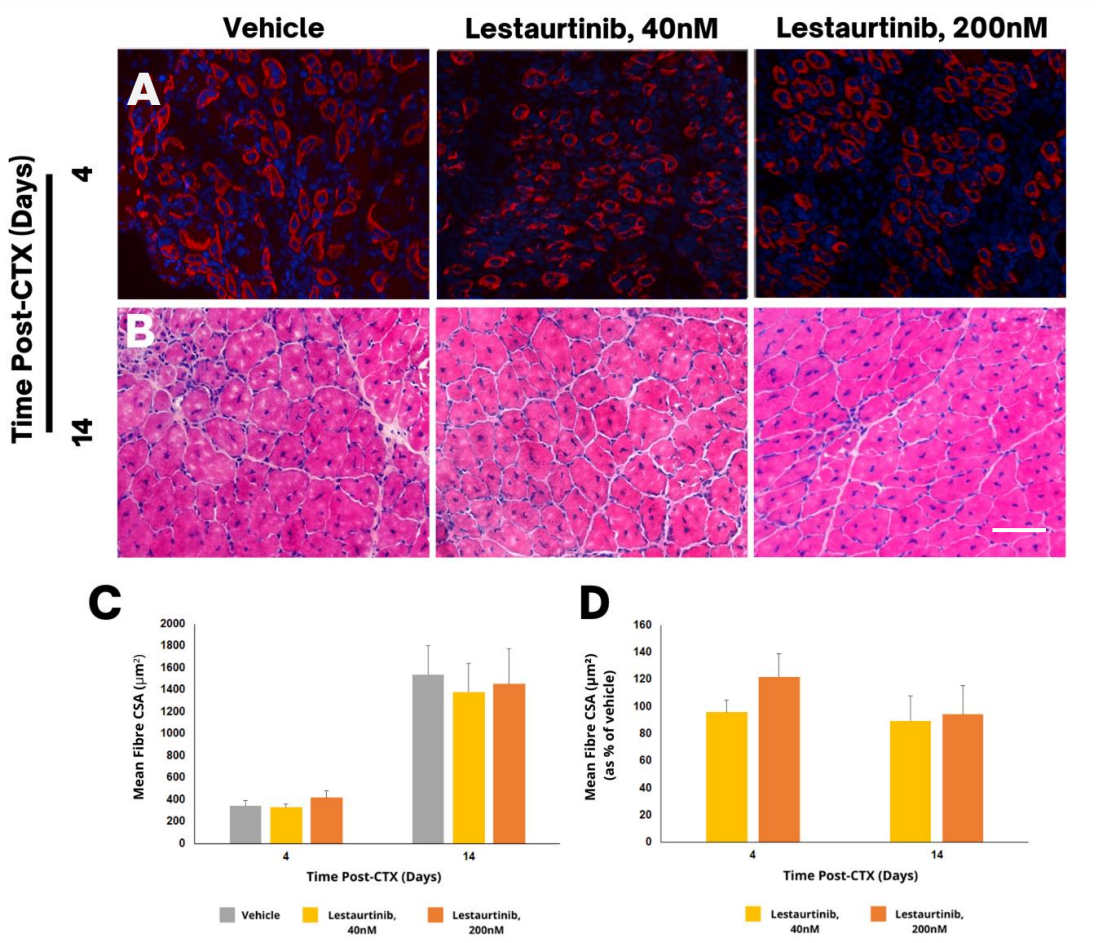


Figure 4. *Myofibre CSA of lestaurtinib-treated muscle is unaffected. (**A**) Regenerating myofibres were identified via embryonic myosin heavy chain (emb-MyHC; red) at 4 days post-CTX (**B**) and via hematoxylin and eosin (H&E) cryosections of the TA muscle at 14 days post-CTX. (**C**) There was no significant difference in myofibre CSA between vehicle-treated and lestaurtinib-treated muscle at either time point or treatment concentration as indicated by absolute values and (**D**) a representation of lestaurtinib-treated mean fibre CSA as a percentage of vehicle-treated mean fibre CSA values. n = 5 per group. Data presented are means ± standard deviation. Scale bar represents 50 µm.*

3.2 Assessment of Collagen-Positive Area

Collagen secreted in damaged muscle helps repair connective tissue but excess collagen deposition, as seen in some diseased muscles, leads to formation of fibrotic scar tissue. Excessive muscle fibrosis is characterized by attenuated regeneration (Chen & Li, 2009; Fukushima et al., 2001; Krause et al., 2013; Mann et al., 2011; Segawa et al., 2008); thus, faster collagen clearing may decrease time to full muscle functionality and promote faster healing. As such, an objective of this study was to determine whether lestaurtinib could improve collagen clearance by measuring the summed area of collagen I-positive structures following muscle damage. To assess the effect of lestaurtinib treatment on collagen I levels following muscle damage, the proportion of area exhibiting positive staining for the collagen I isoform was quantified over time (Fig. 5A).

Following CTX-induced muscle damage, collagen I content within lestaurtinib-treated muscle was significantly reduced when compared to vehicle-treated muscle (main effect of treatment: $p = .009$; Fig.5B). A significant interaction between treatment and recovery time point following damage was observed (interaction effect: $p = .033$). A Bonferroni adjustment was performed to compare effects between lestaurtinib-treatment concentrations (40nM versus 200nM). Results demonstrated that accumulation of collagen I was significantly reduced at the 200nM level at both 7 (post-hoc: $p = .047$) and 14 days post-CTX (post-hoc: $p = .004$; Fig. 5B), though neither treatment level differed significantly from the vehicle treatment at any time point.

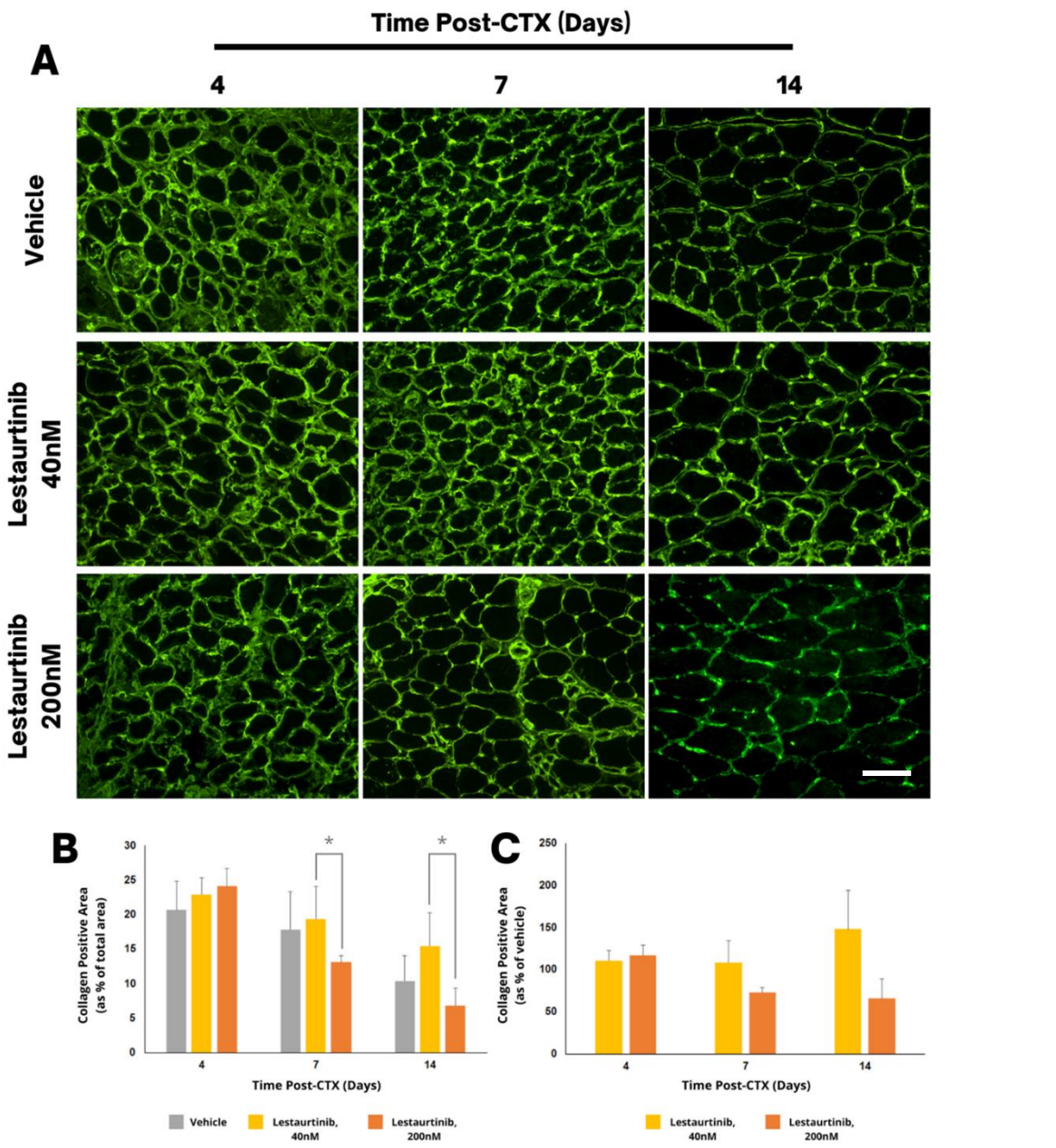


Figure 5. *Collagen I expression is reduced in lestaurtinib-treated muscle. (**A**) Immunostaining of collagen I (green) at each time point following damage. (**B**) Collagen I-positive area in the lestaurtinib-treated leg was observed to be less when compared to the vehicle-treated leg (main effect of treatment: p = .009). A significant interaction between treatment and recovery time point following damage was observed (interaction effect: p = .033). (**C**) When comparing effects between lestaurtinib-treatment concentrations only (40nM versus 200nM), simple main effect post-hoc analysis demonstrated significantly reduced accumulation of collagen I at a concentration of 200nM at both 7 (post-hoc: p = .047) and 14 days post-CTX (post-hoc: p = .004). * indicates significant differences detected by a simple main effects post-hoc analysis. n = 5 per group. Data presented are means ± standard deviation. Scale bar represents 50 μm.*

3.3 Assessment of emb-MyHC Expression

To assess the effect of lestaurtinib treatment on emb-MyHC expression following muscle damage, the proportion of area exhibiting positive staining for emb-MyHC was quantified at 4 and 7 days post-CTX (Fig. 6A). There was no difference in emb-MyHC expression between the vehicle-treated TA muscles and lestaurtinib-treated TA muscles at either time point or treatment concentration following damage (main effect of treatment: $p = .2$; Fig. 6B).

Number of emb-MyHC-positive objects was also investigated at 4 days post-CTX. The number of emb-MyHC-positive objects per mm^2 was determined (Fig. 7A). No significant difference was found in number of objects/mm^2 between vehicle- and lestaurtinib-treated muscles at either treatment concentration (main effect of treatment: $p = .871$; Fig. 7B).

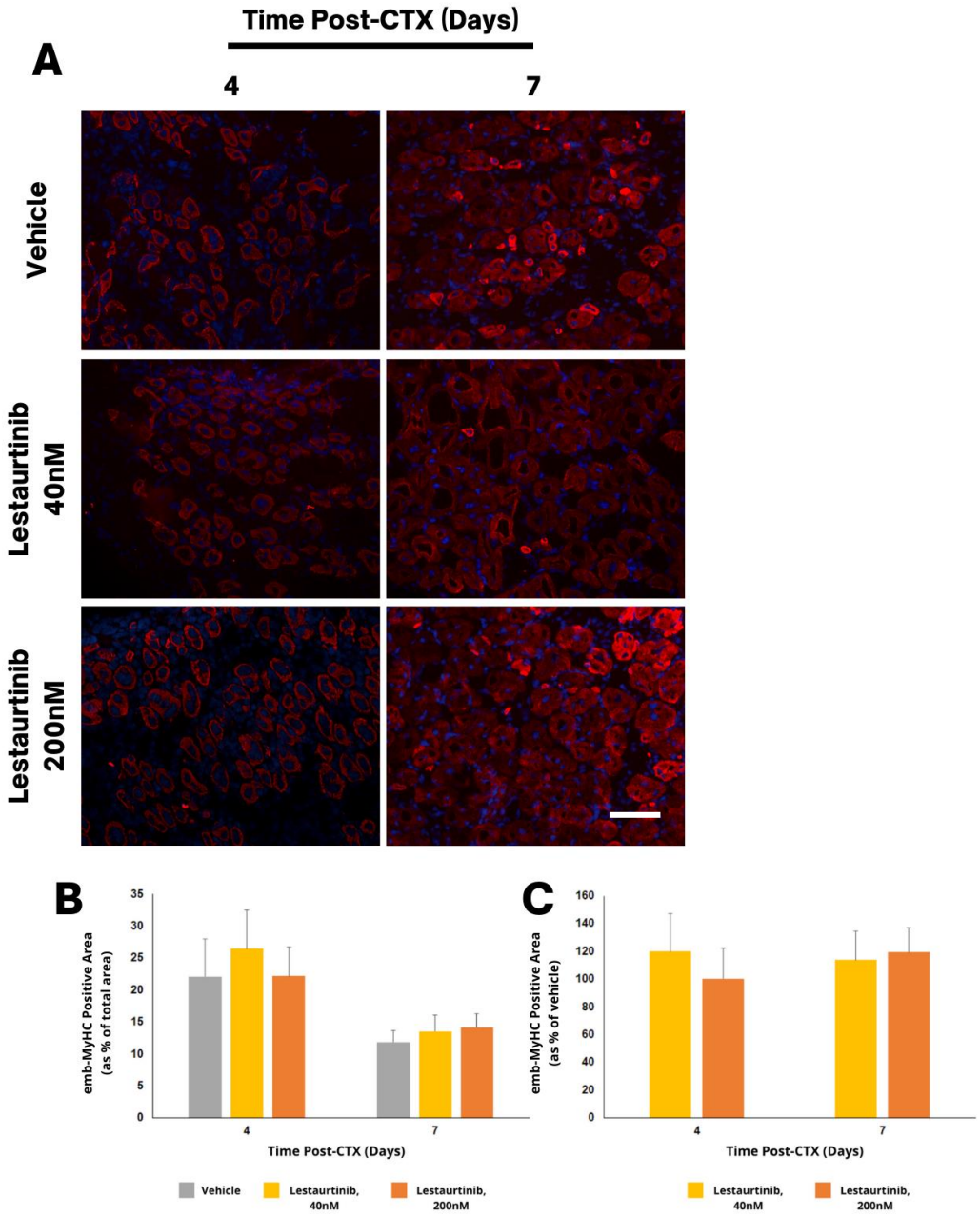


Figure 6. *No difference in emb-MyHC expression in lestaurtinib-treated muscles. (**A**) Immunostaining of emb-MyHC (red) and DAPI (blue) used to identify emb-MyHC in regenerating regions. (**B**) There was no significant difference in emb-MyHC positive area in the regenerating regions of vehicle- and lestaurtinib-treated muscle at either time point or either concentration as indicated by absolute values and (**C**) a representation of lestaurtinib-treated emb-MyHC positive area as a percentage of vehicle-treated emb-MyHC positive area values. n = 5 per group. Data presented are means ± standard deviation. Scale bars represent 50 μm.*

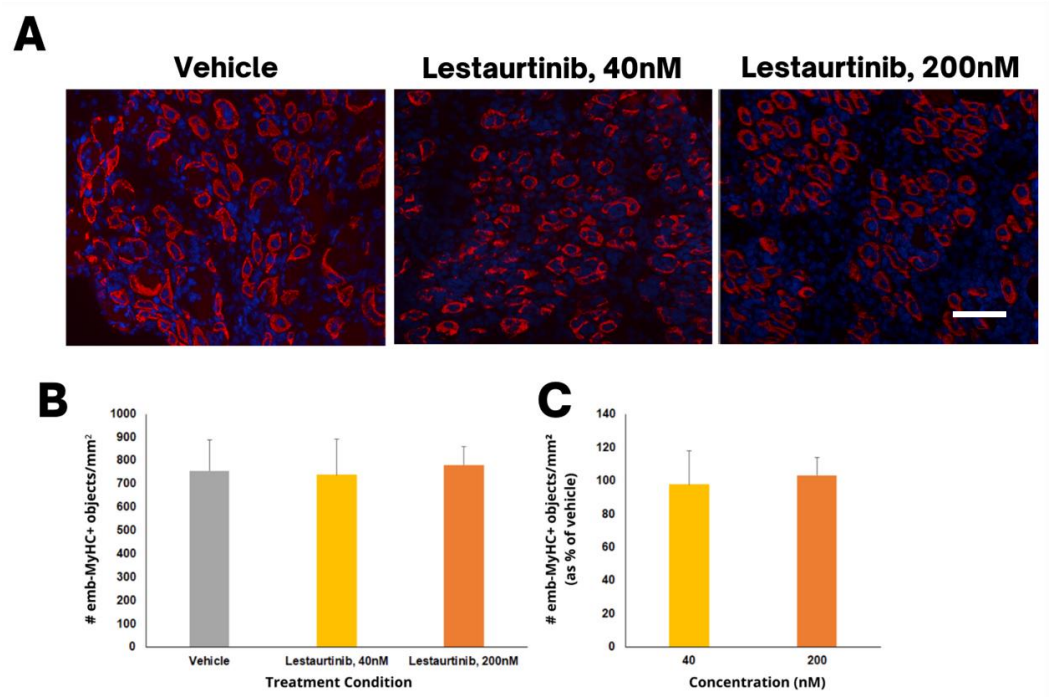


Figure 7. *No difference in number of emb-MyHC-positive objects/mm^2 in lestaurtinib-treated muscles at 4 days post-CTX damage. (**A**) Immunostaining of emb-MyHC (red) and DAPI (blue) used to identify emb-MyHC-positive objects in regenerating regions. (**B**) There was no significant difference in emb-MyHC-positive objects/mm^2 in the regenerating regions of vehicle- and lestaurtinib-treated muscle at 4 days post-CTX damage as indicated by absolute values and (**C**) a representation of lestaurtinib-treated emb-MyHC-positive objects/mm^2 as a percentage of vehicle-treated emb-MyHC-positive objects/mm^2 values.. n = 5 per group. Data presented are means ± standard deviation. Scale bars represent 50 μm.*

4. DISCUSSION

Aging and other myopathic disease states can negatively impact the regenerative capacity of skeletal muscle, ultimately leading to functional impairments. In the case of cancer cachexia, loss of muscle mass contributes to loss of overall body weight, which is negatively associated with length of survival time for many individuals with cancer (Fearon et al., 2006). As such, there is considerable interest in developing novel treatments, such as drug therapies, that may ameliorate symptoms associated with pathophysiological states such as aging, cancer cachexia, obesity, or myopathy, possibly by encouraging satellite cell renewal, activation, proliferation, and/or differentiation. Drug repurposing is one way to expedite development of such novel treatments. However, to date, there are only a limited number of studies investigating the effects of novel or repurposed drug treatments on the acute responses to damage in muscle tissue (Deasy et al., 2002; Gehrig & Lynch, 2011; Hu et al., 2002; Miyatake et al., 2016; Rotter et al., 2008; Sakakima et al., 2006; Wada et al., 2017). In 2015, a compound screen on proliferating myoblasts found that the drug lestaurtinib appeared to induce differentiation as noted by the formation of small myotubes. Follow-up *in vitro* experiments confirmed that under particular conditions, lestaurtinib application resulted in myotubes that were larger, more branched, and more nuclei per myotube (Appendix B; Krause et al., 2015). Based on the results of this pilot study, the current project was designed to assess the affects of lestaurtinib *in vivo*. Single dose lestaurtinib-treated mouse muscle was assessed at 4, 7, and 14 days following CTX-induced muscle damage as these time points encompass several major regenerative events including collagen synthesis, and proliferation, fusion, and differentiation of myoblasts into new myofibres. The findings presented here demonstrate that several aspects of the regeneration process appear to be unaffected by lestaurtinib treatment *in vivo* in the first two weeks following skeletal muscle damage.

4.1 Skeletal Muscle Fibre Size Following Lestaurtinib Treatment

In several myopathies, muscle fibres tend to atrophy following repeated bouts of dysfunctional regeneration; thus, a faster return to normal fibre size or overall greater fibre size following muscle damage may be of benefit. Lestaurtinib treatment was hypothesized to increase skeletal muscle fibre size following damage and/or stimulate an accelerated return to normal fibre size. H&E stains and emb-MyHC stains were used to assess muscle fibre CSA at 4 and 14 days post-CTX damage (Fig. 4A, B). No difference between vehicle- and lestaurtinib-treated muscle CSA was observed at either time point of the regeneration process (Fig. 4C), suggesting that treatment with lestaurtinib under these experimental conditions does not appear to increase skeletal muscle fibre size following damage, nor does it appear to promote a faster return to normal muscle fibre size following damage.

Preliminary cell culture experiments revealed that lestaurtinib is a myotube-promoting compound when applied to myoblasts on differentiation media. However, muscle regeneration is a complex system, and the effects of lestaurtinib on other factors that affect the process *in vivo* remain unknown. This could be one possible reason why lestaurtinib did not appear to increase skeletal muscle fibre size following damage. Muscle damage causes the release of various growth factors into the extracellular space that may be endogenous to the muscle itself or may be secreted by other cell types at the damage site, such as neutrophils and macrophages. Factors such as HGF, FGF, myostatin, TGFβ, and IGF are critically important during muscle repair, and especially in the recruitment of satellite cells (Karalaki et al., 2009). For example, IGF-I can induce muscle hypertrophy by increasing myotube size and protein synthesis (Bark et al., 1998). During muscle regeneration, IGF-I increases the proliferation potential of satellite cells by enhancing the expression of specific intracellular mediators (Chakravarthy et al., 2000; Florini et al., 1996). It

also stimulates differentiation by inducing *myogenin* expression, which allows myotube formation and maturation later in myogenesis (Aguiar et al., 2013; Florini et al., 1991). However, IGF-I would not have been part of the cell culture condition in the preliminary experiments; thus, if lestaurtinib had any inhibitory effects on it, the results would not have been reflected *in vitro*.

Similarly, Mrf4 is also involved in the maturation of myotubes by allowing reorganization of myofilaments and central nuclei migration to the periphery of the cell. MyoD in the activated satellite cell contributes to the activation of *MRF4* expression found in the later stages of regeneration (Megeney et al., 1996). Several factors coordinate to regulate the expression of both (Zanou & Gailly, 2013). Thus, if lestaurtinib had any unseen effects on factors outside of the satellite cell that impact their recruitment, regulate the expression of MRFs, or otherwise contribute to the maturation of myofibres following damage, fibre growth could have been unaffected or possibly even stunted. Indeed, this seems to be the case with the 40 nM lestaurtinib treatment at 14 days post-CTX damage (Fig. 4C).

4.2 Alterations to Collagen I Content Following Lestaurtinib Treatment

Collagen synthesis is important following muscle damage as it is the primary structural component of the ECM, thus allowing for the restoration of contractile force transmission as well as the organization of newly formed muscle fibres (Aumailley & Gayraud, 1998). Lestaurtinib treatment was hypothesized to lead to faster reduction in collagen I following muscle damage. Collagen I was investigated as this isoform makes up most of the intramuscular collagen content. It was found that lestaurtinib-treated muscle displayed less collagen I in regenerating regions of damaged TA (Fig. 5B). A simple main effects post-hoc analysis was performed to compare effects between lestaurtinib-treatment concentrations (40nM versus 200nM) at each recovery time point. Results demonstrated that accumulation of collagen I was significantly reduced at the 200nM level

at both 7 and 14 days post-CTX (Fig. 5B), though neither treatment level differed significantly from the vehicle treatment at any time point. These divergent effects on collagen I content suggest that each drug level exhibited opposing effects on collagen synthesis and/or degradation at 7 and 14 days post-CTX. Together, these data suggest that single dose treatment of 40 or 200 nM of lestaurtinib at 3 days post-injury does not augment muscle repair, although the 200 nM dose may reduce collagen accumulation. The opposing effect of each drug treatment level could be potentially explained by looking more closely at the processes that affect collagen synthesis and degradation in a regenerating muscle fibre.

Muscle regeneration requires the activation, migration, and proliferation of fibroblasts (Murphy et al., 2011), which synthesize a wide variety of growth factors and ECM components, including collagen I, to increase intramuscular connective tissue (Goldberg & Green, 1964). In addition to direct synthesis of ECM components, muscle repair also requires degradation of the ECM by muscle-associated proteolytic molecules called matrix metalloproteases (MMPs; Chen & Li, 2009). Collagen degradation is initiated extracellularly by MMPs (Nagase & Woessner, 1999), and an increase in MMP expression accelerates collagen breakdown. In addition, tissue inhibitors of metalloproteases (TIMPs) inhibit MMP activity and are often activated together with MMPs, indicating simultaneous stimulation and inhibition of degradation. As such, TIMPs are assumed to act as regulators of degradation termination to ensure a limited amount of degradation (Chen & Li, 2009).

While there are several other factors that contribute to ECM remodelling, it is possible that in the case of the divergent effects of 40 nM and 200 nM, the differing levels of the drug may have had opposing effects on either fibroblast, MMP, or TIMP activity. For example, 40 nM lestaurtinib may have been enough to increase activation, migration, or proliferation of fibroblasts, thereby

contributing to an increase in collagen I synthesis following damage, while, in contrast, a dosage at the 200 nM level could have had an opposing effect, possibly contributing to increased fibroblast cell death, and therefore less collagen I synthesis. Similarly, it is possible that the 40 nM dose increased the activity of TIMPs, thereby contributing to decreased degradation of collagen I at 7 and 14 days post-CTX damage. It is also possible that the 200 nM level increased the activity of MMPs, thereby contributing to increased degradation of collagen I at 7 and 14 days post-CTX damage.

4.3 Alterations to emb-MyHC Expression Following Lestaurtinib Treatment

As a marker of newly formed myotubes, greater emb-MyHC expression signifies increased myotube formation in regenerating muscles. Lestaurtinib treatment was hypothesized to promote increased myotube formation in regenerating skeletal muscle. Sections were stained for emb-MyHC at 4 and 7 days post-CTX damage (Fig. 6A). Results from the present study showed that no difference between vehicle- and lestaurtinib-treated muscle emb-MyHC expression was observed by day 7 of the regeneration process (Fig. 6B), indicating that treatment with lestaurtinib does not promote increased myotube formation in regenerating skeletal muscle under these experimental conditions.

There are several reasons why increased expression of emb-MyHC might not have been observed. First, it is possible that treatment with lestaurtinib did increase myotube formation, but simultaneously exhibited opposing effects elsewhere, resulting in no observed difference by 4 or 7 days post-CTX damage. Similarly, if myoblast fusion was accelerated, it is possible that examining the muscle tissue at 4 days post-CTX damage may have been too late to determine a difference in fusion. In either of these cases, expression of emb-MyHC might not necessarily reflect an increase in myoblast fusion or an accelerated fusion time. To determine if this is the

case, future studies could quantify the number of Pax7-positive cells following treatment with lestaurtinib. A reduced number of Pax7-positive cells could indicate accelerated differentiation of myoblasts, despite there appearing to be no difference in the expression of emb-MyHC by 4 days post-damage.

4.4 Translation of *in vitro* results into *in vivo* application

One explanation for a general lack of significant results in the present study could be poor translation between preliminary cell culture experiments and the live model. It is possible that lestaurtinib affects many other proteins and processes within muscles beyond what was discovered in the initial compound screen and what is known from other previous studies. Several different extracellular matrix proteins, growth factors, cytokines and their downstream signalling pathways regulate the skeletal muscle regeneration process. While lestaurtinib could have positively affected some factors that contribute to muscle hypertrophy, collagen degradation, or emb-MyHC expression, it could have simultaneously affected other factors that impacted these processes as well; thus, a kind of "balancing" effect could have occurred, with the overall effect being no significant observable changes in any of the dependent variables measured. Furthermore, in the cell culture experiments, the drug was applied to isolated myoblasts in media designed to enhance differentiation. Thus, the impetus for differentiation was already present and myoblast location could be targeted exactly. On the other hand, achieving the same results in a live model carries several more unpredictable external factors to contend with.

5. LIMITATIONS

5.1 Controls

Using the contralateral leg in the same animal as a control might have been ineffective or produced unwanted results. Although the dosage size was very small (50 µL) compared to the overall blood volume of an average sized mouse (~1.5 – 2.5 mL; John Hopkins University, n.d.), without baseline values of each dependent variable prior to treatment, it is difficult to determine whether an IM injection of lestaurtinib in one leg could have possibly affected regeneration in the contralateral "control" leg.

5.2 Route of Administration & Dosing

Information on the bioavailability of lestaurtinib within mice and humans via IM injection is lacking. Thus, it is difficult to determine whether IM injection was the appropriate route of administration to use for this present study. While the data from the pilot studies suggested dosage concentrations of between 40 nM and 200 nM, this was in a controlled *in vitro* environment and these doses were applied directly to myoblasts already in differentiation media. As such, it is possible that doses delivered into the regenerating muscle via IM injection could have diffused out of the muscle more rapidly than expected or was not injected completely into the damaged area. Perhaps a higher dosage is needed to achieve effects similar to those observed in the pilot *in vitro* studies.

Another solution for this in future studies might be successive doses throughout the day to ensure that a consistent level of drug is always circulating through the muscle. This would decrease the likelihood that the drug has diffused out of the muscle before it was able to affect the regenerative process. Additionally, with consistent successive doses, other variables could be measured over time that would not be possible with a single dose; for example, lestaurtinib's

effect on muscle regeneration following repeated bouts of exercise with the drug already circulating within the muscle. Ensuring a consistent level of drug within the muscle could possibly be achieved with an oral dose, administered within food or water. However, one would need to be cautious about potential systemic effects in this case. When taken orally, side effects of lestaurtinib include nausea, diarrhea, constipation, fatigue, dizziness, and headache (Knapper et al., 2006). Though generally reported in less than 20% of cases, these effects would still need to be monitored and would add another level of complexity to any future studies.

5.3 Differences in the Regulation of Human and Mouse Myogenesis

Human satellite-like cells (huSMPs) have been used in several studies to investigate the differences that exist between human and mouse myogenic programs (Bareja et al. 2014). First, downregulation of *Pax7* expression takes much longer in huSMPs than mouse satellite cells. Expression is still detectable in up to 50% of huSMPs after nearly one week in culture; conversely, mouse satellite cells hardly express any *Pax7* by this time (Olguin & Olwin, 2004; Zammit et al., 2004). Similarly, it takes three days in culture for MyoD to be widely detected in huSMPs, while the vast majority of mouse satellite cells have already expressed *MyoD* by this time (Yablonka-Reuveni, & Rivera, 1994; Zammit et al., 2004). Second, during activation and differentiation, mouse satellite cells and huSMPs differ in the temporal expression of several genes. For example, expression of *myogenin* is increased in mouse satellite cells as early as 2 – 3 days post-activation, suggesting an accelerated ability to differentiate. While huSMPs show an earlier increased expression of *MyoD* RNA over a 5-day culture, they do not show increased expression of *myogenin* RNA, and thus differentiate more slowly than mouse satellite cells. Third, huSMPs and mouse satellite cells do not exhibit the same regulatory relationships between MRFs. Recall that Pax7 regulates *Myf5* expression in mouse satellite cells (Kawabe et al., 2012; McKinnell et al., 2008).

As expected, *Pax7* knockdown in mouse satellite cells reduces expression of *Myf5*. In contrast, *Pax7* knockdown in huSMPs does not significantly alter *Myf5* expression. Similarly, knockdown of *MyoD* in human satellite cells has no effect on *Myf5* expression, whereas Myf5 protein is increased in myoblasts of *MyoD*-null mice (Sabourin et al, 1999). These findings highlight the existence of fundamental differences in the regulation of human and mouse adult myogenesis through the divergence of their satellite cells. When developing drugs that affect satellite cells and regeneration, such differences should be taken into consideration.

6. FUTURE DIRECTIONS

Future studies should aim to determine whether lestaurtinib has an effect on accelerating differentiation or myotube formation that might not necessarily reflect through emb-MyHC expression. To do so, future studies could attempt to quantify the number of Pax7-positive cells still present within the muscle after lestaurtinib treatment between 2 and 3 days following damage. As previously mentioned, Pax7 is indicative of both quiescent and activated satellite cells, but the number of Pax7-positive cells decreases as differentiation begins and myotubes begin to form (Zammit et al., 2006). A decreased number at an earlier time point could suggest accelerated myotube formation, while an increase could suggest that the drug promotes satellite cell proliferation (Zammit et al., 2004). Similarly, future studies could quantify number of nuclei present per myofibre after lestaurtinib treatment at 14 days following muscle damage. This result would reflect the number of myoblasts that attempted to integrate into each new myofibre, and as such serve as another indication of potentially enhanced fusion (Shenkman et al., 2010).

Additionally, the preliminary cell culture experiments with lestaurtinib report that pre-treating proliferating myoblasts with lestaurtinib prior to switching to differentiation media results in less overall cell death (Appendix B). Future studies could attempt a similar pre-treatment model, where the drug is circulated within the muscle for up to 4 hours prior to inducing damage, or within 24 hours of inducing damage (before differentiation has begun).

While the present study focused mostly on lestaurtinib's effects on satellite cell differentiation, future studies could attempt to observe how proliferation and/or self-renewal might be affected instead. There is evidence to suggest that inhibition of JAK/STAT signalling enhances symmetric expansion of satellite cells (Doles & Olwin, 2014; Moresi et al., 2019; Price et al., 2014; Tierney et al., 2014), and lestaurtinib is a known inhibitor of JAK/STAT signalling (Santos et al.

2010). Inhibitor studies in mice have demonstrated that inhibition of either JAK or STAT3 increases the number of satellite cell divisions by ~2-fold and reverses muscle regeneration defects in aged and dystrophic mice (Moresi et al., 2019; Price et al., 2014). This is a desirable effect, as aging and dystrophy impair the myogenic potential of satellite cells through a reduced capacity for satellite cell self-renewal. It is possible that treatment with lestaurtinib could produce similar results in future studies.

Similarly, aging has been shown to increase the activation of JAK/STAT signaling in satellite cells, which inhibits their capacity to undergo symmetric stem cell expansion. As such, pharmacological inhibition of JAK/STAT signalling could serve as a therapeutic approach to enhancing muscle regeneration through promotion of enhanced satellite cell proliferation. This hypothesis was recently tested on C57BL/6J aged mice. Following CTX injury and intramuscular injection of JAK/STAT inhibitors (5,15 DPP and Tyr AG), an increase in myofibre size was observed in aged skeletal muscle. Conversely, the proportion of emb-MyHC-positive fibres in aged muscle decreased from 83% ± 10% (vehicle) to 48% ± 7.3% (5,15 DPP) and 40% ± 12% (Tyr AG), suggesting inhibition of differentiation. At the same time, there was evidence of an increased percentage of satellite cells (~100%) in aged muscle, as well as satellite cells lacking *MyoD* expression (Pax7-positive/MyoD-negative). Both muscle regeneration and functional performance were enhanced following treatment (Price et al., 2014). This study suggests that lestaurtinib may show more promising results if tested on a different population. While it did not appear to affect regeneration in young, healthy muscle, future studies may want to determine if any there are any positive effects when tested on aged or myopathic muscle.

7. CONCLUSION

Overall, data from this study suggests that under the specific experimental conditions outlined here, the regeneration process observed in skeletal muscle following acute damage appears to be unaffected by lestaurtinib treatment. However, the possibility that lestaurtinib has the potential to augment skeletal muscle regeneration under different experimental conditions (oral dosing, different dosage levels, etc.) should not be discounted. At the very least, this study has shown that intramuscular injection of lestaurtinib into regenerating skeletal muscle fibres is relatively safe in mice and does not produce any overwhelming negative effects within 14 days of administration. As such, future studies may wish to probe other mechanisms by which lestaurtinib might influence muscle regeneration, including its effects on satellite cell proliferation and the potential to augment muscle regeneration through the stimulation of expansion of the satellite cell pool. Doing so may aid in the development of a drug therapy that could potentially mitigate the negative effects of regeneration dysregulation in certain states such as aging and cancer cachexia.

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APPENDICES

Appendix A

Pilot Study – Screening Methods

1. Myoblast Acquisition from Mice

Myoblasts were isolated from neonatal CD1 mouse hindlimb skeletal muscle using a modified version of the method described by Rando & Blau (1994). Briefly, neonatal CD1 mice were euthanized and dissected to acquire hindlimb muscle. Non-muscle tissue was dissected and discarded. Muscle tissue was rinsed and then manually minced using dissection scissors in Hank's buffered salt solution (HBSS) for ~2 minutes. Tissue slurry was then enzymatically digested using a collagenase/dispase solution for a total of ~25 minutes at 37˚C, periodically triturating the slurry using a 5 ml pipet until the solution could easily pass through a 1 ml pipet. Enzymatic digestion was halted by briefly centrifuging the solution to collect the muscle debris and cells, removing the enzyme solution, and then adding 20% fetal bovine serum (FBS) in Ham's F-10 basal media. Resuspended muscle debris and cells in the FBS/F-10 media were then strained through a 70 μm cell strainer. To further purify the myoblasts, a cell sort was performed as per Yi & Rossi (2011).

To label myoblasts, an antibody for α-7 integrin with an AlexaFluor 647 flurophore (Yi & Rossi, 2011) was incubated with the strained cell solution at 1:500 concentration in 10% goat serum in phosphate-buffered saline (PBS). α-7 integrin-positive cells were then cell-sorted and plated on type I collagen-coated plates at a density of 10,000 cells/ml of proliferation media (20% FBS in Ham's F-10 basal media with 2.5 ng/ml human bFGF and antibiotics). Myoblasts were passaged at 70% confluence and used for experiments after 4-5 passages. Myoblasts were incubated at 37˚C, 5% CO_2.

2. Screen on Myoblasts Identifying Lestaurtinib and Others as Myotube-Promoting Compounds

Myoblasts were seeded in 96-well collagen coated plates with a density of 1000 cells per well in 100 µL of myoblast proliferation media. After 24 hours, each well was administered a 50 µL feed of the same media plus a 1 µM dose of a compound from either the OICR kinase inhibitor library (400 different compounds), OICR tool compound library (160 different compounds), NIH Clinical Collection (480 different compounds), or Prestwick Chemical Library (1120 different compounds). Each 96 well plate had a lane of cells that received the same volume of compound vehicle only (dimethyl sulfoxide, 0.1%). After 48 hours, 15 µL of Alamar Blue was added to each well and incubated for an additional 24 hours and then read on a fluorescent plate reader (λ_{540} excitation/λ_{590} emission). Wells were rinsed with PBS, cells were fixed with paraformaldehyde (4%), stained with Hoechst 33258, and visually examined for prominent changes in number and/or morphology. The library screens were run in duplicate except for the Prestwick Chemical Library (single well).

Compounds that appeared to induce proliferation, cell death, or differentiation were noted, particularly if different compounds with overlap in known protein targets caused similar effects. No themes emerged amongst pro-proliferation compounds; however, myoblast cell death was consistent in response to several compounds that inhibited the following targets: PI3 kinase, mTOR, GSK-3, or polo-like kinases. Several compounds appeared to induce fusion and differentiation. In myoblasts, this is noted as an elongation of cell morphology, alignment with other myoblasts, and by appearing to fuse with other myoblasts to form immature myotubes thus producing cells with multiple nuclei. These compounds were K-252a, CEP-701 (lestaurtinib), SB218078, and Go6976 (Fig. 8). Common protein targets amongst these compounds were various PKC isoforms and the Trk receptors.

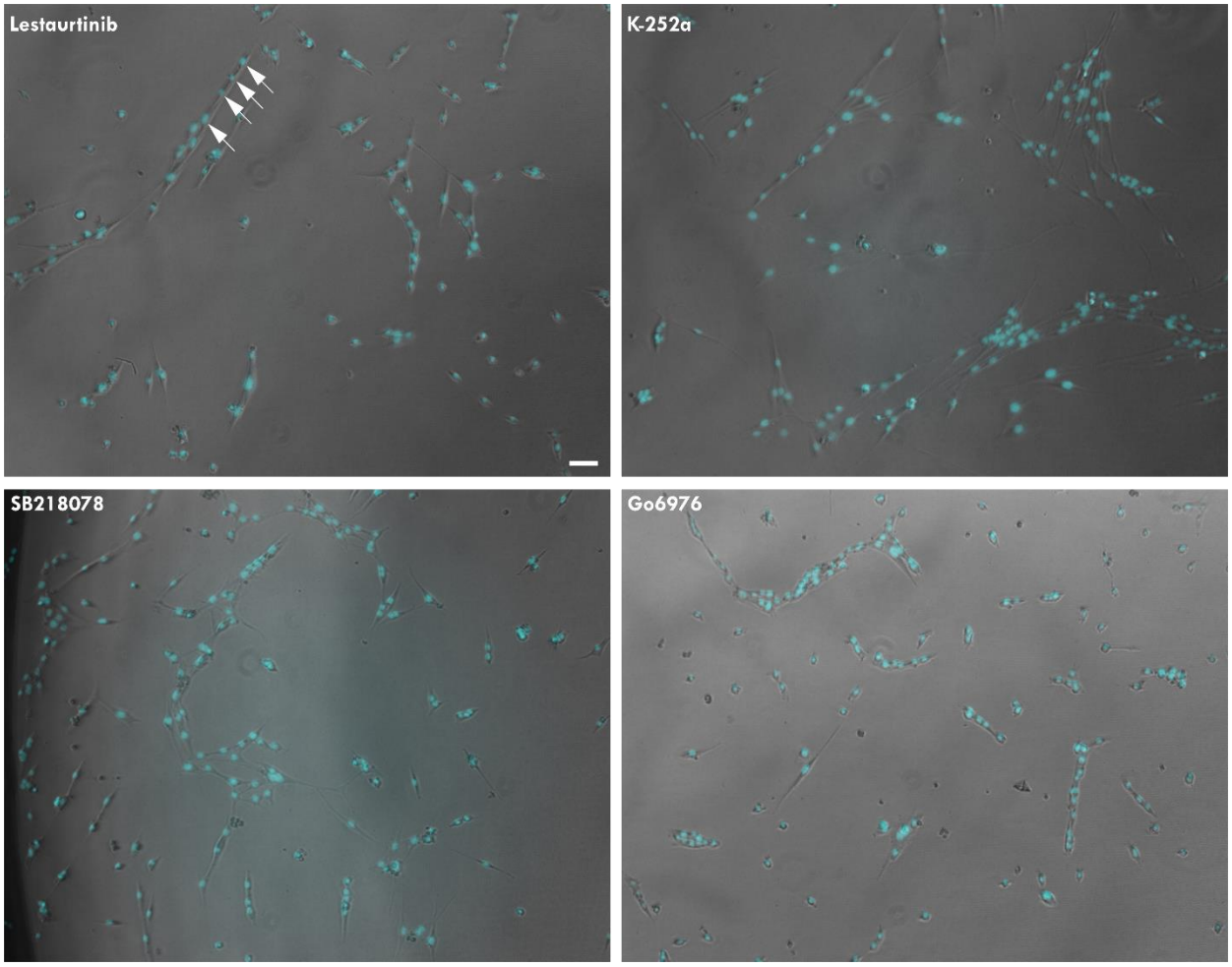

Figure 8. *Several compounds from the OICR kinase inhibitor library appear to induce myoblast alignment, fusion, and differentiation. The compounds lestaurtinib, K-252a, SB218078, and Go976 induce myotube formation despite proliferation media including FBS and bFGF. All compounds were administered at 1 µM. Arrows point to Hoechst-stained nuclei aligning to form a myotube. Scale bar represents 50 µm.*

Appendix B

Pilot Study – Cell Culture Experiments

1. Effect of Lestaurtinib on Myoblasts in Proliferation Media

After the initial screen, lestaurtinib was selected for further study. First, using myoblasts (6000 per well in 96 well plate) in proliferation media, time-lapse video was captured to illustrate the effect of 1 µM lestaurtinib (similar to the original drug screen). Despite taking place in proliferation media, lestaurtinib appeared to induce morphological changes in the myoblasts, and possibly fusion and differentiation (Fig. 9). Myoblasts appeared to be "seeking" fusion partners.

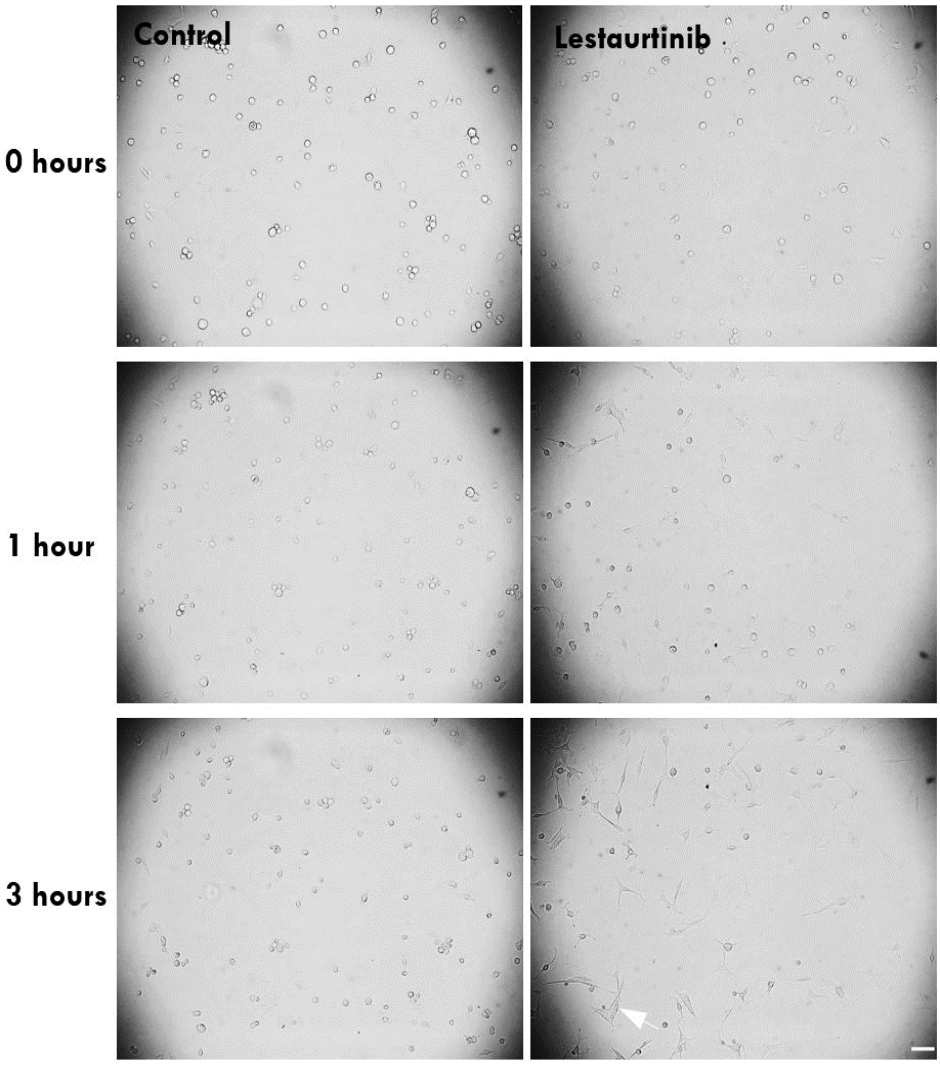

Figure 9. *Time lapse of myoblasts exposed to 1 µM lestaurtinib. Arrow denotes cells that have begun to morphologically change and cluster together despite incubation in myoblast proliferation media. Scale bar represents 100 µm.*

2. Effect of Lestaurtinib on Myoblasts in Differentiation Media

To this point, all testing was done on myoblasts in proliferation media. Thus, the experimenters sought to determine if lestaurtinib would exhibit more potent effects on myoblasts undergoing differentiation in myoblast differentiation media (high glucose DMEM, 1% insulin-transferrin-selenium supplement). Myoblasts were seeded at 3500 cells per well and given 2 hours to settle and adhere, then incubated in differentiation media with lestaurtinib for 48 hours. The following lestaurtinib doses were administered: 0 (DMSO vehicle), 1.6 nM, 8 nM, 40 nM, 200 nM, and 1 µM. At the 200 nM dose, some myotubes appeared larger, more branched, and had more total nuclei (Fig. 10) but this was not apparent at lower doses. The 1 µM dose induced a high degree of cell death, while no changes were observed with doses lower than 40 nM.

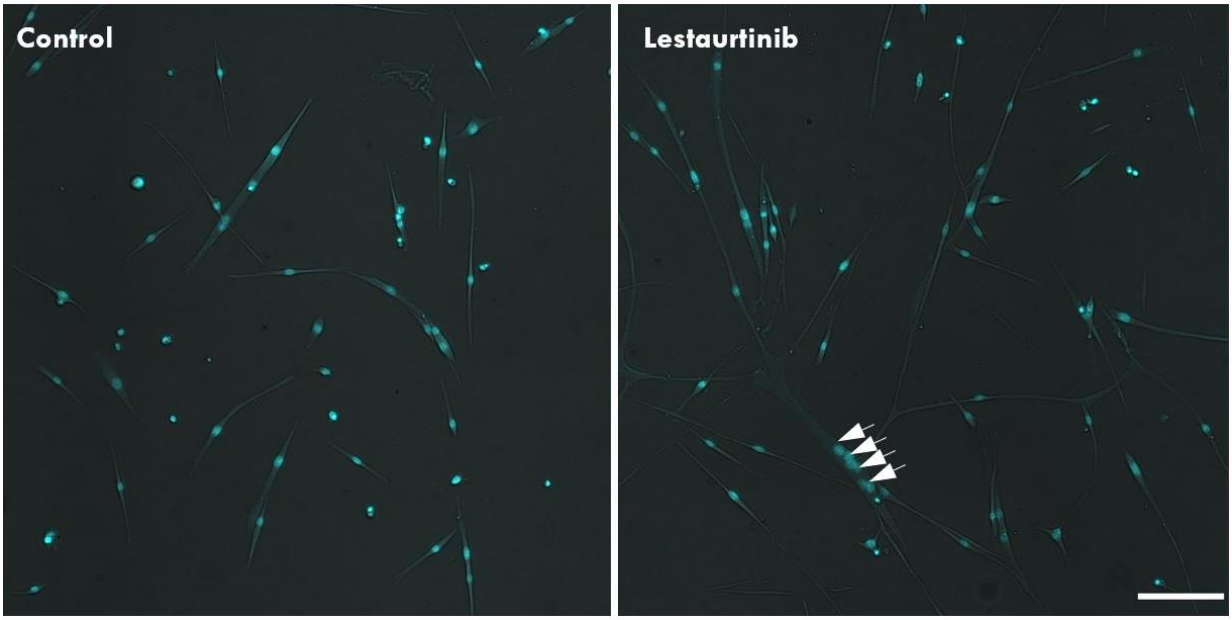

Figure 10. *The effect of lestaurtinib (200 nM) treatment on myoblasts in differentiation media. Scale bar represents 100 µm.*

3. Effect of Lestaurtinib on Pre-treated Myoblasts

Despite lestaurtinib appearing to induce myoblast differentiation, there was greater myoblast cell death under many of the conditions tested by this point. In an attempt to induce differentiation while minimizing cell death, a pre-treatment model was employed. Myoblasts in proliferation media were exposed to lestaurtinib in a limited duration (200 nM, 4-hour exposure) followed by a 12-hour period of differentiation (in differentiation media). The pre-treatment condition resulted in longer myotubes with more nuclei per myotube (Fig. 11), suggesting that a brief dose of lestaurtinib enhances myogenic fusion.

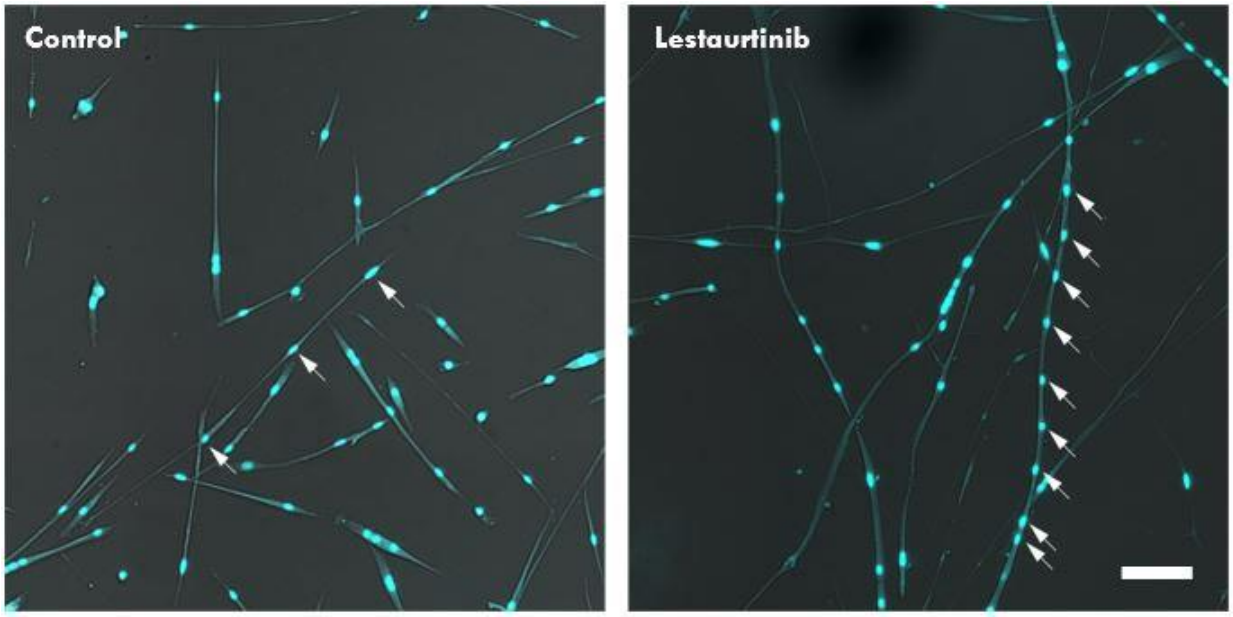

Figure 11. *The effect of pre-treating proliferating myoblasts with 200 nM of lestaurtinib for 4 hours prior to 12 hours of differentiation. Scale bar represents 100 µm.*

To assess how lestaurtinib might be influencing myoblast differentiation, proliferation was assessed. Myoblasts growing in proliferation media were treated for 16-hour with 1 µM lestaurtinib, fixed with 4% PFA, and stained for Ki67, a marker of cell proliferation. It was found that lestaurtinib reduces the number of cells expressing Ki67 (Fig. 12), suggesting that proliferation is slowed.

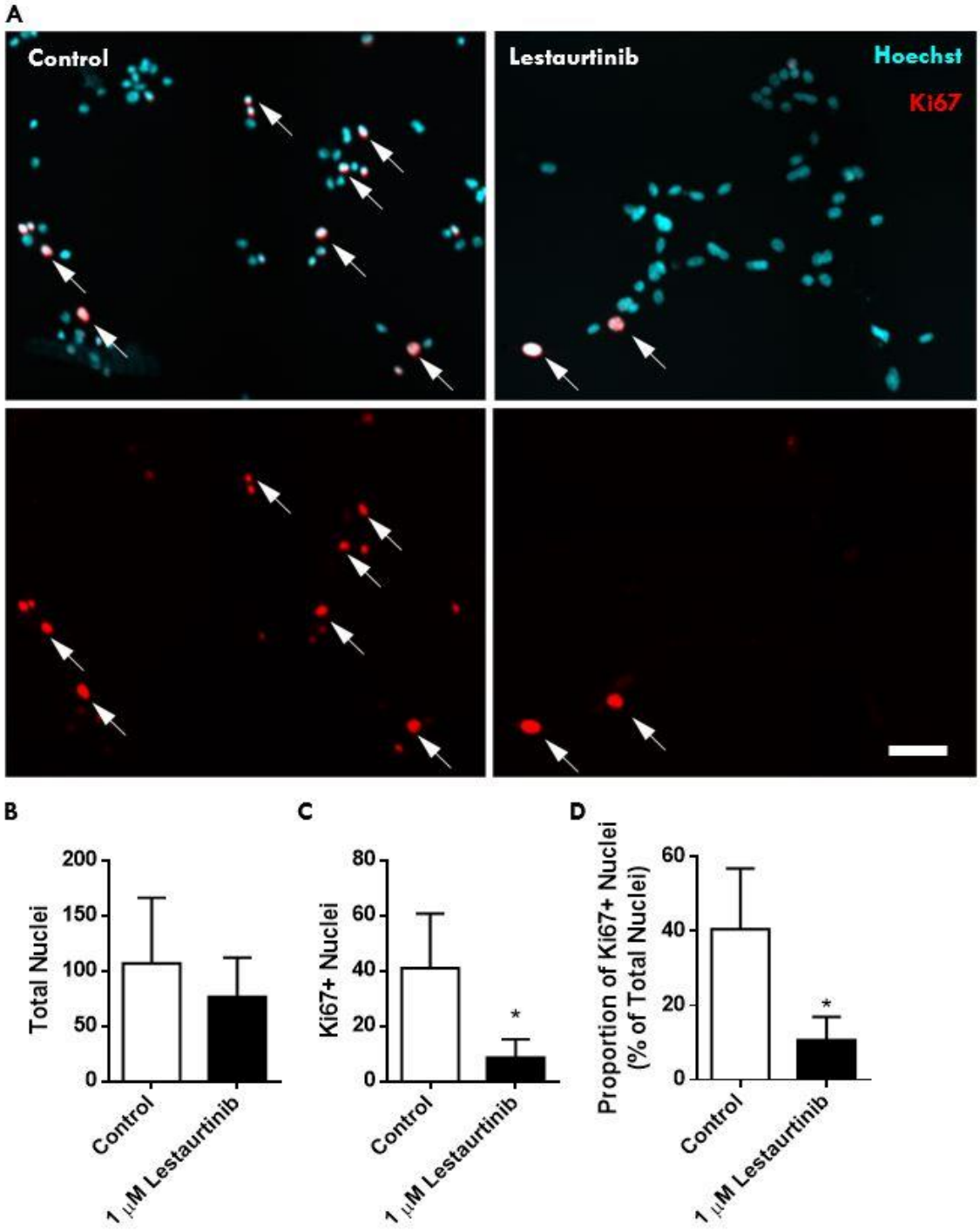

Figure 12. *Myoblast proliferation is slowed by lestaurtinib treatment. Ki67 staining (A) revealed that although total nuclei number did not decrease (B: one-tailed t test, p=0.1787), the number of Ki67+ cells (C: one-tailed t test, p<0.05) and the percentage of Ki67+ cells (D: one-tailed t test, p<0.05) were significantly decreased (n = 5 for B-D). Scale bar represents 50 µm.*

Appendix C

Hematoxylin and Eosin (H&E) Staining Protocol

1. Air dry sections for 15 minutes at room temperature.
2. *Hematoxylin stain:* Apply Harris modified hematoxylin to each muscle section using a Pasteur pipette. Cover each section completely.
    a. Leave on for 3 minutes.
3. Briefly rinse slides in beaker of ddH2O.
4. Place slides in Coplin jar and run under cool tap water for 3 minutes.
5. Rinse slides with ddH2O for 2 minutes. Wipe off excess water with a Kim wipe.
6. *Eosin stain:* Apply eosin solution to each muscle section (1% w/v Eosin Y disodium salt in ddH2O + 0.1% v/v glacial acetic acid).
    a. Leave on for 10 minutes.
7. Briefly rinse slides in beaker of ddH2O.
8. Place slides in Coplin jar and run under cool tap water for 3 minutes.
9. Rinse slides with ddH2O for 2 minutes. Wipe off excess water with a Kim wipe.

Perform the following steps inside fume hood.

10. *Dehydrate:* submerge slide into 95% ethanol for 10 seconds, followed by 100% ethanol for 10 seconds.
11. *Clear:* submerge slide into xylene for 10 seconds.
12. *Mount:* wipe off excess xylene using a Kim wipe and apply permount along with the coverslip before xylene completely dries.
13. Remove from slides from fume hood and let set on a horizontal surface for 15+ minutes.

Appendix D

Table 3. List of antibodies and working concentrations

	Host	Company	Catalogue	Concentration
Collagen I	Rabbit	Abcam	ab34710	1:200
emb-MyHC	Mouse	DSHB	F1.652	1:1

Appendix E

Collagen Staining Protocol

DAY 1

1. Air dry sections for 15 minutes at room temperature
2. Circumscribe sections using a hydrophobic pen
3. *Fix:* each section in 2% paraformaldehyde (PFA) for 5 minutes in fridge
4. Rinse 3x (2 minutes each) with 1x phosphate buffered saline (PBS)
5. *Block:* Apply 5% normal goat serum (Ngs) + 0.1% Triton-X100 in PBS to each section
    a. Incubate covered at room temperature for 40 minutes
6. Remove block. Rinse 3x (2 minutes each) with 1x PBS
7. *Primary Antibody* : Apply rabbit antibody (1:200) in block (5% Ngs + 0.1% Triton-X100 in PBS) on positive sections only. Apply PBS on negative control sections
    a. Incubate 16-20 hours in a humidified chamber at 4°C (fridge)

DAY 2

1. Remove primary antibody and PBS
2. Rinse 3x (2 minutes each) with PBS
3. *Secondary Antibody* : Apply 1:1000 goat anti-rabbit (GaR 488) IgG secondary antibody and 1:1000 DAPI to all sections
    a. Cover at room temperature for 1 hour in simulated dark room
4. Remove secondary antibody. Immediately rinse once with PBS
5. Rinse 3x (2 minutes each) with PBS
6. *Mount :* Apply fluoromount and coverslip. Set for 15 minutes on horizontal flat surface protected from light

Appendix F

emb-MyHC Immunostaining Protocol

Day 1

1. Air dry slides at room temperature for 15 minutes
2. Circumscribe sections using a hydrophobic pen
3. *Block 1*: Apply block to all sections (90 µL of M.O.M. blocking reagent stock solution per 2.5 mL PBS). Incubate for 1 hour at room temperature.
4. Remove block
5. Rinse 3x (1 minute each) in 1x PBS
6. *Block 2:* Apply 10% NGS + 1.5% bovine serum albumin (BSA) + 0.2% Triton-X100 in PBS to each section. Incubate for 1 hour at room temperature.
7. *Primary Antibody*: Remove block. Apply mouse antibody with block (1:1). Incubate 16-20 hours in a humidified chamber at 4°C (fridge).

Day 2

8. Remove primary antibody
9. Rinse 5x (1 minute each) in 1x PBS
10. *Secondary Antibody*: Apply 1:1000 goat anti-mouse IgG secondary antibody and 1:1000 DAPI to all sections. Place in a dark chamber for 1 hour at room temperature.
11. Remove secondary antibody
12. Rinse 3x (1 minute each) in 1x PBS
13. *Mount:* Apply fluoromount and coverslip. Set for 15 minutes on a horizontal surface protected from light.

Appendix G

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