THE EFFECT OF ANTIRETROVIRALS ON MYOBLAST PROLIFERATION, MIGRATION AND DIFFERENTIATION

Wanani Nonhlanhla Sibanda

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ABSTRACT

Successful antiretroviral (ARV) treatment is associated with suppression of HIV viral load and the reduction of clinical disease progression. Despite marked improvements in ARV medication, side effects from long-term treatment, such as loss of muscle mass do occur. The mechanism by which ARVs affect muscle mass is unclear, however, published *in vitro* data suggests a negative effect on myoblast fusion during differentiation. The objective of this study was therefore to determine the effect of ARVs on processes required for successful myogenesis; these included proliferation, migration during wound repair, and differentiation.

C2C12 mouse skeletal myoblasts and human primary culture skeletal (HSk) myoblasts were incubated with Zidovudine (nucleoside reverse transcriptase inhibitor-NRTI), Tenofovir (nucleotide reverse transcriptase inhibitor-NtRTI) or Ritonavir (protease inhibitor-PI) at a concentration range of 0.01 µM to 10 µM. Proliferation was determined using crystal violet and migration was analyzed using a 2D wound healing assay. The commitment of myoblasts into the myogenic lineage was assessed via the expression of the transcription factor Pax7. Differentiation was measured by assessing the fusion index of multinucleated myotubes.

C2C12 myoblast proliferation was observed to increase significantly in response to Tenofovir (1 µM and 10 µM). In HSk cells however, proliferation was observed to decrease significantly in response to Tenofovir (1 µM). Zidovudine had no consistent effect on C2C12 proliferation at any dose tested, but caused a decrease in HSk myoblast proliferation (0.01 µM and 0.1 µM); however this was statistically non-significant. A small dose-dependent increase in C2C12 and HSk cell number, although not significant, was seen in response to Ritonavir. Wound closure results revealed both dose-dependent and time-dependent effects of Tenofovir and Zidovudine on human myoblast migration, with significant decreases in the rate of wound closure (4-7 hours) noted at 0.1 µM and 0.01 µM doses respectively. Zidovudine had no significant effect on migration while Ritonavir (0.01 µM) was observed to significantly increase percentage wound closure of human myoblasts, suggesting an increased ability to migrate during wound repair. Differentiation results indicated a decrease in myoblast fusion in response to all three ARVs. However only Ritonavir was shown to negatively affect myosin heavy chain expression. Further research into the exact mechanism of decreased fusion is required.

To our knowledge, this study is the first to suggest that selected ARVs may significantly influence myoblast regeneration capabilities by modulating myoblast proliferation, migration, differentiation

and fusion, and thereby decrease their myogenic capability. Extended human myoblast studies on differentiation could confirm this hypothesis.

PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Biochemistry, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, from March 2011 to March 2013, under the supervision of Dr. C. U. Niesler.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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I, Wanani Nonhlanhla Sibanda, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.

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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTUION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication.

Sibanda W.N., Pillay T. and Niesler C.U. Effects of antiretrovirals on myoblast proliferation, migration and differentiation [Contributed original data, analysis and interpretation for the majority of the paper]. In preparation.

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LIST OF CONFERENCE CONTRIBUTIONS

Sibanda W.N. and Niesler C.U. Muscle atrophy: Is long-term antiretroviral treatment a causative agent? Presented at the 39th Annual Conference of the Physiology Society of Southern Africa (PSSA), University of the Western Cape, South Africa; 28-31 August 2011.

Sibanda W.N., Pillay T. and Niesler C.U. *In vitro* effects of antiretrovirals on myogenesis. Presented at the South African Society of Biochemistry and Molecular Biology/Federation of African Societies of Biochemistry and Molecular Biology (SASBMB/FASBMB) Congress, Drakensberg, South Africa; 29 January - 1 February 2012.

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ABBREVIATIONS

ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
ARVs	Antiretrovirals
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix (proteins)
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CC_{50}	50% cytotoxicity concentration
CCR5	Chemokine co-receptor 5
CDKs	Cyclin-dependant kinases
CXCR4	Chemokine (C-X-C motif) receptor 4
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified eagle serum
DMSO	Dimethyl sulfoxide
DNA pol gamma	DNA polymerase gamma
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EIs	Entry inhibitors
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
G0 phase	Quiescent phase
G1 phase	Gap 1/Growth phase
G2 phase	Gap 2/pre-mitotic phase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HAART	Highly active antiretroviral therapy
HeLa	Human epithelioid carcinoma cells
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HSCs `	Hematopoietic stem cells
HSk	Human skeletal (myoblasts)
IC_{50}	Concentration that produces 50% inhibition
IGFs	Insulin-like growth factors
MAP	Mitogen-activated protein
MDSCs	Muscle-derived stem cells
MEF2	Myocyte enhancer factor 2
MHC	Myosin heavy chain
MMP	Matrix metalloproteinase
M-phase	Mitotic phase
MRFs	Myogenic regulatory factors
mtDNA	Mitochondrial DNA

NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
NtRTIs	Nucleotide reverse transcriptase inhibitors
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PI3K	Phosphatidylinositol 3-kinase
PIC	Protease inhibitor cocktail
PIs	Protease inhibitors
Rb	Retinoblastoma susceptibility gene
RIPA	Radioimmunoprecipitation assay (buffer)
RNA	Ribonucleic acid
RT	Reverse transcriptase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of mean
SP	Side population
S-phase	Synthesis phase
SR	Sarcoplasmatic reticulum
TGF-β	Transforming growth factor-β
WHO	World Health Organisation

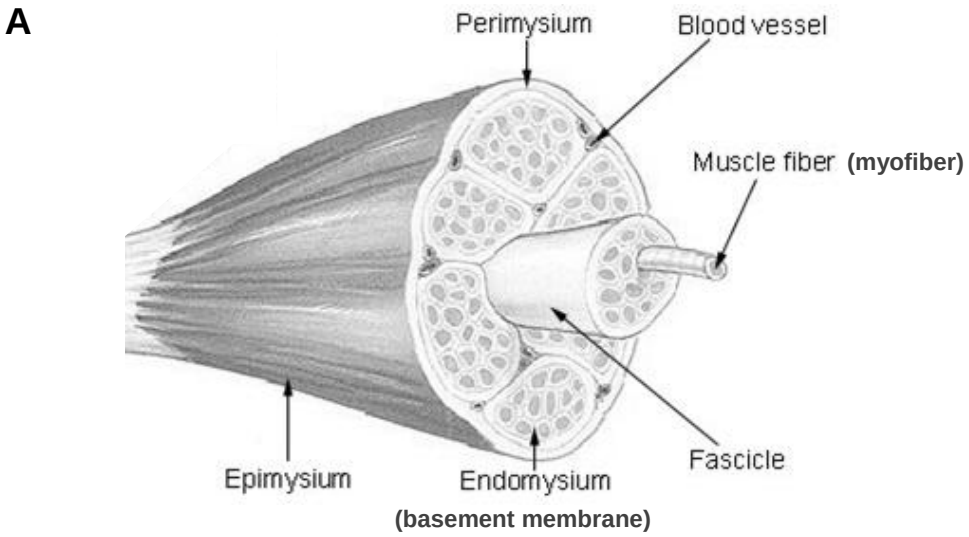
CHAPTER 1:

LITERATURE REVIEW

1.1. Skeletal muscle

Adult skeletal muscle presents the largest tissue in the human body, composing 40-50% of total human body mass (Huard *et al*. 2003). It consists of muscle cells, networks of nerves, blood vessels and connective tissue that connect individual muscle fibers into bundles which then form the complete muscle. The function of skeletal muscle is primarily to facilitate movement, enable breathing and maintain posture (Boldrin and Morgam, 2002).

The adult skeletal muscle cellular unit is the myofiber (Figure 1.1A) (Zammit *et al*., 2004). Newly formed multinucleated fibers exhibit central nucleation and once the nuclei move to the sub-sarcolemmal position, they are then termed myofibers (Huard *et al*., 2003; Charge and Rudnicki, 2004). These basic structural elements, which are essentially large syncytial cells (myofibrils), contain hundreds of myonuclei within a continuous cytoplasm (sarcoplasm). The interior of the myofibers contain basic contractile cellular subunits called sarcomeres (Figure 1.1B) (Collins *et al*., 2005). Sarcomeres consist of thick myosin filaments that interact with thin actin filaments in response to neuromuscular signals. During muscle contraction and relaxation, the relative sliding of thick and thin filaments is brought about by "cross bridges"- parts of the myosin molecules which extend from the myosin filaments and interact with the thin filaments, transporting them by a rowing action (Figure 1.1B). The sarcomere region containing the actin filament is the I band; the region where actin and myosin overlap is the A band; the region within the A band where the I band and A band do not overlap is referred to as the H zone-it consists of myosin only. The Z disc bisects the I band and the distance between one Z disc and the next marks the length of the sarcomere (Figure 1.1B) (Lieber, 2002). During this process ATP (adenosine triphosphate) is hydrolysed to ADP (adenosine diphosphate); hydrolysis of ATP provides the energy for contraction (Huxley and Niedergerke 1954; Lymn and Taylor, 1971; Rayment *et al*. 1993; Lieber, 2002). In response to an action potential signal, the cell depolarizes, leading to the release of calcium ions (Ca^{2+}) from the sarcoplasmatic reticulum (SR). The released Ca^{2+} ions induce ATP-driven interactions between myosin and actin leading to the shortening of the sarcomere and thus, muscle contraction (Inoue *et al*., 2002; Brooks, 2003).



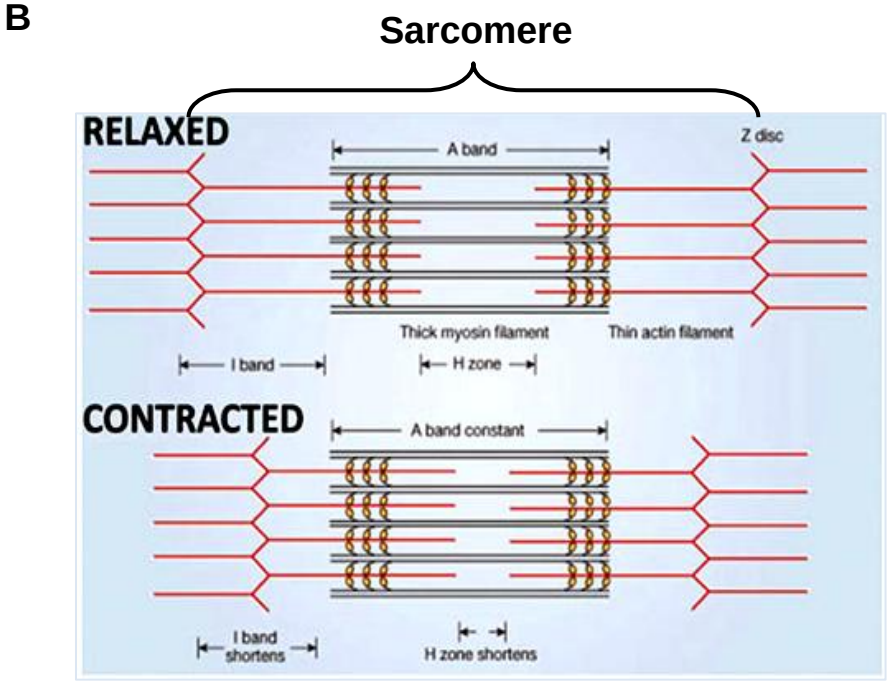


Figure 1.1 Skeletal muscle structure. A. The fibrous outer layer surrounding the complete muscle is the epimysium; the perimysium surrounds each myofiber bundle (fascicle); the endomysium is the basement membrane that surrounds individual myofibers. **B**. Each myofiber contains contractile subunits called sarcomeres, which in turn consist of thick myosin and thin actin filaments. These filaments give skeletal muscle its cross striated appearance. Muscle contraction occurs by the mutual sliding of these two sets of filaments over each other. The A band is where actin and myosin overlap; the I band contains actin only; the H-zone contains myosin and the Z disc marks the start of a sarcomere (Huxley and Niedergerke 1954; Lieber, 2002; Inoue *et al*., 2002).

1.1.1. Muscle stem cells

Adult skeletal muscle contains satellite cells, which are a population of undifferentiated mononuclear myogenic cells that represent committed myogenic stem cells (Seale *et al*., 2000). Other stem cells and progenitors capable of myogenesis include side population (SP) cells, pericytes, mesoangioblasts (blood vessel-associated stem cells), $CD133^+$ stem cells, muscle-derived stem cells (MDSCs), and $CD34^+$ hematopoietic stem cells (HSCs) (Table 1.1) (Beauchamp *et al*., 2000; Charge and Rudnicki, 2004; Maclean *et al*., 2012). Optimal repair of muscle following injury or disease, requires that an ideal stem cell population be a) present in accessible post-natal tissues, b) able to differentiate into skeletal muscle *in vivo* and c) able to reach skeletal muscle at site or through a systemic route (blood vessel system) (Ten Broek *et al*., 2010).

Satellite cells conform to the definition of a stem cell, in that they have the ability to self-renew throughout the lifetime of the organism (Seale and Rudnicki, 2000). They are also the primary cells involved in skeletal muscle regeneration and are therefore the best candidates for the therapy of injured or diseased muscle (Whalen *et al*., 1990;Zammit *et al*., 2006; Bentzinger *et al*., 2013). However, their inability to cross the endothelial lining of the blood vessels in skeletal muscle, makes them less ideal for systematic delivery during transplantations (Palacios and Puri, 2006 and Dellavalle *et al*., 2007). Muscle SP cells (Table 1.1) on the other hand, are a subset of stem cells with the capacity to function in various tissues from which they are isolated (Hirschmann-Jax *et al*., 2004). They have hematopoietic potential and have also been shown to take the role of satellite cells in Pax7 null mice (Asakura *et al*., 2001). Unlike satellite cells, pericytes (Table 1.1), which are mesenchymal contractile cells that wrap around endothelial cells of blood vessel walls (Bergers and Song, 2005), express myogenic markers only when associated with differentiated myotubes; they can colonize skeletal muscle and generate many muscle fibers (Dellavalle *et al*., 2007). Mesoangioblasts (Table 1.1) are multipotent progenitors of mesodermal tissues that exhibit many similarities to pericytes, and have the potential to progress down the endothelial or mesodermal lineages (Sampaolesi *et al*., 2006). Mesoangioblasts can self-renew extensively *in vitro* (Cossu and Bianco, 2003; Sampaolesi *et al*., 2006). Their myogenic potential is limited as they are unable to colonize muscle due to incomplete adhesion to myofibers; they therefore seep out into the surrounding tissue (Galvez *et al*., 2006). HSCs (Table 1.1) found in the mammalian blood system, can circulate in the bloodstream and are able to cross the endothelial lining of blood vessels (Ten Broek *et al*.,2010). HSCs are able to contribute to the satellite cell pool and appear to play a bigger role in muscle regeneration compared to the other non-muscle derived stem cells due to their developmental plasticity (LaBarge and Blau, 2002). $CD133^+$ stem cells (Table 1.1) circulate in the bloodstream and can differentiate *in vitro* into muscle cells. CD133 is expressed in hematopoietic

stem and muscle progenitor cells (Pellacani *et al*., 2011). $CD133^+$ stem cells are able to migrate through blood vessel walls and have a high potential for treating skeletal muscle injury and disease. Intra-muscularly injected human $CD133^+$ cells have been shown to have a greater regenerative capacity than injected human myoblasts, and therefore have great potential for use in treating diseased and injured muscle (Negroni *et al*. 2009). MDSCs (Table 1.1) are potential precursors of satellite cells, but are not restricted to muscle or other mesenchymal tissues (Huard *et al*., 2003). Transplantation of MDSCs into skeletal muscle has been found to give better results compared to satellite cell transplantation to repair muscle damage in Duchenne muscular dystrophy (DMD) (Torrente *et al*., 2003). MDSCs have prolonged proliferation, immune tolerance and a strong tendency for self-renewal *in vivo*, following their systemic transplantation (Qu-Petersen *et al*., 2002; Peault *et al*., 2007). In animal models, MDSCs have also been shown to help reconstitute damaged peripheral nerve cells and vascular cells such as endothelial cells (Meng *et al*., 2011; Staack and Rodriguez, 2011). However, there is insufficient information regarding their *in vivo* long-term self-renewal capacity (Boldrin and Morgan, 2007).

Table 1.1 Stem cells that may play a role in skeletal muscle regeneration. Their origin, localization, lineage potential and function is indicated.

Cell type	Origin	Localization	Lineage Potential	*In vivo* Function	References
Satellite cells (myoblasts)	Mesoderm	Between basal lamina and sarcolemma	Myogenic	Regeneration of muscle fibers in injured muscle	Zammit *et al*. (2006), Le Grand and Rudnicki, (2007)
Side population (SP)	Somatic	Interstitial; also associated with blood vessels	Myogenic, hematopoietic	Unknown	Asakura *et al*. (2002), Hirschmann-Jax *et al*. (2004)
Pericytes	Ectoderm, mesoderm	Periphery of capillary and microvessels	Myogenic, osteogenic, adipogenic, chondrogenic	Blood vessel flow; Angiogenesis	Bergers and Song, (2005)
Mesoangioblasts	Mesoderm; walls of blood vessels	Associated with microvessel walls	Myogenic, osteogenic, adipogenic, cardiogenic	Unknown	Cossu and Bianco (2003); Sampaolesi *et al*. (2006)
Hematopoietic stem cells (HSCs)	Embryonic endothelium	Bone marrow	Myogenic, myelogenic, lymphogenic	Production of blood cells	LaBarge and Blau, 2002
CD133+ cells	Mesoderm	Myofiber close to blood vessels	Myogenic, hematopoietic, endothelial	Angiogenesis; hematopoiesis	Torrente *et al*. (2003); Pellacani *et al*. (2011)
Muscle-derived stem cells (MDSCs)	Unknown	Myofiber periphery closely associated with blood vessels	Myogenic, osteogenic, hematopoietic, cardiogenic, chondrogenic	Unknown	Huard *et al*. (2003)

1.1.2. Satellite cells

Satellite cells were identified over 40 years ago through electron microscopy and first described by Mauro (1961). During embryonic development, specific signals and cellular events allow the satellite cells to become committed to the myogenic lineage. They are known to provide extra nuclei for post-natal muscle growth during muscle repair and regeneration (McGeachie Grounds, 1987; Hill, *et al.*, 2003; Morgan and Partridge, 2003).

Satellite cells are located in their niche between the continuous layer of the basal lamina and sarcolemma of the myofiber (Mauro, 1961) (Figure 1.2) and are positioned along the entire length of the myofiber (Muir *et al.*, 1965). Satellite cells also have a relatively high nucleus-to-cytoplasm ratio and contain very few organelles (Hawke and Garry, 2001; Charge and Rudnicki, 2004). Of particular interest is that the nuclei of the satellite cells are much smaller and contain more heterochromatin than the myonuclei of the myofibers, which are located at the peripheral (or sub-sarcolemmal) region of the myofiber (Schultz and McCormick, 1994; Hawke and Garry, 2001, Charge and Rudnicki, 2004; Terada *et al.*, 2010).

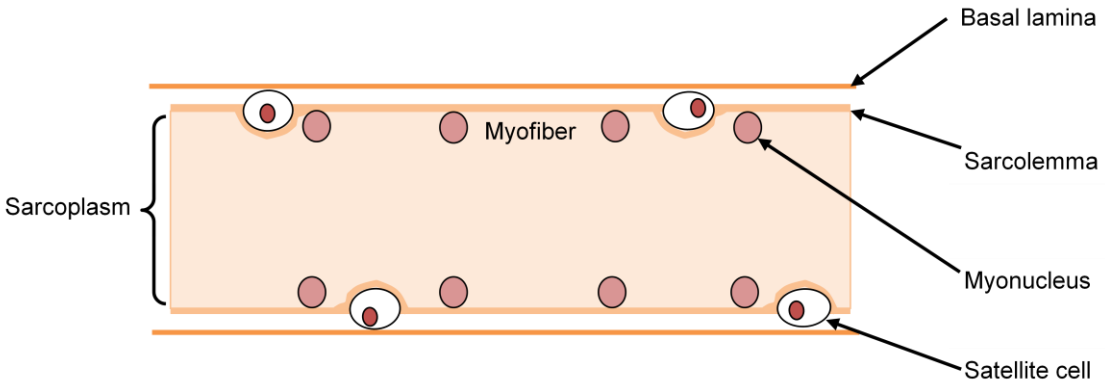


Figure 1.2: Localization of satellite cells. This image illustrates a longitudinal view of a myotube revealing the localization of satellite cells between the basal lamina (basement membrane) and sarcolemma of the myofiber. Events such as muscle fiber injury may trigger mitotic activation of normally quiescent satellite cells. Satellite cells migrate to, differentiate and fuse with damaged fibers, leading to formation of regenerated fibers [Adapted from literature in Section 1.1.2; Muir *et al.*, 1965; Charge and Rudnicki, 2004; Terada *et al.*, 2010].

In normal adult muscle tissue, satellite cells are quiescent and are activated during normal growth, exercise, injury and in degenerative diseases such as Duchenne muscular dystrophy (Kuang and Rudnicki, 2008; Maclean *et al.*, 2012). These precursor cells undergo a series of proliferative steps prior to terminal differentiation and fusion with new or growing myofibers. Muscle growth in terms of fiber size is also mediated by satellite activation as well as increases in protein synthesis

(Rosenblatt *et al*., 1994). Following activation, some satellite cells return to the quiescent phase (known as self-renewal) to replenish the pool of satellite cells on the developing myofiber (Figure 1.3B). It is important to realize that at the end of the G1 phase of the cell cycle, just before entry into the S-phase, the satellite cell responds to signaling molecules to either divide and continue through the S-phase, or exit the cell cycle (and enter G0) to commit to myogenic differentiation or quiescence. On the molecular level, muscle-specific regulatory factors are up-regulated and control satellite cell activation and the subsequent myogenic processes (McGeachie and Grounds, 1987; Zammit *et al*., 2004; Bentzinger *et al*., 2012).

1.2. Mechanisms and mediators of myogenesis

Specific signals during embryonic development control the expression of myogenic regulatory factors (MRFs), which are a group of highly conserved muscle-specific transcription factors that affect the downstream expression of structural and functional proteins (Fernando *et al*., 2002; Palacios and Puri, 2006; Wanschitz *et al*., 2013). In order to enhance transcription, MRF proteins require secondary interactions with other muscle transcription factors such as myocyte enhancer factor 2 (MEF2) - a group of MADS-box regulators (conserved sequence motifs whose domain binds to DNA sequences that are highly similar to the motif) (Yun and Wold, 1996; Fernando *et al*., 2002). It is the basic domain of the MRFs that mediates DNA binding (Massari and Murre, 2000). It is important to note that there are primary and secondary MRFs. The commonly known primary MRFs include the basic helix-loop-helix (bHLH) proteins, myogenic differentiation 1 (MyoD) and myogenic factor 5 (Myf5) (Figure 1.3) (Cossu, *et al*., 1996; Burattini *et al*., 2004). Muscle regulatory factor 4 (Mrf4) and myogenin (Figure 1.3), also bHLH proteins, are secondary MRFs (Massari and Murre, 2000). The helix–loop–helix motif is required for heterodimerization with E proteins that mediate the recognition of genomic E boxes, a motif found in the promoters of many muscle-specific genes (Massari and Murre, 2000).

1.2.1. Activation and Proliferation

Pax7 (Figure 1.3B) expression is believed to be essential for the activation of satellite cells. Pax7 is a paired box transcription factor (Oustanina *et al*., 2004; Zammit *et al*., 2006) and is expressed in both quiescent satellite cells and their proliferating progeny (Yablonka-Reuveni, 1999; Seale *et al*., 2000; Zammit and Beauchamp, 2001). The origin and exact role of Pax7 in satellite cells is unknown (Zammit *et al*., 2006; Seale *et al*., 2000), however, it has been reported that homozygous Pax7 null mutant mice completely lack muscle satellite cells and display reduced muscle growth (Seale *et al*., 2000). Another study suggests that muscle stem cells can become satellite cells

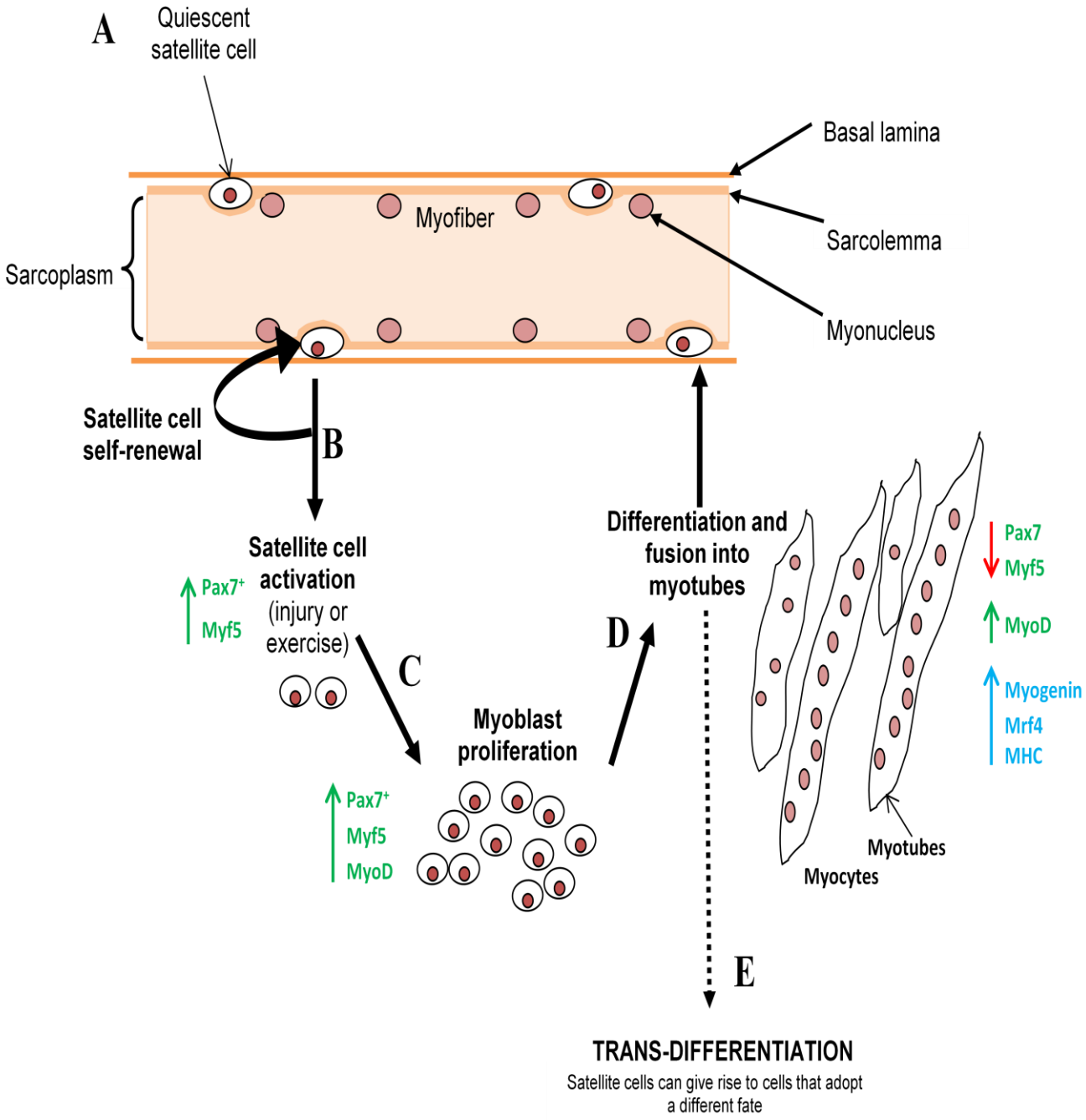


Figure 1.3 Summary of myogenesis and its regulatory factors. A. Satellite cells are quiescent and the majority are Pax7^{+}. **B.** During activation, the satellite cells co-express Pax7 and the primary MRF (green) Myf5. **C.** Activated satellite cells, now termed myoblasts, proliferate and a subset return to the quiescent phase in a process of self-renewal. MyoD expression is up-regulated and Pax7 levels are maintained. **D**. Upon differentiation myoblasts exit the cell cycle and fuse to form multinucleated myofibers. This requires Pax7 expression levels to be down-regulated. Expression of Myf5 is also down-regulated, whilst MyoD, together with the secondary MRFs (blue), myogenin, MRF4 and MHC are up-regulated. **E**. Muscle stem cells can also trans-differentiate to other cell types [Diagram adapted from literature in sections 1.2.1 and 1.2.2, (Zammit *et al*., 2006; Buckingham, 2007; Francetic and Li, 2011)].

through activation of Pax7 (Buckingham, 2007). Furthermore, Pax7 single knock-out mice fail to thrive and most of them die within 2–3 weeks after birth (Mansouri *et al*., 1996). Based on the post-natal decline in the number of satellite cells, it is proposed that Pax7 has a crucial function in the renewal, activation and myogenic commitment of satellite cells (Oustanina *et al*., 2004). A more recent study demonstrated muscle regeneration following transplantation of adult $Pax7^{+}$ satellite cells into Pax7 null mice (Sambasivan *et al*., 2011). However, injection of diphtheria toxin, (which is known to inhibit protein synthesis) into these mice in combination with muscle injury by exercise and injected myotoxins caused significant failure in skeletal muscle regeneration. This effect was sustained over 3 weeks with no recovery (Sambasivan *et al*., 2011). Interestingly, loss of myofibers as result of toxin injection into the tibialis anterior muscle of adult mice, was accompanied by the presence of adipocytes as observed with Oil Red O staining. The findings suggest that satellite cells have a very high regenerative potential as muscle regeneration was rescued following the transplantation (Sambasivan *et al*., 2011).

The primary MRFs, MyoD and Myf5 (Figure 1.3C), are rapidly up-regulated following satellite cell activation (Bornemann and Schmalbruch, 1994; Fernando *et al*., 2002). Specification to the myogenic lineage is likely due to DNA de-methylation at regulatory regions of MRFs which are then expressed in actively proliferating cells prior to differentiation (Massari and Murre, 2000). To assess and determine their functions, MRF null mutation studies have been used (Francetic and Li, 2011). MyoD null mice display no negative effects on skeletal muscle development; these mice are viable and have normal physiological and morphological skeletal muscle features. However, the expression of Myf5 in these mice is increased and prolonged and the mice display a somewhat delayed embryonic myogenesis (Rudnicki *et al*., 1992; Yun and Wold, 1996). The Myf5/MyoD double null mutant fetus lacks muscle tissue (Braun *et al*., 1992); no muscle precursors or myoblast populations are produced and these mice die shortly after birth. The complete lack of myogenin and skeletal muscle thereafter, emphasizes the importance of these two proteins in myogenesis (Rudnicki *et al*., 1993 and Palacios and Puri, 2006). Interestingly, these mutants display elevated levels of fat, raising the possibility that some cells adopt an adipogenic fate (Figure 1.3E). This would imply that the determining gene products of Myf5 and MyoD are essential in promoting the survival, proliferation and myogenic capability of myoblasts (Yun and Wold, 1996). It is important to note that myoblast activation and proliferation are usually a response to muscle growth or injury and appear to be important processes in the ability of muscle to regenerate (Charge and Rudnicki, 2004).

1.2.1.1 Migration

Skeletal muscle fibers are surrounded by a basil lamina (Section 1.1.2, Figure 1.2) composed predominantly of type IV collagen, laminin, fibronectin and heparin sulfate proteoglycan, which contribute to the complex system of the skeletal muscle extracellular matrix (ECM) (Gillies and Lieber, 2011). A key process for muscle regeneration is the migration of activated satellite cells along the basement membrane to the injured site (Friedl and Brocker, 2000). In some instances, the muscle injury may result in the rupture of the basement membrane; myoblast migration would then occur within the interstitial tissue surrounding the damaged myofibers (Friedl and Brocker, 2000; Jarvinen *et al*., 2004). Degradation and subsequent remodeling of the ECM components may largely determine myoblast migration and their ability to facilitate regeneration. Matrix metalloproteases (MMPs) are essential regulatory molecules necessary for the formation, degradation and remodeling of the ECM (Chen and Li, 2009). In skeletal muscle, MMPs play an important role in the homeostasis and maintenance of myofiber functional integrity by breaking down ECM components and regulating skeletal muscle cell migration and differentiation (Chen and Li, 2009). Some of the responsible proteases that play a role in skeletal muscle repair have been identified and include MMP-1, MMP-2, MT1-MMP and MMP-9 (Nishimura *et al*., 2008).

During migration, the leading front of the cell produces extensions called lamellapodia. The leading front together with the rear of the cell interact in a binding and contracting manner, allowing the cells to migrate towards the signal (e.g. a wound). This typically involves detachment and attachment of focal adhesion points to the ECM surface. Thus, in addition to changes in the ECM, migration also involves changes to the cytoskeleton and cell-substrate adhesions (Kaibuchi *et al*., 1999; Hakkinen *et al*., 2011). A number of intracellular signaling molecules control these changes. Of particular interest are the Ras homologous gaunosine triphophatases (Rho GTPases). Members of this protein group include the Rho family which are associated with the focal adhesion assembly and contraction during migration, as well as the Rac and Cdc42 families, which are required at the leading front of the cell to regulate lamellapodia formation and migratory direction (Nobes and Hall, 1995; Ridley, 2001).

1.2.2. Differentiation and Fusion

Once myoblasts are committed to the myogenic lineage, Pax7 is no longer transcriptionally active and its expression is down-regulated (Figure 1.3D). Differentiation of myoblasts to myotubes is initiated via an increase in MyoD expression levels, while Myf5 expression decreases (Oustanina *et al*., 2004; Zammit *et al*., 2006). At this stage, the cells express myogenic regulatory factor 4 (Mrf4) and myogenin, which are the secondary MRFs that mark differentiation (Figure 1.3D). Myogenin and Mrf4 are more directly involved in the differentiation process and trigger the expression of myotube-specific proteins such as myosin heavy chain (MHC). Studies confirm that mice lacking myogenin have very poorly developed skeletal muscle tissue even though myoblasts are present, suggesting that while myogenin plays a critical role in terminal differentiation of myotubes to myofibers, it is not required for initially establishing the myogenic lineage (Berkes and Tapscotts, 2005).

Mrf4 encodes a predicted 27 kDa protein that contains the conserved *basic/myc-like* domain common to MyoD, myogenin, and Myf5 (Rhodes and Konieczny, 1989). Although Mrf4 knock-out mice are viable, they tend to develop abnormal intercostals muscles (Vivian *et al*., 2000). Mrf4 is expressed transiently during embryonic development of mice, immediately following Myf5 expression (Berkes and Tapscott, 2005). Its expression then wavers and is observed to be highly up-regulated again in differentiating muscle fibers. Thus, its expression pattern suggests potential roles in both myogenic commitment and terminal differentiation (Berkes and Tapscott, 2005). Complete myogenesis can be partially rescued in myogenin single knock-out embryos; in a positive auto-regulatory loop mechanism, Mrf4 can substitute for myogenin to activate its own expression and promote myofiber formation (Zhu and Miller, 1997). In embryonic stem cells lacking myogenin, fully differentiated muscle fibers can be generated by over-expression of Mrf4, but not MyoD (Zhu and Miller, 1997; Myer *et al*., 2001).

Terminally differentiating myotubes express muscle-specific proteins such MHC (Figure 1.3D) (Lassar *et al*., 1994; Yablonka-Reuveni *et al*., 1999; Zammit *et al*., 2004). MHC is the motor protein of muscle thick filaments. The α-helical rods of MHC form coiled dimers and assemble into the backbones of muscle thick filaments. The globular head of each MHC molecule protrudes from the thick filament and binds the actin of thin filaments in an ATP-dependent fashion. Conformational changes occur within the heads when they are attached to the thin filaments; these result in muscle contraction (Cooke, 1986; Rayment and Holden, 1994). MHC is the most important protein subunit found in skeletal muscle cells since it contains the molecular motor. Lack of MHC results in motion being impossible, in mammals and other species (Kohn and Myburgh,

2006). Most organisms produce different muscle MHC isoforms and expression patterns are both temporally and spatially regulated, suggesting that isoforms have specific characteristics necessary for key muscle properties (Emerson and Bernstein, 1987; Bottinelli, 2001). Human skeletal muscle expresses three isoforms; MHC I, MHC IIa, and MHCIIx (Kohn and Myburgh, 2006). Small mammals express a fourth isoform, MHC IIb. The different isoforms determine fiber contractile speed and influence the rate of ATP utilization within muscle cells (Kohn and Myburgh, 2006). Alterations of MHC isoforms in human muscle with exercise have been shown to serve as an important mechanism for muscle adaptation to exercise. In one study, MHC isoforms from muscle biopsy samples of males, 3 days before and 7 days after specific training protocols, were determined and analyzed via SDS-PAGE and RT-PCR. Following investigation of the effects of different strength-training protocols on MHC isoform expression, the composition of MHC isoforms were found to vary and it was suggested that this was due to different training methods and the types of muscles studied (Liu *et al*. 2003). Alterations of MHC isoform composition in muscle serve as an important mechanism for muscle adaptation to exercise. This further implies that the correct MHC isoform is required for myofibril stability and muscle function (Liu *et al*. 2003). Other studies have shown that in mice, muscles with different functions undergo distinct programs of myosin isoform transitions during post-natal muscle development (Agbulut *et al*., 2003). This study revealed that the pattern of MHC expression is similar in all skeletal muscles, but the time in which mature phenotypes are established, differs.

Several classes of trans-membrane proteins have been implicated in the fusion process of myocytes to myotubes and myofibers, including members of the cadherin family (Kramerova *et al*., 2006). M-cadherin, a Ca^{2+} dependent cell adhesion molecule, acts in the organization and maintenance of muscle tissue (Zeschnigk *et al.*,1995). It is present in quiescent as well as activated satellite cells, but not in other cell types in skeletal muscle (Wernig *et al*., 2004). M-cadherin mRNA can be detected in a small set of quiescent satellite cells, but studies reveal it is at constantly low levels in these cells. In normal adult muscle, M-cadherin is localized on the sarcolemma underneath the satellite cells (Irintchev *et al*., 1994; Bornemann and Schmalbruch, 1994). Its expression is then up-regulated on satellite cells following activation and subsequent induction of myotube formation in culture (Moore and Walsh, 1993; Wernig *et al*., 2004). Synthetic peptides that bind to the extracellular domain of M-cadherin and interfere with homophilic interactions are able to block myoblast fusion in a dose-dependent manner (Zeschnigk *et al*., 1995). RNA interference studies have confirmed the importance of M-cadherin for fusion of cultured myoblasts (Charrasse *et al*., 2006). M-cadherin silencing was analyzed via Western blotting during myogenic differentiation of C2C12 myoblasts. A decrease in M-cadherin was observed, but N-cadherin, α-tubulin, myogenin, MHC and troponin T were not affected, showing that these genes are induced independently of M-

cadherin. However, myotube formation was prevented (Charrasse *et al*., 2006). Changes in M-cadherin levels lead to changes in fusion; specifically the down-regulation of M-cadherin causes inhibition, while up-regulation of M-cadherin results in an enhanced fusion process (Zeschnigk *et al*., 1995). Interestingly however, mice lacking M-cadherin can develop normal skeletal muscle suggesting that other cadherins (e.g. E-cadherin) can compensate for the lack of M-cadherin *in vivo* (Hollnagel *et al*., 2002).

1.2.3. Self-renewal of satellite cells

It has been suggested that a functional pool of stem cells is maintained throughout life via self-renewal, as the steady-state number of the satellite cells does not change between adulthood and old age in mice (McGeachie and Grounds, 1995). This observation was further supported by the maintained stability of telomeres in satellite cells from young and old human muscle (Decary *et al*., 1997; O'Connor *et al*., 2009). Interestingly, although the satellite cell population is constantly replenished, contradictory studies have shown a decline in satellite cell numbers and reduced proliferation capacities during aging in mice and humans (Bischoff, 1994, Seale and Rudnicki, 2000; Brack *et al*., 2005). Although the molecular mechanism for self-renewal is not well understood, it has been suggested that the asymmetric division of satellite cells gives rise to a committed myogenic progenitor and a 'repopulating self' (Jan and Jan, 1998). On the other hand, Myf5-expressing satellite cells which do not upregulate MyoD may co-exist in regenerating muscle and thus allow the replenishment of the satellite cells (Sabourin *et al*., 1999).

1.3. Signalling pathways regulating myogenesis

There are many known growth factors and cytokines that induce satellite cell activation. These signaling molecules play an important role in regulating skeletal muscle development. For example, the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway positively regulates myogenic differentiation. It involves activation by insulin and insulin-like growth factors (IGFs) which stimulate the terminal differentiation of myoblasts. This is supported by studies that showed IGF-1 promotes embryonic skeletal muscle development (Lui *et al*., 1993). In addition, another study showed that the PI3K/Akt pathway is crucial and mediates the stimulatory effect of IGFs on myogenin expression during muscle differentiation (Xu and Wu, 2000). Furthermore, increased levels of IGF-1 result in an increase in muscle mass due to augmentations in muscle protein and DNA content (Charge and Rudnicki, 2004). In the absence of the IGF-1 receptor signaling, mice die due to incomplete development of muscle (hypoplasia) and respiratory failure due to muscular weakness (Trennery *et al*., 2001; Rehfeldt *et al*., 2009).

The JAK–signal transducer and activator of transcription (STAT) pathway represent one of the best-characterized cellular signaling pathways. It is well established that the JAK–STAT pathway plays essential roles in haematopoiesis (O'Shea *et al*., 2002). Several lines of evidence suggest that the JAK–STAT pathway may have a role in myogenic differentiation. In regenerating rat muscles, proliferating myoblasts were found to contain higher levels of phosphorylated (i.e. active) STAT3 (Kami and Senba, 2002). Another study using murine models further demonstrated that JAK1–STAT1–STAT3 signaling interactions are not only required for myoblast proliferation, but that they also serve as checkpoints to prevent premature differentiation (Sun *et al*., 2007). Furthermore, knockdown of *JAK1* in primary and immortalized myoblasts induced differentiation accompanied by a reduction in cell proliferation (Kami and Senba, 2002; Sun *et al*., 2007; Trennery *et al*., 2011).

The p38 MAP kinases have also been implicated in cell differentiation. Of the four isoforms of p38 (α, β, γ, δ), the phosphorylation and activity of α and β isoforms are gradually induced during the differentiation of myoblasts (Wu *et al*., 2000; Xu *et al*., 2002). A C2C12 cell line expressing a dominant negative form of the p38 activator (MKK3) failed to form myotubes following 4 days of differentiation, suggesting a key role for p38 in differentiation (Cabane *et al*., 2003). In this study, the inhibition of cell cycle and myogenic markers (p21 and MyoD) and disorganization of the cytoskeleton of the differentiated cells was demonstrated. Using phalloidin staining, the structural muscle proteins myosin and actin were seen to be at much lower levels in the transformed compared to control cells (Cabane *et al*., 2003).

During the fusion of myocytes into multinucleated myotubes, cell cycle activators such as cyclins and cyclin-dependant kinases (CDKs) are down-regulated, whereas cell cycle inhibitors such as the retinoblastoma susceptibility gene (Rb) and the proteins, p21, p27, and p57 are up-regulated. In particular, Rb and p21 trigger the transition to subsequent phases of the cycle and have also been shown to play an important role in the growth arrest of differentiating myoblasts (Kitzmann *et al*. 2001; van den Heuvel, 2005). M-calpain has been shown to be important in the cytoskeletal reorganization during myoblast fusion. M-calpain belongs to a family of calcium-dependent intracellular non-lysosomal cysteine proteases (Charge and Rudnicki, 2004; Colby-Germinario *et al*., 2004). M-calpain activity was observed to increase during fusion *in vitro* and conversely, fusion was prevented by inhibiting its activity with calpastatin, a specific inhibitor of m- and μ-calpains (Dedieu *et al*., 2002). A potential target of M-calpain is the intermediate filament desmin. Both M-calpain and desmin are present as fusion begins. It has been shown that desmin concentration decreases as myoblasts fuse. M-calpain could be involved in myoblast fusion via

desmin cleavage and thus positively facilitate differentiation (Elamrani *et al*., 1995; Dourdin *et al*. 1999 and Keren *et al*., 2006).

1.4. Skeletal muscle repair and maintenance

Following muscle injury, satellite cells are also stimulated by extracellular components and growth factors such as hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and transforming growth factor-β (TGF-B) to proliferate, migrate along the basement membrane to the site of injury, differentiate and either fuse with existing fibers or fuse to form new fibers *de novo*, thereby regenerating damaged or degenerating fibers (Grounds and Yablonka-Reuveni, 1993; Bischoff, 1994). It is important to note that there are two main forms of muscle injury, namely *in situ* necrosis, which is a localized injury and shear injury, which implies local deformity of the muscle tissue (Ehrhardt and Morgan, 2005). *In situ* necrosis involves the rapid cell death of the skeletal muscle fiber and may be triggered in muscle myopathies. The basement membrane remains intact. Organelles are degraded and the injury produces an inflammatory response in which neutrophils rapidly invade, followed by macrophages (Jarvinen *et al*., 2007). Fibre degeneration with subsequent influx of leucocytes into the damaged area predominates in the first few days. Regeneration begins once the phagocytic inflammatory cells clear necrotic tissue (Hill *et al*., 2003). This phase of muscle remodeling is characterized by activation of undifferentiated skeletal muscle precursor cells or satellite cells. Cell adhesion molecules, for example M-cadherin have been shown to be expressed in myoblasts and on myotubes during the regeneration process (Irintchev *et al*. 1994; Qu-Petersen *et al*., 2002).

Shear injury, on the other hand, is a more severe type of muscle injury that results in the tearing or breaking of the basement membrane. The interstitial ECM components then enter the muscle fiber and initiate the wound repair process (Jarvinen *et al*., 2007). Additionally, following muscle tissue injury, fibroblasts enter the wound and synthesize collagen and elastin thus playing a critical role in wound healing. They differentiate into contractile and secretory myofibroblasts that enter the damaged muscle tissue and contribute to muscle repair during wound healing. It is important to note that increased fibrosis can severely impair muscle function and ECM protein secretion can become excessive, as observed during scarring (Hinz *et al*., 2007; Wynn, 2008).

Satellite cells mediate skeletal muscle tissue growth and function to repair and revitalize muscle tissue upon injury or due to heavy bouts of exercise For effective restoration of structure and function during repeated injury, the population of quiescent satellite cells is replenished by the process of self-renewal as described earlier (section 1.2.3) (Le Grand and Rudnicki, 2007). A number of diseases cause skeletal muscle tissue damage (Mann *et al*., 2011). Muscle atrophy (muscle wasting) can result from lack of muscle movement and use, in which case it is called

disuse atrophy. It is often caused by being bedridden or diseases such as rheumatoid arthritis and involves damage to the peripheral nervous systems. When the nerves are damaged, the most common symptoms are muscle weakness and muscle wasting associated with pain and eventual muscle loss. Muscle dystrophy on the other hand involves the degeneration of muscle due to genetic defects (Gomes *et al*., 2001; Owczarek *et al*., 2005; Wang *et al*., 2010). DMD is a disease of concern that is characterized by muscle weakness and pain, muscle wasting and eventual muscle loss. This disease arises due to the absence of dystrophin- protein that binds muscle cells, keeping them intact (Mann *et al*., 2011).

Acquired immunodeficiency syndrome (AIDS), especially in the later stages of the disease, can result in skeletal muscle wasting (Dudgeon *et al*., 2006). Prior to the introduction of antiretrovirals (ARVs), weight loss and tissue wasting were commonly observed complications of HIV/AIDS. The AIDS wasting syndrome is defined to be the involuntary loss of more than 10% of a person's body weight, especially muscle, accompanied with excessive nausea and diarrhea for more than 30 days (Dworkin and Williamson, 2003). Interestingly, increasing numbers of studies are showing that adverse side effects of ARVs themselves can be implicated in the causes of muscle tissue wasting. ARVs may cause defects in muscle, preventing normal cellular repair and replacement, and eventually causing autoimmunity, muscle wasting and death (Lewis *et al*., 1992; Colby-Germinario *et al*., 2004; Oliverio *et al*., 2005; Scruggs and Dirks Naylor, 2008). We speculate that long-term treatment with ARVs may decrease the myogenic capacity of satellite cells, leading to a decrease in maintenance and repair, and eventual loss of muscle mass.

1.5. Antiretroviral (ARV) therapies

A number of ARV therapies are used to treat human immunodeficiency virus (HIV) infection, but sadly, to date, none can cure HIV/AIDS and those individuals undergoing treatment have to do so for life. The introduction of highly active antiretroviral therapy (HAART), a treatment paradigm using three or more ARVs in combination, has led to a significant decline in HIV-associated mortality (Ledergerber *et al*., 1999; Porco *et al*., 2004; McMahon *et al*., 2009). The goals of ARV therapy include the maximal and durable suppression of HIV replication, restoration and preservation of immune function, improvement in quality of life and ultimately, the reduction in HIV-related morbidity and mortality (Carr and Cooper, 2000).

1.5.1. Classes of ARVs

ARVs are broadly classified by the phase of the HIV cycle that they inhibit (Bean, 2005; Sungkanuparph, 2010). Each class of ARVs interrupts HIV replication at a different point in the viral cycle (Figure 1.4). The classes of ARVs are listed as follows:

- Nucleoside reverse transcriptase inhibitors (NRTIs)
- Nucleotide reverse transcriptase inhibitors (NtRTIs)
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
- Protease inhibitors (PIs)
- Entry inhibitors (EIs)
- Integrase inhibitors

The first antiretroviral drug to be licensed, Zidovudine, became available in 1987 (Arts and Hazuda, 2012; WHO (Management of infection): accessed November 2012). Since then, numerous ARVs have been introduced. The U.S Food and Drug Administration (FDA) (2011) indicate that there are currently 35 ARVs used in the treatment of HIV. As already outlined, patients with HIV infection usually receive combinations of ARVs, and these combinations include compounds from more than one group or class, often two nucleoside analogue reverse transcriptase (RT) inhibitors and one non-nucleoside analog or protease inhibitor (Velasco-Hernandez *et al*., 2002). The rationale for combination ARV therapy is the same in adults and children: to target at least two points in the viral cycle. ARV drug combination therapies defend against resistance by suppressing HIV replication as much as possible (Gulick *et al*., 1997; Velasco-Hernandez *et al*., 2002). In addition, ARV regimens need to be as safe, effective, tolerable, stable and as convenient as possible. Thus, there are on-going studies to determine the best combinations of different ARV classes (Velasco-Hernandez *et al*., 2002).

1.5.2. ARVs mechanism of action

HIV is an enveloped retrovirus that contains two copies of viral genomic RNA in its core (Figure 1.4). In addition to the copies of RNA, the viral core also contains three enzymes required for HIV replication; a reverse transcriptase, integrase, and protease. The first step in the HIV replication cycle is the interaction between the envelope proteins of the virus (gp14 and gp120) and specific surface receptors (e.g. CD4 receptor) of the host cell (Figure 1.4). This binding causes the chemokine co-receptors, CXCR4 or CCR5 of the host cell, to interact with the CD4 receptor, resulting in conformational changes in the viral envelope proteins (Figure 1.4). This ultimately allows in the fusion of the viral envelope and the host cytoplasmic membrane. Fusion creates a pore through which the viral capsid enters the cell. Following entry into the cell, the viral reverse transcriptase enzyme catalyses the conversion of viral RNA into DNA (Figure 1.4). This viral DNA enters the nucleus and becomes inserted into the chromosomal DNA of the host cell (integration). This process is facilitated by the viral integrase (Figure 1.4). Expression of viral genes then leads to production of precursor viral proteins. These proteins and viral RNA are assembled at the cell surface into new viral particles and leave the host cell by a process called budding. During budding, they acquire the outer layer and envelope. At this stage, the protease cleaves the precursor viral proteins into their mature products (Figure 1.4). If this final phase of the replication cycle does not take place, the released viral particles are immature and non-infectious and thus fail to initiate the replication cycle in other susceptible cells. (Lapadat-Tapolsky *et al*., 1993; Emerman, 1996; Warnke *et al*., 2007; Arts and Hazuda, 2012).

The life cycle of HIV can be as short as 36 hours, from the moment the virus enters the cell, through replication, assembly and release of more viral particles. HIV lacks proof reading enzymes to correct errors made when its RNA is transcribed into DNA by reverse transcription. Thus, its short life cycle and high error rate cause it to mutate rapidly, resulting in the high genetic variability of HIV (Boyer *et al*., 1992).

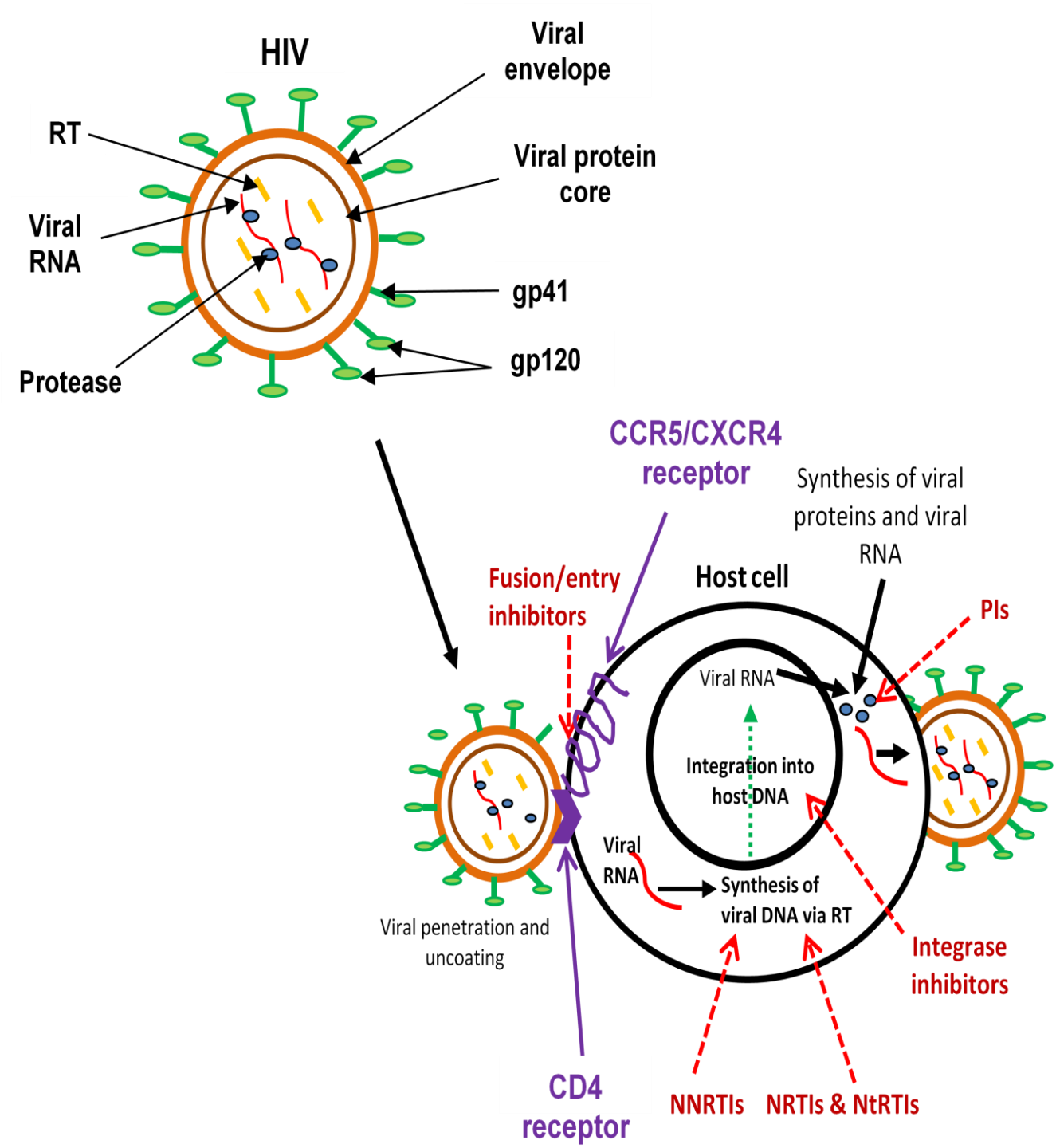


Figure 1.4 Mechanism of action of antiretrovirals. HIV enters the host cell. The viral envelope proteins, gp41 and gp120 initially interact with host cell receptors CD4, which then interacts with CXCR4 or CCR5 co-receptor, to allow fusion of the viral envelope to the host membrane. Once inside the host cell, HIV replication occurs. ARVs target specific stages of HIV replication. The viral attachment (fusion/entry) to the host cell can be inhibited by using drugs that either mimic the virus or the host cells receptors, causing the virus to attach to the drugs instead of the cell or prevent attachment to host cell. Viral replication can be inhibited by non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs and NtRTIs) and integrase inhibitors. During the assembly and maturation stage, protease inhibitors (PIs) prevent cleavage of the viral proteins thereby inhibiting the maturation phase of the virus. Dotted red lines indicate point of action of specific ARV. [Diagram adapted from literature in section 1.5.2, Lapadat-Tapolsky *et al.*, 1993; Arts and Hazuda, 2012].

During viral attachment to the host cell, viral proteins need to bind to receptors and proteins on the surface of the host cell. If that link cannot be established, the virus will not enter the cell. This attachment can be prevented by using a drug that mimics the host cells receptors thereby preventing viral attachment (e.g. Maraviroc and Enfuvirtide) (Table 1.2) (Briz *et al*., 2006).

There are three main ways in which the viral replication can be interrupted: via polymerase inhibitors, integrase inhibitors and RT inhibitors (Table 1.2) (Lapadat-Tapolsky, *et al*., 1993). Nucleoside analogues that inhibit the RT, such as Zidovudine, are the reference ARVs in HIV/AIDS infected patients (Table 1.2). These compounds are analogues of naturally occurring deoxyribonucleotides (e.g. thymidine) needed to synthesize the viral DNA and they compete with the natural deoxyribonucleotides for incorporation into the growing viral DNA chain. The analogues become phosphorylated and incorporated into both viral and host nuclear and mitochondrial DNA (Poirier *et al*., 2004; Oliverio *et al*., 2005). The phosphorylated drugs incorporate into nascent DNA chains and fail to form 5'-3' phosphodiester bonds, as they lack the necessary moieties required for DNA chain extension; DNA elongation is consequently terminated (Bean, 2005; Lewis *et al*., 1992). Zidovudine has been used in the treatment of cancer as most malignant cancers express the enzyme telomerase, which has reverse transcriptase activity; thus it is also prone to the inhibitory effects of Zidovudine (Scrugg and Dirks Naylor, 2008). Interestingly, satellite cells that maintain the muscular system display robust telomerase activity. Conversely, telomerase activity rapidly declines upon differentiation of satellite cells into myoblasts, and remains relatively high in old muscle stem cells. NRTIs may therefore affect satellite cell number via telomerase activity (O'Connor *et al*., 2009). Tenofovir, is an acyclic nucleotide analog with potent *in vitro* and *in vivo* antiretroviral activity and its lipophilic prodrug, Tenofovir disoproxil fumarate, is an approved oral agent for treatment of HIV infection (Cihlar *et al*., 2002). Nucleotide analogues such as Tenofovir (Table 1.2) would most likely have a similar mechanism of action to Zidovudine as they both inhibit the RT enzyme. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) also inhibit RT, but act by binding non-competitively to the enzyme (Table 1.2). The binding causes a conformational change in the structure of the enzyme and affects the catalytic activity of the enzyme thereby blocking the HIV-1 replication by inhibiting the active site (De Clercq, 1998).

Some ARVs target the maturation and release stage. New viral proteins need to be packaged into the viral coat. The mature viral particles are activated and the viral copies are released from the host cell. Viral proteins are generated in long strips that are digested by the protease enzyme (Lapadat-Tapolsky, *et al*., 1993; Warnke *et al*., 2007). Thus protease inhibitors such as Ritonavir (Table 1.2), would prevent the cleaving of the viral proteins.

Table 1.2: Classes of approved ARVs and their mechanisms of action (Gulick, 1997; Warnke *et al*., 2007)

Class of ARV	Examples	General Mode of Action
Nucleoside Reverse Transcriptase Inhibitors (NTRIs)	***Zidovudine (AZT)** Lamivudine (3TC) Emtricitabine (FTC) Stavudine (d4T)	Incorporated into the viral DNA. Competes with nucleosides/ nucleotides and prevents transcription of viral RNA to DNA.
Nucleotide Reverse Transcriptase Inhibitors (NtRTIs)	***Tenofovir (TDF)**	Same as NTRIs
Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Efavirenz (EFV), Nevirapine (NVP) Etravirine Rilpivirine	Prevent HIV production by binding directly onto RT (non-competitively) and preventing transcription.
Protease Inhibitors (PI)	***Ritonavir (RTV),** Lopinavir (LPV) Amprenavir (APV) Indinavir (IDV)	Bind to viral protease and prevent correct cleavage of viral proteins. Prevents successful assembly of HIV virus and its release from infected cells
Fusion/Entry Inhibitors	Maraviroc (MVC) Enfuvirtide (T-20)	Bind to gp41 region of HIV and prevent virus-cell fusion
Integrase Inhibitors	Raltegravir	Prevent the integration of viral DNA into the DNA of the infected cell.

*** ARVs used in current study**

Maturation inhibitors are currently being investigated and are aimed at preventing HIV from proper assembly, maturation, protective outer coat formation and emergence from human cells. Inhibiting this step would be an entirely new way of halting or inhibiting HIV replication. There are currently no Food and Drug Administration (FDA) approved HIV maturation inhibitor drugs available (Salzwedel *et al*., 2007). Vivecon is a maturation inhibitor which is in Phase II of clinical trials in antiretroviral-naive HIV-positive patients. Previous trials have found that Vivecon may be effective for those resistant to NNRTIs and PIs ([#]Aidsmap website, HIV Treatments Directory, 2011).

Among currently available ARVs, the first-line ARV therapy option to be optimized and used for adults includes Tenofovir[$]/Lamivudine/Efavirenz (Table 1.2) or Tenofovir[$]/Emtricitabine/ Efavirenz in fixed-dose combination. For children, heat-stable formulations of Lopinavir/Ritonavir (Table 1.2), dispersible tablets of Zidovudine/Lamivudine and tablets of a dispersible fixed dose of Tenofovir/Lamivudine/Efavirenz combination are the first-line formulations used. Progressive phasing out of more toxic drugs, such as Stavudine (Table 1.2) are underway (WHO (New progress and guidance on HIV treatment), 2010). Recently, Tenofovir has been proven effective in protecting woman from HIV infection. The Centre for the AIDS Program of Research in South

[#]Available at: http://www.aidsmap.com/resources/treatmentsdirectory/A-to-Z-of-investigational-drugs/page/1331192/
[$]Zidovudine is often substituted for Tenofovir

Africa (CAPRISA) 004 trial studied the usefulness and safety of a 1% vaginal gel formulation of Tenofovir. They found HIV infection to be reduced by 39% (Karim *et al*., 2010). Tenofovir (NtRTI), Zidovudine (NRTI) and Ritonavir (PI) were the selected ARVs for the current study. Their structures can be seen in Figure 1.5. The structures of Zidovudine and Tenofovir enable them to be incorporated into the HIV DNA. Ritonavir on the other hand prevents correct cleavage of viral proteins by binding to the viral protease. Table 1.3 summarizes the plasma peak concentrations (C_{max}) of the ARVs used in the current study.

Table 1.3: Peak plasma concentrations (C_{max}) of Tenofovir, Zidovudine and Ritonavir

ARV Drug	**Recommended Dose (mg)**	**C_{max} (µM)**	**Literature Reference**
Tenofovir (NtRTI)	300 (daily)	0.8 - 1.3	Barditch-Crovo *et al*., 2001
Zidovudine (NRTI)	600 (daily)	1.5 – 10;	Langtry *et al*., 1989
Ritonavir (PI)	600 (twice daily)	0.4 - 20	Lorenzi *et al.*, 1997;Chittick *et al.*, 2006;

A

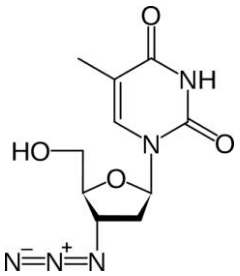


Figure 1.5 (A) Zidovudine (NTRI) (Weight = 267.2413) is a dideoxy-nucleoside compound in which the 3'-hydroxy group on the sugar moiety has been replaced by an azido group. This modification prevents the formation of phosphodiester linkages which are needed for the completion of nucleic acid chains. The compound is a potent inhibitor of HIV replication, acting as a chain-terminator of viral DNA during reverse transcription (Drug Bank website, 2012 [Available at www.drugbank.ca/]).

B

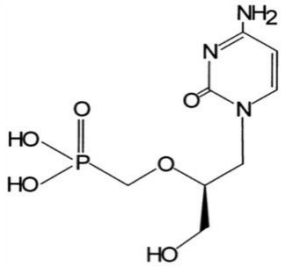


Figure 1.5 (B) Tenofovir (NtRTI) (Weight = 287.2123**)**, a dideoxy-nucleotide analogue reverse transcriptase inhibitor which blocks reverse transcriptase, the enzyme crucial to viral production in HIV-infected people. It does this by competing with the natural substrate deoxy-adenosine 5'-triphosphate and, like Zidovudine, it lacks a 3'-hydroxyl group on the deoxy-ribose moiety, resulting in DNA chain termination (Drug Bank website, 2012 [Available www.drugbank.ca/]).

C

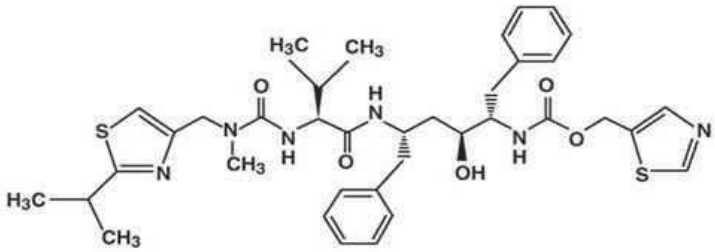


Figure 1.5 (C) Ritonavir (PI) (Weight = 720.944) blocks the HIV-1 protease by binding to the protease active site thus inhibiting the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles (Drug Bank website, 2012 [Available at www.drugbank.ca/]).

1.6. Theories underlying the effect of ARVs on skeletal muscle

Significant reductions have been observed in rates of death and suffering following potent ARV regimen implementations. Despite this, prolonged use of ARVs has a number of side effects. Some of these complications include patients experiencing muscle pain, muscle damage and loss of muscle mass (Carr and Cooper, 2000). Although it is known that myopathy is present in advanced HIV/AIDS, it is necessary to acknowledge the association of myopathy with ARV treatment itself (Scruggs and Dirk Naylor, 2008). The proposed mechanisms by which ARVs may affect muscle mass are discussed below.

1.6.1. Effect of ARVs on Mitochondrial Toxicity

Some ARV medications are thought to affect muscle due to excessive production of lactate (lactic acidosis). Mitochondria, are the "power-houses" of cells, converting oxygen, fat and sugars to produce energy (ATP) during cellular respiration. The highest numbers of mitochondria are found in nerve, liver and muscle cells. Mitochondrial toxicity arises due to damage that decreases the number of mitochondria. One of the most common signs of mitochondrial toxicity is muscle weakness (myopathy). The muscles fail to obtain sufficient energy and consequently have to obtain their energy anaerobically. This ultimately creates lactic acid as a waste product which then causes muscle pain. Thus, lactic acidosis results when people with mitochondrial toxicity have high lactic acid levels in their blood. (Carr and Cooper, 2000; Foli *et al*., 2001).

Mitochondria contain the enzyme DNA polymerase gamma (DNA pol gamma) that is solely responsible for mitochondrial DNA (mtDNA) replication. This enzyme is very similar to the RT enzyme found in the HIV virus in that they both function to synthesize DNA. This unfortunately means that the ARVs used to inhibit viral RT can also inhibit DNA pol gamma (Yarchoan *et al*., 1989). Consequently, few mitochondria are produced, less energy is available and, for muscle cells specifically, this could be the underlying reason as to how myopathy develops due to long-term ARV treatment. Studies have shown that mitochondrial toxicity can cause muscle weakness in individuals prescribed with Zidovudine (Brinkman *et al*., 1998; Masanes *et al*., 1998). Patients with Zidovudine-induced myopathy suffer from progressive muscle pain and weakness and this is reflected by increased serum concentrations of creatine kinase (an indicator of muscle damage) (Scruggs and Dirks Naylor, 2008). Morphological changes in mitochondria, cytochrome *c* oxidase deficiency as well as reductions in mtDNA have been detected in muscle tissue from HIV-infected patients with Zidovudine-induced myopathy (Birkus *et al*., 2002). Furthermore, Zidovudine can reduce the capacity of the respiratory chain in mitochondria without depleting mtDNA (Martin *et al*., 1994; Birkus *et al*., 2002). Other studies on mitochondrial function of human muscle cells

exposed to Zidovudine have yielded mixed results. Mitochondrial dysfunction was reported to be detectable when skeletal muscle cells from HIV/AIDS patients were exposed to concentrations similar to those observed in the serum of Zidovudine receivers (Table 1.3) (Lamperth *et al*., 1991). Another group failed to demonstrate any dysfunction until exposure to a concentration of 5 mM, 1000 times higher than the ID_{50} dose of 5 μM (Gallicchio *et al*., 1992; Herzberg *et al*., 1992). No decrease of mtDNA content and no mtDNA deletions in Zidovudine-exposed muscle cells was noted. It was proposed that the effect of Zidovudine-induced myopathy may result from a decrease in proliferation of muscle cells, rather than inhibition of mtDNA replication (Herzberg *et al*., 1992).

Tenofovir on the other hand, is less likely to be associated with mitochondrial toxicity than other NRTIs (Birkus *et al*., 2002). Uptake of Tenofovir (3 μM-300 μM) into HepG2 human liver cells is very low (100-fold) compared to the lipophilic oral prodrug, Tenofovir disoproxil (0.3 μM and 3 μM) (Birkus *et al*., 2002). Similar results were observed using primary human skeletal myoblasts at the same dose of Tenofovir and Tenofovir disoproxil after 9 days of proliferation (Birkus *et al*., 2002). Interestingly, the proliferating skeletal muscle cells did not show any decrease in mtDNA levels following 9 days exposure to 300 μM Tenofovir or Zidovudine. These results suggest that the potential for Tenofovir to interfere with mitochondrial function is low. The *in vitro* cytotoxicity of Tenofovir in HepG2 human liver cells versus human skeletal muscle cells has been compared with other NRTIs (Zidovudine, Zalcitabine, Lamivudie and Didanosine) (Cihlar *et al*., 2002). Tenofovir, following 8-day incubation with proliferating HepG2 cells, showed a weak inhibition of the cell growth as indicated by 50% cytotoxicity concentration (CC_{50}) of 399 μM. In comparison, the CC_{50} of Zidovudine was 57 μM. In skeletal myoblasts, Tenofovir, with CC_{50} of 870 μM (CC_{50} of Zidovudine was 497 μM), also showed weak inhibition of cell growth after 6 days incubation. These studies provide evidence of the low *in vitro* cytotoxicity of Tenofovir; this is consistent with the tolerability profile of Tenofovir in patients infected with HIV/AIDS (Cihlar *et al*., 2002).

Previous studies have indicated that the effect of therapeutic PIs on mitochondrial function could contribute to lipodystrophy syndrome. It was suggested that a lack of cleavage of mitochondrial proteases could result in non- or dysfunctional mitochondrial proteins (Mukhopadhyay *et al*., 2002). Interestingly, the PI, Ritonavir, may have beneficial effects on mitochondrial function in skeletal muscle (Miro *et al*., 2006). This was suggested following a study on patients whose ARV drug combinations were changed from one NRTI, to an Enfuvirtide/Tenofovir/Saquinavir/Ritonavir combination over 24 weeks. Their study suggests that Ritonavir (used as a booster drug in combination with Saquinavir) may have resulted in improvements on mitochondrial functionality. In summary, mitochondrial toxicity, which is associated with a decrease in the number of mitochondria leading to muscle weakness, may be caused by NRTIs.

1.6.2. Effect of ARVs on cell cycle genes

As outlined earlier, the mode of action of Tenofovir and Zidovudine involves the termination of DNA synthesis following their incorporation into newly synthesized DNA. On a cellular level, the inhibition of DNA synthesis caused by chain termination consequently prevents effective cell cycle regulation (Barditch *et al*., 2001; Bean, 2005).

Studies using human epithelioid carcinoma (HeLa) cells report that when Zidovudine (125 µM-500 µM) is incorporated into nuclear DNA, it induces an up-regulation of cyclin D1. This is accompanied by a down-regulation of cyclin D1-associated inhibitors (p18 and p57) and the G1-S checkpoint gene, p21, and ultimately causes an arrest of cells in the S-phase. A consistent decrease in the percentage of cells in the G0/G1 phase accompanied the Zidovudine-induced accumulation of cells in the S-phase following 24 hour treatment with Zidovudine (Oliverio *et al*., 2005). This would result in decreased proliferation.

Earlier studies compared the effects of Zidovudine on proliferation, differentiation and lipid accumulation in cultured human muscle cells (Benbrika *et al*., 1997). It was observed that after 6 and 10 days exposure (0-5000 µM for Zidovudine and 0-1000 µM for Didanosine and Zalcitabine) there was a time-dependent decrease in proliferation as the ARV drug concentration increased**.** No change in differentiation of the myoblasts into myotubes at day 2 and day 4 was detected, but differentiation was decreased after 6 days exposure to >200 µM Zidovudine. A progressive increase in lipid droplet accumulation was also observed in the myoblasts after 10 days (Zidovudine 5000 µM). As previously discussed (section 1.2) elevated levels of fat were demonstrated in Myf5/MyoD double null mice mutants, suggesting the possibility that some cells had adopted an adipogenic fate (Braun *et al*., 1992; Yun and Wold, 1996). Zidovudine may possibly alter the gene products of Myf5 and MyoD; hence the decrease in myogenic differentiation and increase in adipogenesis. An interesting observation made by Masanes *et al.* (1998) was that muscle atrophy persisted long after Zidovudine treatment was withdrawn, however it was ultimately reversible (Miro *et al*., 1997).

Tenofovir has recently been shown to cause loss of bone mineral density during HIV/AIDS treatment (Grigsby *et al*., 2010). Tenofovir (50 nM to 500 µM) was analyzed for its effect on *in vitro* primary osteoblast viability. The highest doses of Tenofovir (50 µM, 250 µM and 500 µM) were found to significantly reduce cell viability. The lower doses however, (50 nM, 500 nM and 5 µM) had no effect on cell viability (Grigsby *et al*., 2010). This gives an indication of the effect that Tenofovir might have on the viability of muscle cells. More recently, Truvada, an HIV reverse transcriptase inhibitor, has been shown to induce DNA damage and cell cycle arrest in HeLa cells

(Bruning *et al*., 2012). Truvada is a combination of the adenosine analog Tenofovir as well as the cytidine analog Emtricitabine (NRTI). Thus, it is suggested that Tenofovir affects gene expression and induces cell cycle arrest in human cancer cells. The combination of both drugs caused the greatest effect by blocking cell clone formation of the Hela cells and strongly interfered with the morphology and genomic integrity of the cells. Severe DNA damage by high drug concentrations (>100 µM) directly results in the induction of apoptosis. Truvada was observed to induce apoptosis and cause accumulation of cell cycle-promoting proteins such as cyclin B and phosphocdk1. Cyclin B1 is a regulatory protein involved in mitosis and is expressed during G2/M phase of the cell cycle; its accumulation would decrease proliferation (Bruning *et al*., 2012). Interestingly, anti-tumor activity was predominantly caused by Tenofovir (100 µM).

1.6.3. The effect of ARVs on proteinases and their role in reducing muscle mass

Protease activity is required for myotube formation during myoblast differentiation. Calpains are calcium-dependent cysteine proteases that are found in all mammalian cells. The *in vitro* differentiation of rat muscle cells (L6 myoblasts) requires the activities of M-calpain (Colby-Germinario *et al*., 2004) and inhibition of calpain reduces myotube formation (Grounds and Yablonka-Reuveni, 1993; Barnoy *et al*., 1996). Published *in vitro* data suggests an effect on rat and mouse myoblast fusion by Ritonavir (10 µM and 20 µM) and Indinavir (10 µM and 20 µM) (Colby-Germinario *et al*., 2004). In addition, Ritonavir (10 µM and 20 µM) decreased calpain activity in myoblasts *in vitro*. Compared to control cultures, L6 and C2C12 myoblast models did not fuse to form myotubes following treatment with both protease inhibitors. This data suggests that ARV protease inhibitors may contribute to initiating or maintaining muscle wasting by reducing myoblast fusion into myotubes (Colby-Germinario *et al*., 2004).

Lysosomal endopeptidases are also believed to play an important role in protein catabolism. They consist of cysteine proteinases such as cathepsins B, H, L as well as the aspartic proteinase cathepsin D (Takio *et al*., 1983; Dufour *et al*., 1987). Differentiation of L6 and C2 cell lines have also been shown to require the activities of cathepsin B, H and L (Colella *et al*., 1986; Colby-Germinario *et al*., 2004). The presence of cathepsins within muscle fibers can be quantified by histochemical and immunohistochemical studies, although at very low levels (Stauber and Ong, 1982). It has been demonstrated that the activities of lysosomal cysteine proteinases increase during myogenic differentiation (McElligott and Bird, 1981; Kirschke *et al*., 1983). Interestingly, the HIV protease is an aspartic protein and so Ritonavir targets aspartic proteases, including for instance cathepsin D.

Figure 1.6 summarizes the reported effects of Zidovudine, Tenofovir and Ritonavir on the different stages of myogenesis. Zidovudine studies were carried out in human muscle cells (Herzberg *et al*., 1992; Benbrika *et al*., 1997), whereas the effect of Tenofovir was analyzed on HeLa cells Bruning *et al*., 2012). To date, no studies on the effect of Tenofovir on differentiation of muscle cells have been published. Ritonavir has been observed to decrease fusion of C2C12 myoblasts (Colby-Germinario *et al*., 2004).

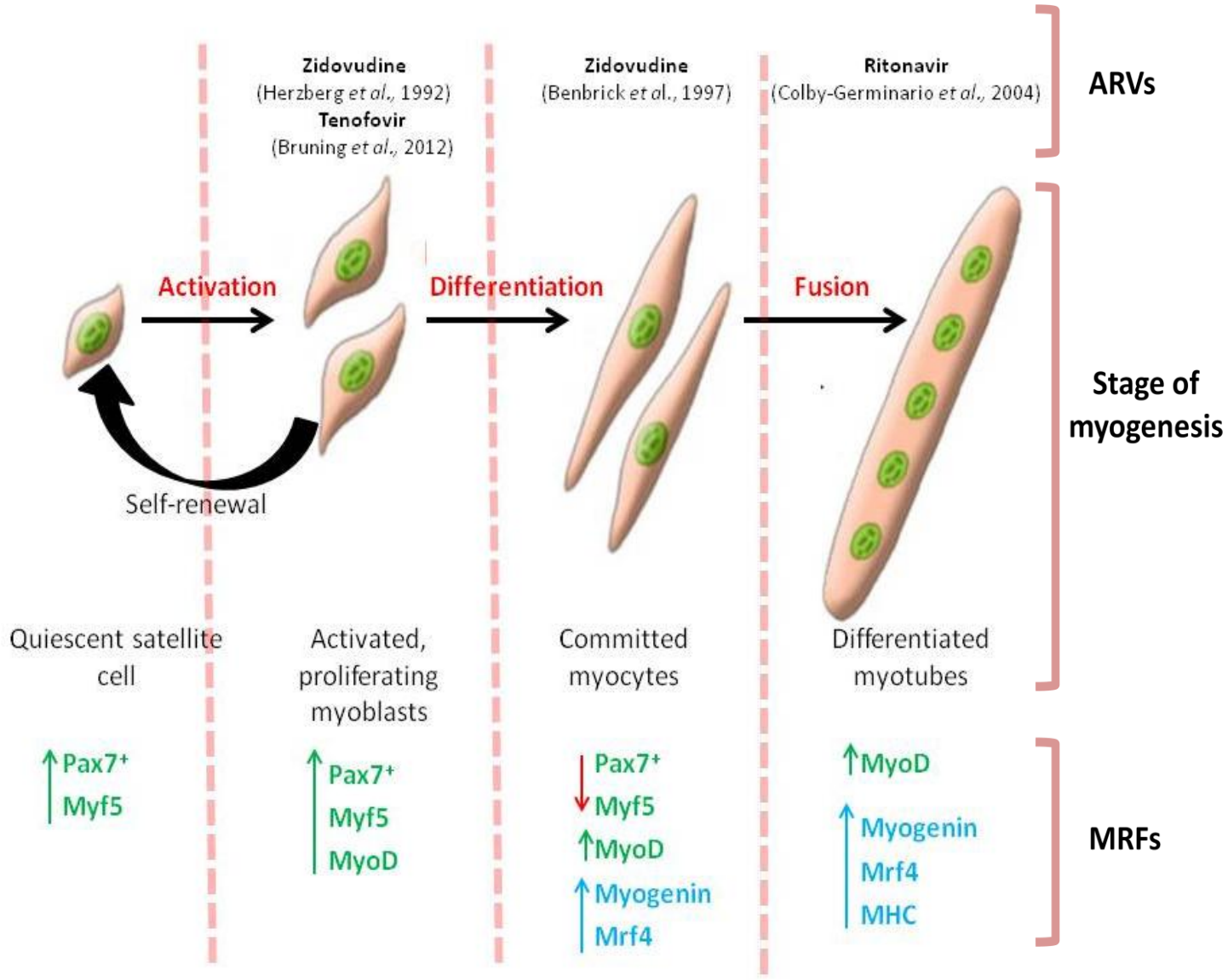


Figure 1.6: Negative effects of Zidovudine, Tenofovir and Ritonavir on myogenesis. The effects of the ARVs on myogenesis, as reported in the literature (section 1.6.3) are shown. Zidovudine and Tenofovir reduce proliferation of Hela and human muscle cells respectively; Zidovudine decreases differentiation in human muscle cells and Ritonavir reduces fusion of rat myoblasts.

1.7. Aims of study

Based upon the observations of ARV drug-associated reduction in muscle mass we sought to further explore the basis for this clinical observation. The objective of the current study was therefore to determine the effect of Zidovudine, Tenofovir and Ritonavir, on processes required for successful myogenesis, including:

- proliferation of skeletal myoblasts
- migration during wound repair
- commitment to differentiation and subsequent myocyte fusion into myotubes

C2C12 mouse myoblasts and human primary culture myoblasts (HSk) were used as the muscle stem cell models. The ARV drug concentrations (range: 0.01 μM to 10 μM) were physiologically relevant according to the peak plasma levels (Table 1.3).

CHAPTER 2:

GENERAL METHODS AND MATERIALS

The following general methods chapter provides additional information to the experimental protocols employed in Chapters 3 and 4, Section 3.2 and 4.2 respectively. It expands on methodologies used in the results chapters and is referred to in Chapter 3 and 4.

2.1 Cell Culture

2.1.1. Murine skeletal myoblast cell culture

The C2C12 murine myoblasts (donated by Professor Anna-Mart Engelbrecht, Department of Physiological Sciences, University of Stellenbosch) were used as the main muscle model cell line, representing activated satellite cells. The C2C12 cells, a diploid sub-clone of the C2 cell line (Blau *et al.*, 1985) are capable of proliferating, differentiating and fusing into multinucleated myotubes.

C2C12 myoblasts were cultured and maintained in growth media which contained 90% Dulbecco's Modified Eagle's Medium (DMEM, Highveld, cat. CN3726), supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco, cat. 10500), 2% (v/v) Penicillin-Streptomycin (PenStrep, LONZA, cat. DE17-602E) and 2% (v/v) L-Glutamine (LONZA, cat. 6230). Media was changed every 48 hours. Cells were maintained in a humidified incubator (Innova CO-170) at 37^{o}C and 5% CO_2 and passages 11 - 16 were used in all experiments.

2.1.2. Human skeletal myoblast primary culture

The human skeletal (HSk) myoblasts (Lonza, cat. CC2561) were used as a model of activated human satellite cells. The HSk myoblasts were cultured and maintained in growth media containing 80% HAMS F10 media (Gibco, cat. 31550-015), supplemented with 20% FBS, 2% (v/v) PenStrep, 2% L-Glutamine and basic fibroblast growth factor (FGF, Promega, cat. G5071, 2.5 ng/ml). Media was replenished every 48 hours and cells were maintained in a humidified incubator (Shel lab) at 37^{o}C and 5% CO_2. Passages 1 - 4 were used in all experiments. Primary culture HSk myoblasts were observed to take at least two days to adhere to culture flasks prior to proliferation.

Subsequent experimental protocols were repeated a minimum of three times (n = 3) to verify the results obtained, to refine the experimental observations and to generate acceptable data. Each 'n' was performed in duplicate or in triplicate.

2.1.3. Induction of Differentiation

To induce differentiation of C2C12 and HSk myoblasts into myotubes, cells were grown to 80% confluence in their respective growth media. Media was then changed to differentiation media containing DMEM or HAMS F10 (for C2C12 and HSk cells respectively), supplemented with 1% (v/v) Horse serum (Invitrogen, cat. 16050-130), 2% PenStrep and 2% L-Glutamine. Cells were allowed to differentiate for 7 days.

2.2. Preparation of Antiretrovirals (ARVs)

The antiretrovirals (ARVs) were donated and obtained in powder form from Professor Tahir Pillay of the College of Health Sciences, UKZN. Stability data on drugs in solution indicated that the study drugs, Tenofovir (a nucleotide reverse transcriptase inhibitor, NtRTI) and Zidovudine (a nucleoside reverse transcriptase inhibitor, NRTI) were soluble in PBS, whereas Ritonavir (a protease inhibitor, PI) was soluble in DMSO. Stock concentrations (50 mM) were made up and diluted in their respective media to the working concentrations of 0.01 µM, 0.1 µM, 1 µM and 10 µM, for all experiments. Media containing the ARVs was made up fresh for each experiment. Normal growth media ± 0.001% DMSO acted as controls for all activation and migration experiments, whereas normal differentiation media ± 0.001% DMSO was used as controls for all differentiation experiments.

2.3. Proliferation of Myoblasts: Crystal Violet Assay

Crystal violet is a dye that intercalates double stranded DNA of viable cells. Following 24 hour treatment with the ARVs (0.01 µM - 10 µM) the cells were washed with PBS (5 minutes). Methanol (Sigma, cat. 24229) was required to fix the cells; 0.2 % (w/v) crystal violet (Sigma, cat. C-3886) was made up in methanol and used to simultaneously fix and stain the cells for 10 minutes (Figure 2.1). The solution was then removed and the cells carefully washed three times with dH_2O and allowed to air dry. Stained cells were solubilised for 10 minutes in 1% SDS (Merck, SAAR5823610EM) and 100 µl of the solubilised solution was transferred to a new 96-well plate. The absorbance of the solubilised solution was measured at 595 nm using the FLUOoptima micro 96-well plate reader. 1% SDS was used as the blank control. Experiments were done in duplicate.

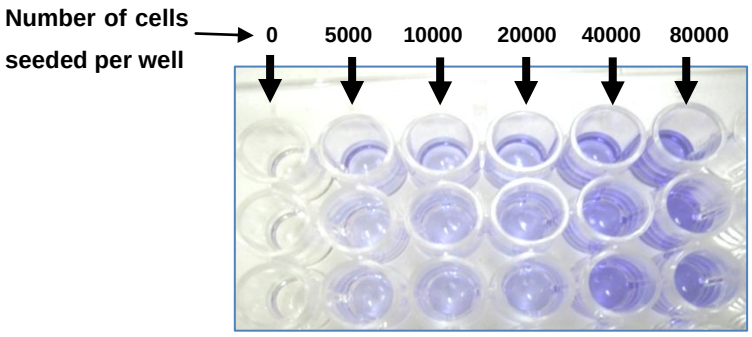


Figure 2.1 Crystal violet assay. A 96-well plate showing that with an increase in cell number, an increase in crystal violet dye staining was observed.

2.4. Migration of myoblasts: 2D *in vitro* wound healing assay

To assess the effects of the ARVs on wound closure during muscle repair, migration of C2C12 and HSk myoblasts was assessed over a 7 hour period.

Cells were pre-treated with ARVs for 16 hours prior to wounding. "Wounds" were created by scratching the confluent monolayer of cells using a sterile 200 µl loading tip. Debris was washed off with sterile PBS and media containing ARVs was added. The cells were then incubated for 7 hours and wound images taken at 0, 4 and 7 hours, using a Motic 3.0 megapixel camera (40X magnification, Olympus CKX41). Experiments were done in duplicate. Percentage wound closure was determined by tracing an outline of the 'wounded' area (Figure 2.2) using the Motic 2.0 image analysis software, and calculating the area of the wound at the specific time intervals.

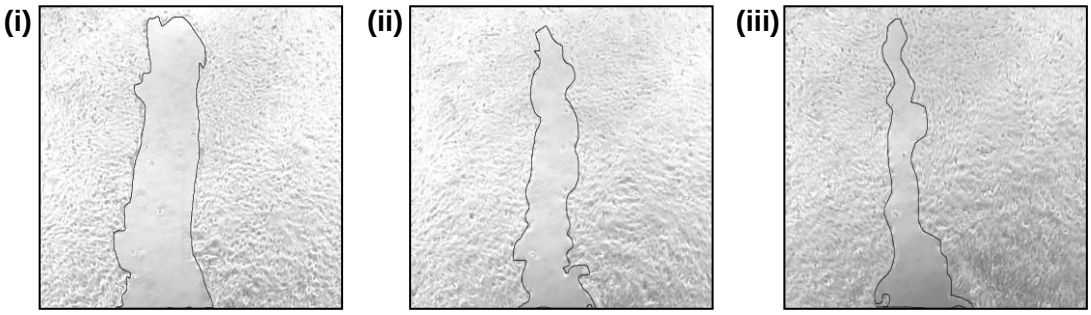


Figure 2.2: Representative images illustrating wound closure observed over 7 hours due to migrating C2C12 myoblasts. (i) 0 hours, (ii) 4 hours and (iii) 7 hours. The wound healing assay mimics the process whereby activated myoblasts respond to injury, and migrate to facilitate wound repair.

The percentage wound closure was determined using the equation below:

$$\frac{\text{[Wound area (0 hrs) – Wound area (}n\text{ hrs)]}}{\text{Wound area (0 hrs)}} \times 100 = \%\ \text{Wound Closure}$$

The rate of migration was also determined from the 0-4 hour and 4-7 hour period, by calculating the line of best fit for each experiment. The rate was represented as percentage wound closure per hour.

2.5. Differentiation of myoblasts

2.5.1. Immunocytochemistry: Pax7 and Myosin Heavy Chain

To determine the percentage $Pax7^+$ cells and fusion index (via MHC staining), cells were treated with ARVs (1 µM) and then briefly washed with PBS (5 minutes) and fixed with 4% paraformaldehyde for 15 minutes. Following 2 x 5 minute washes with PBS, the cells were then washed in 5% (v/v) donkey serum (Sigma, cat. D9663) for 1 hour, at room temperature, to block non-specific binding of the antibodies. The cells were then incubated with specific primary antibodies: mouse monoclonal anti-MHC (Hybridoma Bank, MF-20; 1:200 dilution) and mouse monoclonal anti-Pax7 (Abcam, cat. ab55494; 1:500 dilution) (Table 2.1) overnight at room temperature followed by wash steps with PBS (5 x 6 minutes). The cells were then incubated in the dark at room temperature with their specific fluorescent-tagged secondary antibodies: Dylight488 donkey anti-mouse (Jackson, cat. 715-515-151; 1:800 dilution) and Dylight594 donkey anti-mouse (Jackson, cat. 715-485-151; 1:1000 dilution) (Table 2.2) for 1 hour. This was followed by a careful wash with PBS (5 x 6 minutes) before adding the nuclear stain, Hoechst (10 mg/ml, SIGMA, cat. B2661; 1:2000). Prior to mounting the coverslips onto glass slides using movial (6 ul), the cells were washed again with PBS (4 x 5 minutes) and were then ready to be viewed. Images of the specimens were captured with the Zeiss LSM 710 NLO ConforCor 3 confocal microscope and analyzed using the Zen 2009 Light Edition and ImageJ software. Experiments were done in duplicate.

Table 2.1 Optimized primary antibodies and dilutions used for confocal microscopy

Primary Antibody	Size (kDa)	Host Species	Dilution	Supplier
Pax7	~57	Mouse	1:500	Abcam
MHC	~200	Mouse	1:200	Hybridoma Bank

Table 2.2 Optimized secondary antibodies and dilutions used for confocal microscopy

Secondary Antibody	Host Species	Dilution	Supplier
Dylight488 anti-mouse	Donkey	1:800	Jackson
Dylight594 anti- mouse	Donkey	1:1000	Jackson
Hoechst 33342 (nuclear stain)	-	1:2000	Sigma

PBS was used to dilute all antibodies to their optimal concentrations.

2.5.2. Total Protein isolation

C2C12 and HSk cells were allowed to differentiate for 7 days in the presence of ARVs (1 µM). The cells were trypsinized and pelleted for 5 minutes at a speed of 100 x *g*. The supernatant was discarded. The pellets were resuspended and washed in cold PBS (2 x 5 minutes) prior to lysing for 1 hour in the presence of 1 µl protease inhibitor cocktail (PIC[#], Sigma, cat.P8340), and 100 µl Radioimmunoprecipitation assay (RIPA[##)] buffer for cell lysis (pH 8, Sigma, cat. R0278), in order to release the proteins. The amounts of PIC and RIPA buffer added were adjusted to compensate for cell number variation. The cells were further ruptured by a sonication process set at a speed of 13 on the VirSonic 60 machine.

[#]PIC consists of 104 mM AEBSF-[4-(2-Aminoethyl)benzenesufonyl fluoride hydrochloride], 80 µM aprotinin, 4 mM bestatin hydrochloride, 1.4 mM E-64-[N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide], 2 mm leupeptin hemisulfate salt and 1.5 mM pepstatin A

[##]RIPA buffer consists of 50 mM Tris-HCl, 150 mM sodium chloride, 1 % Igepal CA-630, 0.5 % sodium deoxycholate and 0.1 % sodium dodecyl sulfate (SDS)

2.5.3. Bradford protein assay

The Bradford assay is a colorimetric protein assay based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 (Sigma, cat. B-0770); under acidic conditions (perchloric acid, 2 % w/v) the red form of the dye is converted into a blue form that binds to the protein being assayed. This assay was used to determine the soluble protein concentrations of the control and ARV-treated samples.

A standard curve was generated using bovine serum albumin (BSA, 1 mg/mL, Roche, cat. 10 735 086 001) as the standard protein. This involved mixing diluted aliquots of BSA with PBS and the Coomassie dye solution (SIGMA, cat. B0770) (Table 2.3), incubating the mixture for 2 minutes and determining the absorbance at 595 nm using a spectrophotometer (Ultrospec II E, LKB Biochrom). To determine protein concentrations of experimental samples, 5 µl of each sample was mixed with 95 µl PBS and 900 µl Coomassie solution, absorption was measured and compared to the standard curve (Figure 2.3). All sample measurements were carried out in triplicate.

Table 2.3 Bradford assay protocol

BSA standard protein (µl)	PBS (µl)	Coomassie dye solution(µl)
5	95	900
10	90	900
20	80	900
30	70	900
40	60	900

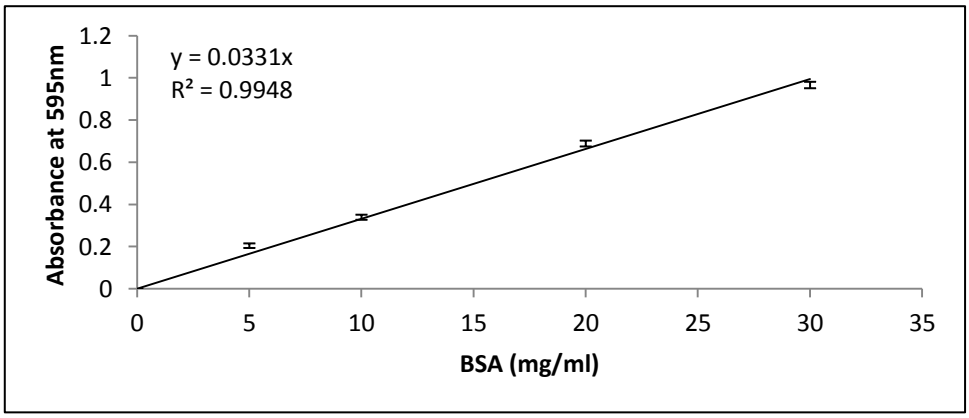


Figure 2.3 Bradford standard curve. The absorbance at 595 nm of diluted BSA standard protein from a 1 mg/ml stock solution

2.5.4. Protein separation by SDS-PAGE

The protein concentrations as determined by the Bradford assay described above (Section 2.5.3) were prepared for separation by denaturing them in an equal volume of reducing sample treatment buffer and boiling the mixture for 2 minutes. 30 µg of protein was loaded onto the gels alongside the molecular weight protein marker (Peqlab, cat. 27-2210). The protein samples were then resolved in a reducing 12.5% SDS polyacrylamide gel at 18 mA/gel. These gels were run using the BioRad electrophoresis unit (BioRad Mini-PROTEAN 3 Cell). To observe the separated proteins, the gels were stained with Coomassie G-250. Alternatively, the gels were used for Western blotting.

2.5.5. Western Blot Analysis

Western blotting involved the transfer (blotting) of separated proteins from the SDS-PAGE process described in section 2.5.4 onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences). The blotting process was carried out in the fridge ($4^{o}C$) for 16 hours at 200 mA. Prior to the blots (membranes) being probed with specific antibodies to detect the proteins of interest, the non-specific binding sites on the blots were first blocked for 1 hour using 5% (w/v) fat-free milk made up in PBS. The membranes were then subjected to incubation with mouse monoclonal anti-MHC primary antibody (Hybridoma Bank, MF-20; 1:200 dilution) overnight. The internal control used was rabbit monoclonal anti-GAPDH (Cell Signaling, cat. 2118; 1:4000 dilution). The membranes were subsequently washed in PBS and incubated in HRPO-linked rabbit anti-mouse secondary antibody (Dako, cat. P0260, 1:8000 dilution) or HRPO-linked goat anti-rabbit (Dako, cat. P0448) for 1 hour at room temperature. The primary and secondary antibodies and their respective dilutions are summarized in Table 2.4 and Table 2.5. Following further washes in PBS, the antibody-antigen protein complexes were detected using an enhanced chemiluminescence (ECL) kit (Immun-Star^{TM} Western C^{TM} Chemiluminescent Kit, BioRad) according to the manufacturer's instructions and images of the protein bands were captured using the SYNGENE G:Box Chemi-XR5 machine (Vacutec).

Table 2.4 Optimized primary antibodies and dilutions used for Western blotting

Primary Antibody	Size (kDa)	Host Species	Dilution	Supplier
MHC	~200	Mouse	1:200	Hybridoma Bank
GAPDH (Internal control)	~37	Rabbit	1:4000	Cell Signaling

Table 2.5 Optimized secondary antibodies and dilutions used for Western blotting

Secondary Antibody	Host Species	Dilution	Supplier
Polyclonal anti-mouse-HRP	Rabbit	1:8000	Dako
Polyclonal anti-rabbit-HRP	Goat	1:4000	Dako

2.6. Statistical analysis

All data was analyzed using the 14th Edition of the GenStat software program (VSN International). All parametric data was analyzed using the paired Student T-test. Non-parametric data was analyzed using the Mann Whitney-U test. Values of $p < 0.05$ were considered to be statistically significant in comparison to the controls. All data were represented as mean ± SEM.

CHAPTER 3:

EFFECTS OF ANTIRETROVIRALS ON THE PROLIFERATION AND MIGRATION OF C2C12 AND HSk MYOBLASTS

3.1 Introduction

Skeletal muscle has the remarkable ability to initiate a rapid and extensive repair process via satellite cell activation, ultimately preventing the loss of muscle mass. Notably, the proliferation of activated myoblasts provides a sufficient source of new myonuclei for muscle repair, allowing for new muscle fiber formation (Charge and Rudnicki, 2004). The early phase of muscle injury is usually accompanied by the activation of inflammatory cells resulting in macrophage infiltration, platelet aggregation and deposition of a fibrin clot at the site of injury (Friedl and Brocker, 2000). Activated satellite cells then migrate along the myofiber to the site of injury, where they proliferate and subsequently differentiate into myotubes. Activated satellite cells are characterized by expression of Pax7 and the primary MRFs, MyoD and Myf5 (Section 1.2.1), which promote their progression to terminal differentiation (Charge and Rudnicki, 2004). Signals that trigger migration of myoblasts to the site of injury during wound repair should be mutually exclusive with proliferation. Newly formed multinucleated myotubes fuse to the existing damaged myofiber or to other differentiated myotubes to facilitate wound repair (Peault *et al*., 2007; Mastroyiannopoulos *et al*., 2012). Myoblasts need to increase in number to facilitate efficient muscle regeneration; failure to do so would ultimately result in loss of muscle mass. Studies have shown that long-term ARV treatment can affect proper muscle development. Effects on proliferation and migration may to some extent explain the loss of muscle mass seen during long-term ARV treatment regimens.

ARVs are categorized based on their mode of action. Tenofovir and Zidovudine for example, are both reverse transcriptase inhibitors. They are analogues of the nucleotide, adenosine, and the nucleoside, thymidine, respectively. Thus, their mechanism of action involves competing with normal nucleotides/nucleosides for their incorporation into the HIV genome (Velasco-Hernandez *et al*., 2002; Warnke *et al*., 2007). Consequently, Tenofovir and Zidovudine prevent DNA synthesis and inhibit viral activity. On a cellular level, inhibition of DNA synthesis in myoblasts would affect proliferation. The expression and regulation of cyclin D1 and B1 have been shown to increase in human cells exposed to Zidovudine and Tenofovir respectively (Oliverio *et al*., 2005; Bruning *et al*., 2012), thereby decreasing their proliferation. Some ARVs are classified as protease inhibitors; these compounds inhibit the successful processing of proteins needed for a functioning virus. As

outlined in section 1.2.1.1, proteases are known to be required to facilitate extracellular matrix (ECM) remodeling for the migratory process of myoblasts (Dedieu *et al.*, 2003). Ritonavir is a protease inhibitor and may therefore influence the migratory process of myoblasts.

Skeletal muscle fibers are surrounded by a basement membrane composed of various proteins such as polysaccharides and proteoglycans that make up the skeletal muscle ECM (Section 1.2.1.1) (Friedl and Brocker, 2000; Gillies and Lieber, 2011). To access the injured site, satellite cells need to migrate either across the basement membrane and within the interstitial tissue surrounding the damaged myofibers (Section 1.2.1.1). This is predominantly regulated by proteases such as matrix metalloproteinases (MMPs) (Nishimura *et al*., 2008; Chen and Li, 2009). Furthermore, members of the calpain family which are also called calcium activated neutral cysteine proteases, are suggested to cleave myofibrillar proteins involved in cell adhesion such as, α-actinin, desmin and vimentin, aiding in cell migration (Dedieu *et al*., 2003). Myoblast migration has been shown to be drastically reduced when the expression of μ- and m-calpain is decreased. It is also important to note that these calpains are suggested to be localized at focal adhesion sites, which are specialized regions of the plasma membrane that allows cellular contact with ECM components (Dedieu *et al*., 2003).

To date, the effects of ARVs during muscle wound repair have not been investigated. We investigated the effects of three commonly used ARVs, Tenofovir, Zidovudine and Ritonavir, on the proliferation and migration of C2C12 and HSk myoblasts. To clarify the influence of ARV exposure on myoblast proliferation, we used the crystal violet assay, whilst changes in migration were investigated using the *in vitro* wound healing assay.

3.2. Methods and Materials

3.2.1. Proliferation of C2C12 and HSk myoblasts in response to ARVs

The effects of Tenofovir, Zidovudine and Ritonavir on the proliferation of activated myoblasts were investigated using the crystal violet assay as previously described (Section 2.3). A dose response was established at a range of 0.01 µM to 10 µM.

Standard curve: C2C12 and HSk myoblasts were seeded into 24-well plates at increasing cell densities: 5 000, 10 000, 20 000 40 000 and 80 000 cells/well. After 24 hours, the growth media was replenished with fresh media. Following a further 24 hours, cells were subjected to the crystal violet staining procedure described in section 2.3 and the results plotted in Figure 3.1. Experiments were done in duplicate.

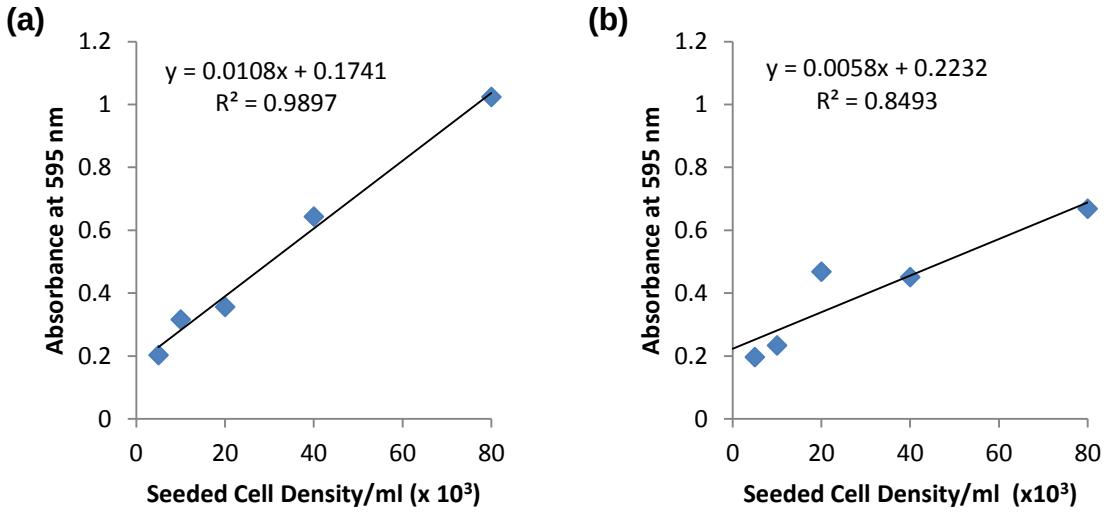


Figure 3.1: Crystal violet standard curves. The curves show the proportional increase of crystal violet absorbance against the increasing (a) C2C12 and (b) HSk cell number seeded.

ARV treatment: C2C12 cells were seeded in growth media at 20 000 cells/well in a 24-well plate for 24 hours. HSk cells were seeded at 40 000 cells/well in a 24-well plate, but were allowed to adhere for 2 days prior to treatment. The ARVs were then added to the media at their final working concentrations (0.01 µM - 10 µM) and cells allowed to proliferate in the treated media for a further 24 hours. Growth media ± 0.001 % DMSO was used as controls for all experiments. Cells were subjected to crystal violet staining (Section 2.3.) and cell number determined from the standard curve (Figure 3.1).

3.2.2. Migration of C2C12 and HSk myoblasts in response to ARVs

To determine the effects of Tenofovir, Zidovudine and Ritonavir on myoblast migration, a dose response was carried out (0.01 µM - 10 µM), using a 2D *in vitro* wound healing assay.

C2C12 and HSk cells were seeded at cell densities of 40 000 and 50 000 cells/well respectively and then pre-treated with the ARVs for 16 hours. Control cells were not pre-treated. "Wounds" were then created by scratching the confluent monolayer of cells (Section 2.4). The cells were incubated over a 7 hour period, in the presence and absence of the ARVs. The percentage wound closure was determined by calculating the area of the wound at 0, 4 and 7 hours (Figure 2.2 and Section 2.4), using the Motic 2.0 image analysis software. The rate of migration was also determined over a 0-4 hour and a 4-7 hour period (Section 2.4).

The flow diagram below (Figure 3.1.1) summarizes the basic outline of the proliferation and migration experimental protocols described above.

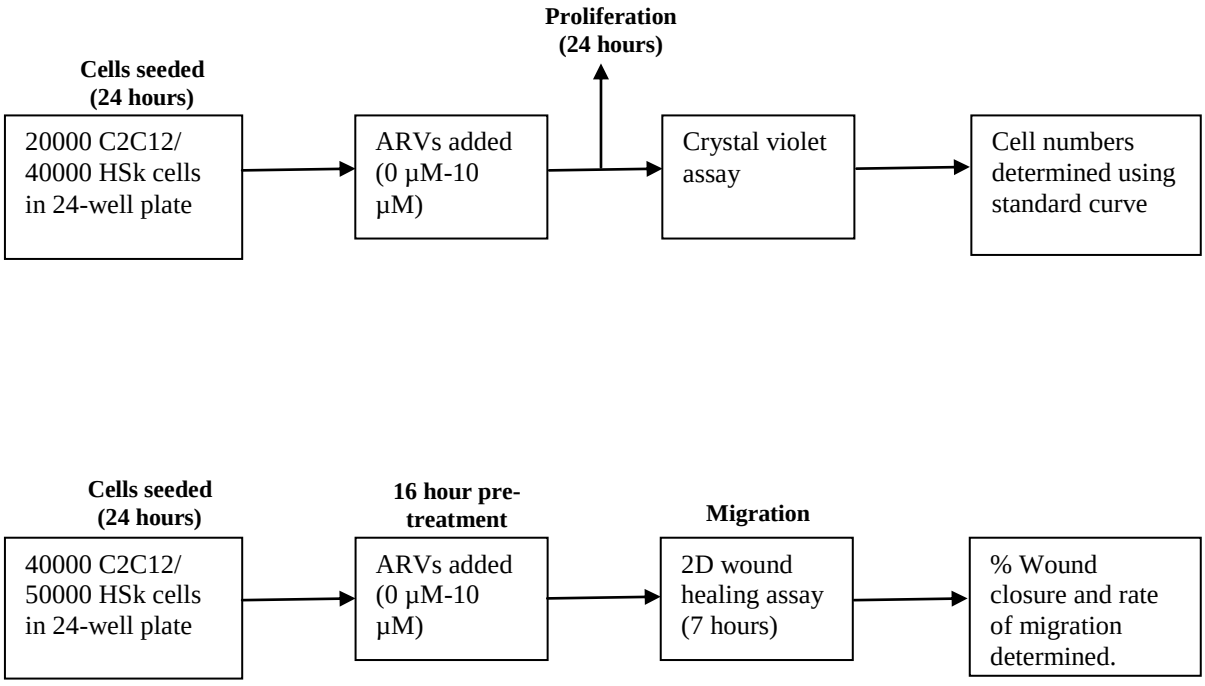


Figure 3.1.1 Flow diagram illustrating the experimental protocols used to investigate the effect of ARV drugs on myoblast proliferation and migration.

3.3. Results

3.3.1. Tenofovir has differential effects on C2C12 versus HSk myoblast proliferation

The crystal violet assay was used to determine the effect of the ARVs on myoblast proliferation. Tenofovir was found to have a differential effect on the proliferation of C2C12 versus HSk myoblasts. It induced a small, but significant increase in C2C12 cell number compared to control at 1 µM and 10 µM doses (140 ± 11.9% and 130 ± 9.18% respectively, $p < 0.05$) (Figure 3.2A) and induced a small, but significant decrease in HSk cell number at 1 µM (90 ± 1.21%, $p < 0.05$) (Figure 3.2B).

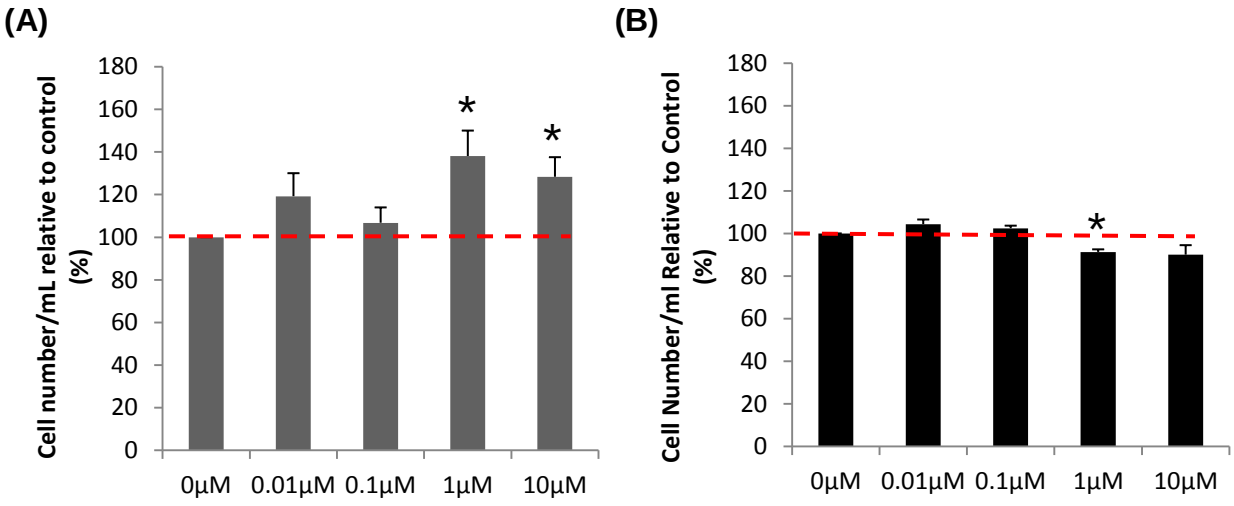


Figure 3.2 Dose response of Tenofovir on (A) C2C12 myoblast and (B) HSk myoblast proliferation. The crystal violet assay was used as described in section 2.3. Crystal violet intercalates and stains DNA of cells. The intensity of staining is directly proportional to the number of cells and hence, the amount of DNA. Cell numbers were determined from the standard curve (Section 3.2). Cell number was expressed as a percentage relative to control. Tenofovir induced significant effects on myoblast proliferation. [*$p < 0.05$, n=6 and n=4 for C2C12 and HSk myoblasts respectively (each experimental repeat was performed in duplicate). Data = Mean ± SEM]. The red dotted line indicates 100%.

3.3.2. Zidovudine does not significantly influence myoblast proliferation

Zidovudine appeared to have no consistent effect on C2C12 myoblast proliferation (Figure 3.3A). A decrease in proliferation was observed at 0.01 µM and 0.1 µM for the HSk myoblasts (Figure 3.3B). No change was observed at higher doses in comparison to the control (Figure 3.3B). Unfortunately any differences observed in response to incubation of myoblasts with Zidovudine were not significant.

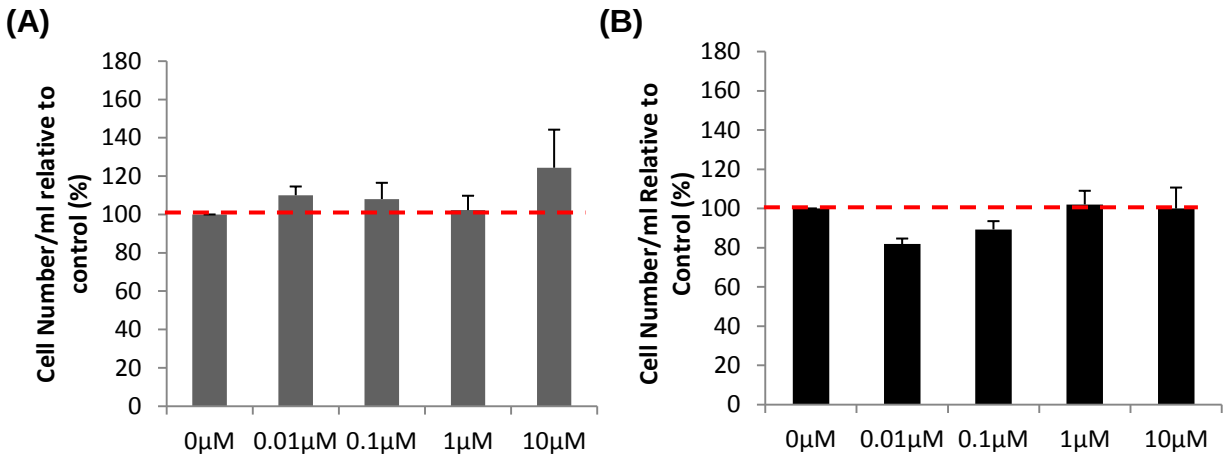


Figure 3.3 Dose response of Zidovudine on (A) C2C12 myoblast and (B) HSk myoblast proliferation. The crystal violet assay was used as described in section 2.3. Crystal violet intercalates and stains DNA of cells. The intensity of staining is directly proportional to the number of cells and hence, the amount of DNA. Cell numbers were determined from the standard curve (Section 3.2). Cell number was expressed as a percentage relative to control. [n=6 and n=4 for C2C12 and HSk myoblasts respectively (each experimental repeat was performed in duplicate). Data = Mean ± SEM]. The red dotted line indicates 100%.

3.3.3. Ritonavir does not significantly increase myoblast proliferation

Ritonavir did not induce any statistically significant change in cell number for either C2C12 (Figure 3.4A) or HSk myoblasts (Figure 3.4B). However, a marginal increase was observed in C2C12 and HSk cell number with increasing doses of Ritonavir, from 0 µM to 10 µM (Figure 3.4).

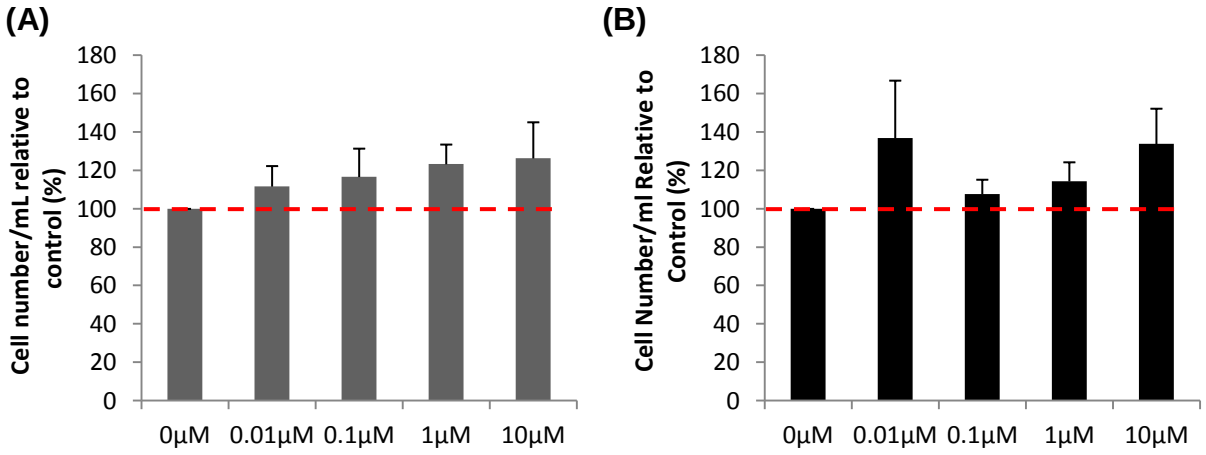


Figure 3.4 Dose response of Ritonavir on (A) C2C12 myoblast and (B) HSk myoblast proliferation. The crystal violet assay was used as described in section 2.3. Crystal violet intercalates and stains DNA of cells. The intensity of staining is directly proportional to the number of cells and hence, the amount of DNA. Cell numbers were determined from the standard curve (Section 3.2). Cell number was expressed as a percentage relative to control. [n=6 and n=4 for C2C12 and HSk myoblasts respectively (each experimental repeat was performed in duplicate). Data = Mean ± SEM]. The red dotted line indicates 100%.

3.3.4. Tenofovir has a dose and time-dependent effect on HSk myoblast migration

Using the *in vitro* wound healing assay, myoblast migration was analyzed at 4 and 7 hours; rate of migration was analyzed at 0-4 hour and 4-7 hour time period, post-wounding. There was a slight increase in the percentage wound closure at 0.01 µM Tenofovir (20 ± 4.22% to 25 ± 5.82%), followed by a slight decrease at 10 µM (20 ± 4.22% to 17 ± 3.95%) compared to control (Figure 3.5A and B). This was observed at both time intervals (Figure 3.5B). These changes were however statistically non-significant. A similar trend was observed upon analysis of the rate of migration from 0-4 hours [Figure 3.5C(i)]. However, from 4-7 hours, only the 10 µM dose maintained its negative effect on the rate of migration [Figure 3.5C(ii)].

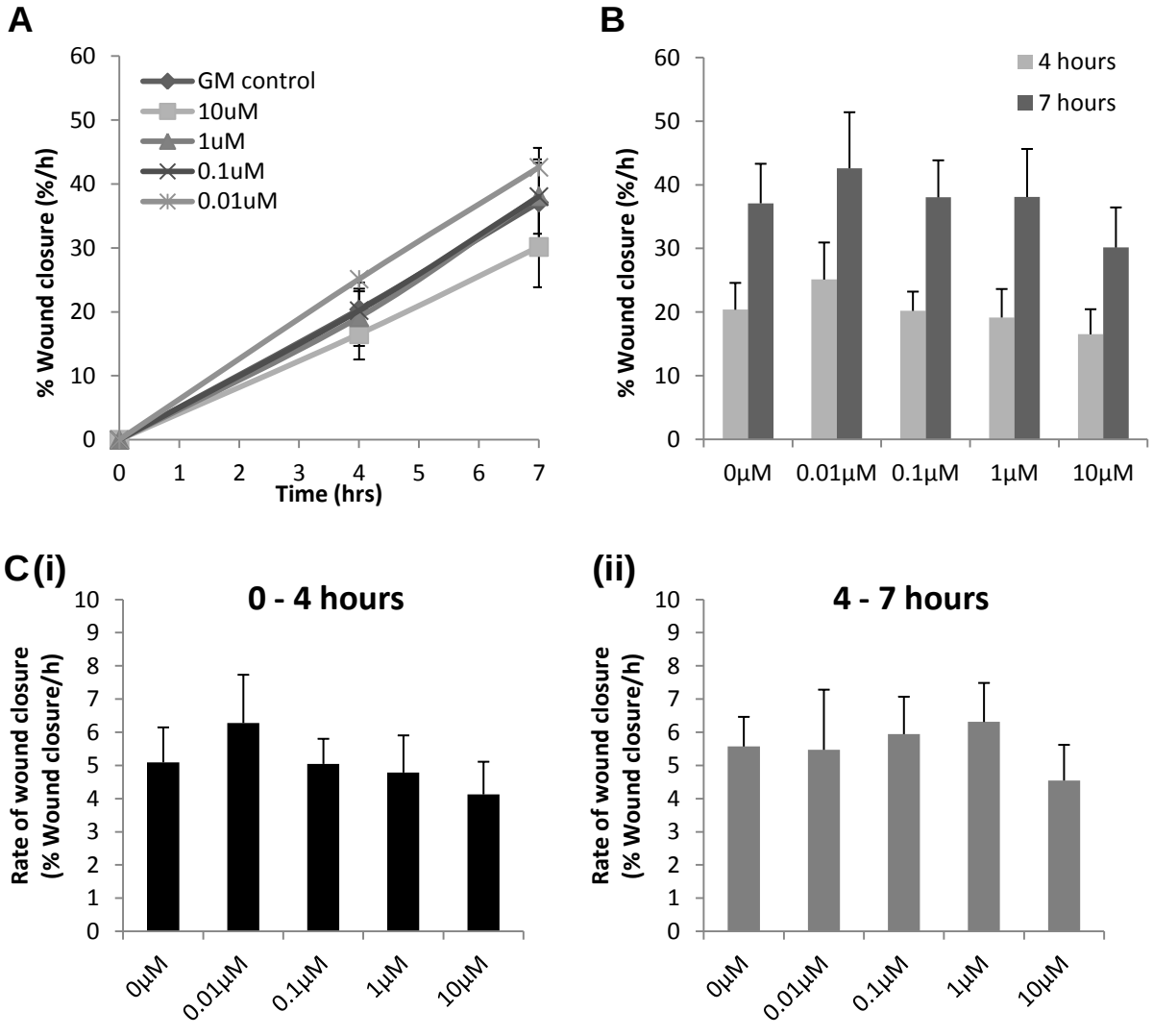


Figure 3.5 Dose response of Tenofovir on C2C12 myoblast migration. The scratch assay was used as described in section 2.4. The concentration of Tenofovir ranged from 0.01 µM - 10 µM. Growth media served as the control. **(A)** Percentage wound closure was determined at 4 and 7 hours as described in section 2.4. **(B)** Comparison of percentage wound closure at 4 versus 7 hours **(C)** The rate of migration at the time intervals **(i)** 0-4 hours versus **(ii)** 4-7 hours were compared. [n = 6 (each experimental repeat was performed in duplicate), Data = Mean ± SEM].

In HSk myoblasts 0.01 µM Tenofovir induced a marginal increase in percentage wound closure compared to control at 7 hours post-wounding (31 ± 7.20% to 36 ± 2.17%, $p<0.05$) (Figure 3.6B). A small, non-significant decrease in percentage wound closure at 10 µM dose at 4 hours post-wounding was observed compared to control 31 ± 7.20% to 27 ± 3.42% respectively) (Figure 3.6B). Following 7 hours, the decrease was still maintained and a decrease in wound closure was observed at 0.1 µM (Figure 3.6B). Analysis of rate of migration revealed different profiles during early (0-4 hours) and later (4-7 hours) migration (Figure 3.6A and C). At 0-4 hours, a small non-significant increase in percentage wound closure was observed at 0.1 µM (10 ± 2.77% to 16 ± 4.90%) and also at 1 µM (10 ± 2.77% to 13 ± 2.97%), compared to control [Figure 3.6C(i)]. At 4-7 hours however, a significant decrease ($p < 0.05$) in the rate of migration was observed in response to 0.1 µM Tenofovir [Figure 3.6C(ii)]. Interestingly, unlike in C2C12 cells, Tenofovir appears to cause a rapid increase in the rate of wound closure in HSk cells, between 4 and 7 hours compared to 0 and 4 hours [Figure 3.6A, C(i) and C(ii)].

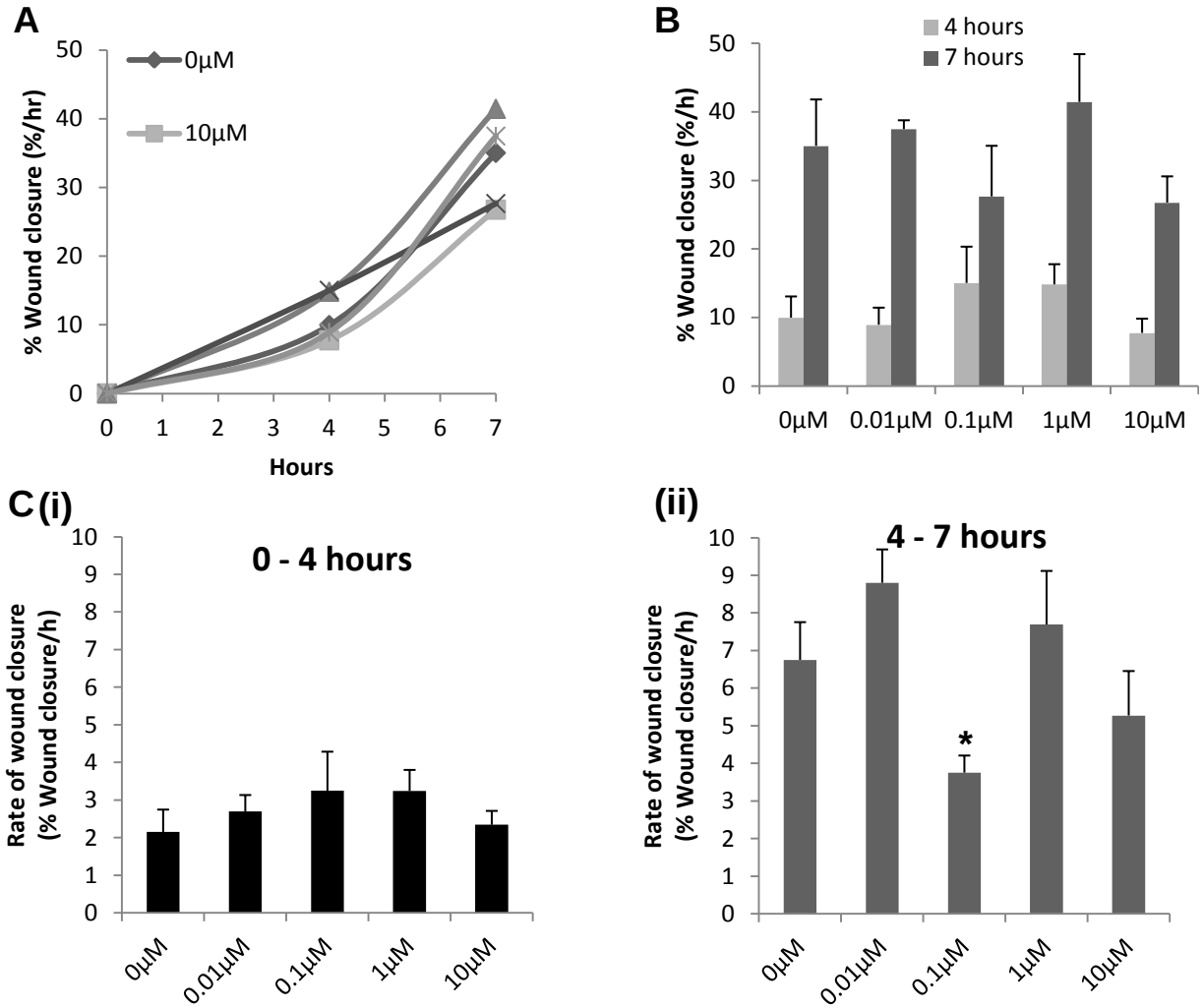


Figure 3.6 Dose response of Tenofovir on HSK myoblast migration. The scratch assay was used as described in section 2.4. The concentration of Tenofovir ranged from 0.01 µM - 10 µM. Growth media served as the control. **(A)** Percentage wound closure was determined at 4 and 7 hours as described in section 2.4. **(B)** Comparison of percentage wound closure at 4 versus 7 hours **(C)** The rate of migration at the time intervals **(i)** 0-4 hours versus **(ii)** 4-7 hours were compared. Tenofovir (0.1 µM) showed a significant decrease at the 4-7 hour interval ($p < 0.05$). [*$p < 0.05$, n = 5 (each experimental repeat was performed in duplicate), Data = Mean ± SEM].

This suggests not only a dose-dependent, but also a time-dependent effect of Tenofovir on myoblast migration.

3.3.5. Zidovudine significantly affects HSk myoblast migration but not C2C12 migration

Zidovudine had no effect on the percentage wound closure of C2C12 myoblasts (Figure 3.7A and B) or their rate of migration (Figure 3.7C) at all doses tested, compared to the control.

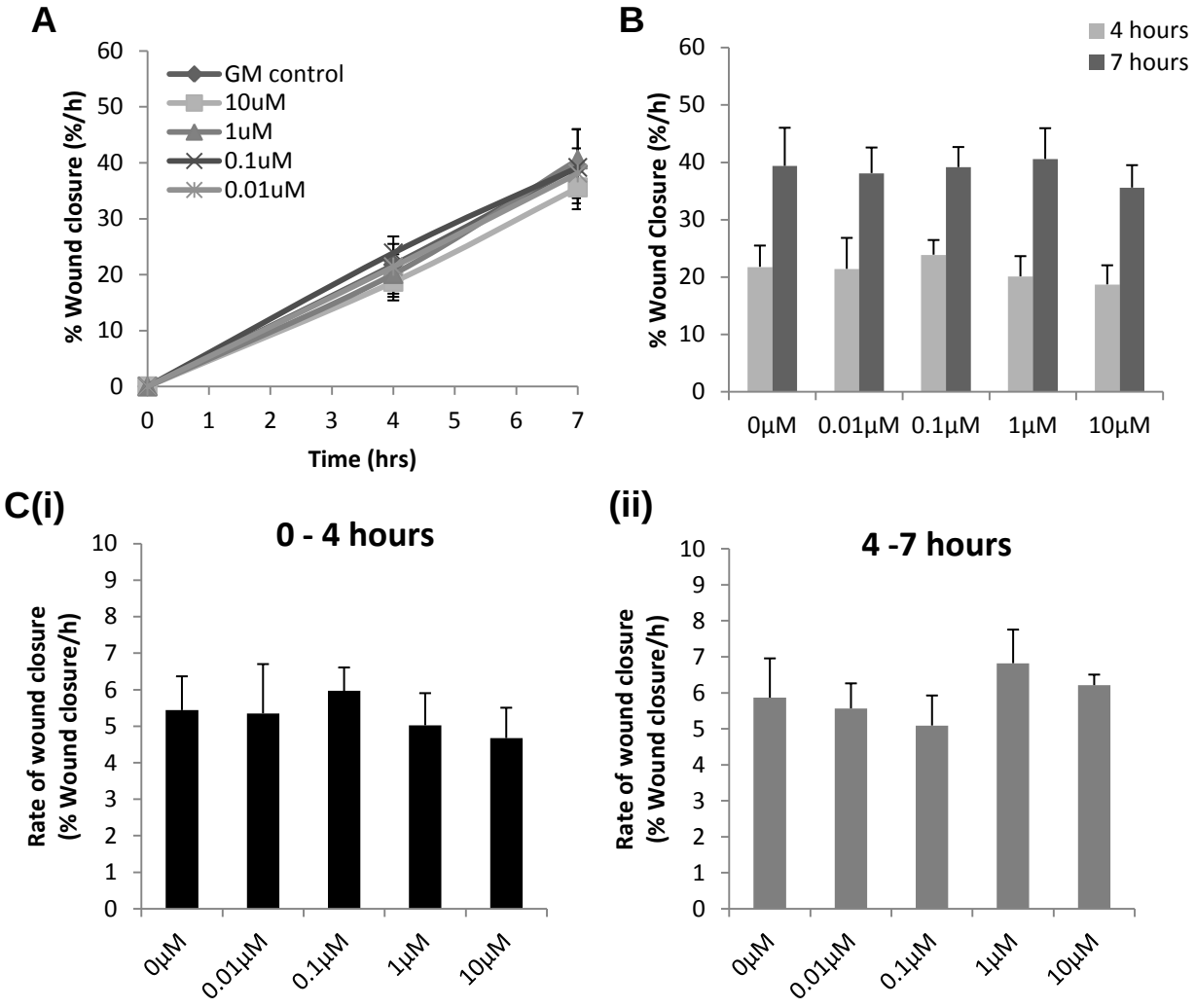


Figure 3.7 Dose responses of Zidovudine on C2C12 myoblast migration. The scratch assay was used as described in section 2.4. **(A)** The concentration of Zidovudine ranged from 0.01 µM - 10 µM. Growth media served as the control. **(A)** Percentage wound closure was determined at 4 and 7 hours as described in section 2.4. **(B)** Comparison of percentage wound closure at 4 versus 7 hours **(C)** The rate of migration at the time intervals **(i)** 0-4 hours versus **(ii)** 4-7 hours were compared. [n = 6 (each experimental repeat was performed in duplicate), Data = Mean ± SEM].

Zidovudine had a significant effect on HSk myoblast migration. There was a marginal decrease in percentage wound closure at the 0.01 µM dose compared to the control after 7 hours (28 ± 4.27% to 24 ± 4.68%) (Figure 3.8A and B). This trend was also observed with the rate of wound closure post 4 hours, where a significant 6-fold decrease ($p < 0.05$) in rate of migration was observed at 0.01 µM [Figure 3.8C(ii)].

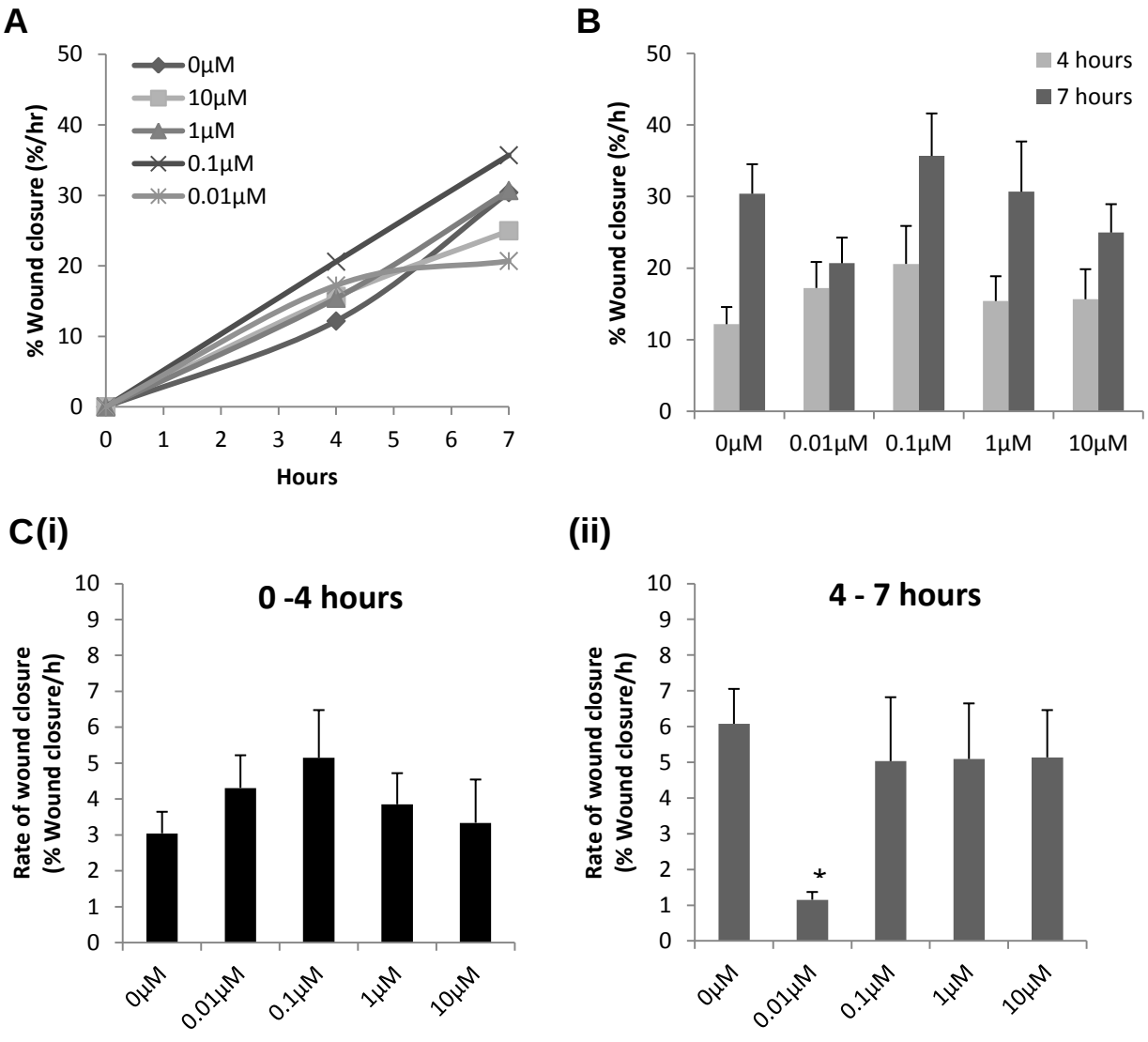


Figure 3.8 Dose responses of Zidovudine on HSk myoblast migration. The scratch assay was used as described in section 2.4. The concentration of Zidovudine ranged from 0.01 µM - 10 µM. Growth media served as the control. **(A)** Percentage wound closure was determined at 4 and 7 hours as described in section 2.4. **(B)** Comparison of percentage wound closure at 4 versus 7 hours **(C)** The rate of migration at the time intervals **(i)** 0-4 hours versus **(ii)** 4-7 hours were compared. Zidovudine (0.01 µM) showed a significant decrease at the 4-7 hour interval ($p < 0.05$).[*$p < 0.05$, n = 5 (each experimental repeat was performed in duplicate), Data = Mean ± SEM].

3.3.6. Ritonavir induces minor changes in myoblast migration

As Ritonavir was reconstituted in DMSO (final concentration 0.001%), we first determined whether DMSO (0.001%) itself, affects migration. No significant changes were observed in percentage wound closure of C2C12 (Figure 3.9A) or HSk myoblasts (Figure 3.9B), in response to 0.001% DMSO compared to the normal growth media control.

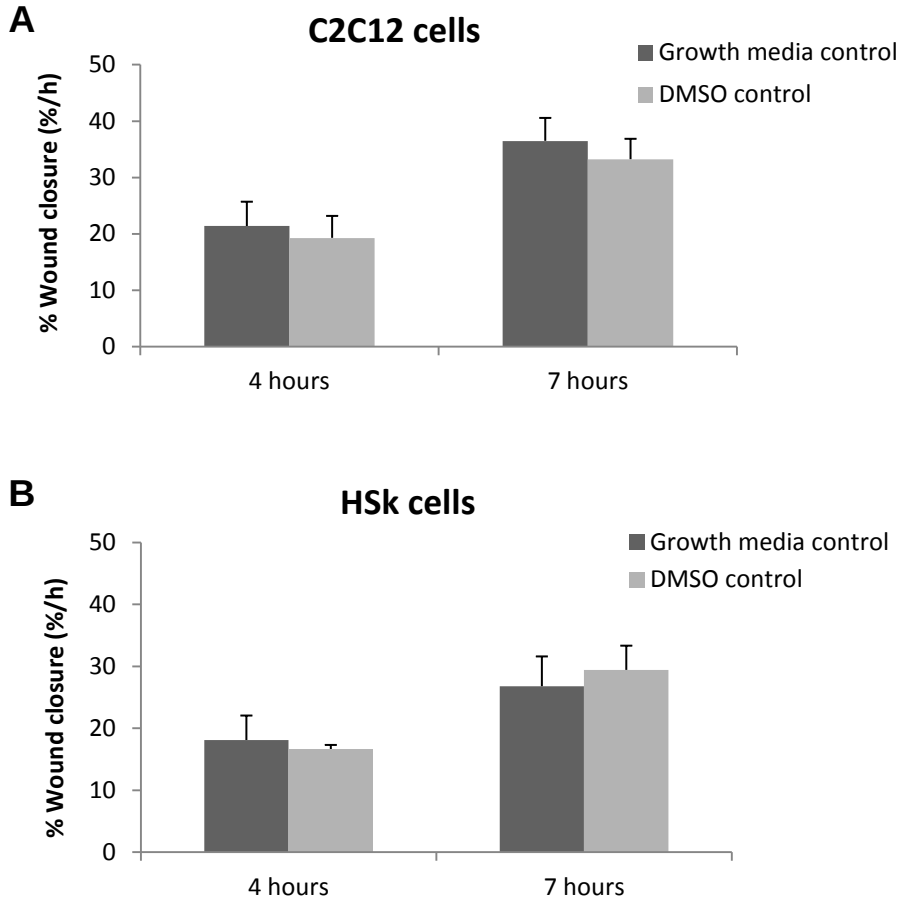


Figure 3.9 A comparison between growth media control and growth media ± DMSO (0.001%). The graphs show that there was no difference between the **(A)** C2C12 control cells and **(B)** HSk control cells. [=6 and n=5 for C2C12 and HSk myoblasts respectively (each experimental repeat was performed in duplicate). Data = Mean ± SEM].

Although Ritonavir (0.01 µM to 1 µM) tended to increase the percentage wound closure of C2C12 myoblasts (1 µM: 39 ± 5.67%) compared to control (33 ± 1.93%), this effect was minor and not significant (Figure 3.10A and B). The highest dose (10 µM) did not have any effect on percentage wound closure compared to the control (Figure 3.10B). The effect of Ritonavir on the rate of wound closure at 0-4 hours [Figure 3.10C(i)], was observed to follow a similar trend to percentage wound closure. At 4-7 hours however, the rate of wound closure appeared to increase slightly (although non-significantly) at all doses analyzed [Figure 3.10C(ii)].

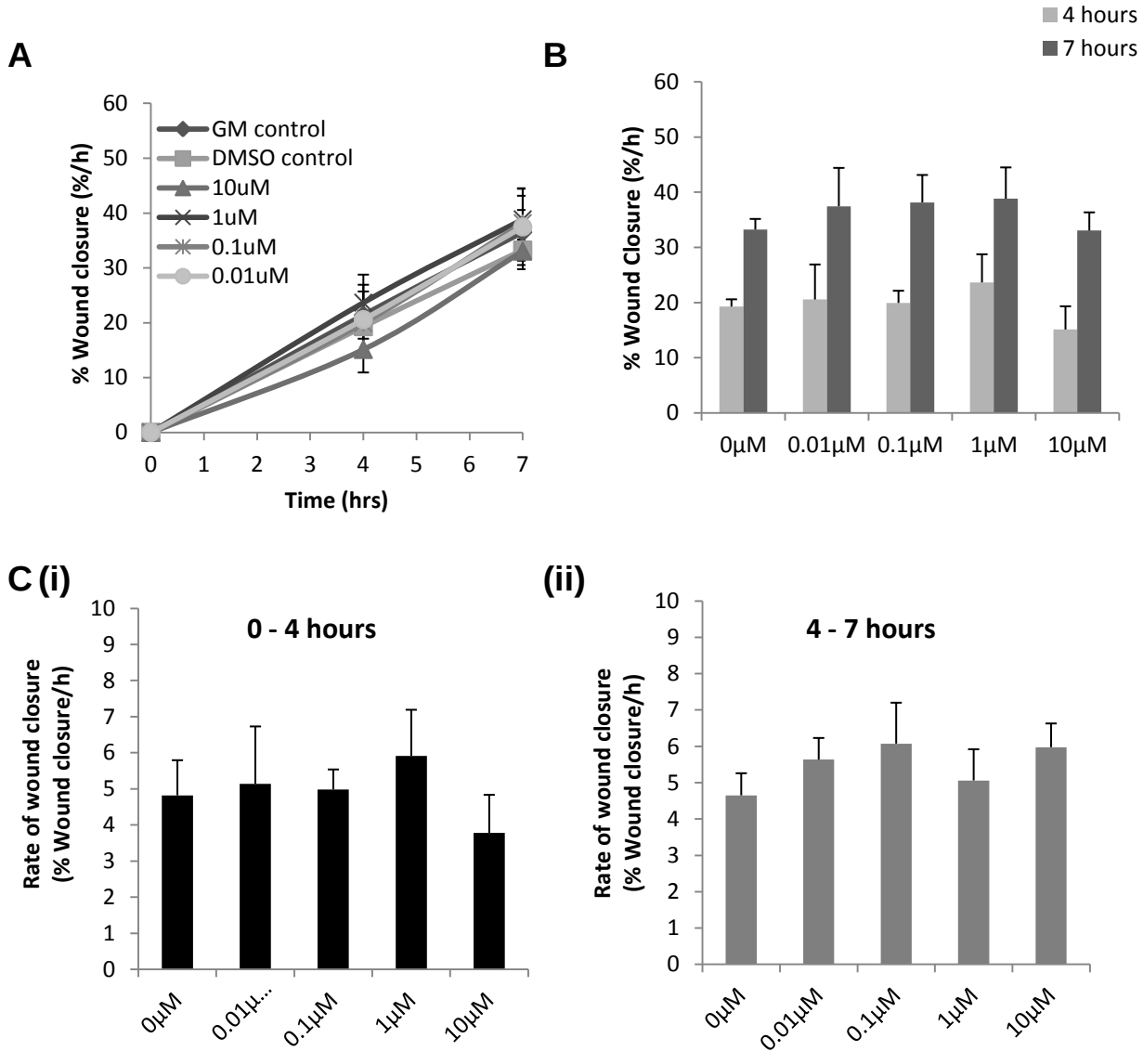


Figure 3.10 Dose responses of Ritonavir on C2C12 myoblast migration. The scratch assay was used as described in section 2.4. The concentration of Ritonavir ranged from 0.01 µM - 10 µM. (0 µM refers to 0.001% DMSO control). **(A)** Percentage wound closure was determined at 4 and 7 hours (Section 2.4). **(B)** Comparison of percentage wound closure at 4 versus 7 hours **(C)** The rate of migration was then determined from this data. The time intervals **(i)** 0-4 hours versus **(ii)** 4-7 hours were compared. [n = 6 (each experimental repeat was performed in duplicate), Data = Mean ± SEM].

In HSk myoblasts, Ritonavir induced a significant increase in wound closure compared to control (12 ± 1.68% to 24 ± 4.36%) following 4 hours at 0.01 µM ($p < 0.05$). Thereafter, a decrease in wound closure was observed with increasing concentrations (Figure 3.11B). A small increase in percentage wound closure 7 hours post-wounding was observed at 0.1 µM compared to control (29 ± 5.20% to 33 ± 5.55%) (Figure 3.11A and 3.11B) and decrease at 10 µM (29 ± 5.20% to 25 ± 7.14%) (Figure 3.11B); however these changes were statistically non-significant. During early wound closure (0-4 hours), the rate of migration was non-significantly increased in response to 0.01 µM [Figure 3.11C(i)]; however, at the same dose, this effect was decreased during late wound closure (4-7 hours) [Figure 3.11C(ii)].

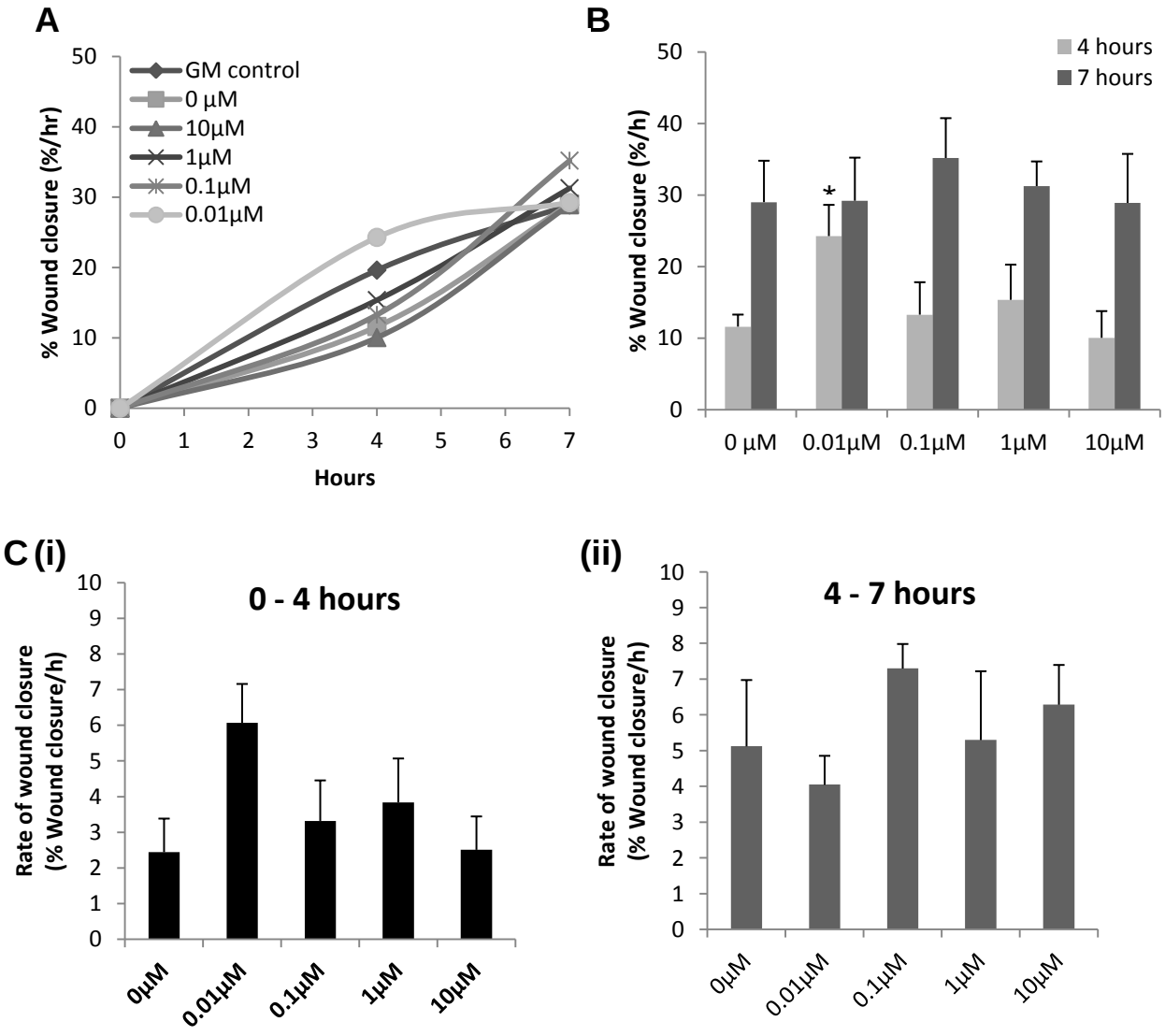


Figure 3.11 Dose responses of Ritonavir on HSk myoblast migration. The scratch assay was used as described in section 2.4. The concentration of Ritonavir ranged from 0.01 µM - 10 µM. (0 µM refers to DMSO control). **(A)** Percentage wound closure was determined at 4 and 7 hours (Section 2.4). Ritonavir (0.01 µM) significantly increased wound closure ($p < 0.05$). **(B)** Comparison of percentage wound closure at 4 versus 7 hours. Ritonavir (0.01 µM) showed significant increase 4 hours post-wounding ($p < 0.05$). **(C)** The rate of migration was then determined from this data. The time intervals (i) 0-4 hours versus (ii) 4-7 hours were compared. [*$p < 0.05$, n = 5 (each experimental repeat was performed in duplicate), Data = Mean ± SEM].

3.4. Discussion

ARV therapy consists of the combination of at least three ARVs to maximally suppress HIV replication and stop the progression of the disease. The most common drug combination consists of two NRTIs (e.g. Zidovudine, Tenofovir or Lamivudine) and one NNRTI (e.g. Efavirenz) or a "boosted" protease inhibitor (e.g. Ritonavir) (Section 1.5.2, Table 1.2) (Velasco-Hernandez *et al.*, 2002; McMahonan *et al*., 2009). Revised guidelines and recommendations for future use of ARVs for adults and adolescents are currently being developed (WHO (New progress and guidance on HIV treatment), 2010). Unfortunately, long-term ARV treatment can cause a number of adverse side effects. The adverse side effect of particular interest to the current study is muscle atrophy; the mechanisms underlying the development of muscle atrophy in response to ARVs are unclear (Authier *et al*., 2005; Hong-Brown *et al*., 2012).

In the current study, we therefore aimed to determine whether Tenofovir, Zidovudine or Ritonavir affect myoblast proliferation and migration *in vitro*. We used C2C12 mouse myoblasts to initially analyze the effects of these drugs on the proliferative and migratory stages of myogenesis. To mimic conditions in humans as close as possible, we repeated the experiments using HSk myoblasts.

We observed significantly increased C2C12 myoblast proliferation in response to Tenofovir at 1 µM and 10 µM doses (Figure 3.2A). On the other hand, we observed significantly decreased proliferation of HSk myoblasts in response to Tenofovir at 1 µM (Figure 3.2B). This differential effect in mouse versus human myoblasts is of interest. In HSk myoblasts, Zidovudine (0.01 µM and 0.1 µM) was also observed to decrease proliferation (Figure 3.3B). Zidovudine, a reverse transcriptase inhibitor similar to Tenofovir, has been observed to decrease proliferation of human T cells, human and mouse bone marrow cells and human muscle cells (Table 3.1). These results are in agreement with our human myoblasts studies.

Table 3.1 Summary of previous studies on the proliferation of various cell types in response to Zidovudine

Zidovudine dose (µM)	Cell Type	Effect on proliferation	Reference
1000	Human T-cell (H9)	↓	Furnam *et al*., 1986
> 1000	Human T-cell (CEM)	↓	Balzarini *et al*., 1989
5	Human bone marrow	↓	Inoue *et al*., 1989
54 1.5	Human T-cell (CEM); Mouse bone marrow	↓	Mansuri *et al*., 1990
10	Human muscle	↓	Herzberg *et al*., 1992
4-5000	Human muscle	↓	Benbrika *et al.*, 1997

The observed increase in proliferation of C2C12 myoblasts and the decrease observed in HSk myoblasts in the presence of Tenofovir, may have been due to interactions with components of the cell cycle. It is therefore tempting to assume that the expression of cell cycle components in C2C12 myoblasts, which are an immortalized cell line, would be different to primary myoblast cultures. One study has compared the hepatoma cell line, Hepa1–6 with primary hepatocytes to derive functional phenotypes (Pan *et al*., 2009). Using bioinformatics analysis of proteomic phenotypes, they revealed that Hepa1–6 cells were deficient in mitochondria, and they suggested that a re-arrangement of metabolic pathways could drastically up-regulate cell cycle-associated functions for the liver (Pan *et al*., 2009). Both Tenofovir and Zidovudine lack 3'OH ends required for phosphodiester bond formation and consequently prevent reverse transcription of RNA to DNA by reverse transcriptase, resulting in chain termination (Lewis *et al*., 1992; Velasco-Hernandez *et al*., 2002; Warnke *et al*., 2007). Inhibition of successful cellular chain elongation by Tenofovir or Zidovudine may induce growth arrest at the G2/S regulation point resulting in reduced proliferation. Furthermore, telomerase activity is high in satellite cells as well as immortalized C2C12 cells, and is down-regulated during adult myogenesis (O'Connor *et al* ., 2009). Tenofovir and Zidovudine may result in a decrease in telomerase activity causing shortening of telomeres and ultimately satellite cell exhaustion.

Zidovudine has been shown to induce S phase arrest in human epithelial carcinoma (HeLa) cells in a dose-dependent manner (Oliverio *et al*., 2005). Using flow cytometry, a dose-related increase of cells in the S phase, following incubation of 500 µM Zidovudine for 24 hours, from 9.5% (no drug) to 36% was observed. At the same dose, a decrease in cells in G1 phase from 82% (no drug) to 58.5% after 24 hours was noted (Oliverio *et al*., 2005). In the same study, drug combinations with another reverse transcriptase inhibitor, Lamivudine (50 µM) were compared with Zidovudine, and

it was suggested that Zidovudine was responsible for the accumulation of cells in the S phase. An accumulation of cells in the S phase inhibits the progression of cell cycling into mitosis. It was also suggested that Zidovudine induces an up-regulation of cyclin D1 accompanied by a decrease in the regulation of cyclin D1 inhibitors (p21), ultimately causing a decrease in proliferation (Oliverio *et al*., 2005). This may be a mechanism applicable to the HSk myoblasts in response to Tenofovir (1 µM) and Zidovudine (0.1 µM and 0.01 µM) (Figure 3.2B and 3.3B). Other previous studies have also observed decreased proliferation rates of myogenic cells and other cell types when exposed to similar or slightly higher concentrations of Zidovudine (Table 3.1). The current results suggest that decreased myoblast proliferation, in response to Zidovudine and Tenofovir, may contribute to the progression of muscle myopathies (Benbrika *et al*., 1997; Chen *et al*., 2011).

Ritonavir was not observed to affect proliferation or migration significantly. Proliferating cell nuclear antigen (PCNA) expression is high in proliferating cells and is reduced when cells enter the G0 phase of the cell cycle, where the cell adopts the quiescent phase or is signaled to terminally differentiate (Magi and Hubs her, 2003). A study analyzing at the effect of Ritonavir on myotube formation over a four day period, revealed that there were initially similar PCNA protein levels in Ritonavir-treated L6 myoblasts and control cells; these levels decreased similarly with increasing time in fusion media, indicating proliferation arrest. Therefore the decrease in myotube formation observed was not due to continual myoblast proliferation in the Ritonavir-treated cells (Colby-Germinario *et al*., 2004). Further investigations are required to elucidate the effect of Ritonavir on proliferation.

Our investigation into the effects of Tenofovir on the ability of the myoblasts to migrate in response to injury, showed marginal dose-dependent effects on HSk myoblasts (Figure 3.6). Specifically at 0.1 µM, Tenofovir significantly decreased HSk myoblast migration, whereas at higher doses, this effect was not seen (Figure 3.6). Zidovudine decreased the rate of wound closure at lower doses in the myoblasts (Figure 3.7 and 3.8) and significantly so at 0.01 µM for HSk myoblasts (Figure 3.8). Ritonavir (0.01 µM) significantly increased HSk myoblast migration (Figure 3.11B); however minor, non-significant changes were seen in response to Ritonavir in rate of wound closure (Figure 3.10).

In most published studies using Zidovudine (Table 3.1), the concentrations of Zidovudine were extremely high (e.g. 5000 µM) in comparison to the concentrations used in the current study (0-10 µM). Distribution of drugs in tissues varies and is dependent on blood perfusion, tissue binding and permeability of cell membranes. In most tissues, the rate at which a drug is distributed is determined primarily by perfusion. In poorly perfused tissues, such as muscle, distribution is very slow (Engel *et al*., 2008). Published peak plasma concentrations are summarized in section 1.6,

Table 1.3. The concentrations of the ARVs used in our study, although far lower than used in other reports, were based on these peak plasma levels. Furthermore, high drug concentrations often exert cytotoxic effects on muscle cells and may cause mitochondrial damage (Benbrika *et al*., 1997).

In summary, human and mouse myoblasts respond differently to incubation with ARVs. In response to Tenofovir and Zidovudine, proliferation of human skeletal myoblasts was seen to decrease, whereas mouse myoblast proliferation increased. This observed difference may also relate to the immortal versus primary culture characteristics of C2C12 versus HSk myoblasts. Ritonavir was not seen to significantly affect proliferation in either mouse or human myoblasts. Migration of human myoblasts was significantly increased in response Ritonavir, and was significantly decreased in response to Tenofovir and Zidovudine.

CHAPTER 4:

EFFECT OF ANTIRETROVIRALS ON DIFFERENTIATION OF MYOBLASTS

4.1 Introduction

The progression of HIV/AIDS is known to be associated with severe muscle wasting (Dworkin and Williamson, 2003). Treatment with ARVs such as Zidovudine, while limiting the expansion of HIV, can also be linked to muscle wasting and toxic mitochondrial myopathies (Scruggs and Dirks Naylor, 2008). ARVs have been shown to affect key proteins involved in muscle myofiber elongation during myogenesis. A few studies suggest that ARV protease inhibitors (PIs), Lopinavir and Indinavir, impair protein synthesis in C2C12 myocytes (Hong-Brown *et al*., 2012). It was hypothesized that ARVs may affect targets including AMP and MAP signaling kinases, although the mode of action of ARVs has not been well characterized (Hong-Brown *et al*., 2012). Although the impact of ARV treatment on muscle mass has not strongly been recognized by previous epidemiological studies (Mache *et al*., 2000), such an impact seems plausible in practice, as suggested by the marked decrease in size of muscle biopsies in HIV-infected individuals (Authier *et al*., 2005).

The progression of satellite cells through myogenesis is regulated to a large extent by Pax7 (Tedesco *et al*., 2010 and Holguin and Visconti, 2012). Prior to activation, quiescent satellite cells express Pax7, a paired box transcription factor involved in the control of muscle development and maintenance of the population of satellite cells (Seale *et al*., 2000 and 2004; Zammit *et al*., 2006). Following activation, myoblasts upregulate and express Pax7 together with Myf5 and MyoD (primary MRFs), which are genes encoding basic helix loop helix (bHLH) transcription factors (Section 1.2.1). Myf5 regulates myoblast proliferation rate and homeostasis (Legman and Rudnicki, 2007), whereas MyoD determines the differentiation potential of activated myoblasts (Legman and Rudnicki, 2007; Giannakopoulos *et al*., 2011). Terminal differentiation of skeletal muscle myoblasts requires that the cells exit from the cell cycle in G1 phase and enter into the growth-arrested post-mitotic state (G0 phase) (Mastroyiannopoulos *et al*., 2012). MyoD causes cells to exit the cell cycle thus halting their proliferation. MyoD has been shown to regulate the progression at the G1 phase of the cell cycle by enhancing the transcription of p21 - a cyclin-dependent kinase (CDK) inhibitor protein that binds to and inhibits the activity of cyclin-CDK2 or -CDK1 complexes. MyoD activity, in turn, is inhibited by CDKs (Lassar *et al*., 1994; Rae *et al*., 1994; Zhang *et al*., 1999). At the onset of differentiation, Pax7 expression is down-regulated and the secondary MRFs, myogenin, Mrf4 and Myosin Heavy Chain (MHC) are up-regulated (Section

1.2.2) indicating that myoblasts have irreversibly exited the cell cycle (Zhu and Miller, 1997; Zammit *et al*., 2004; Berkes and Tapscott, 2005).

Under proliferative conditions, the percentage $Pax7^+$ cells reflects the myogenic potential of the population. However, one must remember that in response to differentiation cues, Pax7 expression must ultimately decrease. A study demonstrated that $Pax7^+$ cells are the selected source of regenerative myonuclei following injury (Leper *et al*., 2011). Consistent with their findings, a parallel study showed that muscle regeneration in mice fails when most of the $Pax7^+$ cells are ablated; when combined with muscle injury caused by exercise, there was a marked loss of muscle tissue and failure to regenerate skeletal muscle (Sambasivan *et al*., 2011). In conjunction with MHC expression, analysis of $Pax7^+$ cell number can provide insight into the effect of ARVs on myogenesis.

A few studies have evaluated the effect of nucleoside reverse transcriptase inhibitors (NRTIs) on differentiating myotubes. The effect of Zidovudine on differentiating human muscle cells has been investigated and it was found that differentiation (day 6) was decreased in response to Zidovudine (250 µM) (Herzberg *et al*., 1992). It has been suggested that Zidovudine (10 µM) may induce premature S-phase arrest, which would ultimately result in reduced myogenic capability (Oliverio *et al*., 2005). Moreover, exposure of cultured human myotubes to Zidovudine (250 µM) decreased the number of myotubes, induced destructive changes in the mitochondria (swelling, lamellar inclusions and multiple concentric cristae) and increased fat droplets (Semi no-Mora, 1994). In another study, the myotoxicity of Zidovudine was compared in uninfected versus ts1 retrovirus (mutant of Moloney murine leukemia virus) infected mouse skeletal muscle cells. The Zidovudine half inhibitory concentration (IC_{50}) for myoblast proliferation was determined to range from 2-5 µM and was significantly less than the Zidovudine IC_{50} for muscle cell differentiation (100 µM) (Waclawik *et al*., 1999). The study revealed that infection with ts1 retrovirus did not significantly shift the IC_{50} of Zidovudine for either proliferation or differentiation; furthermore, no depletion of mtDNA at toxic concentrations (>100 µM) of Zidovudine was observed. More recently, a study found that Zidovudine did not affect myoblast and myotube cell numbers, but decreased mtDNA levels in HSk myoblasts and myotubes (Saitoh *et al*., 2008). The concentration of Zidovudine (7.1 µM) used was based on the average peak steady-state levels in human plasma (C_{max} determined during antiretroviral therapy) (Saitoh *et al*., 2008). The study concluded that the decrease in mtDNA was due to either a decrease in replication or an increase in mtDNA degradation. However, it may also have been due to other pathways, such as reactive oxygen species production, uncoupling proteins, and depletion of deoxyribonucleotide triphosphate pools in muscle mitochondria. In contrast, no decrease of mtDNA content was found in Zidovudine-treated muscle

cells in the earlier study by Hertzberg *et al*., 1992). They proposed that Zidovudine induces a decrease in proliferation of muscle cells, rather than inhibition of mtDNA replication.

To date, Tenofovir has not been shown to affect the differentiation of muscle cells. Tenofovir-treatment of HepG2 human liver cells and primary human skeletal muscle cells proved to be weakly cytotoxic compared to NRTIs currently used for the treatment of HIV. It was revealed that Tenofovir inhibited the proliferation of HepG2 cells after 8 days and skeletal muscle cells after 6 days of proliferation. Cytotoxicity concentration values were 398 µM and 870 µM respectively, indicating concentrations that result in the death of 50% of the cells (CC_{50}); Zidovudine showed lower CC_{50} values (0.06–5 µM) in these two cell types (Cihlar *et al*., 2002).

Ritonavir (10 µM) has been shown to reduce calpain activity in L6 myoblasts (Germinario-Colby *et al*., 2004). Calpain is a calcium (Ca^{2+}) -dependent intracellular protease required for myotube formation (Huang and Wang, 2001). MyoD and Myogenin and their downstream target, MHC, were however expressed at similar levels in both the control and drug-treated cells. Ritonavir is a protease inhibitor that inhibits the HIV protease, an aspartyl protease (Wensing *et al*., 2010). It is therefore possible that Ritonavir inhibits aspartic proteins in muscle cells. For example, in myoblasts, caspases, which are cysteine-dependent-Aspartic-directed proteases play a role in myogenesis. Caspase 3, an apoptotic protease is required for muscle differentiation. A study revealed that inhibition of caspase 3 activity or homologous deletion of caspase 3 leads to reduction in both myotube and myofiber formation as well as decreased expression of muscle-specific proteins (MyoD, Myogenin) (Fernando *et al*., 2002).

The above information is persuasive in suggesting that the formation of multinucleated myotubes may be the stage in myogenesis most affected by ARVs. In the current study, the effect of Tenofovir (1 µM), Zidovudine (1 µM) and Ritonavir (1 µM) was tested on the differentiation of myoblasts into myotubes.

4.2. Methods and Materials

4.2.1. Percentage Pax7$^+$ cells

C2C12 and HSk myoblasts were cultured in their respective growth media (section 2.1.1 and 2.1.2) on glass coverslips in 24-well plates. Cells were then treated with Tenofovir (1 µM), Zidovudine (1 µM) and Ritonavir (1 µM) respectively. Control cells were cultured in media ± 0.001% DMSO. Incubation with ARVs occurred either for 24 hours under proliferative conditions or for 1 or 7 days in differentiation media. Media ± ARVs was changed daily. The cells were then fixed with 4% paraformaldehyde and blocked with 5% donkey serum before being exposed to mouse anti-Pax7 primary antibody (Section 2.5.1, Table 2.1) overnight. The cells were then washed with PBS and incubated in Dylight488 donkey anti-mouse secondary antibody for 1 hour (Section 2.5.1, Table 2.2). Hoechst (Section 2.5.1, Table 2.2) was added to stain the nuclei prior to capturing images of the cells with the Zeiss LSM 710 NLO ConfoCor confocal microscope and analyzing them to quantify the percentage of Pax7$^+$ cells under proliferative conditions as well as at day 1 and day 7 of differentiation. The percentage Pax7$^+$ cells were calculated by counting the number of cells that expressed nuclear Pax7 and dividing this number by the total number of cells x 100 (Image J software).

4.2.2. MHC Protein Expression

C2C12 cells were seeded in growth media at 100 000 cells/well in T25 culture flasks until 70% confluent. Differentiation was induced by the addition of differentiation media (Section 2.1.3). Tenofovir (1 µM), Zidovudine (1 µM) and Ritonavir (1 µM) were added to this media. Differentiation media ± 0.001 % DMSO served as controls for all experiments. The differentiation media ± ARVs was changed daily. Brightfield images using the Olympus CKX41 were taken at day 7; protein lysates were prepared and protein concentrations determined by the Bradford assay (Section 2.5.3). Lysates from treated and non-treated differentiated cells were separated on a reducing 12.5% SDS-PAGE gel at 18mA/gel using the BioRad electrophoresis unit kit (Section 2.5.4). The separated proteins were transferred (blotted) from the gel onto the nitrocellulose membrane as described in section 2.5.5. Primary antibodies recognizing MHC (Section 2.5.5, Table 2.4) and an internal control, GAPDH, as well as secondary HRPO-linked donkey anti-mouse and anti-rabbit antibodies (Section 2.5.5, Table 2.5) were used to probe for the proteins.

4.2.3. Fusion of C2C12 myoblasts

30 000 C2C12 cells were seeded onto glass cover slips in each well within a 24-well plate and allowed to proliferate until they reached 70% confluence in growth media. The growth media was then replaced with differentiation media containing either 1 µM Tenofovir, Zidovudine or Ritonavir. Differentiation media ± 0.001 % DMSO was used as controls. Cells were allowed to differentiate for 7 days. The differentiation media ± ARVs was replenished daily. On day 7, brightfield images using the Olympus CKX41 microscope were taken. Cells were then fixed with 4% paraformaldehyde and blocked in 5% donkey serum (Section 2.5.1). The cells were then incubated with mouse anti-MHC primary antibody (Section 2.5.1, Table 2.1) overnight and then with Dylight594 donkey anti-mouse secondary antibody (Section 2.5.1, Table 2.2). The nuclei were stained with Hoechst (Section 2.5.1, Table 2.2) prior viewing. Images of the specimens were captured and analyzed as described in section 2.5.1.

4.2.4. Determination of fusion index

Fusion indices were used to determine the extent of fusion in the presence and absence of the ARVs. Myotubes were classified as MHC-expressing cells with two or more nuclei. The fusion index is therefore the number of nuclei in myotubes with two or more nuclei, divided by the total number of nuclei x 100. Single cells that expressed MHC (known as myocytes) were also counted and compared to the number of myotubes. Analysis of myocyte versus myotube numbers gave insight into the ability of ARVs to affect fusion, but not MHC expression.

The flow diagram below (Figure 4.1.) summarizes the basic outline of the proliferation differentiation experimental protocols described above.

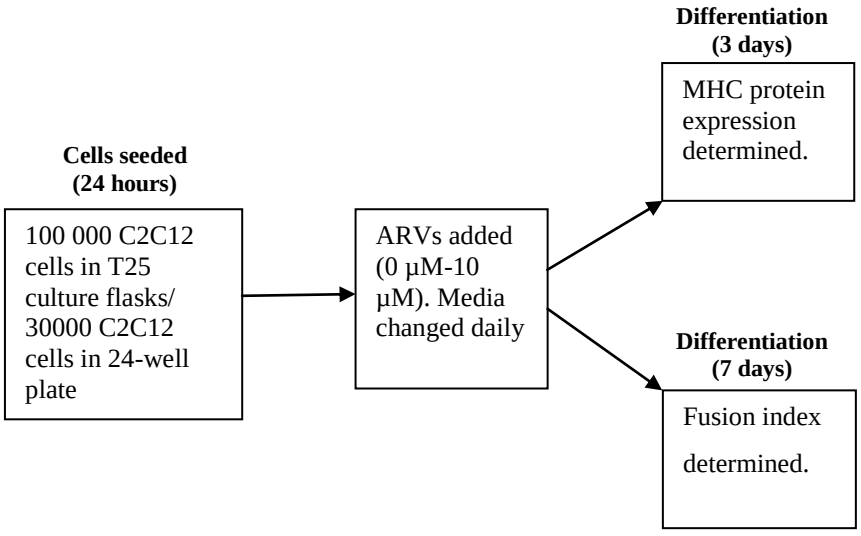


Figure 4.1 Flow diagram illustrating the experimental protocols used to investigate the effect of ARV drugs on myoblast differentiation.

4.3. Results

4.3.1. Tenofovir and Zidovudine have differential effects on % Pax7$^+$ myoblasts under proliferating conditions

Under normal growth conditions, the number of Pax7$^+$ C2C12 and HSk myoblasts in response to the presence and absence of the reverse transcriptase inhibitor drugs, Tenofovir and Zidovudine (1 µM), was analyzed by immunocytochemistry and confocal microscopy. The number of Pax7$^+$ myoblasts was quantified using Image-J software (Section 4.2.1).

Under proliferating conditions, Tenofovir (1 µM) increased the percentage Pax7$^+$ C2C12 cells by almost 50% in comparison to the control (17 ± 4.5% to 29 ± 7.0%; Figure 4.1A), although this was not significant. Zidovudine had no effect on the percentage Pax7$^+$ C2C12 cells (Figure 4.2A). The effect of Tenofovir on human muscle cells was the opposite compared to C2C12 cells (Figure 4.2B). The percentage of Pax7$^+$ HSk myoblasts decreased, although non-significantly, compared to the control, from 55 ± 3.4% to 42 ± 12.4%. Zidovudine significantly decreased the number of Pax7$^+$ HSk myoblasts (55 ± 3.4% to 31 ± 7.0%), compared to control (Figure 4.2B). Interestingly, 55% HSk myoblasts expressed nuclear Pax7, whereas only 15% C2C12 myoblasts expressed nuclear Pax7 (Figure 4.2A versus 4.2B).

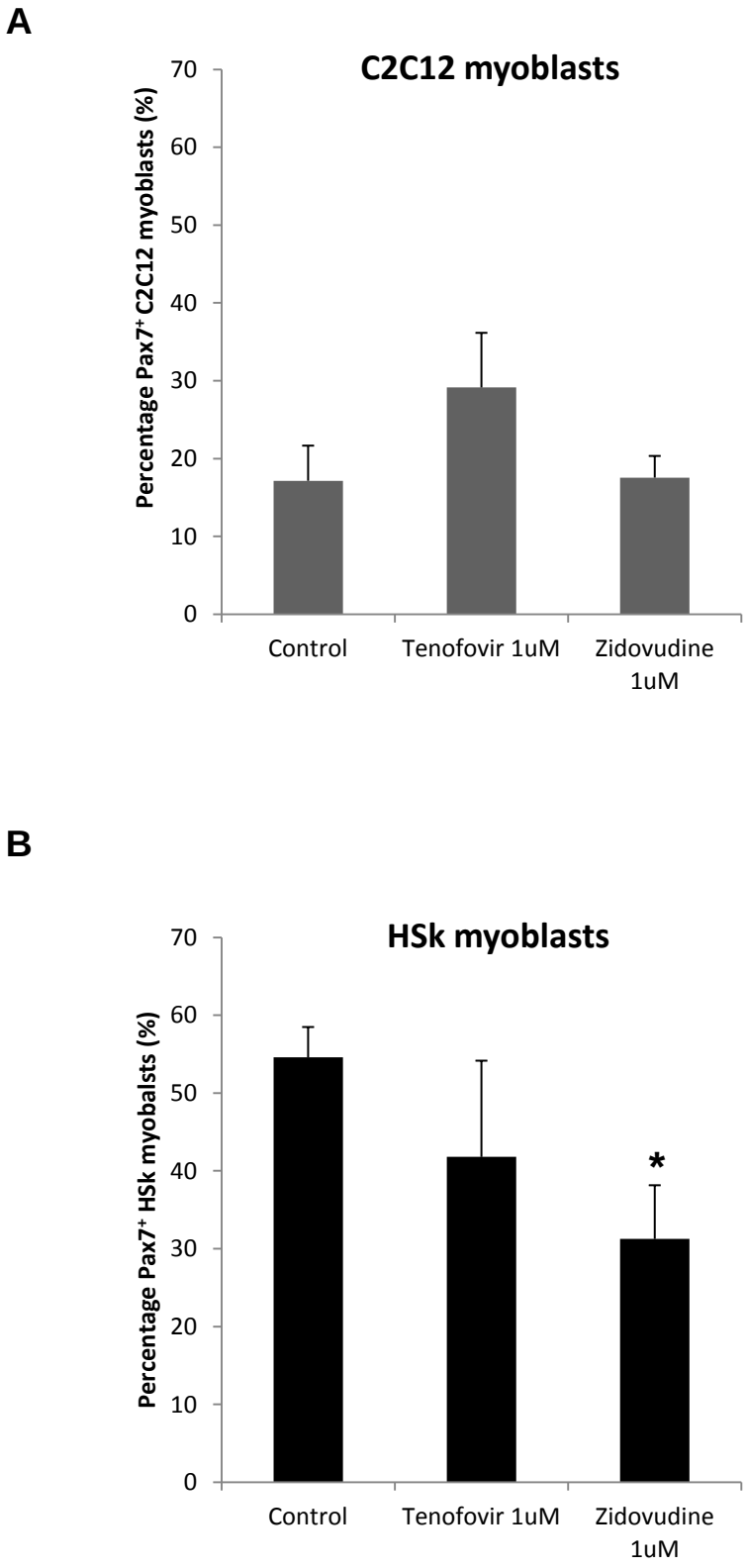


Figure 4.2: Percentage Pax7$^+$ myoblasts under proliferating conditions in response to Tenofovir and Zidovudine. C2C12 myoblasts and HSk myoblasts, were cultured in growth media ± Tenofovir and Zidovudine (1 µM). Immunocytochemistry to detect Pax7 was performed on the myoblasts. The percentage Pax7$^+$ cells were determined as described in section 4.2.1 for C2C12 myoblasts **(A)** and HSk myoblasts **(B).** Zidovudine significantly decreased % Pax7$^+$ HSk myoblasts ($p<0.05$). [n=6 and n=4 for C2C12 and HSk myoblasts respectively (each experimental repeat was performed in duplicate), *$p<0.05$, Data = Mean ± SEM].

4.3.2. Ritonavir does not affect % Pax7$^+$ myoblasts under proliferating conditions

The % Pax7$^+$ C2C12 and HSk myoblasts in response to Ritonavir (1 µM) under growth conditions was also analyzed by immunocytochemistry and confocal microscopy. The number of Pax7$^+$ myoblasts was quantified as described in section 4.2.1. In both C2C12 and HSk myoblasts, Ritonavir did not affect the percentage Pax7$^+$ myoblast number under proliferating conditions. (Figure 4.3A and B) compared with DMSO control.

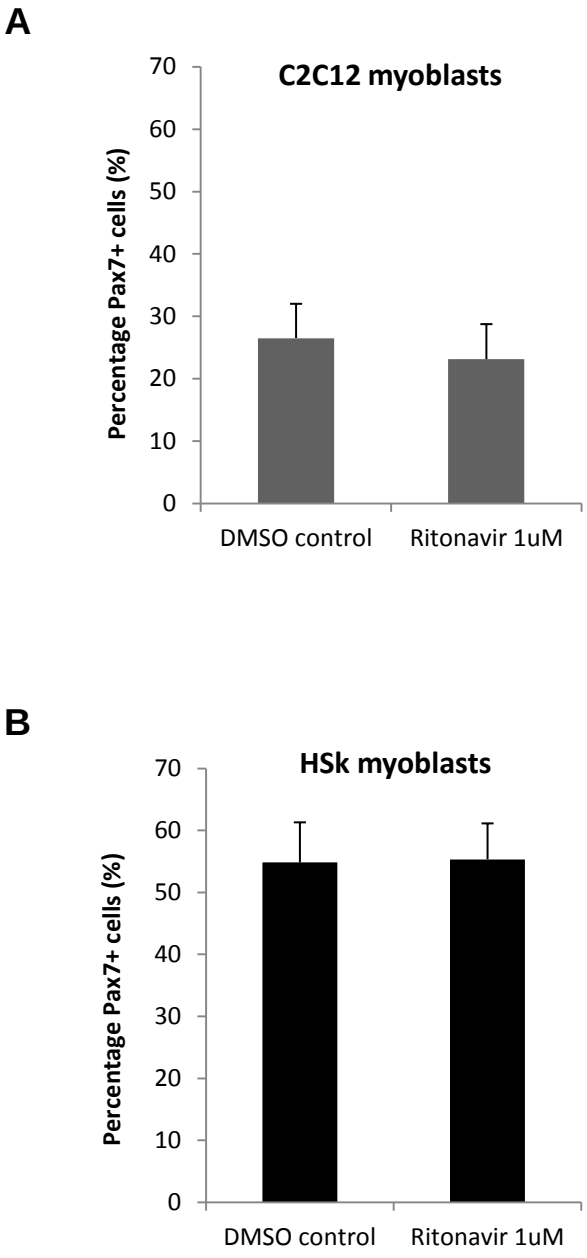


Figure 4.3: A. Percentage Pax7$^+$ myoblasts under proliferating conditions in response to Ritonavir. C2C12 myoblasts and HSk myoblasts were cultured in growth media ± Ritonavir (1 µM). Immunocytochemistry to detect Pax7 was performed on the myoblasts. The percentage Pax7$^+$ cells were determined (Section 4.2.1) for C2C12 myoblasts **(A)** and HSk myoblasts **(B).** [n=6 and n=4 for C2C12 and HSk myoblasts respectively (each experimental repeat was performed in duplicate), Data = Mean ± SEM].

4.3.3. % Pax7$^+$ myoblasts are not affected by Tenofovir, Zidovudine or Ritonavir under differentiating conditions

C2C12 myoblasts were grown to 80% confluence in growth media and subsequently induced to differentiate for 1 or 7 days in differentiation media ± Tenofovir, Zidovudine and Ritonavir (1 µM).

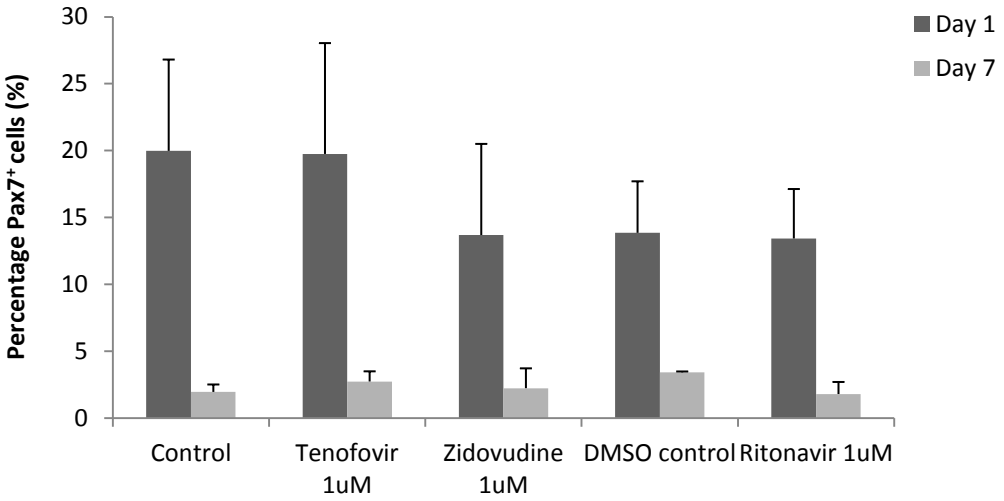


Figure 4.4: Comparison of Pax7$^+$ C2C12 myoblasts at day 1 and day 7 of differentiation. Myoblasts were cultured in differentiation media ± Tenofovir, Zidovudine and Ritonavir (1 µM). The percentage Pax7$^+$ cells were determined (Section 4.2.1) at the two time points (day 1 and day 7). [n=4 and n=3 for day 1 and day 7 respectively (each experimental repeat was performed in duplicate), Data = Mean ± SEM].

In the absence of ARVs, there was an overall decrease in the percentage of Pax7$^+$ C2C12 cells from day 1 to day 7 of differentiation (20 ± 6.83% to 3 ± 0.76%). The decrease in % Pax7$^+$ cells suggests that the cells have committed to differentiate. The addition of ARVs did not significantly alter the percentage Pax7$^+$ cells at either day 1 or day 7 (Figure 4.4).

4.3.4. Tenofovir, Zidovudine and Ritonavir reduce fusion of C2C12 myotubes

To investigate the extent to which ARVs affect the regeneration of skeletal muscle, C2C12 myoblasts were grown to 80% confluence in growth media and induced to differentiate for 7 days in differentiation media ± Tenofovir, Zidovudine and Ritonavir (1 μM). Brightfield and confocal images were taken to assess the effects of ARVs on differentiation. Terminal myoblast differentiation appeared to be negatively affected in response to all three ARVs as indicated by the decrease in number and size of multinucleated myotubes in comparison to control (Figure 4.5A). In addition, the fusion index was decreased in all treated cells (Figure 4.5B); however, this effect was only significant in response to Tenofovir (16 ± 5.20% to 10 ± 4.51%, $p<0.05$).

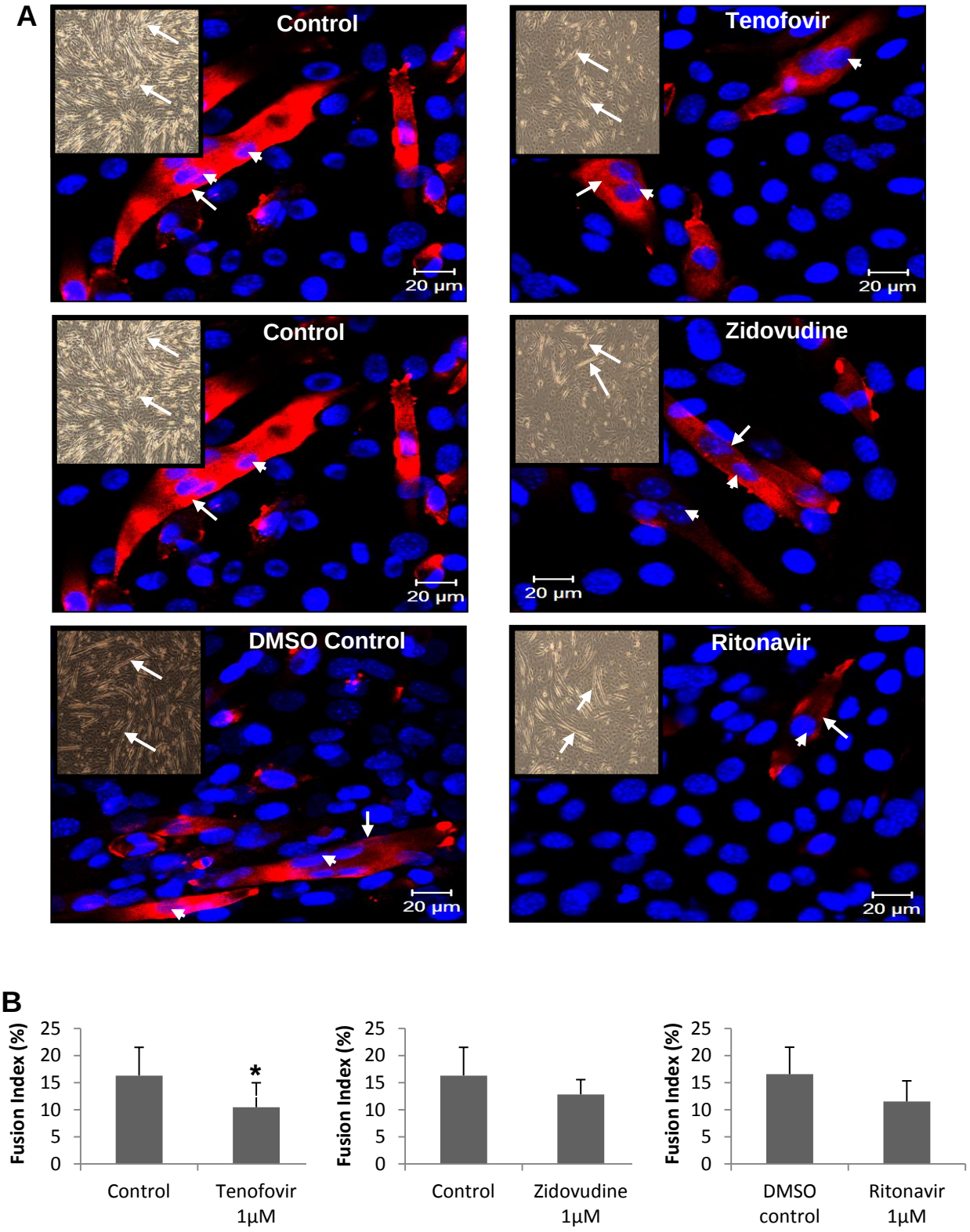


Figure 4.5: Determination of fusion index. Cells were cultured in differentiation media ± ARVs for 7 days. (**A)** Following 7 days of differentiation, brightfield images (inserts) as well as confocal images show fewer multinucleated myotube formation in response to all three ARVs (1 µM) compared to control (arrows point to MHC^{+} myotubes and myocytes; arrowheads point to nuclei of myotube/myocytes). **(B)** The fusion index (%) was determined from the confocal images (Section 4.2.3). Tenofovir significantly reduced the fusion of myotubes ($p<0.05$) [n=7 (each experimental repeat was performed in duplicate), *$p<0.05$, Data = Mean ± SEM].

4.3.5. Myocytes versus myotubes

We next determined the total protein expression of MHC in control versus ARV-treated myoblasts. Total MHC expression in Tenofovir- and Zidovudine-treated cells [T and Z] was similar to control [C^1] (Figure 4.6). Ritonavir [R] reduced the total MHC expression in C2C12 myotubes compared to control [C^2] (Figure 4.5). Interestingly, two MHC bands were observed following separation by SDS-PAGE (Section 4.2.2), indicating that two isoforms of MHC were expressed.

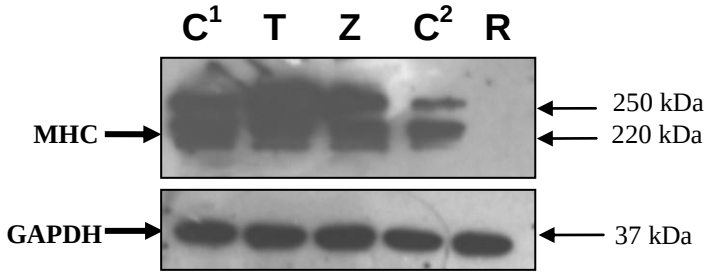


Figure 4.6: The effect of Tenofovir, Zidovudine and Ritonavir on total MHC expression during myogenic differentiation. C2C12 myoblasts were allowed to differentiate for 3 days in the presence of Tenofovir (1 µM), Zidovudine (1 µM) and Ritonavir (1 µM). 30 µg of protein determined by the Bradford assay (Section 2.5.3) was loaded for protein separation (Section 2.5.2). MHC expression was assessed by Western blot analysis and ECL (Section 2.5.5) [Differentiation media control = C^1, Tenofovir-treated cells [T], Zidovudine-treated cells [Z], DMSO control [C^2] and Ritonavir-treated cells (R).] GAPDH served as the internal control.

Despite total MHC protein expression being similar (Figure 4.6) in Tenofovir- or Zidovudine-treated cells compared to control cells, fusion was observed to decrease (Figure 4.5). We therefore went onto quantify the number of MHC^+ multinucleated myotubes versus MHC^+ mono-nucleated myocytes, under control and ARV-treated conditions (Figure 4.7). It was observed that in response to all three ARVs, the number of myotubes decreased, whereas myocyte number increased (Figure 4.7).

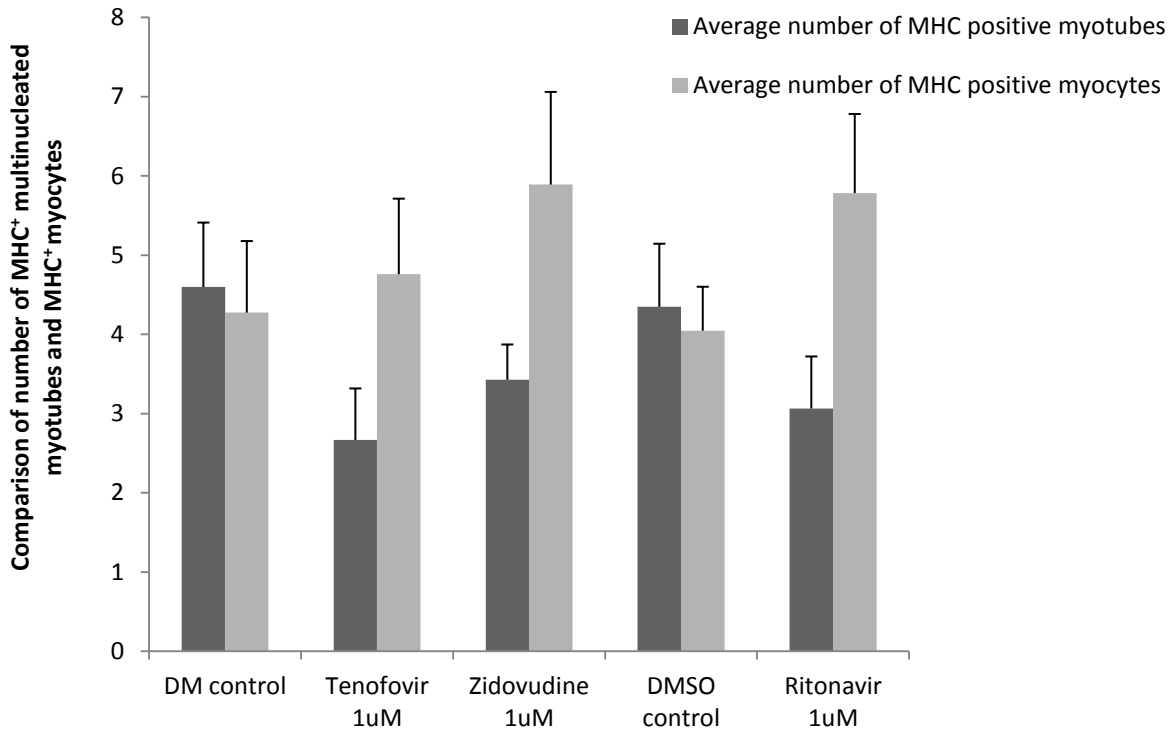


Figure 4.7: Comparison of the number of MHC$^+$ multinucleated myotubes to MHC$^+$ mononucleated myocytes. Following confocal microscopy analysis, the number of MHC$^+$ myotubes and myocytes was determined. It was observed that there were more MHC$^+$ myocytes than MHC$^+$ myotubes in the treated cells compared to control.

4.4. Discussion

In response to injury, adult skeletal muscle undergoes regeneration which typically involves activation of satellite cells (myoblasts) to proliferate and differentiate into myocytes which subsequently fuse to form multinucleated myotubes (Charge and Rudnicki, 2003). Pax7 is a good satellite cell marker that indicates the progression of these cells toward the myogenic lineage (Seale *et al*., 2000; Oustanina *et al*., 2004; Guardiola *et al*., 2012). A number of conditions such as diseases or intense bouts of exercise can lead to degeneration or loss of skeletal muscle. An adverse consequence from long-term ARV treatment is loss of muscle mass. In the current study, we investigated the effect of Zidovudine, Tenofovir and Ritonavir on skeletal muscle myogenesis.

In C2C12 myoblasts, Tenofovir increased % $Pax7^+$ cells during proliferation, whereas Zidovudine and Ritonavir had no observable effect; no statistically significant differences were observed. Tenofovir had the opposite effect on the HSk myoblasts compared to C2C12 myoblasts. A decrease in % $Pax7^+$ cells was observed. Furthermore, Zidovudine significantly decreased % $Pax7^+$ HSk cells (Figure 4.2B). However, Tenofovir, Zidovudine and Ritonavir did not affect the myogenic commitment of the myoblasts, as the % $Pax7^+$ cells decreased similarly from day 1 to day 7 of differentiation in control and ARV-treated conditions.

Tenofovir, Zidovudine and Ritonavir (1 μM), were seen to reduce fusion of myoblasts into myotubes (Figure 4.5) significantly so in response to Tenofovir (Figure 4.5B). Interestingly, the total MHC protein expression was greatly reduced in Ritonavir-, but not Tenofovir- or Zidovudine-treated cells (Figure 4.6). MHC is expressed shortly prior to fusion and is commonly used to identify terminally differentiated myotubes (Gianakopoulos *et al*., 2011 and Mastroyiannopoulos *et al*., 2012). However, subsequent to MHC up-regulation, other proteins must be up-regulated to ensure fusion (Charrasse *et al*., 2006). It has been shown that the size of individual muscles is determined largely by the number and size of its constituent myofibers. The diameter can be affected by factors such as age, exercise and the effects of diseases and drugs (Rayne and Crawford, 1975). In our current study, differentiated cultures from the ARV-treated cells versus non-treated cells were morphologically different; shorter myotubes were observed in ARV-treated cells (Figure 4.5A).

Our results suggest that ARVs may affect myogenesis on the level of MHC expression (Ritonavir) as well as fusion (Tenofovir, Zidovudine and Ritonavir). In addition, there may be effects on

isoform expression, however, our current methodology was not sensitive enough to detect these differences accurately (Kohn and Myburgh, 2006).

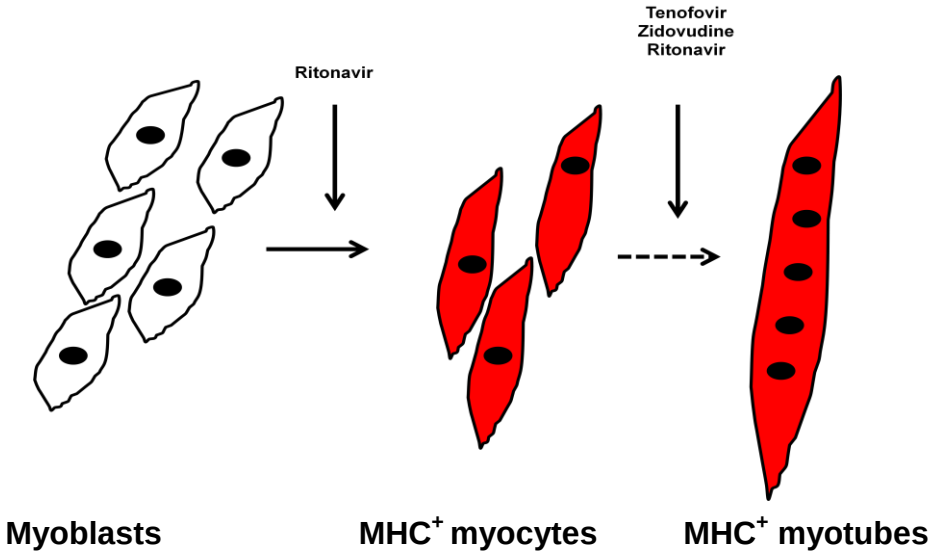


Figure 4.7: ARVs reduce fusion of myocytes. Upon satellite cell activation, activated myoblasts exit the cell cycle and are committed to the myogenic lineage. MHC expression (red) marks the onset of terminal differentiation; it is detected in mononucleated cells and then in multinucleated cells. Ritonavir appears to negatively affect MHC expression prior to fusion of myocytes into myotubes; all three ARVS reduce fusion of myotubes compared to control.

In summary, in the current study we show that all three ARVs decreased the fusion of myocytes into myotubes. Ritonavir was additionally seen to decrease the expression of MHC in differentiated myoblasts. Pax7 expression during differentiation was not significantly affected in response to the ARVs tested.

CHAPTER 5:

CONCLUSION AND FUTURE WORK

ARVs are used to alleviate the HIV/AIDS diseases. As patients' life expectancies increase with ARV treatment, a number of complications such as muscle myopathies have emerged (Pan *et al*., 2006). Tenofovir, Zidovudine and Ritonavir are ARVs used in the first-line of ARV therapy. The mode of action of these ARVs on muscle development is not clearly understood; hence the objective of this exploratory study was to investigate the effects of Tenofovir, Zidovudine and Ritonavir, on processes required for successful myogenesis. The key findings of the current study are summarized in Table 5.1 for mouse and human studies respectively.

Table 5.1 Summary of key findings (A green arrow indicates an increase; a red arrow indicates a decrease; a dash indicates no effect, in response to ARVs).

	Tenofovir	Zidovudine	Ritonavir	
C2C12	↑*	↑	↑	Proliferation
HSk	↓*	↓	↑	
C2C12	—	—	—	Migration
HSk	↓*	↓*	↑*	
C2C12	—	—	↓	MHC
C2C12	↓*	↓	↓	Fusion

* Significant findings

An effect of Zidovudine and Tenofovir on the proliferation of various cell types has been observed (Herzberg *et al*., 1992; Bruning *et al*., 2012). In our investigations, it was interesting to find that Tenofovir and Zidovudine decreased proliferation of human myoblasts, significantly so with Tenofovir (1 µM) (Figure 3.2B and 3.3B). These results are consistent with previously published *in vitro* data demonstrating marked inhibition of cultured human muscle cell proliferation in the presence of Zidovudine (10 µM) (Herzberg *et al*.,1992 and Benbrika *et al*., 1997). At the same dose of Tenofovir, a small but statistically significant increase was observed in C2C12 cell numbers (Figure 3.2A). Our current study shows that mouse and human myoblast proliferation is differentially affected by Tenofovir; suggesting that Tenofovir may differentially influence cell cycle genes as previously shown for Zidovudine (Oliverio *et al*., 2005). The increase in proliferation observed with the mouse myoblasts could explain the decreased differentiation, as the process of proliferation and differentiation are mutually exclusive. On the other hand, the decreased proliferation observed in human myoblasts would also negatively affect the subsequent myotube formation, thereby also contribute to a decrease in muscle mass.

In a heterogeneous pool of activated C2C12 myoblasts, some are $Pax7^+$ whilst a sub-population do not express detectable Pax7 protein (Olguin and Olwin, 2004), implying that a subset of these myoblasts have exited the cell cycle. It has been proposed that all the cells of the myogenic lineage are derived from the $Pax7^+$ population of myogenic progenitors. Previous experiments provide evidence that acute injury-induced muscle regeneration in the hind limb is entirely dependent on the resident $Pax7^+$ cell population (Seale *et al.*, 2004; Messina and Cossu *et al*., 2009; Weber *et al*., 2012). However, a decrease in the expression of Pax7 is required to promote terminal differentiation during regenerative myogenesis. This was confirmed in our results where, in response to differentiation cues, the % Pax7 myoblasts was drastically reduced. In addition, a study revealed that the population of $Pax7^+$ cells within a pool of activated myoblasts, ranges between approximately 25-53% (Olguin and Olwin, 2004). Interestingly, our studies show that HSk myoblasts have more $Pax7^+$ cells (55%) than C2C12 myoblasts (15%) (Figure 4.2A and B), suggesting that the HSk myoblasts may have a more rapid response to muscle injury that immortalized muscle cells (Lepper *et al*., 2012).

In response to Tenofovir, Zidovudine and Ritonavir, there was a decrease in fusion following 7 days of differentiation (Figure 4.5). These results correlate with previous data whereby Zidovudine was observed to induce a dose-related decrease of cultured human muscle differentiation (Benbrika *et al*., 1997). It was interesting to observe that regardless of similar MHC expression in Tenofovir- and Zidovudine-treated cells compared to control, there was less myotube formation in treated cells compared to control. MHC expression often begins within 2 or 3 days of differentiation and is

detected in mononucleated cells and at later time points, is detected in myotubes. MHC^+ mononucleated myocytes are cells that have withdrawn from the cell cycle prior to phenotypic differentiation and fusion (Andres and Walsh, 1996). Interestingly, both MHC expression and fusion were not detected in Ritonavir-treated cells (Figure 4.5 and 4.6). The current results are also consistent with studies in rats that show that myotube fusion is decreased by Ritonavir (Colby-Germinario *et al*., 2004).

As mentioned earlier, immortalized cells possess high levels of telomerase activity. Immortalized cells have telomeres with a constant length; the telomerase adding back DNA at each cell division. In contrast, primary myoblasts (the immediate progeny of satellite cells) have decreased levels of telomerase activity (O'Connor *et al*., 2009). Interestingly, during *in vitro* terminal differentiation of either human or murine cells, telomerase activity is down-regulated and this coincides with fusion of myotubes *in vivo* (O'Connor *et al*., 2009). In addition, there is little difference in the proliferative capacity between young and old cells, but there is a decline in the ability of aged cells to repair and maintain skeletal muscle *in vivo* (Chakravarthy *et al*., 2001; O'Connor *et al* ., 2009). Future *in vivo* studies could include correlating telomerase activity in satellite cells with markers of differentiation, in the presence and absence of ARVs.

The main downfall of *in vitro* experimental systems is the difficulty in extrapolating and relating the results to the actual biology of the living organism. Hence the need to diverge from over-interpreting results from *in vitro* studies, which can lead to incorrect conclusions about the organisms biology (Tiffany-Castiglioni *et al*., 1999). However, the complexity of living organisms makes it difficult to identify and investigate their basic functions and interactions (Brandon *et al*., 2003). Thus, the main advantage of *in vitro* work is that it provides a simplified outline of the processes under investigation. Additionally, many biotechnology companies thoroughly consider the advantage of *in vitro* research studies before *in vivo* research. Mainly because the costs and production times for *in vitro* research are much more less than that of *in vivo* research. Often enough, the *in vitro* experiments provide additional information (toxicity, metabolism, permeability) that animal testing may not. On the other hand, many candidate drugs that are effective *in vitro* may be ineffective *in vivo* because of the way in which the drug was delivered or toxic effects that were not represented in the initial *in vitro* studies (Tiffany-Castiglioni *et al*., 1999; Staton *et al*., 2004).

Mice are often the main model system of experimental tools for a number of *in vitro* studies. Studies using mice have yielded great insight into the workings of the human biological system. Reports on the sequencing of human and mice genomes, reveal that about 300 genes appear to be unique to one species or the other. There are however, a number of genetic similarities between

mice and humans that have lead to the tendency of allowing some differences to be ignored (Waterston *et al*., 2002). About 65 million years of evolution has passed, and this might suggest that a number of significant differences may exist. Thus by making such assumptions, there is the risk of overlooking aspects of human responses to challenges that do not occur or cannot be modeled in mice. With all this in mind, together with the relative ease in which mice can now be genetically manipulated to mimic human biological systems, mouse models will continue to provide important information for many years to come (Mestas and Hughes, 2004).

Further limitations to this study were that the intracellular drug concentrations used for this study may not reflect the concentrations *in vivo*, although the drug levels in culture were designed to approximate those used clinically. In addition, the duration of treatment of 2 to 7 days may not reflect the drug effect in patients receiving long-term ARV therapy. It is highly recommended that in future studies, cells should be exposed to the ARVs for longer time periods to help better understand the way in which the drugs work, and experiment the effects of higher concentrations as observed for other cell types. Furthermore, combination studies were not investigated and therefore this project does not reflect the combination drug therapies currently used in the clinics.

The study of human skeletal muscle cells is generally considered one of the most clinically relevant *in vitro* indicators of potential mitochondrial damage in patients, because mitochondrial toxicity often presents with muscular symptoms, including muscle atrophy, weakness, fatigue and cardiomyopathy (Saitoh *et al*., 2008). In our study, in addition to using C2C12 murine muscle cells, we used human skeletal muscle cells to provide an appropriate experimental model that has human physiological relevance. The differences in responses observed between mouse and human myoblasts may be species-related or due to the immortal nature of the C2C12 cells versus the primary culture HSk myoblasts. Future studies using primary culture mouse myoblasts could elucidate this. The results of this current study suggest, but do not prove, that ARVs affect myogenesis by decreasing the fusion of differentiated myocytes, thereby leading to a decrease in skeletal muscle mass. Despite the limitations, the models used in this study are consistent with clinical data. The results give further understanding of molecular mechanisms that could lead to muscle wasting in HIV/AIDS patients who are on long-term ARV treatment.

Finally, an increase in adipose deposition between myotubes may be a result of long-term treatment with ARVs, causing shorter myotube formation and allowing more fat droplets to be deposited in between the muscle cells. This could be an underlying reason as to why muscles of HIV-infected patients becomes "fatty" (Owczarek *et al*., 2005). A number of studies that have been carried out on the cross-talk between muscle and fat cells is of particular interest with regards to ARV treatment and its associated metabolic side effects. The net consequence of this involves

progressive atrophy and loss of individual muscle fibers, associated with infiltration of fat and other non-contractile material, which causes a reduction in muscle quality (Bonaldo and Sandri, 2013). It would be fascinating to observe if blocking myogenesis in muscle cells would enable successful trans-differentiation of these cells into adipocytes in response to Tenofovir, Zidovudine or Ritonavir.

In conclusion, we have shown that Tenofovir, Zidovudine and Ritonavir, in clinically relevant concentrations, have an impact most notably on myoblast proliferation and fusion into myotubes, as well as on myoblast migration. These data are consistent with previous studies that show that chronic use of ARVs could lead to muscle wasting. This exploratory study gives scientific evidence that further research into effects of ARVs on skeletal muscle stem cells is warranted.

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APPENDIX

CONFERENCE ATTENDANCE

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1. **39th Annual Conference of the Physiology Society of Southern Africa (PSSA)**

Muscle atrophy: Is long-term antiretroviral treatment a causative agent?

Sibanda, W. N and Niesler, C. U.
School of Life Sciences

Successful antiretroviral (ARV) treatment is associated with the suppression of HIV viral load and the reduction of clinical disease progression. However, despite marked improvements, side effects from long-term therapies, such as loss of muscle mass do occur. The mechanism by which ARVs affect muscle mass is unclear, however, published *in vitro* data indicates an effect on myoblast fusion during differentiation. The objective of this study is therefore to determine the effect of ARVs on other processes required for successful myogenesis, such as proliferation and migration. Zidovudine (nucleoside reverse transcriptase inhibitor-NRTI), Tenofovir (nucleotide reverse transcriptase inhibitor-NtRTI) and Ritonavir (protease inhibitor-PI) were utilized at a concentration range of 0.01 µM to 10 µM. C2C12 cells were used as the model myoblast cell line. Proliferation was determined using crystal violet, whereas migration was analyzed using 2D wound healing assay. Short-term incubation with all three ARVs had no significant effect on proliferation or migration. However, following long-term incubation, proliferation was increased in response to all three ARVs. Furthermore, migration was observed to have increased in response to Zidovudine and Tenofovir, but not Ritonavir. To our knowledge, this study is the first to suggest that chronic incubation with selected ARVs may significantly influence myoblast proliferation and migration. An increase in these processes may result in a depletion of the satellite cell population, thereby contributing to a decrease in muscle mass. Studies to analyze the self-renewal capability of myoblasts in response to ARVs are underway.

2. **South African Society of Biochemistry and Molecular Biology/Federation of African Societies of Biochemistry and Molecular Biology (SASBMB/FASBMB) Congress**

In vitro effects of antiretrovirals on myogenesis

Sibanda, W. N., Pillay T. and Niesler, C. U.
School of Life Sciences

Successful antiretroviral (ARV) treatment is associated with the suppression of HIV viral load and the reduction of clinical disease progression. Despite marked improvements to ARV treatments, side effects from long-term therapies, such as loss of muscle mass do occur. The mechanism by which ARVs affect muscle mass is unclear, however, published *in vitro* data indicates an effect on myoblast fusion during differentiation. The objective of this study is therefore to determine the effect of ARVs on processes required for successful myogenesis; these include self-renewal capability of myoblasts, proliferation, migration and differentiation. C2C12 mouse myoblasts and human primary culture myoblasts were incubated with Zidovudine (nucleoside reverse transcriptase inhibitor-NRTI), Tenofovir (nucleotide reverse transcriptase inhibitor-NtRTI) or Ritonavir (protease inhibitor-PI) at a concentration range of 0.01 µM to 10 µM. Self-renewal was assessed via the expression of the transcription factor Pax7 and proliferation determined using crystal violet. Migration was analyzed using a 2D wound healing assay, whereas differentiation was measured by assessing the fusion index. Pax7 nuclear localization, as well as myoblast proliferation, was observed to increase in response to all three ARVs in C2C12 cells. However, migration increased in response to Zidovudine only. Preliminary differentiation results indicate a decrease in fusion of C2C12 myoblasts into myofibers. To our knowledge, this study is the first to suggest that selected ARVs may significantly influence myoblast self-renewal capabilities through prolonged Pax7 nuclear expression and thereby decrease their myogenic capability. Human myoblast studies to confirm this hypothesis are underway.