Differential Sensitivity to Toxicity of Statins: A Comparison of Liver and Muscle Cell Lines

by

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Abstract

Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis, and are widely used to treat hypercholesterolaemia, a risk factor for cardiovascular disease. Myopathy (muscle toxicity) is an adverse effect that is thought to occur due to the secondary effects of inhibiting HMGCR; however the mechanism of this myopathy is not fully understood. Therefore, the aim of this study was to use two human cell lines as an *in vitro* model for comparing the effects of a variety of statins on both cholesterol synthesis and protein prenylation between the liver, the intended target tissue for therapeutic benefit, and skeletal muscle, the major site of toxicity.

Initially, the differentiation of RD rhabdomyosarcoma muscle cells was characterised in terms of morphological and molecular changes that occur, using microscopy and TaqMan analysis respectively, and a 5-day cell differentiation period was chosen for use in later experiments. The initial hypothesis to then be tested was that the process of protein prenylation exhibits circadian variation, as has already been shown for cholesterol synthesis, and this might enable temporal separation between the cholesterol-lowering efficacy and the toxicity of statins. However, the ability of the RD cell line to establish and maintain circadian rhythmicity *in vitro* could not be verified and so the circadian contributions to cholesterol metabolism and protein prenylation in liver and muscle in this model could not be investigated.

Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, significant, dose-dependent reductions in cell viability were observed in both liver and muscle cells on treatment with each of the statins ($P = 0.0041$, atorvastatin; $P < 0.0001$, all other statins, two-way analysis of variance (ANOVA)). However, greater cell toxicity was observed with the more lipophilic statins, simvastatin (acid), lovastatin (acid) and cerivastatin, and also in RD muscle cells compared to Huh7 liver cells. Taken together, these data represent the first comprehensive comparative analysis of statin toxicity in human liver and muscle cell lines. Cholesterol metabolism was then examined in these cells: Although cholesterol was present in all cell types, the levels in RD cells were somewhat lower than in Huh7 cells, probably due to much lower levels

of HMGCR as measured at both the transcript and protein level; and 48 hour treatment with 10 μ M simvastatin and pravastatin, and 1 μ M cerivastatin, seemed to lead to a decrease in total cellular cholesterol.

Subsequently, protein was extracted from Huh7 and RD cells treated with simvastatin, cerivastatin or pravastatin, and then subjected to analysis by western blot to detect any changes in the prenylation status of selected small GTPases. Inhibition of prenylation was found by directly analysing changes in the unprenylated form of Rap1A, using a specific antibody, and by examining the reduction in membrane association of H-Ras, M-Ras and Rab1A. Treatment with simvastatin and cerivastatin inhibited the prenylation of Rap1A, and seemed to also affect membrane association of the small GTPases H-Ras, M-Ras and Rab1A. In both muscle and liver cells the inhibition of prenylation of Rap1A, a Ras-related GTPase, was 10-fold more sensitive to treatment with cerivastatin than simvastatin. Inhibition of Rap1A prenylation in RD muscle cells was also 10-fold more sensitive to simvastatin and 3-fold more sensitive to cerivastatin treatment than in liver cells. Hydrophilic pravastatin, which showed the lowest toxicity in MTT assays, did not affect the prenylation of any of the small GTPases examined, as far as was detectable using western blot analysis.

In conclusion, this work has shown that RD muscle cells show both greater sensitivity to the toxic effects of statins and preferential disruption of protein prenylation in response to statin treatment when compared to Huh7 liver cells. This further supports a mechanism whereby disrupting production of intermediates in the cholesterol synthesis pathway, including the isoprenoids used in protein prenylation, is a significant cause of statin-induced muscle toxicity. This study also highlights the importance of examining the effects of drugs at both the target site of action and at the site of toxicity, rather than only studying one site in isolation, in order to gain insight into the mechanism of adverse drug effects.

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I have always loved science and studying the natural world, flitting between loves of biology, chemistry and maths. The path of a PhD has been in my mind for a long time, however it was only after having worked as a research assistant for a while that I felt ready (ha-ha) to take one on. I realised that I missed academia, and the challenge of wrestling with a problem to try and find the answer. Little did I realise how big a challenge it would turn out to be! There are so many people who have been such a huge support to me along this PhD path, who I would just like to take a moment to say thank you to.

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Psalm 23

(New Living Translation)

The Lord is my shepherd; I have all that I need.

He lets me rest in green meadows;

He leads me beside peaceful streams.

He renews my strength.

He guides me along right paths,

bringing honour to his name.

Even when I walk through the darkest valley,

I will not be afraid,

for you are close beside me.

Your rod and your staff

protect and comfort me.

You prepare a feast for me

in the presence of my enemies.

You honour me by anointing my head with oil.

My cup overflows with blessings.

Surely your goodness and unfailing love will pursue me

all the days of my life,

and I will live in the house of the Lord

forever.

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Abbreviations

3T3-L1	Mouse Preadipocyte Cell Line
4S	Scandinavian Simvastatin Survival Study
ACTB	β -Actin
ADME	Absorption, Distribution, Metabolism and Excretion
AMPK	Adenosine Monophosphate–Activated Protein Kinase
ANOVA	Analysis of Variance
APS	Ammonium Persulphate
ATCC	American Type Culture Collection
Bcl-2	B-cell lymphoma 2
BisTris	Bis-(2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane
BMAL1	Brain and Muscle Arnt-Like Protein-1 (protein)
<i>Bmal1</i>	Reference to the mouse or rat gene; also used in generic statements
<i>BMAL1</i>	Reference specifically to the human gene
BME	β -Mercaptoethanol
bp	Base Pairs
BSA	Bovine Serum Albumin
β -TrCP	Transducin Repeat Containing Protein
BTS	British Toxicology Society
C2C12	Mouse Myoblast Cell Line
Ccg	Clock Controlled Gene
cDNA	Copy DNA
Ceriva	Cerivastatin
CK1	Casein Kinase 1
CLOCK	Circadian Locomotor Output Cycles Kaput
CoQ10	Coenzyme Q10 (also known as ubiquinone)
CRY	Cryptochrome
CYP	Cytochrome P450
CYP3A4	Cytochrome P450, Family 3, Subfamily A, Polypeptide 4
CYP7A1	Cholesterol-7 α -Hydroxylase
CYP8B1	Sterol-12 α -Hydroxylase
DBP	D site of Albumin Promoter (Albumin D-box) Binding Protein
DEC1	Deleted in Oesophageal Cancer 1

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
ECACC	European Collection of Cell Cultures
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
FBS	Foetal Bovine Serum
FBXL3	F-box and Leucine-Rich Repeat Protein 3
FDA	US Food and Drug Administration
FOXO1	Forkhead Box O1
FPP	Farnesylpyrophosphate
FT	Farnesyltransferase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDP	Guanosine Diphosphate
GGPP	Geranylgeranylpyrophosphate
GGT-I	Geranylgeranyltransferase type I
GGT-II	Geranylgeranyltransferase type II
GSK	GlaxoSmithKline
GTP	Guanosine Triphosphate
GTPase	Guanine Nucleotide-Binding Protein
HepG2	Human Hepatoblastoma Cell Line
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
HMGCR	3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase
HPLC	High Performance Liquid Chromatography
H-RAS	v-Ha-Ras Harvey Rat Sarcoma Viral Oncogene Homolog
Huh7	Human Hepatocellular Carcinoma 7 Cell Line
HRP	Horse Radish Peroxidase
ID	Inhibitor of Differentiation
IGF-1	Insulin-Like Growth Factor 1
JUPITER	Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin
kDa	Kilodaltons
LC-MS	Liquid Chromatography–Mass Spectrometry

LDH	Lactate Dehydrogenase
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LXR	Liver-X-Receptor
MAPK	Mitogen-Activated Protein Kinase
MCK	Muscle Creatine Kinase
MEK	Mitogen-Activated Protein Kinase Kinase
MHC	Myosin Heavy Chain
M-RAS	Muscle RAS Oncogene Homolog
MRF	Myogenic Regulatory Factor
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)2,5-Diphenyl Tetrazolium Bromide
MYH3	Myosin Heavy Chain 3
MYOD1	Myogenic Differentiation 1
MYOG	Myogenin
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NEAA	Non-Essential Amino Acids
NPAS2	Neuronal PAS Domain Protein 2
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
OAT	Organic Anion Transporter
OATP	Organic Anion Transporting Polypeptide
PAR	Proline and Acidic Amino Acid–Rich
PBS	Phosphate Buffered Saline
PER	Period
PI3K	Phosphatidylinositol 3-Kinase
PP	Pyrophosphate
Prava	Pravastatin
PVDF	Polyvinylidene Difluoride
qPCR	Quantitative Polymerase Chain Reaction
RD	Human Embryonic Rhabdomyosarcoma Cell Line
RD-U	Undifferentiated RD Cells
RD-D	Differentiated RD Cells
REP	Rab Escort Protein
REV-ERB α	Reverse Erythroblastosis Virus α

RIPA	RadiolImmunoPrecipitation Assay
RNA	Ribonucleic Acid
ROR	Retinoic Acid-Related Orphan Nuclear Receptor
RRE	Retinoic Acid-Related Orphan Receptor Response Element
rRNA	Ribosomal RNA
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCF	Skp1-Cul1-F-Box-Protein
SCN	Suprachiasmatic Nucleus
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Sec-tRNA	Selenocysteine tRNA
SEM	Standard Error of the Mean
SEPN1	Selenoprotein N
Simva	Simvastatin (acid)
SLCO1B1	Solute Carrier Organic Anion Transporter Family, Member 1B1
SNP	Single Nucleotide Polymorphism
SREBP	Sterol Regulatory Element Binding Protein
T25	25cm ² Vented Cell Culture Flask
T75	75cm ² Vented Cell Culture Flask
TAE	Tris Acetate EDTA
TBP	TATA-Binding Protein
TBST	Tris-Buffered Saline–Tween 20
TCA	Trichloroacetic Acid
TCEP	Tris(2-carboxyethyl)phosphine HCl
TEMED	Tetramethylethylenediamine
TPA	12-O-Tetradecanoyl Phorbol-13-Acetate
tRNA	Transfer Ribonucleic Acid
UK	United Kingdom
US	United States
V/V	Volume per volume
W/V	Weight per Volume
WOSCOPS	West of Scotland Coronary Prevention Study

1 Introduction

1.1 Statins

1.1.1 Clinical Use

Cardiovascular diseases are the leading cause of death worldwide, causing 27% and 32% of all deaths of men and women respectively (World Health Organisation 2008). Lowering cholesterol in the form of low-density lipoprotein (LDL) is a common strategy in the fight against cardiovascular disease since an elevated concentration of cholesterol in this form is known to be one of the most important risk factors (Brown and Goldstein 2004).

The statin class of drugs is currently the first line of treatment for hypercholesterolaemia, after numerous large clinical trials have demonstrated the benefits of their use (Liao 2002; Ong 2005). In particular, the 4S trial (Scandinavian Simvastatin Survival Study 1994) and the West of Scotland Coronary Prevention Study (WOSCOPS) (Shepherd et al. 1995) were two trials in the 1990s that were very significant in first demonstrating the advantages of statin treatment (Ong 2005). It has been shown that statins are very effective at lowering blood LDL levels (Weng et al. 2010); in short-term studies LDL cholesterol reductions of 17–55 % have been measured in patients, depending on the dose and the particular statin used (Table 1-1). Widespread use of statins has also led to a decrease in the rates of morbidity and mortality due to atherosclerosis (Stossel 2008).

Statin	Dose (mg/day)	LDL Cholesterol (%)
Lovastatin	20	-29
	40	-32
	80	-48
Simvastatin	10	-28
	20	-35
	40	-41
	80	-46
Pravastatin	10	-19
	20	-24
	40	-34
Fluvastatin	20	-17
	40	-23
	80*	-36
Atorvastatin	10	-38
	20	-46
	40	-51
	80	-54
Rosuvastatin ^{1,2}	5	-42
	10	-47
	20	-53
	40	-54
Pitavastatin ¹	1	-34
	2	-42
	4	-47
	8	-55
Cerivastatin	0.3	-31
	0.4	-36
	0.8	-45

* Extended-release formulation (-36%); 40mg twice daily (-32%)

Table 1-1 Percentage change in plasma concentration of low density lipoprotein (LDL) cholesterol with approved doses of eight statins in patients with hypercholesterolaemia

Data from (Chong et al. 2001), other than where indicated, obtained from short-term studies ranging from 4-10 weeks in duration. ¹ Data from (Mukhtar et al. 2005), obtained from studies of 6 weeks (rosuvastatin, 20mg and 40mg) or 12 weeks (pitavastatin) in duration. ² Data from (Blasetto et al. 2003) obtained from a study of 12 weeks in duration (rosuvastatin, 5 mg and 10mg).

Statins are used to treat both those with established cardiovascular disease (secondary prevention) and those who are healthy but designated 'high risk' of a cardiovascular event (primary prevention). However, treatment is continually expanding to include those at lower risk (Hlatky 2008). This is partially due to the results of several recent clinical trials, which have demonstrated the benefits of the use of statins in primary prevention (Brugts et al. 2009). One such example is the Justification for the use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER); in which comparison with a placebo group showed a reduction in the number of major cardiovascular events, in healthy men and women with low LDL levels but elevated levels of the inflammatory biomarker high-sensitivity C-reactive protein who were treated with rosuvastatin (Hlatky 2008; Ridker et al. 2008). There is currently debate over the potential benefits of statin treatment even for those who are considered at low risk and would not be considered for such intervention under current guidelines (Mihaylova et al. 2012; Taylor et al. 2011).

For this reason, as well as from looking at recent trends (Stagnitti 2008), the use of statins seems set to only continue to increase in the coming years. Therefore, it is all the more important to have an in-depth understanding of the mechanisms by which these drugs exert their cholesterol-lowering and other non-cholesterol related effects on the body, and to understand the factors regulating these effects.

1.1.2 Currently Available Statins

Since the first inhibitor of cholesterol synthesis was discovered by Akira Endo in 1977 (Steinberg 2006), a range of related compounds have been isolated and synthesized, and collectively these cholesterol lowering drugs are known as the statins. The currently available statins can be divided into two classes – those isolated from fungi or derived from fungal products (type 1 statins) and those which are completely synthetic (type 2). Those in the former category include lovastatin, simvastatin and pravastatin. Those in the latter category include fluvastatin, atorvastatin, rosuvastatin, pitavastatin and cerivastatin (Shitara and Sugiyama 2006). Generic names and the most common UK brand names of statins are listed in Table 1-2.

Generic Name	Brand Name (Manufacturer)
Atorvastatin	Lipitor (Pfizer)
Cerivastatin ¹	Lipobay (Bayer)
Fluvastatin	Lescol (Novartis)
Lovastatin	Mevacor (Merck), Altocor, Altoprev
Pitavastatin ²	Livazo, Alipza (Kowa Pharmaceutical)
Pravastatin	Lipostat (Bristol-Myers Squibb)
Rosuvastatin	Crestor (AstraZeneca)
Simvastatin	Zocor (Merck), Simvador

Table 1-2 Generic and most common brand names of statins in the United Kingdom

Information taken from the British National Formulary (Joint Formulary Committee 2008).
¹ Cerivastatin was withdrawn from the worldwide market in 2001 (Bellingham 2001); ² Pitavastatin is not yet commercially available in the UK (Kowa Pharmaceutical Europe 2011).

Pitavastatin is the newest statin; it has been available in Japan since 2003 (Chapman 2010) and is now being marketed as Livazo in an increasing number of countries around the world, with the first European launch having taken place in Spain in 2011 (Kowa Pharmaceutical Europe 2011). In July 2004 simvastatin became available over-the-counter in the United Kingdom (UK), as a 10mg/day dose, with a licence for sale to those at moderate (10-15%) ten-year risk of a first coronary event (National Institute for Health and Clinical Excellence 2006). This controversial decision made the UK the first country to have a non-prescription

statin (Abrams 2005). Cerivastatin was withdrawn from the worldwide market in 2001 and is therefore no longer commercially available (Bellingham 2001).

1.1.3 Mechanism of Action

Cholesterol plays a vital role in the body and is essential for normal cell function: It is a major constituent of eukaryotic cell membranes, where its sterol ring structure provides rigidity (Figure 1-1). This means that the amount of cholesterol present in a membrane is important in determining the properties of the membrane. Cholesterol is also the precursor to steroid hormones (such as oestrogen, progesterone, testosterone and glucocorticoids), vitamin D and bile salts. However, in excess it can also contribute to the build up of lipid deposits and hardening of the arteries in atherosclerosis, which is thought to often lead to cardiovascular disease (Shitara and Sugiyama 2006; Voet and Voet 2004).

Figure 1-1 The structure of cholesterol

Cholesterol has a polar head group, a sterol ring structure that provides rigidity to cell membranes, and a nonpolar hydrocarbon tail. (Voet and Voet 2004).

There are two main sources of cholesterol in the body: cholesterol from the diet and cholesterol that has been synthesized internally. Although almost all cells are

able to synthesize cholesterol, the liver is the main site of cholesterol biosynthesis in the body and is therefore the main target organ of all of the statins (Catapano 2007).

Cholesterol biosynthesis is a complex pathway involving many enzymes and intermediates. All statins act by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR) (Figure 1-2), which catalyses the first committed, and rate-limiting, step of cholesterol biosynthesis (Shitara and Sugiyama 2006). The resulting decrease in *de novo* cholesterol synthesis leads to upregulation of the LDL receptor (LDLR), through the activation of cholesterol-sensing transcription factor sterol regulatory element binding protein (SREBP) (Brown and Goldstein 1997). The increase in LDLR then leads to increased uptake of cholesterol, in the form of LDL, from the bloodstream into cells, resulting in an overall reduction in plasma LDL levels (Brown and Goldstein 2004).

Figure 1-2 The conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate is catalysed by the enzyme HMG-CoA reductase (HMGCR)

This reaction is a reduction, requiring NADPH (nicotinamide adenine dinucleotide phosphate), and is the rate-limiting step in the biosynthesis of cholesterol. It is also the step where statins exert their effect by acting as competitive inhibitors of HMGCR. (Voet and Voet 2004).

The mevalonate pathway (Figure 1-3), the branched pathway that cholesterol biosynthesis is a part of, uses common precursors to also synthesize a number of essential isoprenoids other than cholesterol. These include dolichol, isopentenylpyrophosphate, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). Statin inhibition of HMGCR affects the

level of all common precursors and hence levels of these other isoprenoids. It is this which is thought to underlie the major adverse effects of statins (Baker 2005).

Figure 1-3 The mevalonate pathway: biosynthesis of cholesterol and other essential isoprenoids

Statin inhibition of the rate-limiting enzyme HMGCR affects levels of not only cholesterol but also of sec-tRNA, ubiquinone, dolichol and prenylated proteins. Dolichol synthesis also uses isopentenyl-PP, but arrow not shown for diagram simplicity. Figure produced using the following references: (Baker 2005; Goldstein and Brown 1990; Lane and Beese 2006; McTaggart 2006; Shitara and Sugiyama 2006; Vekrellas et al. 2009; Voet and Voet 2004).

1.1.4 Similarities and Differences between the Statins

All of the statins have an HMG-like moiety, enabling them to tightly bind to the catalytic site of HMGCR and block access for the substrate HMG-CoA, resulting in potent, competitive inhibition of HMGCR (Figure 1-4). However, there are structural differences between statins that result in differences in their physiological properties (Table 1-3). This includes differences in their modes of binding, which can be seen from the crystal structures that have been solved for a portion of the enzyme bound to each of six different statins (Istvan and Deisenhofer 2001). In general the newer statins exhibit a higher affinity for HMGCR and have more potent inhibitor effects (Shitara and Sugiyama 2006).

Figure 1-4 Chemical structures of the different statins

All of the statins have an HMG-like moiety, which can either be in the lactone form (simvastatin and lovastatin) or in the open acid form (all other statins). However, there are also many differences in structure which account for the different physiological properties between statins. Figure reproduced from (Shitara and Sugiyama 2006).

Statin	Simvastatin (Simvastatin acid)	Lovastatin (Lovastatin acid)	Cerivastatin	Fluvastatin	Atorvastatin	Pitavastatin	Rosuvastatin	Pravastatin
logD (pH 7.0) ¹	4.4 (1.88)	3.91 (1.51)	2.32	1.75	1.53	1.5		-0.47
logD (pH 7.4) ¹			1.50 - 1.75	1.00 - 1.25	1.00 - 1.25		-0.25 - -0.50	-0.75 - -1.00
Lipophilicity	Lipophilic	Lipophilic	Lipophilic	Lipophilic	Lipophilic	Lipophilic	Hydrophilic	Hydrophilic
Uptake ²								
Passive diffusion ?	Yes (OATP1B1)	Yes (OATP1B1)	Yes OATP1B1	Yes Unknown	? OATP1B1	? OATP1B1 (OATP1B3)	No OATP1B1 OATP1B3	No OATP1B1
Active transport ?					OATP2B1 (L/M)		OATP2B1 (L/M)	OATP2B1 (L/M) OATP1A4 (M) OAT3(M)
Metabolism ³	CYP3A4 CYP3A5	CYP3A4	(CYP3A4) CYP2C8	CYP2C9	CYP3A4	(CYP3A4) CYP2A9 CYP2C9 Minimally; slow	CYP2C9 Minimally	CYP3A4 Sulphation Minimally
Half-life (hours) ⁴	3	3 - 4	2 - 3	< 1	14	11	19	1.8
Bioavailability (%) ⁴	< 5	< 5	60	24	12	> 60	20	17
Year introduced ⁵	1991	1987	1997 (withdrawn 2001)	1993	1996	2003 (Japan) 2011 (EU)	2003	1991

Table 1-3 Summary of the major differences between properties of the statins

¹ (Shitara and Sugiyama 2006)

² (Kitamura et al. 2008; Knauer et al. 2010; Sakamoto et al. 2008; Shitara and Sugiyama 2006; Takeda et al. 2004)

³ (Chong et al. 2001; Mukhtar et al. 2005; Vaklavas et al. 2009)

⁴ (AstraZeneca 2007; Chong et al. 2001; Mukhtar et al. 2005)

⁵ (AstraZeneca 2007; Recordati 24 October 2008; Staffa et al. 2002)

LogD values reflect the lipophilicity of a statin, with simvastatin and lovastatin being the most lipophilic (yellow), and rosuvastatin and pravastatin the least lipophilic (orange). The newest statins, atorvastatin, pitavastatin and rosuvastatin, appear to have the longest half-lives in the body. Bioavailability refers to the percentage of the drug that becomes available at the target site in the body, for example after absorption, and uptake refers to hepatic transport other than where noted: L/M=liver and muscle, M=exclusively muscle transport. OATP = organic anion transporting polypeptides; OAT = organic anion transporter; CYP = Cytochrome P450.

The statins exhibit varying degrees of lipophilicity and therefore have different uptake mechanisms (Table 1-3). Simvastatin and lovastatin are the only statins that are administered as prodrugs in an inactive form, containing a lactone ring that is converted into the open acid form in the body. This means that simvastatin and lovastatin are the most lipophilic; they can cross lipid bilayers by passive diffusion and can therefore become widely distributed to a variety of tissues. In contrast, pravastatin and rosuvastatin are hydrophilic and rely on transporters for uptake into hepatocytes. The other statins have intermediate physicochemical properties (Hamelin and Turgeon 1998). Studies have demonstrated that transport of statins is mediated by various members of the organic anion transporting polypeptide

(OATP) and organic anion transporter (OAT) families (Hsiang et al. 1999; Sakamoto et al. 2008; Takeda et al. 2004; Tokui et al. 1999). In particular OATP1B1, a transporter exclusive to liver, contributes to varying degrees to the hepatic uptake of many of the statins (Kitamura et al. 2008).

The statins are also metabolised differently by the liver (Table 1-3). The cytochrome P450 (CYP) family is a large family of haem-containing monooxygenase enzymes that catalyse many reactions involved in drug metabolism. CYP3A4 (cytochrome P450, family 3, subfamily A, polypeptide 4) is thought to be involved in the metabolism of approximately half of all drugs in current use (Takiguchi et al. 2007), and this includes simvastatin, lovastatin and atorvastatin (Bolego et al. 2002). Cerivastatin is metabolised by CYP2C8 as well as CYP3A4 and fluvastatin, alone amongst the statins, is metabolised by CYP2C9. Pitavastatin, rosuvastatin and pravastatin undergo minimal metabolism by CYP enzymes and are therefore not susceptible to many of the metabolism drug-drug interactions in the same way as other statins (Shitara and Sugiyama 2006). Both cerivastatin and atorvastatin have metabolites that contribute towards their lipid-lowering effects (Chong et al. 2001).

All of these differences impact on statin prescribing choices, depending on the needs of each individual patient based on their risk profile (Chong et al. 2001). They potentially also impact upon the incidence of adverse effects (Bolego et al. 2002).

1.2 The Problem of Statin-Induced Myopathy

1.2.1 Myopathy and its Incidence amongst Statin Users

Statins are generally considered to be effective and relatively safe drugs, and are well tolerated by most (Law and Rudnicka 2006). However some patients do experience side effects, the most significant of which is myopathy. Myopathy is a general term used to refer to any disease of the muscles, which encompasses everything ranging from mild fatigue and minor muscle aches, pains, cramps and

weakness to complete collapse of the muscle structure in rhabdomyolysis, which requires hospitalisation and in rare cases can prove fatal (Harper and Jacobson 2007).

Muscle complaints, including rhabdomyolysis, have been reported with all statins (Bolego et al. 2002) and rhabdomyolysis-related death has been reported with all statins other than fluvastatin (Table 1-4). It was the high number of deaths caused by rhabdomyolysis following treatment with cerivastatin in the US that prompted its withdrawal from the market in 2001 (Bellingham 2001). After cerivastatin, the next highest number of fatal cases has been reported in patients taking lipophilic drugs simvastatin and lovastatin; and in fact, due to increased risk of myopathy in general the highest dose of 80mg simvastatin is now no longer recommended, other than in patients who have already had this treatment for more than 12 months with no adverse muscle effects (Gotto and Moon 2012). Fluvastatin and pravastatin seem to produce the fewest cases of rhabdomyolysis; however these statins are also less effective at lowering blood cholesterol than some of the other statins (Table 1-1).

	<u>Lovastatin</u>	<u>Pravastatin</u>	<u>Simvastatin</u>	<u>Fluvastatin</u>	<u>Atorvastatin</u>	<u>Cerivastatin</u>	<u>Total</u>
Date Approved	08/1987	10/1991	12/1991	12/1993	12/1996	06/1997	-
Fatal Cases of Rhabdomyolysis	19	3	14	0	6	31	73
No. prescriptions dispensed since marketing began (million)	99.197	81.364	116.145	37.392	140.360	9.815	484.273
Reporting Rate (per 1 million prescriptions)	0.19	0.04	0.12	0	0.04	3.16	0.15

Table 1-4 Reported cases of fatal rhabdomyolysis for all statins dispensed in the United States from when these products were launched until 2001

Fatal rhabdomyolysis has been reported with all statins, other than fluvastatin, however different statins have different reporting rates. The highest reporting rate of rhabdomyolysis was with cerivastatin, prompting its withdrawal from the market in 2001, with the next highest number of cases seen with simvastatin and lovastatin. Table reproduced from: (Staffa et al. 2002). Reported fatal cases of rhabdomyolysis are those reported to the US Food and Drug Administration (FDA) before 2001 and where death resulted either directly or indirectly from rhabdomyolysis. Reporting rate is the number of fatal cases divided by the number of prescriptions.

Myalgia, muscle weakness and aches without elevation of creatine kinase, has been estimated to occur in 10-15% of all statin patients (Mohaupt et al. 2009) and rhabdomyolysis incidence has been estimated at 3.4 per 100,000 person-years (with statins other than cerivastatin) (Law and Rudnicka 2006). However, it is difficult to accurately estimate the incidence of all statin-induced myopathy as it is well recognised that the less severe muscle effects are under-reported, and that incidence of myopathy in controlled clinical trials is artificially depressed, partly because many of those who would be more prone to statin-induced myopathy are not included in these trials (Vaklavas et al. 2009). It has also been suggested that cases of rhabdomyolysis are similarly under-reported (McAdams et al. 2008).

1.2.2 Factors Affecting the Incidence of Statin-Induced Myopathy

Statin-induced myopathy is known to be dose-dependent (Law and Rudnicka 2006) and any interaction which leads to an increase in statin exposure is a risk factor (Kitamura et al. 2008; Knauer et al. 2010; Sidaway et al. 2009).

The lipophilicity of statins such as simvastatin and lovastatin has been suggested to account for their higher incidence of adverse muscular side effects, due to their high rates of passive diffusion into all tissue, including muscle (Vaklavas et al. 2009). However, transporters such as OAT1, OATP1A4 and OATP2B1 have been reported to be expressed in muscle tissue and recognise statins as substrates, and could therefore play a role in determining muscle statin exposure (Table 1-3). The importance of these transporters in the development of muscle toxicity is not yet entirely clear (Knauer et al. 2010; Shitara and Sugiyama 2006; Sidaway et al. 2009), although it has been shown that some genetic polymorphisms affecting transporters, and therefore drug disposition, can affect patient risk of developing myopathy. One such example is a single nucleotide polymorphism (SNP) in the solute carrier organic anion transporter family, member 1B1 (*SLCO1B1*) gene, encoding the transporter OATP1B1, that has been associated with a higher risk of myopathy in response to simvastatin treatment (Link et al. 2008; Voora et al. 2009). Due to these findings a diagnostic test has now been launched, enabling

the use of *SLCO1B1* genotyping to identify those at high risk (Isis Innovation 2012).

Myopathy risk is also increased by drug interactions that can occur when statins are administered as part of combination therapy, particularly with drugs that can affect statin metabolism and therefore statin exposure (Knauer et al. 2010). Many drugs, including cyclosporine, azole antifungals, macrolides and calcium channel blockers, can inhibit the activity of CYP3A4, an enzyme important for the metabolism of many of the statins (Table 1-3). In fact, it has been estimated that interactions with drugs known to inhibit CYP3A4 activity underlie 60% of rhabdomyolysis cases associated with simvastatin, lovastatin or atorvastatin (Vaklavas et al. 2009). Similarly, fluvastatin may interact with inhibitors of CYP2C9, such as omeprazole and ritonavir (Chong et al. 2001), and with other substrates of CYP2C9, such as warfarin, fluconazole and diclofenac (Bolego et al. 2002).

The risk of rhabdomyolysis is substantially higher on co-treatment with a statin and a fibrate, particularly gemfibrozil (Harper and Jacobson 2010; Law and Rudnicka 2006; Mastaglia and Needham 2012). Gemfibrozil has been shown to inhibit CYP2C8, one of the enzymes involved in the metabolism of cerivastatin, which could account for the higher incidence of rhabdomyolysis in patients treated with both cerivastatin and gemfibrozil. Gemfibrozil is also a substrate of CYP3A4 and can inhibit the glucuronidation of statins (Prueksaritanont et al. 2002).

There are many patient characteristics which are thought to affect myopathy risk. These include several underlying medical conditions that can predispose a patient towards developing statin-induced myopathy, such as hepatic dysfunction, hypothyroidism and metabolic muscle diseases. Women and those of more advanced age are thought to be at higher risk of experiencing statin-induced myopathy (Harper and Jacobson 2010). In addition lifestyle choices, such as

performing strenuous exercise and the consumption of alcohol, can also increase risk.

Time of dosing may impact upon tolerability of statins, as has already been demonstrated with a number of other drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and cancer therapeutics, due to the impact of circadian rhythms on wide-ranging aspects of physiology (Levi and Schibler 2007). This will be discussed further in section 1.4.

1.3 The Mechanism of Statin-Induced Myopathy

There continues to be much debate over the precise mechanism of statin-induced myopathy. Co-treatment with statin and mevalonate, the product of HMGCR, has been shown to prevent the development of myopathy *in vivo* (Westwood et al. 2008) and so the current consensus seems to be that the decreased production of essential isoprenoids in the mevalonate pathway is the most likely underlying cause of myopathy (Baker 2005; Mastaglia and Needham 2012; Sathasivam 2012; Vaklavas et al. 2009). There is varying evidence for the involvement of each of the products and intermediates of this pathway (Vaklavas et al. 2009).

1.3.1 Decreased Membrane Cholesterol

There is evidence to suggest that a decreased level of cholesterol is not in itself the cause of myopathy, although this was initially thought to be the case (Baker 2005; Nishimoto et al. 2003). Skeletal myopathy is associated with mevalonic aciduria, a disorder caused by deficiency of mevalonate kinase which is an enzyme important at an early stage of the cholesterol biosynthesis pathway (Figure 1-3). In contrast, syndromes caused by deficiencies in terminal enzymes (distal enzymopathies) in cholesterol biosynthesis do not show myopathy among their clinical manifestations (Baker 2005).

In addition, inhibitors of squalene synthase do not cause muscle toxicity *in vitro* (Flint et al. 1997) and they have in fact shown some protective effect on statin-induced myopathy (Nishimoto et al. 2007). Unlike statins, they do not lead to a

decrease in mevalonate (Elsayed and Evans 2008) since squalene synthase is the first enzyme downstream of the branch point in the mevalonate pathway and hence is the first enzyme solely committed to cholesterol synthesis (Charlton-Menys and Durrington 2008). There has therefore been considerable interest in squalene synthase inhibitors (Nishimoto et al. 2007; Nishimoto et al. 2003; Tavridou et al. 2006) however TAK-475 (Iapaquistat), the only squalene synthase inhibitor to have progressed as far as phase III clinical trials in Europe and the United States, was recently discontinued from clinical development (Takeda 28 March 2008).

Consequently, it seems more likely that myopathy results from reductions in products and intermediates of the mevalonate pathway other than cholesterol. This means that potential candidates could include ubiquinone, dolichol, selenocysteine tRNA and the isoprenoids farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP).

1.3.2 The Role of Ubiquinone

Ubiquinone (also known as coenzyme Q10, coQ10) is synthesized from GGPP, in a multistep side-branch of the mevalonate pathway (Figure 1-3), and its role in myopathy is unclear. CoQ10 acts as an electron carrier during oxidative phosphorylation and so is essential for mitochondrial energy production (Traustadottir et al. 2008). It has therefore been proposed that statin inhibition of coQ10 production could lead to myopathy due to muscle mitochondrial dysfunction (Marcoff and Thompson 2007; Sathasivam 2012).

It is well known that statins decrease plasma levels of coQ10, however it has yet to be shown conclusively that levels are also lowered in muscle and that lower muscle mitochondrial coQ10 leads to myopathy (Marcoff and Thompson 2007). Several clinical trials investigating the effects of coQ10 supplementation alongside statin therapy have proved inconclusive (Schaars and Stalenhoef 2008) and evidence thus far seems to suggest that coQ10 depletion is only likely to play a

major role in myopathy in patients who already have depleted levels of coQ10 due to pre-existing conditions (Oh et al. 2007; Schaars and Stalenhoef 2008).

1.3.3 Dolichol and Glycosylation

Statins have been shown *in vitro* to affect N-linked glycosylation (Mullen et al. 2010; Siddals et al. 2004), the most common form of glycosylation in mammals (Martin-Rendon and Blake 2003). Glycosylation, the addition of oligosaccharides to polypeptides to form glycoproteins, is an important co-translational covalent modification for the correct biological functioning of many proteins. Dolichol, a product of the mevalonate pathway, is vital in this process as it acts as a carrier for the 14 sugar oligosaccharide that is the precursor of all N-linked oligosaccharide chains (Baker 2005; Veklavas et al. 2009).

It is thought that a reduction in dolichol synthesis could result in myopathy due to an impaired response to growth factors such as insulin-like growth factor-1 (IGF-1). IGF-1 is well known to have proliferative effects in skeletal muscle, as well as a role in muscle fibre regeneration (myoregeneration) after injury (Barton et al. 2012), and statins have been shown to affect the N-linked glycosylation and processing of IGF receptors in adipocytes (Siddals et al. 2004).

Many other cellular proteins could additionally be affected, for example the glycoprotein dystroglycan which mediates communication between the extracellular matrix and the cytoskeleton. This process is thought to also be important in myoregeneration (Cohn et al. 2002) and defects in posttranslational processing of dystroglycan have been linked to a variety of muscle disorders (Martin-Rendon and Blake 2003; Muntoni et al. 2004).

1.3.4 Selenoproteins

There are estimated to be approximately 25 human selenoproteins, which are proteins containing selenium usually in the form of selenocysteine (Muttenthaler and Alewood 2008). Selenocysteine is considered to be the 21st amino acid in ribosome-mediated protein synthesis (Stadtman 1996) and its incorporation into

proteins in selenoprotein synthesis is mediated by selenocysteine tRNA (Sec tRNA). Sec tRNA inserts a selenocysteine where there is a UGA codon in the messenger RNA. UGA is usually a stop codon; therefore incorrect decoding by sec tRNA can lead to premature termination of translation and production of truncated proteins.

It is known that isopentylation of adenosine 37 in sec tRNA is important for efficient decoding. This modification is catalysed by the enzyme isopentenyl pyrophosphate: tRNA isopentenyltransferase and uses isopentenyl-PP, an intermediate of the mevalonate pathway, as a substrate. Lovastatin treatment has been shown to lead to a reduction of sec tRNA with this modification and to reduce the ability of cells to synthesize selenoproteins (Warner et al. 2000).

Evidence for the potential importance of this selenoprotein deficiency for statin myopathy is that mutations in the gene for selenoprotein N (*SEPN1*) have been linked to a range of congenital muscular disorders. Selenoprotein N is an endoplasmic reticulum glycoprotein postulated to be important for regulating intracellular calcium release via the ryanodine receptor intracellular calcium release channel; its correct function is therefore thought to be important for muscle fibre formation (myogenesis) and for myoregeneration (Jurynek et al. 2008; Petit et al. 2003).

Further evidence is provided by reports that statin-induced myopathy shares many similarities with myopathy resulting from selenium deficiency (Moosmann and Behl 2004). In addition, statin treatment has been shown to lead to a reduction in the activity of the selenoprotein glutathione peroxidase, leading to increased oxidative stress in human HepG2 liver cells (Kromer and Moosmann 2009). It is therefore possible that deficiency in selenoprotein synthesis could likewise lead to an increased vulnerability to oxidative stress in muscle.

1.3.5 Protein Prenylation

Protein prenylation is known to be a very important process physiologically and it is thought that prenylated proteins account for up to 2% of total cellular protein. Prenylation involves the post-translational covalent addition of the prenyl groups farnesyl (15-carbon) or geranylgeranyl (20-carbon) to cysteine residues at or near the C-terminus of proteins via thioether linkages. The addition of these lipophilic groups is thought to be essential for the biological function of many proteins, by enabling them to anchor to cell membranes and hence specifying their cellular localisation. This is important for cellular protein-protein interactions and membrane-associated protein trafficking (McTaggart 2006). These prenyl groups are donated by the isoprenoids farnesylpyrophosphate (FPP) or geranylgeranylpyrophosphate (GGPP) respectively and, since both are intermediates in the mevalonate pathway, statin use is thought to lead to a decrease in protein prenylation.

There are three distinct protein prenyltransferase enzymes that catalyse the addition of prenyl groups to proteins: farnesyltransferase (FT), geranylgeranyltransferase-I (GGT-I) and geranylgeranyltransferase-II (GGT-II). FT and GGT-I both catalyse the addition of a prenyl group to the cysteine of a C-terminal CAAX protein motif, (where C is cysteine, A is an aliphatic residue and X can be a wide variety of different amino acids (McTaggart 2006)). They are therefore known as the CAAX prenyltransferases. They are zinc metalloenzymes and heterodimers that share an α subunit but have different β subunits. It is thought that the β subunits of these enzymes contain the substrate binding sites (Casey and Seabra 1996) and that it is these different β subunits which provide the different substrate specificities (Terry et al. 2006). GGT-II (also known as Rab GGT) consists of distinct α and β subunits. In a complex with Rab escort protein (REP) it catalyses the addition of geranylgeranyl groups onto two cysteine residues in the C-terminal recognition sequences CC or CXC, or a range of other similar C-terminal motifs (Maurer-Stroh et al. 2003).

Substrates of FT include small G protein Ras and the nuclear lamins, and substrates of GGT-I include Ras-related GTPases, such as those of the Ras, Rap, and Rho subfamilies, and the γ subunits of heterotrimeric G-proteins (Casey and Seabra 1996; Lane and Beese 2006). GGT-II catalyses the addition of prenyl groups onto small G proteins of the Rab subfamily (Casey and Seabra 1996; Leung et al. 2006). There is increasing evidence to suggest that disruption of the prenylation of these various targets plays an important role in statin-induced myopathy (Blanco-Colio et al. 2002; Matzno et al. 2005; Sakamoto et al. 2011).

1.3.5.1 Lamins

Lamins are intermediate filament proteins that are the main structural component of the nuclear lamina, adjacent to the inner nuclear membrane. In addition to its structural role, the nuclear lamina has important roles in chromatin organisation, cell division and gene transcription (Davies et al. 2009), and lamins are thought to also mediate the targeting of integral membrane proteins to the inner nuclear membrane (Wilson 2000). The importance of functional lamins for proper muscle function is emphasised by the fact that mutations in the gene coding for lamins A and C are known to cause a variety of muscle disorders, including Emery-Dreifuss muscular dystrophy (Wilson 2000).

Lamins are substrates of FT (Lane and Beese 2006) and they undergo post-translational modification by farnesylation, which is thought to be important for the targeting of the lamins to the inner nuclear membrane (Davies et al. 2009). Lamins B1 and B2 are farnesylated (Farnsworth et al. 1989; Rusinol and Sinensky 2006) and prelamin A, a precursor to lamin A, undergoes farnesylation-dependent processing to form the functional protein (Davies et al. 2009). It has been shown *in vitro* that treatment with lovastatin inhibits processing of prelamin A (Beck et al. 1990), results in nuclear accumulation of prelamin A (Sinensky et al. 1994) and prevents the assembly of lamin A into the nuclear lamina (Lutz et al. 1992). It is unclear how disruption of lamin function could lead to myopathy, but it is possible that resulting disruption of nuclear stability could make muscle cells less able to

sustain the forces from muscle contraction and more susceptible to apoptosis (Lammerding et al. 2004).

1.3.5.2 Small G Proteins

Small G-proteins, or small GTPases (guanine nucleotide-binding proteins), are monomeric proteins that cycle between an inactive guanosine diphosphate (GDP) bound state and an active guanosine triphosphate (GTP) bound state and have important roles in cellular signal transduction. Small G-proteins are a major target for prenylation. There are known to be over 100 small G-proteins in eukaryotes, divided into superfamilies and subfamilies. The most well studied of the small G-proteins are those in the Ras superfamily, including the Ras, Rho and Rab subfamilies (reviewed by (McTaggart 2006)).

1) Ras small GTPases

Ras small G proteins have key roles in many signalling cascades, transducing receptor activation to effects on gene transcription and cellular proliferation. The best known of these is the mitogen-activated protein (MAP) kinase cascade. In this cascade, upon activation of a tyrosine kinase receptor Ras becomes activated and can bind to Raf proteins. These in turn phosphorylate and activate MEK (mitogen-activated protein kinase kinase), which then phosphorylates and activates MAP kinase (MAPK). MAPK then translocates to the nucleus where it activates various transcription factors (Matozaki et al. 2000; Vojtek and Der 1998).

Ras proteins are further substrates of FT (Casey and Seabra 1996), with the exception of K-RasB which can undergo geranylgeranylation by GGT-I in the presence of FT inhibitors (Whyte et al. 1997). The farnesylation of Ras is vital for its membrane localisation, and therefore also for its activation and function in downstream signal transduction (Dirks and Jones 2006). *In vitro* experiments have shown a reduction in Ras farnesylation in mouse myoblasts on simvastatin treatment, which could be rescued by co-incubation with farnesol (Mullen et al. 2010), and in rat skeletal myoblasts treated with a range of statins membrane-

bound Ras was activated and detached, leading to cell death by apoptosis (Matzno et al. 2005).

ii) Rho Subfamily GTPases

Members of the Rho subfamily, such as Rho, Rac and cdc42, are involved in regulating cytoskeleton organisation. Their roles include regulating the formation of actin stress fibres and focal adhesions, as well as regulating microtubule stability. They can also have effects on gene expression (Fukata et al. 2003; Matozaki et al. 2000). Rho family proteins are substrates of GGT-I and usually undergo geranylgeranylation. Exceptions to this include proposed prenylation by FT, in addition to GGT-I, when these proteins are overproduced (Casey and Seabra 1996) and RhoB, which can be either farnesylated or geranylgeranylated by GGT-I (Armstrong et al. 1995).

There is varying evidence for the importance of disrupted prenylation of Rho proteins in statin-induced myopathy. *In vitro* treatment with statins leads to fragmentation of actin and collapse of the microtubule network (Bifulco et al. 1993; Liao 2002; Matzno et al. 1997) which can be reversed by mevalonate treatment (Bifulco et al. 1993; Liao 2002). In rat primary neurons, statin treatment has been shown to cause a decrease in membrane-bound Rho A as well as disruption of the microtubule network (Meske et al. 2003). Simvastatin and fluvastatin treatment similarly led to a change in localisation of Rho A from the membrane to the cytosol and apoptosis in rat myoblasts, which could be rescued on co-addition of GGPP. A Rho-kinase inhibitor mimicked the statin-induced apoptosis and a similar change in Rho A localisation was observed in rat skeletal muscle *in vivo* (Itagaki et al. 2009). It has also been reported that myopathy resulting from treatment with colchicine, a drug that disrupts microtubule dynamics, results in similar pathology to statin-induced myopathy (Baker et al. 2004).

Moreover, statins have been shown to induce apoptosis *in vitro* in a range of different cells (Dirks and Jones 2006). One study has provided evidence that treatment of vascular smooth muscle cells with statins *in vitro* leads to a

downregulation of the anti-apoptotic protein Bcl-2 (B-cell lymphoma 2), via inhibition of the prenylation of Rho A, and that the resulting apoptosis can be rescued by addition of mevalonate, FPP or GGPP (Blanco-Colio et al. 2002). However, in contrast to this, another study found levels of membrane-bound Rho to be unaffected by statin treatment in rat myoblasts. The authors therefore suggested that statin-induced apoptosis of skeletal muscle is not caused directly by depletion of Rho, and they proposed that depletion of Rho may only be important in mediating the pleiotropic effects of statins in other tissues (Matzno et al. 2005).

iii] Rab Subfamily GTPases

Small G-proteins in the Rab subfamily are important in organelle biogenesis and intracellular trafficking of vesicles. There are numerous different isoforms, each of which has a different intracellular localisation and regulates a different trafficking step. Rab proteins are geranylgeranylated by GGT-II, which is an important modification for Rab protein localisation and therefore function (Leung et al. 2006).

Recent studies have demonstrated that inactivation of Rab proteins could play a crucial role in statin-induced morphological changes in muscle. Treatment with perillyl alcohol, an inhibitor of GGT-I and GGT-II, has been shown to mimic the effect of statins in isolated rat skeletal myofibres by inducing vacuolisation, degeneration and swelling of organelles. These effects could be rescued by co-addition of GGPP but not FPP, and could not be reproduced by specific inhibition of GGT-I or FT (Sakamoto et al. 2007). More recently, specific small molecule inhibition of GGT-II has been shown to produce similar effects to simvastatin treatment in mouse myotubes, in lowering the levels of cellular ATP (Wagner et al. 2011), whilst fluvastatin has been shown to inhibit translocation of Rab1A to the membrane in rat skeletal myofibres, an effect which could be rescued on co-treatment with GGPP. Treatment with Brefeldin A, a specific suppressor of the trafficking step regulated by Rab1A, reproduced the effects of vacuolation and myofibre cell death seen with fluvastatin treatment (Sakamoto et al. 2011).

Therefore, although there is debate over which of the small G-proteins are the most important, (Matzno et al. 2005; Sakamoto et al. 2007) it does seem very likely that reduced prenylation plays an important role in statin-induced myopathy, most likely through the resulting disruption of muscle fibre stability, apoptosis of muscle fibres and defects in myoregeneration. However, the majority of studies described here only examine the effects of statins on prenylation in muscle, as a site of statin toxicity, and do not compare this to effects in the liver, as the target organ of the statins. They also tend to only use one or a small number of statins.

1.3.6 Pleiotropic Effects of Statins

Although reductions in the other isoprenoids of the mevalonate pathway may be the main cause of statin-induced myopathy it is thought that these effects, particularly those effects on the reduced prenylation of small G proteins (Liao and Laufs 2005), may also contribute to the variety of pleiotropic effects observed. These pleiotropic effects of the statins are independent of cholesterol lowering and include reduced inflammation (Liang et al. 2006), decreased oxidative stress (Makabe et al. 2010; Schupp et al. 2008), improved endothelial function (Jacobson 2008), vasorelaxant effects (Yaktubay Dondas et al. 2011), anti-thrombotic effects (Jacobson 2008; Mason 2006) and effects on improving stability of atherosclerotic plaques (Abela et al. 2011; Mason 2006).

Therefore, although the statins are used primarily as lipid lowering drugs, there has been evidence indicating that they may also prove useful for treating conditions other than hypercholesterolaemia. There has been interest in the use of statins as anti-cancer agents, to be used in combination with chemotherapy or radiation. This is because statins have been shown to have anti-proliferative effects, to inhibit pathways associated with the invasion and metastasis of cancer cells and also to induce apoptosis in a variety of malignant tumour cells, including acute myelogenous leukaemia cells, possibly through decreased geranylgeranylation of proteins. Whereas apoptosis of healthy muscle fibres leading to myopathy is an unwanted side-effect, the apoptosis of cells in certain diseased tissue may prove

beneficial for treating cancers. These anti-tumour effects have been demonstrated both *in vitro* and *in vivo* in animal models (Chan et al. 2003; Xia et al. 2001). Statins could be used to help overcome multidrug resistance, a significant problem during cancer treatment, through their inhibition of efflux transporters (Sieczkowski et al. 2010). It has also been suggested that statins may prove useful as a treatment for numerous other medical conditions, including asthma (Chiba et al. 2010), multiple sclerosis (Kuipers et al. 2006), their use may lead to a decreased risk of Alzheimer's disease (Cordle et al. 2005; Ostrowski et al. 2007) and they may be beneficial for patients with heart failure (Gullestad et al. 2007).

In summary, although the use of statins can be greatly beneficial, the reality of muscle toxicity continues to represent a problem in terms of both patient health and compliance. Therefore, it is important to fully understand the mechanism of this toxicity in order to find ways to reduce or avoid it whilst maintaining therapeutic doses. Should a patient experience myopathy, this is often managed by switching them either to a different statin or to a lower dose of the same statin (Harper and Jacobson 2010). However, as previously mentioned in section 1.2.2, there may also be potential for simply managing the problem of myopathy temporally, by attempting to time treatment with respect to circadian biology.

1.4 Circadian Rhythms

Daily rhythms are known to regulate a multitude of biological processes including the daily sleep-wake cycle, body temperature, feeding, hormone secretion, glucose homeostasis, cell-cycle progression and metabolism (Takahashi et al. 2008). These circadian rhythms are endogenously generated, persist in the absence of external cues and have a period of approximately 24 hours (circa = about; Diem = a day) (Hirota and Fukada 2004). They are beneficial to organisms as they allow their behaviour to be anticipatory of and adaptive to regular and predictable daily environmental changes (Wu et al. 2010). The ubiquitous nature of this regulation also has implications for the efficacy and toxicity of xenobiotics (Baraldo 2008; Levi and Schibler 2007).

1.4.1 Chronotherapy

Chronotherapy is the name given to the optimising of treatment by taking into account the temporal changes in drug pharmacokinetics and pharmacodynamics, as well as in the condition being treated, usually with the result of increased efficacy and/or reduced toxicity (Levi and Schibler 2007). Its importance in clinical practise is gradually being acknowledged (Baraldo 2008; Zmrzljak and Rozman 2012), and there are several cases where changing the timing of treatment is already making a difference in terms of both efficacy and toxicity (Levi and Schibler 2007). One such example is the decreased toxicity when changing to evening dosing of the NSAIDS (Non-steroidal anti-inflammatory drugs) indomethacin and ketoprofen in patients with hip or knee osteoarthritis. This is thought to be due to 24-hour variations in both the pharmacokinetics of these drugs and in joint pain. Greater efficacy and reduced toxicity has also been observed in response to colorectal cancer treatment administered using a chronomodulated time schedule as opposed to a constant rate infusion (Levi and Schibler 2007; Takahashi et al. 2008). In addition, there are numerous examples of where the potential for chronotherapy is currently being investigated, including treatment for asthma (Ohdo 2010), for hypertension (Portaluppi and Smolensky 2010) and treatment

with a variety of chemotherapeutic agents (Innominato et al. 2010; Ohdo 2010; Shukla et al. 2011).

The case of statins could potentially be another example of where changing the time of dosing may prove beneficial. It is known that in humans cholesterol synthesis peaks in the night/early morning (midnight - 4:00 am) (Gälman et al. 2005; Jones and Schoeller 1990; Parker et al. 1982) and so it is thought that for maximum LDL-lowering efficacy it is best for statins with short half-lives (simvastatin, lovastatin, fluvastatin, cerivastatin) to be taken in the evening, but that the statins with longer half-lives, such as atorvastatin and rosuvastatin, can be taken in either the morning or evening (Martin et al. 2002; Muck et al. 2000; Plakogiannis and Cohen 2007; Plakogiannis et al. 2005). However, most of these studies looking into the effect of temporal dosing of statins focus primarily on temporal changes in their LDL-lowering efficacy, and do not also examine temporal changes in statin related muscle toxicity. It is therefore important to fully understand circadian changes that occur at the molecular level and how these changes might relate to statin treatment and the mevalonate pathway.

1.4.2 The Molecular Basis of Mammalian Circadian Rhythms

Molecular clocks are responsible for the generation of circadian rhythms, where the molecular mechanism underlying oscillations within the molecular clock is thought to involve many transcriptional – translational feedback loops (Figure 1-5). Key in these feedback loops are the basic helix-loop-helix-PAS transcription factors BMAL1 (brain and muscle arnt-like protein-1) and CLOCK (circadian locomotor output cycles kaput) (Takahashi et al. 2008).

Figure 1-5 The mammalian molecular oscillator

In a network of transcriptional - translational feedback loops the BMAL1: CLOCK heterodimer activates transcription of core clock genes *Per*, *Cry*, *Rev-erba*, and *Rora* by binding to E-box elements. These gene products then activate or repress their own transcription through effects on BMAL1 and/or CLOCK (RRE = retinoic acid-related orphan receptor response element). BMAL2 and NPAS2 are alternative dimerisation partners for CLOCK and BMAL1 respectively. Post-translational modifications, such as phosphorylation by CK1δ, CK1ε and AMPK and degradation mediated by SCF ubiquitin E3 ligases FBXL3 and β-TrCP, ensure that the cycle has a period of approximately 24 hours. The core clock machinery also regulates the expression of many other clock-controlled genes (*Ccga*) which have roles in numerous biological processes. Figure produced using (Crumbley and Burris 2011; Crumbley et al. 2010; Ko and Takahashi 2006; Kohsaka and Bass 2007; Lamia et al. 2009; Shi et al. 2010; Virshup and Forger 2007).

Heterodimeric complexes of BMAL1 and CLOCK (BMAL1: CLOCK) activate transcription of the genes encoding period homologues (*Per 1-3*) and cryptochrome (*Cry1 and Cry2*) via binding to E-box elements (CACGTG) within enhancer and promoter sequences (Albrecht 2012). As PER and CRY proteins accumulate, they form protein complexes and translocate back to the nucleus where they repress their own transcription by interacting with the BMAL1: CLOCK complex (PER/CRY loop, bottom of Figure 1-5). The resulting decrease in PER and CRY protein levels, then allows BMAL1 and CLOCK to activate transcription of *Per* and *Cry* genes again, and the cycle continues (Levi and Schibler 2007). NPAS2 (neuronal PAS domain protein 2) has been reported to also dimerise with BMAL1 to form an active transcriptional complex in the absence of CLOCK (DeBruyne et al. 2006; DeBruyne et al. 2007), and likewise BMAL2 can act as an alternative dimerisation partner for CLOCK (Sasaki et al. 2009; Shi et al. 2010).

BMAL1: CLOCK also activates expression of the transcription factors *Rora* (retinoic acid-related orphan nuclear receptor α) and *Rev-erba* (reverse erythroblastosis virus α) (Ko and Takahashi 2006). ROR α and REV-ERB α then compete to bind to retinoic acid-related orphan receptor response elements (RREs) in the promoters of *Bmal1* (Preitner et al. 2002) and *Npas2* (Crumbley et al. 2010), whereupon ROR α activates transcription and REV-ERB α represses transcription (ROR/REV-ERB loop, top of Figure 1-5). REV-ERB α has also been shown to regulate the expression of *Clock* (Crumbley and Burris 2011) and to interact with PER2 thereby coordinating these two feedback loops (Schmutz et al. 2010).

In addition, there are a number of other important feedback mechanisms. DBP (D site of albumin promoter (albumin D-box) binding protein) and E4BP4 are members of the PAR (proline and acidic amino acid-rich) basic leucine zipper transcription factor family. They antagonistically compete for D-box elements (Mitsui et al. 2001) and regulate expression of *Per1* (Yamaguchi et al. 2000) as well as many clock controlled genes (ccg) (Noshiro et al. 2007). Basic helix-loop-helix transcription factors DEC1 (deleted in oesophageal Cancer 1) and DEC2 have been suggested

to act as negative feedback regulators, as *Dec1* expression is controlled by BMAL1:CLOCK, and DEC1 and DEC2 inhibit BMAL1:CLOCK function (Hirota and Fukada 2004; Honma et al. 2002)

Post-translational modifications, such as phosphorylation and ubiquitination, modify the activity and stability of clock proteins to ensure that a complete cycle takes approximately 24 hours (Hirota and Fukada 2004). The phosphorylation of PER proteins by casein kinase 1 epsilon and casein kinase 1 delta (CK1 ϵ and CK1 δ) (Akashi et al. 2002; Lowrey et al. 2000; Xu et al. 2005) has been shown to regulate both their nuclear translocation (Akashi et al. 2002; Takano et al. 2004; Vielhaber et al. 2000) and their stability (Meng et al. 2008). Degradation is determined by subsequent recognition of phosphorylated PER proteins by the Skp1-Cul1-F-Box protein (SCF) ubiquitin E3 ligases β -transducin repeat containing protein 1 (β -TrCP1) and β -TrCP2 (Ohsaki et al. 2008; Reischl et al. 2007; Shirogane et al. 2005). Stability of the CRY proteins is regulated through phosphorylation by adenosine monophosphate-activated protein kinase (AMPK) (Lamia et al. 2009) and by the SCF ubiquitin E3 ligase F-box and leucine-rich repeat protein 3 (FBXL3) (Busino et al. 2007; Godinho et al. 2007; Siepka et al. 2007) (Figure 1-5). It has been suggested that CRY and BMAL1 may also be substrates of CK1 ϵ (Eide et al. 2002).

1.4.3 Hierarchy of the Mammalian Circadian Timing System

A master clock resides in the suprachiasmatic nucleus (SCN) within the anterior hypothalamus in the brain (Ko and Takahashi 2006). This clock is synchronised with the external 24-hour day via a number of environmental cues, or zeitgebers, the most important of which is thought to be light (Dibner et al. 2010; Hirota and Fukada 2004).

However, it has also been shown that there are molecular clocks in many peripheral tissues that regulate local rhythms (Yamazaki et al. 2000; Yoo et al. 2004), including in the liver (Oishi et al. 2005), heart (Leibetseder et al. 2009),

adipose tissue (Zvonic et al. 2006), the vasculature (Reilly et al. 2007) and skeletal muscle (Zhang et al. 2009). These peripheral clocks are thought to be self-sustaining (Tahara et al. 2012; Yoo et al. 2004) but entrained both by neural and humoral signals originating from the master clock in the SCN (Hirota and Fukada 2004; Yamazaki et al. 2000) as well as by feeding (Damiola et al. 2000; Yamajuku et al. 2012). *In vitro* a serum pulse is able to synchronise the endogenous rhythms of a population of cells in culture (Balsalobre et al. 1998) and to date circadian rhythmicity has been observed in a wide variety of cell types (Balsalobre et al. 1998; Chalmers et al. 2008; Huang et al. 2009; Lemos et al. 2007; Li et al. 2008; Maronde and Motzkus 2003; Otway et al. 2009; Takiguchi et al. 2007).

The output of the clock is driven by the expression of many clock controlled genes (ccg), which show 24 hour cycles in their expression but are not themselves part of the core clock mechanism (Bozek et al. 2009). Circadian cycling has been shown to occur at both the transcript level, where studies have indicated that approximately 10% of genes in a variety of tissues are transcribed in a circadian manner (Akhtar et al. 2002; Panda et al. 2002; Storch et al. 2002), and at the protein level, where 20% of all soluble proteins in mouse liver have been found subject to circadian control (Reddy et al. 2006). Circadian regulation has been shown to have tissue specific differences (Panda et al. 2002; Storch et al. 2002) and to include genes from most functional categories, including metabolism (McCarthy et al. 2007).

1.4.4 Circadian Control of Cholesterol Metabolism

Many of the genes encoding enzymes and other proteins that are involved in the regulation of cholesterol metabolism have been shown to exhibit rhythmic patterns of expression (Figure 1-6).

Figure 1-6 Daily rhythmicity in key enzymes and receptors involved in the regulation of cholesterol levels or statin metabolism

(A) 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) activity in rat liver (Balasubramaniam et al. 1994), (B) low-density lipoprotein receptor (LDLR) expression in rat liver (Balasubramaniam et al. 1994), (C) *Cyp7a* and *Cyp8b* mRNA levels, as measured in rat liver by northern blots (upper panels) and quantitative real time RT-PCR (lower graphs) (Noshiro et al. 2004) and (D) CYP3A4 mRNA levels in HepG2 liver cells (Takiguchi et al. 2007).

HMGCR has been shown to exhibit diurnal variation (Panda et al. 2002). Experiments in mouse liver have shown approximately a 2-fold difference in *Hmgcr* transcript levels between peak and nadir (Oishi et al. 2005) and in rat liver a 6-fold difference in activity between peak and nadir has been reported (Figure 1-6A) (Balasubramaniam et al. 1994). It has also been shown that LDLR expression exhibits diurnal variation (Figure 1-6B) that is independent of cellular cholesterol

levels, where in rat liver LDLR expression has shown a 2-3 fold difference between peak and nadir (Balasubramaniam et al. 1994).

Cholesterol-7 α -hydroxylase (CYP7A1) and sterol-12 α -hydroxylase (CYP8B1) are two enzymes that are important for the conversion of cholesterol into bile acids in the liver, which is the most important route for metabolism and excretion of cholesterol in mammals. Both of these enzymes have been shown to exhibit diurnal mRNA expression patterns (Figure 1-6C) in the livers of both mice and rats (Noshiro et al. 2004; Noshiro et al. 2007) and the expression of *Cyp7a1* has been shown to be regulated by a number of transcription factors, most notably by DBP, DEC2 and REV-ERB α , many of which are directly regulated by the CLOCK:BMAL1 heterodimer (Noshiro et al. 2007) (section 1.4.2).

The circadian rhythmicity of cholesterol metabolism has been further highlighted by *in vivo* studies investigating the output of core clock transcription factors REV-ERB α (Le Martelot et al. 2009) and ROR α (Kang et al. 2007). In mouse liver REV-ERB α was shown to modulate the activity of both the transcription factor SREBP, which regulates the expression of many genes involved in cholesterol metabolism including HMGCR and LDLR (Brown and Goldstein 1997), and of the nuclear receptor liver-X-receptor (LXR), which regulates *Cyp7A1* expression (Le Martelot et al. 2009). ROR transcription factors were found to similarly regulate the expression of *Cyp8b1* (Kang et al. 2007).

It is also of interest to consider that circadian rhythmicity has been observed in CYP3A4, the cytochrome P450 enzyme involved in metabolism of simvastatin, lovastatin, cerivastatin and atorvastatin (section 1.1.4). In synchronised HepG2 liver cells, mRNA levels (Figure 1-6D), protein levels and metabolic activity of CYP3A4 were observed to fluctuate rhythmically with a period of about 24 hours; a 2-fold difference was observed in CYP3A4 transcript level between peak and nadir, whilst increases and decreases in protein expression seemed to correspond to similar oscillations in enzymatic activity, as measured by the 6 β -hydroxylation of

testosterone (Takiguchi et al. 2007). In addition, the expression of *CYP2C8*, an enzyme also involved in the metabolism of cerivastatin, has been reported to be regulated by the core clock transcription factor ROR α (Chen et al. 2009b).

Taking all of this into consideration, it therefore seems likely that other aspects of the mevalonate pathway, for example protein prenylation, may exhibit circadian rhythmicity in the same way as has been demonstrated for cholesterol metabolism.

1.5 Initial Hypothesis and Objectives

The initial hypothesis for this project was therefore that:

- Protein prenylation, postulated to play an important role in statin-induced myopathy, is under circadian variation as has already been shown for other aspects of the mevalonate pathway.

It may therefore prove possible to achieve greater separation between the cholesterol-lowering efficacy of statins and muscle toxicity by changing the time of treatment.

Aims and Objectives:

The initial aim was to use liver and muscle cells in culture as a model system for comparing the effects of temporal statin administration on both cholesterol and isoprenoid biosynthesis. This was to be achieved via the following objectives;

Objective 1: characterise RD muscle cells and their differentiation process

- Induce RD cell differentiation using serum deprivation
- Measure mRNA expression of key differentiation markers
- Confirm expression of core clock genes and key cholesterol metabolism genes

Objective 2: verify the ability of HepG2 (liver) and RD (muscle) cells to establish and maintain a circadian cycle

- Synchronise cells using the serum pulse technique
- Measure mRNA expression of core clock genes and key cholesterol metabolism genes
- Confirm transcript measurements at the protein level through western blotting

Objective 3: assess whether circadian changes in gene expression translate to circadian changes in cellular function.

At key points in the circadian cycle measure:

- HMGCR activity
- cholesterol level
- level of isoprenoids (F-PP and GG-PP)
- prenylated proteins in bound and soluble cell fractions

Objective 4: examine the effect of statin treatment on outputs measured in objectives 2 and 3.

Objective 5: combine all data using computer modelling software to determine whether greater separation can be achieved between efficacy and toxicity.

2 Materials and Methods

2.1 Materials

Unless otherwise stated in Table 2-1, all chemicals and materials were purchased from Sigma-Aldrich (Dorset, UK) and were of molecular biology standard.

Item	Supplier
Cell Culture	
RD cells and 3T3-L1 cells	American Type Culture Collection (Teddington, UK)
HepG2 cells	European Collection of Cell Cultures (Salisbury, UK)
Huh7 cells	Dr S. Hood, GlaxoSmithKline (Ware, UK)
Dulbecco's modified eagle medium (DMEM), foetal bovine serum (FBS), horse serum, penicillin, streptomycin, non-essential amino acids, trypsin-EDTA	Invitrogen GIBCO (Paisley, UK)
Phosphate buffered saline tablets	Oxoid (Basingstoke, UK)
Xenobiotics	
Simvastatin (sodium salt), simvastatin lovastatin (sodium salt), lovastatin pravastatin, fluvastatin	Calbiochem, Merck (Nottingham UK)
Atorvastatin, rosuvastatin	Molekula Limited (Dorset, UK)
Cerivastatin, simvastatin (sodium salt), pravastatin	Sequoia Research Products Limited (Pangbourne, UK)
FTI-277 trifluoroacetate salt, GGTI-2133, perillyl alcohol	Sigma-Aldrich (Dorset, UK)

RNA Analysis	
DNase/RNase free tips and Eppendorf tubes	Axygen (Union City, USA).
Fastrak Pipette Tips (RTPCR)	Alpha Laboratories (Eastleigh, UK)
NucleoSpin II RNA extraction kit	Macherey-Nagel (Düren, Germany)
RNase-free DNase I, 10 x DNase buffer, DNase stop solution, human genomic DNA, mouse genomic DNA	Promega (Southampton, UK)
RNase-free water, dNTPs, random hexamers, Superscript II, DTT, RNase OUT	Invitrogen GIBCO (Paisley, UK)
White Thermo-fast 96 PCR detection plates, ABsolute QPCR ROX mix, ABsolute QPCR seal	Abgene (Epsom, UK)
TaqMan primers and probes	MWG Eurofins (Ebersberg, Germany).
Protein Analysis	
Complete, mini, EDTA-free protease inhibitor cocktail tablets	Roche (Lewes, UK)
ProteoExtract Native Membrane Protein Extraction Kit	Calbiochem, Merck (Nottingham, UK)
Folin-Ciocalteu's phenol reagent	Sigma-Aldrich (Dorset, UK)
Bis-acrylamide stock solution (2% w/v) Acrylamide stock solution (40% w/v)	VWR (Lutterworth, UK)

Novex Sharp Protein Standard	Invitrogen (Paisley, UK)
PVDF Western Blotting Membrane,	Roche (Lewes, UK)
Primary and secondary antibodies (unless otherwise stated)	Santa Cruz Biotechnology (Heidelberg, Germany)
Myogenin antibody	Developmental Studies Hybridoma Bank (Iowa City, USA)
Myosin heavy chain 3 antibody	
β-actin antibody (mouse monoclonal)	Sigma-Aldrich (Dorset, UK)
Lactate dehydrogenase antibody (goat polyclonal)	Abcam (Cambridge, UK)
ECL Plus Western Blotting Detection System	GE Healthcare (Chalfont St Giles, UK)
Cholesterol Quantification	
Amplex Red Cholesterol Assay Kit	Invitrogen Molecular Probes (Eugene, USA)

Table 2-1 Suppliers of specialist materials used in this work

2.2 Methods

2.2.1 Cell Culture of Immortalised Cell Lines and Cell-Based Procedures

Cell culture materials were purchased from Invitrogen GIBCO, (Paisley, UK) unless otherwise stated.

2.2.1.1 RD Cell Culture

The human embryonal rhabdomyosarcoma (RD) cell line is a prototypic, immortalised cell line derived from embryonal rhabdomyosarcoma tissue (McAllister et al. 1969). It is used as an *in vitro* model of skeletal muscle as these cells express a number of muscle-specific proteins (Kobayashi et al. 2008). RD cells were routinely cultured in Dulbecco's modified eagle medium (DMEM) with phenol red containing 4.5g/L glucose and L-glutamine, supplemented with 10% foetal bovine serum (FBS), 100U/ml Penicillin and 100U/ml Streptomycin.

2.2.1.2 Huh7 Cell Culture

The human hepatoma cell line, Huh7, is a differentiated, immortalised liver cell line derived from a hepatocellular carcinoma. These cells produce a variety of plasma proteins and enzymes that would be expected in liver cells *in vivo* (Nakabayashi et al. 1982). Huh7 cells were cultured in DMEM with phenol red containing 4.5g/L D-glucose and L-glutamine, supplemented with 10% FBS, 1% non-essential amino acids (NEAA), 100U/ml Penicillin and 100U/ml Streptomycin.

2.2.1.3 HepG2 Cell Culture

The human hepatoma cell line, HepG2, is a differentiated, immortalised liver cell line derived from a hepatoblastoma (Aden et al. 1979; Knowles et al. 1980). HepG2 cells were cultured in DMEM with phenol red containing 4.5g/L D-glucose and L-glutamine, supplemented with 10% FBS, 1% non-essential amino acids (NEAA), 100U/ml Penicillin and 100U/ml Streptomycin.

2.2.1.4 3T3-L1 Cell Culture

The 3T3-L1 cell line is a murine preadipocyte cell line (Mackall et al. 1976) that has frequently been used to study diabetes, obesity and other metabolic disorders (Carlotti et al. 2004). 3T3-L1 cells were cultured in DMEM with phenol red containing 4.5g/L D-glucose and L-glutamine, supplemented with 10% FBS, 1% sodium pyruvate, 100U/ml Penicillin and 100U/ml Streptomycin.

2.2.1.5 Sub-Culturing Cells

Cells were passaged when they reached approximately 70-80% confluence. The medium was first aspirated from the flasks. Cells were then washed in sterile phosphate buffered saline (PBS) and 3-4ml trypsin-EDTA solution added (for a 75cm² vented tissue culture flask (T75)). After coating the base of the flask, most of the trypsin solution was aspirated off before leaving the flask at 37°C for approximately 5 minutes, or until cells had detached from the bottom of the flask. After cell detachment, cells were resuspended in fresh complete medium, to inactivate the trypsin-EDTA, and pipetted up and down, to break up any cell clumps. The cell suspension was then pipetted into a fresh T75 flask, containing complete medium, to give the required cell split ensuring that the total volume in the flask was 15ml. The precise ratio for cell splitting varied between 1:2 and 1:10, depending on the cell type and the experiment being carried out. Cells were kept in an incubator at 37°C with 5% CO₂.

For a 25cm² vented tissue culture flask (T25), a final volume of 5ml in each flask was used and 1ml of trypsin solution was used to detach cells. In a 6-well plate, the final volume in each well was 2.5ml. All 6-well and 96-well plates were kept in a humidified chamber in an incubator at 37°C with 5% CO₂.

2.2.1.6 Counting Cells using a Haemocytometer

Cells were detached from the culture flask by trypsinisation (section 2.2.1.5) and resuspended in fresh complete medium. After moistening the edges, a cover slip was affixed to the haemocytometer by applying gentle pressure, and 8.5 μ L of cell suspension was pipetted into each chamber. In each chamber, the cells in 3 different large squares were counted and an average taken. After taking any dilutions into account, this value represented the number of cells $\times 10^4$ per ml in the original cell suspension.

2.2.1.7 Storage of Cells in Liquid Nitrogen

For long term storage, cells at an early passage number were detached from the culture flask by trypsinisation (section 2.2.1.5) and counted using a haemocytometer (section 2.2.1.6). Cells were then pelleted by centrifugation at 500g for 3 minutes and resuspended in sufficient freezing medium (90% DMEM, 10% dimethyl sulfoxide, DMSO) to give 1×10^6 cells/ml. 1ml aliquots in cryovials were transferred to the NALGENE cryo freezing container for storage at -80°C for 24 hours, to allow slow freezing, before storage in liquid nitrogen until further use.

2.2.1.8 Recovery of Cells from Liquid Nitrogen

A cryovial, containing 1ml of cell suspension, was thawed from liquid nitrogen in a 37°C water bath. Once thawed, the cell suspension was transferred to a sterile tube containing 9ml of pre-warmed complete medium and centrifuged at 125g for 7 minutes. The supernatant was removed and the cell pellet resuspended in 8ml fresh pre-warmed complete medium, which was then placed in a T25 culture flask. Medium was changed the following day, to remove any remaining traces of DMSO and unattached cells. Once the cells were 70-80% confluent they were trypsinised and transferred to a T75 culture flask.

2.2.1.9 RD cell Differentiation

RD cells were grown to confluence in T25 culture flasks, 6-well plates or 96-well plates and then induced to differentiate by changing the medium to low serum medium (1% FBS; Figure 2-1). In a preliminary experiment, to characterise the differentiation process, cells were grown in T25 flasks and every 2 days cell photographs were taken and RNA extracted for analysis by quantitative RT-PCR (see section 2.2.2). After day 0, low-serum medium was refreshed just before taking cell photographs, in all flasks other than those being used to extract RNA that day, and every day after day 4. Scale bar on the cell photographs was derived from comparison with the literature (Vachon et al. 1996).

Figure 2-1 Protocol for differentiation of RD cells

RD cells were grown to confluence in standard growth medium (10% FBS) and at T = 0 medium was changed to low-serum medium (1% FBS). At indicated 2-day intervals, cells were photographed and total RNA extracted for analysis by quantitative RT-PCR (TaqMan).

As a result of this analysis, it was decided that a period of 5 days in low-serum medium would be used for the differentiation of RD cells for future experiments. In the remainder of this thesis, the term “RD-D” is used to refer to RD cells that have undergone this 5-day differentiation protocol prior to use. “RD-U” refers to undifferentiated RD cells that have not undergone differentiation in low-serum medium prior to use.

2.2.1.10 Synchronising Cells using a Serum Pulse

The serum pulse technique (Figure 2-2 and Figure 2-3) is a way of synchronising the molecular clocks in individual cells in a cell culture, using shock with a high concentration of serum, in order to study their circadian rhythms (Balsalobre et al. 1998).

In an initial pilot study 3T3-L1, HepG2 and Huh7 cells were seeded at densities of 2×10^5 , 1×10^5 and 2×10^5 cells per well respectively on 6-well plates, using 1 plate per time point per cell type. All cells were grown to 2 days post-confluence, after which the medium was changed to medium containing 50% horse serum (T=0). After 2 hours medium was changed to serum-free medium, after which the medium was not changed again for the remainder of the experiment (Figure 2-2). RNA was extracted from 4 wells of 3T3-L1 and HepG2 cells at T=0, 14, 26, 36 and from Huh7 cells at T=0, 12, 18 and 26 and analysed by quantitative RT-PCR (TaqMan; section 2.2.2).

Figure 2-2 Protocol for pilot serum pulse experiment

At indicated intervals total RNA was extracted from A] 3T3-L1 and Huh7 cells, B] Huh7 cells for analysis by quantitative RT-PCR (TaqMan).

Subsequently RD and Huh7 cells were grown in 6-well plates, using 1 plate per time point per cell type: Huh7 cells were seeded at a density of 2×10^5 cells per well and grown to 2 days post-confluence. RD cells were seeded at a density of 3×10^5 cells per well and used immediately following a 24 hour period for cell

attachment (RD-U), and were seeded at 1×10^5 cells per well and allowed to differentiate for 5 days (RD-D; section 2.2.1.9). Medium was changed to medium containing 50% horse serum (T=0). After 2 hours medium was changed to serum-free medium, after which the medium was not changed again for the remainder of the experiment (Figure 2-3). Every 4 hours starting from T = 0, for each cell type, RNA was extracted from 4 wells for analysis by quantitative RT-PCR (TaqMan; section 2.2.2).

Figure 2-3 Protocol for serum pulse of Huh7 and RD cells

At indicated 4-hour intervals total RNA was extracted for analysis by quantitative RT-PCR (TaqMan).

2.2.1.11 Treatment of Cells with Xenobiotics

Cells were seeded as required in 96-well plates or 6-well plates (sections 2.2.1.10, 2.2.1.12, 2.2.3.1, 2.2.4.1), with a final medium volume of 200μl in each well of a 96-well plate, 2.5ml in each well of a 6-well plate or 5ml in each T25 flask. Seeding densities ranged from $2 \times 10^3 - 1 \times 10^4$ in 96-well plates and $1-4 \times 10^5$ in 6-well plates.

After 24 hours, medium was aspirated from each well and replaced with serum-free medium containing either xenobiotic or vehicle control (see Table 2-2), which was prepared as follows: Each xenobiotic was dissolved in a relevant solvent (see Table 2-2) to give a stock solution of 100mM. This was then further diluted as needed, in the solvent, to give concentrations of xenobiotic that were 1000-fold more concentrated than that required in the final medium. These stocks were then diluted in serum-free medium by 1:1000, in order to maintain the percentage of solvent in the final medium at 0.1%. 100μl of xenobiotic/control-containing serum-free medium was added to each well of a 96-well plate, or 2.5ml to each well of a

6-well plate. Note that the sodium salts, of simvastatin and lovastatin, are referred to as their acid forms (in contrast to the lactone form) in subsequent chapters.

Xenobiotic	Solvent
Simvastatin (sodium salt)	DMSO
Simvastatin	Ethanol
Lovastatin (sodium salt)	Ethanol
Lovastatin	DMSO
Cerivastatin	Water
Atorvastatin	DMSO
Fluvastatin	Water
Rosuvastatin	DMSO
Pravastatin	Water
FTI-277	Water
GGTI-2133	DMSO
Perillyl Alcohol	Water

Table 2-2 Solvents used for xenoblotics
 Relevant solvent was also used as the related vehicle control.

2.2.1.12 Assessing Cell Viability using the MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay is a colorimetric assay that is used to quantitatively assess metabolic and cellular viability. The tetrazolium bromide (MTT) salt is reduced by dehydrogenase enzymes, in active mitochondria in living cells, to form a water-insoluble, coloured formazan salt. The amount of formazan generated, and absorbance measured, is directly proportional to the number of viable cells, over the range 200 - 50,000 cells per well (Mosmann 1983).

In these experiments, RD cells were seeded at a density of 2 x 10³ cells per well on 96-well plates and left to differentiate for 5 days in low-serum medium (RD-D; section 2.2.1.9). Huh7 and RD-U cells were seeded on 96-well plates 24 hours prior to experiment, at densities of 5 x 10³ cells per well and 1 x 10⁴ cells per well respectively.

Cells were exposed to various statin concentrations or a vehicle control (see section 2.2.1.11) for 48 hours. 10µl of MTT solution (5mg/ml MTT dissolved in PBS and then filter sterilised) was added to each well for the final 2.5 hours of the 48 hour dosing period. Medium was then replaced with 100µl DMSO per well and the absorbance read at 540nm in the Multiskan RC microplate reader (ThermoFisher Scientific, Loughborough, UK). Absorbance measured was expressed as a percentage of the vehicle control (defined as 100%). At each statin concentration, mean absorbance values were calculated from each independent experiment, which were then combined to generate final data.

2.2.2 RNA Analysis

For RNA work, all tips and Eppendorf tubes used were DNase/RNase free. For TaqMan, non-filter tips and Eppendorf tubes were autoclaved prior to use.

2.2.2.1 RNA Isolation

Total RNA was extracted from 3T3-L1, HepG2, Huh7, RD-U and RD-D cells using the Nucleospin II RNA extraction kit according to the manufacturer's instructions: Cells were first lysed by the addition of the lysis buffer provided (RA1) and the viscosity reduced by passing each lysate through a 21 gauge needle several times. The lysate was then filtered using a provided NucleoSpin Filter, to further reduce viscosity and remove cellular debris. The flow-through from this column was loaded onto a NucleoSpin RNA II Column, in order to bind the RNA to the silica membrane, after the adjustment of the binding conditions with the addition of ethanol. Membrane desalting buffer was then added to the column, followed by a centrifugation step at 11,000g for 1 minute to dry the membrane. Each column was washed with provided wash buffers RA2 and RA3, before the final elution of pure RNA in 60µl RNase-free water.

Total RNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Delaware, USA) using the A260/280 and A260/230 ratios as an indication of RNA quality. For pure RNA the expected 260/280 ratio is

in the region of 1.8-2.0 and the expected 260/230 ratio is approximately 2.0. RNA absorbs light with an absorption peak at 260nm and so if the 260/280 ratio is much lower than this it is an indication that there may be protein contamination in the sample as protein absorbs light at 280nm. If the 260/230 ratio is much lower, this may be an indication of sample contamination by for example thiocyanate ions, which are present in the buffers RA1, RA2 and MDB that are used in the RNA extraction (NanoDrop 2007).

RNA quality was also checked by running 1µg of each sample on a 1% agarose gel, containing ethidium bromide (0.5µg/ml), in 1xTAE buffer (0.04M Tris acetate, 0.01M EDTA). Samples were diluted in RNase free water to 1µg in a total volume of 10µl. This was then mixed with 2µl Orange G loading dye (0.25% Orange G, 50% glycerol) and the full 12µl loaded into the wells. Bands were visualised under UV light using the Gene Genius Bio Imaging System (Syngene, Cambridge, UK). If the RNA was intact and of good quality then sharp bands representing 28S and 18S ribosomal RNA (rRNA) were present on the resulting gel (Figure 2-4), with no obvious low molecular weight smear which would indicate degraded RNA (Applied Biosystems 2009). RNA was stored at -80°C.

Figure 2-4 Example of how an agarose gel can be used to give an indication of RNA quality

RNA samples shown here were extracted from RD muscle cells on days 6 and 8 of differentiation (N=4). Arrows indicate bands representing 28S and 18S rRNA. If RNA is intact these bands should be sharp with more 28S present than 18S (approximately twice as much 28S), as is shown here.

2.2.2.2 DNase Treatment

All RNA samples were DNase treated to remove contaminating genomic DNA. 3µg total RNA, 2µl 10 x DNase buffer and 2µl RNase-free DNase I were added to the volume of RNase-free water required to bring the total reaction mixture to a total volume of 20µl. This reaction mixture was incubated at 37°C for 30 minutes, followed by a heat inactivation step by the addition of 2µl DNase stop solution (20mM EGTA) and incubation at 65°C for 10 minutes.

2.2.2.3 cDNA Synthesis

4µl of the DNA-free RNA solution was added to 1.5µl dNTP mix (in which each dNTP was at a concentration of 10mM), 1.5µl random hexamers (150ng/µl) and 5µl RNase-free water. This was incubated at 65°C for 5 minutes, to denature the RNA, and then cooled on ice for 2 minutes. 8µl of a reverse transcriptase (RT+) mix was then added to each RNA/primer mix, where this RT+ mix contained 4µl 5 x FS buffer (first strand synthesis buffer; supplied with Superscript II Reverse Transcriptase enzyme), 2µl 0.1M DTT, 1µl RNase OUT, 0.75µl water and 0.25µl Superscript II for each reaction. This was then incubated at 25°C for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes. In a similar way, a reverse transcriptase negative (RT-) control was also prepared using each RNA sample, where the RNase OUT and Superscript II had been replaced by RNase-free water. All RT+ and RT- reaction mixes were then diluted by adding 80µl RNase-free water and stored at -20°C.

2.2.2.4 Quantitative Reverse Transcriptase Polymerase Chain Reaction

5µl of either RT+ or RT- sample was pipetted into each well of a white Thermo-fast 96 PCR detection plate followed by 20µl of reaction mix; where this reaction mix was made up beforehand in bulk and contained 1µl forward and reverse primers (each primer at 10pmol/µl), 0.5µl probe (5pmol/µl), 6µl sterile water and 12.5µl ABsolute QPCR ROX mix per reaction. Each plate was sealed with an ABsolute QPCR seal, briefly centrifuged and run on the ABI Prism 7000HT sequence detection system (Applied Biosystems, California, USA) using the cycle: 2 minutes

at 50°C, 15 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Relative transcript expression was calculated by comparison with a standard curve, made up of 10⁵, 10⁴, 10³, 10² and 10¹ copies of human or mouse genomic DNA. For each primer/probe set used, duplicate no template controls (NTC) were also run, where 5µl sterile water was used in place of RT+ or RT-sample. Expression data were normalised, as indicated, to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA (rRNA) or β-actin.

All gene-specific primers and probes used were designed using Vector NTI Advance 11 (Invitrogen) and Primer Express Version 2.0 (Applied Biosystems), and ordered from MWG Eurofins (Ebersberg, Germany). Primer and probe sequences are given in Table 2-3 and Table 2-4. Primer sets were designed to be within an exon as this meant that genomic DNA could be used for creating the standard curve. Genomic DNA is easy to work with and, provided that genomic DNA from the correct species is being used, it should contain all of the target genes in varying amounts, removing the need to create a different standard curve using cDNA for each target gene to be analysed.

Gene	RefSeq	Forward Primer (5'-3')	Reverse Primer (5'-3')	TaqMan Probe (5'-3')	Region Amplified (bp) (relative to RefSeq)	Product Size (bp)
<i>MYH3</i>	NM_002470	GCCACACTGCAGCAGAA	CACACTATCCGCATGCTTCTTC	TCAGCGCGGCCACCATGG	3632 - 3691	60
<i>MYOD1</i>	NM_002478	CCGCCTGAGCAAAGTAAATGA	GCAACCGCTGGTTTGGATT	AGACACTCAAGCGCTGCACGTGGA	580 - 653	74
<i>MYOG</i>	NM_002479	GCTGTATGAGACATCCCCCTACTT	GAGGTGACAGGCAGGTAGTTT	TACCAGGAACCCCGCTTCTATGATG GG	294 - 369	76
<i>HMGCR</i>	NM_000859 / NM_001130996 *	CACATCTCCTGTAGTGACACAAAAG A	CCTCTACTGAATCACATTTCTGGTT ATT	TGACCAGCATAGGTTTACGCTCTACA ACAAATTGT	1218 - 1315 (Variants 1 & 2)	98
<i>LDLR</i>	NM_000527	GTGCTCCAGGAC	AGTCCCGGTCTGAGTCACAGA	GAACTGCCGAGAGATGCACCTCCC AT	492 - 568	77
<i>BMAL1</i>	NM_001178 / NM_001030272 / NM_001030273 *	TGCCTCGTCGCAATTGG	ACCCTGATTTCCCCGTTCA	GGACTGCATTCTCATGTAGTTCCAC AACCA	1342 - 1409 (Variant 1) 1287 - 1354 (Variant 2) 1358 - 1425 (Variant 3)	68
<i>PER1</i>	NM_002616	CCCTTTGGTGACCCCAATG	GCCCCATAAGGATAGCTGGAT	TGGCCTTGGTGCTCCCTAACTATCT GTTG	2974 - 3051	78
<i>PER2</i>	NM_022817	GTCCAGCCCCCACCCTTTC	GGGAAGGAATAACTGGGTAGCA	CTGCCCCCTTTGGCGCTGTCTAT	2908 - 2978	71
<i>CRY1</i>	NM_004075	CGTCAGGAGGGTTGGATTCA	CACAGGTCCCCTCGTGTC	AAGCAAGCAACTGCATGCCCTGGCT A	1901 - 1971	71
<i>REV-ERBα</i>	NM_021724	CTTCAATGCCAACCATGCAT	CCTGATTTTCCAGCGATGT	AGGTAGCCCTCCAGCCACCAACCC	1565 - 1629	65
<i>DBP</i>	NM_001352	CGTGAGGTGTTGATGACCTT	TCGTGGCCAGGAATGCTT	AACCCGACCCAGCTGATCTTGCC	982 - 1050	69
<i>GAPDH</i>	NM_002046	CAAGGTCATCCATGACAACCTTTG	GGGCCATCCACAGTCTTCTG	ACCACAGTCCATGCCCATCACTGCCA	585 - 674	90
<i>18S</i>	NR_003286	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT	TGCTGGCACCAGACTTGCCCTC	450 - 636	187
<i>ACTB</i>	NM_001101	TTCAACACCCCGACCATGTA	CGTCACCGGAGTCCATCAC	CTGTGCTATCCCTGTACGCCCTCTGG C	463-556	94

Table 2-3 Human specific primer and probe sequences

All probes designed with modifications 5' FAM and 3' TAMRA; * Multiple human transcript variants - primer/probe sets designed to pick up all.

MYH3 = myosin heavy chain 3; *MYOD1* = myogenic differentiation 1; *MYOG* = myogenin; *HMGCR* = 3-hydroxy-3-methylglutaryl coenzyme A reductase; *LDLR* = low-density lipoprotein receptor; *BMAL1* = brain and muscle arnt-like protein-1; *PER* = period; *CRY* = cryptochrome; *DBP* = D site of albumin promoter (albumin D-box) binding protein; *GAPDH* = Glyceraldehyde 3-Phosphate Dehydrogenase *ACTB* = β -actin; RefSeq = NCBI (National Center for Biotechnology Information) mRNA reference sequence.

Gene	RefSeq	Forward Primer (5'-3')	Reverse Primer (5'-3')	TaqMan Probe (5'-3')	Region Amplified (bp) (relative to RefSeq)	Product Size (bp)
<i>mPer2</i>	NM_011066	GACGCACACAAAGAAGTAAGG	CTCCGCAGGGCATACTTCA	TCCACCTCCCCTGCAGACAAGAAGG C	484 – 599	116
<i>mDbp</i>	NM_016974	CACCGTGGAGGTGCTAATGA	CATGGCCTGGAATGCTTGA	TGAACCTGATCCCGCTGATCTCGC	981-1051	71
<i>mGapdh</i>	NM_008084	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG	ACCACAGTCCATGCCATCACTGCCA	527-616	90
<i>m18S</i>	X00686	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT	TGCTGGCACCAGACTTGCCCTC	451-637	187

Table 2-4 Mouse specific primer and probe sequences

Probes designed by Dr Daniella Otway, with modifications 5' FAM and 3' TAMRA.

Per = period; *Dbp* = D site of albumin promoter (albumin D-box) binding protein; *Gapdh* = Glyceraldehyde 3-Phosphate Dehydrogenase; RefSeq = NCBI (National Center for Biotechnology Information) mRNA reference sequence.

2.2.3 Protein Analysis

2.2.3.1 Total Protein Extraction using RIPA Buffer

HepG2, Huh7, RD-U and RD-D cells were grown in T25 flasks or 6-well plates and total protein was extracted from cells using RIPA buffer (RadioImmunoPrecipitation Assay Buffer; 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) in 1x PBS, with 1 complete, mini, EDTA-free protease inhibitor cocktail tablet per 10ml buffer). Medium was aspirated off cells and cells were washed twice with PBS before the addition of ice cold RIPA lysis buffer (1ml per T25 flask or 300µl per well of a 6-well plate). Flasks or plates were left on ice for 30 minutes to allow cell lysis. After this time, lysate was homogenised and the DNA sheared using a 25 gauge needle, before being centrifuged for 15 minutes at 15,000g at 4°C. The supernatant from this step contained the total cell protein, which was stored at -20°C for regular use or at -80°C for longer term storage. Each sample was used a maximum of 3 times, to minimise the effects of freeze-thawing. Samples to be used more frequently than this were frozen in aliquots.

2.2.3.2 Membrane Protein Extraction

Huh7, RD-U and RD-D cells were grown in 6-well plates and membrane protein extracted using the ProteoExtract Native Membrane Protein Extraction Kit. Medium was aspirated off the cells and cells were washed twice using 0.8ml ice cold wash buffer (provided in the kit). 4µl Protease Inhibitor Cocktail was added to the wall of each well followed by 0.8ml Extraction Buffer I. Plate was swirled, to ensure mixing and covering of the cells, before incubation on ice for 10 minutes under gentle agitation. After incubation, supernatant enriched in cytosolic proteins was removed and stored at -20°C until use. 2µl Protease Inhibitor Cocktail and 0.4ml Extraction Buffer II was then similarly added to each well, and plates incubated on ice for 30 minutes under gentle agitation. Supernatant, enriched in integral membrane proteins and membrane associated proteins, was then removed and stored at -20°C until use. If cells started to detach from the plate after any wash steps or incubation steps, cell suspension was removed, centrifuged for 15 minutes at 15,000g at 4°C and protocol

continued using the resulting pellet. All cytosolic and membrane samples were also only used a maximum of 3 times, to minimise the effects of freeze-thawing. Samples to be used more frequently than this were frozen in aliquots.

2.2.3.3 Protein Quantification using the Lowry Method

Protein was quantified using the Lowry method (Lowry et al. 1951). This is a 2-step method. Firstly, copper ions complex with the protein, under alkaline conditions. Subsequently, Folin-Ciocalteu's phenol reagent is added and becomes reduced, changing from a yellow to a blue colour, which can be measured spectrophotometrically.

A bovine serum albumin (BSA) standard curve (0 – 250µg/ml) was prepared by dilution of BSA stock solution of 250µg/ml in 0.5M NaOH. 30µl of standards and samples (diluted as necessary in 0.5M NaOH) were pipetted, in triplicate, into separate wells of a clear flat-bottomed 96-well plate. Blanks were also prepared, in triplicate, containing 195µl 0.5M NaOH (or protein extraction buffer at the appropriate dilution). 150µl of freshly prepared copper solution (10ml 2% Na₂CO₃, 0.1ml 1% CuSO₄·5H₂O, 0.1ml 2% KNaC₄H₄O₆·4H₂O) was added to each well of standard or sample and the plate was left to stand, at room temperature, for 10 minutes. Then 15µl of Folin-Ciocalteu's phenol reagent (2x stock diluted 1:1 in distilled water) was added to each well of standard or sample and mixed well. Plate was left for at least 30 minutes, at room temperature, and absorbance read at 490nm on a Bio-Tek ELx800 microplate reader. Values were corrected for background absorbance, by subtracting average absorbance value for the blanks, and protein concentrations were determined by comparison to the standard curve.

2.2.3.4 Concentrating Protein Samples

Samples were concentrated, as needed, prior to analysis. 0.1ml 0.15% deoxycholate was added to each 1ml protein sample. This was mixed using a vortex and left to stand at room temperature for 10 minutes. Then, 0.1ml 72% trichloroacetic acid was added and again mixed by vortexing, followed by centrifugation for 30 minutes at 11,000g at 4⁰C. Supernatant was removed and pellet was resuspended in required volume of NaOH, to allow protein concentration to be remeasured using the Lowry method (section 2.2.3.3). In order to neutralise the alkaline before further analysis, an equal volume of 1M Tris/HCl was then added to each sample.

2.2.3.5 Separation of Proteins by SDS-PAGE

Proteins were separated according to their molecular weight by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). For proteins 40-100 kilodaltons (kDa) in size a resolving gel of 12.5% (12.5% acrylamide, 0.3% bisacrylamide, 0.1% SDS, 0.4M Tris-HCl pH 8.8, 0.16% ammonium persulphate (APS) and 0.16% tetramethylethylenediamine (TEMED)) and a stacking gel of 6% (6% acrylamide, 0.16% bisacrylamide, 0.1% SDS, 0.2M Tris-HCl pH 6.8, 0.24% APS, 0.4% TEMED), made up in 1.5mm glass plates, were used. For proteins less than 40kDa in size, a 20% resolving gel was used (20% acrylamide, 0.53% bisacrylamide, 0.1% SDS, 0.33M Tris-HCl pH 8.8, 0.16% ammonium persulphate (APS) and 0.16% TEMED). Protein samples (amount used specified in individual figure legends) were mixed with an equal volume of loading buffer (60mM Tris-HCl pH 6.8, 12% glycerol, 2% SDS, 0.003% pyronin Y, 0.7M β -mercaptoethanol (BME)) and heat denatured at 60⁰C for 10 minutes before being placed on ice. Denatured protein samples were then loaded onto the gel, alongside 10 μ l Novex Sharp Protein Standard. Gels were run at 120mA in 1x running buffer (25mM Tris-HCl pH 8.3, 192mM glycine, 0.1% SDS) for approximately 1 hour, or until the dye front neared the end of the glass plates.

2.2.3.6 Identification of Proteins by Western Blotting

Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane in 1x transfer buffer (16mM Tris base, 120mM glycine) at 300mA for 90 minutes. The membrane was then left in blocking solution (10% Marvel in 1x Tris-buffered saline – Tween 20 (TBST; 20mM Tris-HCl pH 7.5, 500mM NaCl, 0.1% Tween 20)) on a rotary mixer. Following blocking, the membrane was washed 3 times in 10ml TBST, each for 10 minutes, on a rotary mixer. The membrane was then incubated with primary antibody in TBST containing Marvel (see Table 2-5 for amount), followed by 3 further 10 minute washes in 1x TBST and incubation with secondary antibody (5% Marvel in 1x TBST) at room temperature on a rotary mix. Three further 10 minute washes in 1x TBST were carried out, as before, prior to chemiluminescent detection using the ECL plus western blotting detection reagent. The membrane was incubated with the reagent for 5 minutes and then exposed to x-ray film. Conditions were optimised for each primary antibody; dilutions, amount of Marvel and times of incubation are shown in Table 2-5. All overnight incubation steps were carried out at 4⁰C and all other incubation steps carried out at room temperature.

Antibody	Size (kDa)	Blocking	1°	2°	Exposure (minutes)
MYOG F5D	25	overnight	1:25; overnight 0.5% Marvel	1:20,000; 2hr α -mouse (sc-2005)	1
MYH3 F1-652	224	overnight	1:25; overnight 0.5% Marvel	1:20,000; 2hr α -mouse	15
HMGCR (H-300) sc-33827	90	1 hr	1:400; overnight 0.5% Marvel	1:10,000; 2hr α -rabbit (sc-2004)	1 or 5
FT β (H-300) sc-13965	46	1hr	1:1000; overnight 0.5% Marvel	1:20,000; 1hr α -rabbit	1
GGTase-I β (XX12) sc-100820	42	1hr	1:200; overnight 0.5% Marvel	1:2000; 2hr α -mouse	25
GGTase-II β (17-Q) sc-100819	43	1hr	1:200; overnight 0.5% Marvel	1:2000; 2hr α -mouse	15
H-Ras (M90) sc-53959	21	1hr	1:500; overnight 0.5% Marvel	1:5000; 2hr α -mouse	10
M-Ras (N19) sc-8168	29	1hr	1:750; overnight 0.5% Marvel	1:5000; 2hr α -goat (sc-2020)	30 or 50
Rap1A (C17) sc-1482	21	1hr	1:200; overnight 0.5% Marvel	1:10,000; 2hr α -goat	5 or 20
Rap1 (121) sc-65	21	1 hr	1:200; overnight 0.5% Marvel	1:5,000; 2hr α -rabbit	10 or 20
Rab1A (C-19) sc-311	~25	1hr	1:500; overnight 0.5% Marvel	1:5000; 2hr α -rabbit	10
β -actin A5441	40	overnight	1:2000; 1hr 5% Marvel,	1:20,000; 2hr α -mouse	1-5
LDH ab2101-1	35	overnight	1:1000; 2hr 1% Marvel	1:30,000; 1hr α -goat	30

Table 2-5 Antibody conditions for western blotting

All overnight incubation steps were carried out at 4°C, and all other incubation steps at room temperature. 1° = primary antibody and 2° = secondary antibody; MYOG = myogenin; MYH3 = myosin heavy chain 3; HMGCR = 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDH = lactate dehydrogenase; FT = protein farnesyltransferase; GGTase-I = protein geranylgeranyltransferase type I; GGTase-II = protein geranylgeranyltransferase type II

2.2.3.7 Membrane Stripping and Reprobing

To allow reprobing for β -actin as an internal loading control membrane was stripped of primary and secondary antibodies as follows: Membrane was incubated for 10 minutes on a rotary mix at room temperature with mild stripping buffer (1.5% (w/v) glycine, 0.1% (w/v) SDS, 1% (v/v) Tween 20, pH 2.2). This was repeated with fresh stripping buffer, followed by 2x 10 minute washes with PBS and 2x 5 minute washes with TBST. The membrane was blocked as before (see section 2.2.3.6) and probed with the new antibodies. Each membrane was stripped a maximum of 3 times, due to the loss of protein at each stage.

2.2.3.8 Densitometry

Densitometry was used to determine the relative sizes of protein bands obtained through western blotting (see section 2.2.3.6). Blots were scanned into the computer using a Xerox 2400 flatbed scanner, making sure to scan the images in greyscale and on the highest possible resolution, and saved as jpeg files. The graphics programme Paint was then used to select the area of the blot containing the protein bands of interest, and this selection was saved as a 24-colour bmp file. This bmp file was used in the programme GeneTools (Syngene, Cambridge, UK) to determine relative band sizes. Since this programme allows the user to select the exact area of the band, any background on the film should not interfere greatly with the results. Resulting data were normalised using the associated values obtained for the loading control β -actin.

2.2.4 *Measurement of Total Cellular Cholesterol Content*

In the cell, the majority of cholesterol is found in membranes (Brown and Goldstein 1997). Therefore, in order to quantify cellular cholesterol content, it is first necessary to extract it from these cell membranes. To achieve this, a variation of the Folch method was used (Folch et al. 1957) which is commonly used for the extraction of lipids (Gamble et al. 1978; Khan et al. 2003).

2.2.4.1 Extraction of Lipids from Cells

RD cells were seeded at a density of 2×10^5 cells per well on 6-well plates and left to differentiate for 5 days in low-serum medium (RD-D; section 2.2.1.9). Huh7 and RD-U cells were seeded on 6-well plates 24 hours prior to experiment, at densities of 3×10^5 cells per well and 4×10^5 cells per well respectively. Cells were exposed to various statin concentrations or a vehicle control (section 2.2.1.11) for 48 hours. For control experiments, using only vehicle control, Huh7 cells were seeded in T25 flasks at either 8×10^5 cells per flask or at a range of densities between 4×10^5 and 1×10^6 cells per flask.

After the dosing period, medium was aspirated and cells washed twice in PBS. Cells were removed from the plate by trypsinisation (section 2.2.1.5), using 500 μ l trypsin-EDTA per well. 1ml complete medium was added and the resulting cell suspension transferred to an Eppendorf tube. Cells were pelleted in a micro centrifuge (5 minutes at 500g). The supernatant was removed and discarded, and the pellet washed in PBS, followed by further centrifugation to allow for any disturbance of the cell pellet during wash step. Supernatant was removed and discarded, and 200 μ l of a chloroform-methanol solvent mix (2:1 v/v) was added to each pellet, followed by brief vortexing. Samples were centrifuged for 10 minutes at 18,000g in a micro centrifuge. Supernatant from this step (containing the lipid phase) was transferred to a new Eppendorf tube and dried under vacuum, to remove remaining solvent. Extracted lipids were then resuspended in 200 μ l 1x reaction buffer from the Amplex Red Cholesterol Assay Kit (0.1M potassium phosphate, pH 7.4, 50mM NaCl, 5mM cholic acid, 0.1% Triton X-100). The pellet was resuspended in 0.5M NaOH, and protein quantification carried out using the Lowry method (section 2.2.3.3). All samples were stored at -20°C until use.

2.2.4.2 Cholesterol Quantification

Total cholesterol was quantified using the Amplex Red Cholesterol Assay Kit. In this fluorometric method, cholesterol is oxidised by cholesterol oxidase to produce H₂O₂ and a ketone product. The probe Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) reacts stoichiometrically with the H₂O₂, in the presence

of horse radish peroxidase (HRP), to produce resorufin which can be detected using a fluorometer (Amundson and Zhou 1999; Sutter et al. 2005).

In brief, a cholesterol standard curve (0 – 8µg/ml) and a positive control of 10µM H₂O₂ were prepared by dilutions of the provided components into 1x reaction buffer (see section 2.2.4.1). 50µl of standards, positive control and samples (diluted as necessary in 1x reaction buffer) were pipetted, in triplicate, into separate wells of a 96-well black-bottomed microplate. An Amplex Red working solution was prepared (300µM Amplex Red reagent, 2U/ml HRP, 2U/ml cholesterol oxidase, 0.2U/ml cholesterol esterase) and 50µl added to each microplate well. Each plate was incubated for at least 30 minutes at 37°C, protected from light, and fluorescence was measured in a SpectraMax Gemini XS microplate spectrofluorometer (Molecular Devices, California, USA) (excitation 544nm, emission 590nm). Values were corrected for background fluorescence and cholesterol content determined by comparison to the standard curve. Cholesterol values were then normalised to the associated protein data (see section 2.2.4.1).

2.2.5 Statistical Analysis of Data

Where data from a single experiment was presented, mean and standard deviation (SD) were used where the number of repeat measurements was four or greater, whereas median and range were used where this number of repeats was less than four. Standard error of the mean (SEM) was used to show the precision of the mean, where data was derived from several repeat experiments (Festing and Altman 2002). Statistical significance was determined using the most relevant statistical test, as indicated, where $P < 0.05$ was considered to be significant. In general, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was used to compare data from multiple samples, grouped by a single factor, and unpaired t-test was used to compare data from two samples, for example treated and untreated cells. Two-way ANOVA was used to compare data grouped by two factors. All tests were carried out using GraphPad Prism version 5 (California, USA).

3 Characterisation of Human Liver and Muscle Cell Lines

3.1 Introduction

Circadian rhythms are known to influence a wide variety of biological processes, including many aspects of metabolism. Diurnal variation in cholesterol metabolism has been shown to occur, in part due to circadian transcription of key regulatory genes in this pathway: 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGCR*), the enzyme inhibited by the statins; low-density lipoprotein receptor (*LDLR*); and the cytochrome p450 (CYP) enzymes involved in bile acid synthesis (Zmrzljak and Rozman 2012). Circadian rhythms can also affect the toxicity of xenobiotics, due to rhythmic variation in pharmacokinetic and pharmacodynamic factors, and it has been shown that in certain cases adverse drug effects can be reduced by changing drug dosing schedules accordingly (Levi and Schibler 2007). To examine whether differences exist between the circadian aspects of statin efficacy and toxicity, and whether greater separation can consequently be achieved between the two, the temporal effects of statins were to be studied *in vitro*. Initially the most appropriate model system was identified and characterised prior to further study.

The use of immortalised cell lines is an attractive option for such *in vitro* modelling. Human cell lines have previously been used to model and study the effects of statins; the RD muscle cell line has been used to study the myotoxic effects of statins, while the HepG2 liver cell line was used to look at the cholesterol-lowering effects (Kobayashi et al. 2008). Therefore both of these cell lines are good candidates to model the effects of statin exposure in muscle and liver. In addition, since it is possible to observe rhythmic gene expression in cultured human cells following a serum pulse, it has been suggested that studying the peripheral clocks of human cultured cells provides a good *in vitro* model for studying the molecular oscillator in human tissues (Takiguchi et al. 2007).

The HepG2 liver cell line is derived from a human hepatocellular carcinoma and the rhythmic expression of core clock genes, clock-controlled genes and several

CYP enzymes, has already been demonstrated in this cell line (Koyanagi et al. 2006; Matsunaga et al. 2008; Takiguchi et al. 2007). For this reason it should represent a good model for examining the rhythm-dependent effects of statins on the liver.

Circadian rhythmicity is less well characterised in skeletal muscle, compared to tissues such as liver, although some evidence has been observed (Zhang et al. 2009). Transcriptome profiling in mouse skeletal muscle has revealed 215 transcripts (out of 36,182) with a circadian pattern of expression; this included several important muscle-specific proteins such as the transcription factor myogenic differentiation 1 (*MYOD1*) and the ubiquitin ligase atrogin-1 (McCarthy et al. 2007). Robust circadian rhythms have recently also been demonstrated in the skeletal muscle of rats (Miyazaki et al. 2011), zebrafish (Amaral and Johnston 2012), horses (Martin et al. 2010) and in the Movas-1 mouse smooth muscle cell line (Chalmers et al. 2008).

However, to date circadian rhythmicity has not been studied in RD human skeletal muscle cells. In order to use this muscle cell line as a model for looking at the temporal effects of statin administration, the ability of this cell line to establish and maintain circadian rhythms must first be verified. Therefore, in this chapter the hypothesis that RD cells have molecular clocks that are capable of generating robust circadian gene expression was tested.

3.2 Characterisation of RD Cell Differentiation

The RD muscle cell line is derived from a human embryonic rhabdomyosarcoma, a tumour of skeletal muscle origin, and these cells express a number of muscle-specific genes (Knudsen et al. 1998). The expression of several of these genes only occurs after the initiation of muscle cell differentiation (Knudsen et al. 1998). For this reason, RD muscle cell differentiation is initiated before their use in experiments (Nishimoto et al. 2003), as this will lead to a more accurate representation of skeletal muscle. A variety of different differentiation conditions have been used in the literature (Bouche et al. 2000; Carey et al. 2006; Knudsen et al. 1998; Nishimoto et al. 2003; Vachon et al. 1996) and so before using these cells, the optimum differentiation conditions for this model must be determined.

3.2.1 *Expression of Muscle-Specific Markers*

In order to determine the optimum time required for differentiation, RD muscle cells were differentiated by incubation post-confluence in low (1%) serum for between 2 and 10 days and the morphological and molecular changes were examined (section 2.2.1.9). Cell photographs, taken at 2-day intervals during this differentiation process (Figure 3-1), showed an increasing number of long myotube-like structures as differentiation progressed; the arrows in Figure 3-1 indicate examples of myotube formation occurring by days 2 and 4.

Figure 3-1 Morphological characterisation of RD muscle cell differentiation

RD cells were grown to confluence and then induced to differentiate by a change to low-serum (1%) medium on day 0. Photographs were taken at 2-day intervals to show morphology changes over a 10 day differentiation protocol; scale bar, in photograph from day 0, is approximately 50µm and arrows indicate example myotubes.

However, one property of this particular cell line is that on induction of differentiation these cells do not exit the cell cycle and so do not stop proliferating (Knudsen et al. 1998). Therefore, wells became overgrown in the later days of the time course, meaning it became difficult to monitor the progress of differentiation by microscopy. Due to this difficulty, the expression of several known muscle-specific markers was also measured using TaqMan (Figure 3-2).

Figure 3-2 Molecular characterisation of RD muscle cell differentiation

RD cells were grown to confluence and then induced to differentiate by a change to low-serum (1%) medium on day 0. Total RNA was extracted from cells and reverse transcribed using random hexamers and the enzyme Superscript II Reverse Transcriptase (Invitrogen). Expression of three muscle markers, myogenic differentiation 1 (*MYOD1*), myogenin (*MYOG*) and myosin heavy chain 3 (*MYH3*), was measured by quantitative RT-PCR (TaqMan), using comparison with genomic DNA at known concentrations (copy number) to enable quantification. Values plotted represent mean $\pm$ standard deviation (SD), from a single experiment where N=4. Statistical significance determined by one-way ANOVA followed by Bonferroni post hoc test: ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$ compared to day 0.

TaqMan analysis showed that RD cells express the muscle-specific transcription factors *MYOD1* and myogenin (*MYOG*). A statistically significant 4-fold increase in the expression of *MYOG* was observed between day 0 and day 2 of differentiation ($P < 0.001$, one-way analysis of variance (ANOVA) with Bonferroni post hoc test). This increase occurred in the same time scale as the observed morphological changes. In addition, the level of skeletal muscle-specific myosin heavy chain 3 (*MYH3*) was very low prior to differentiation (day 0); however, there was a statistically significant 7-fold increase in *MYH3* expression between days 2 and 4 of differentiation ($P < 0.01$, Bonferroni post hoc test). *MYOD1* was found to already be expressed at day 0, with no significant change in expression observed early in the differentiation process, although a significant decrease in both *MYOG* and *MYOD1* expression was observed by day 10 ($p < 0.05$ when comparing day 10 to days 2, 4 and 6 for expression of both genes; Bonferroni post hoc test.)

These results indicate that cell differentiation has already begun after incubation for 2 days in low serum medium, when *MYOG* expression increases and myotubes are observed in culture, and that differentiation is complete by day 4, when *MYH3* is being expressed.

Western blot analysis showed that the transcript level changes were mirrored at the level of protein expression, following 5 days of differentiation (Figure 3-3).

Figure 3-3 Protein expression of muscle-specific markers in Huh7 and RD cells

Total protein was extracted from Huh7 cells, undifferentiated RD cells (RD-U) and RD cells following 5 days of differentiation in 1% serum (RD-D). Protein concentration was determined using the Lowry method and 15µg was separated by SDS-PAGE. The levels of myogenin (*MYOG*) and myosin heavy chain 3 (*MYH3*) were detected using specific antibodies, and β-actin was used as a loading control; N=3. Western blot analysis was carried out by Kate Plant and Jenny Spinks.

Limited expression of myogenin was observed in undifferentiated RD cells (RD-U), but greater expression of myogenin protein was observed in cells that had been differentiated for 5 days in low-serum medium. No myogenin was detected in Huh7 liver cells. High MYH3 protein levels were observed in differentiated RD cells, but not in undifferentiated or Huh7 cells. This supports the data from microscopy and TaqMan analysis, and further supports the conclusion that it has been possible to differentiate RD cells here.

3.2.2 Expression of Cholesterol Metabolism-Related Genes

In order to determine whether differentiation would have an effect on cholesterol metabolism, the expression of *HMGCR* and *LDLR* was measured. TaqMan analysis confirmed the expression of both genes in undifferentiated RD cells, and there was no significant change in expression levels during the first 6 days post-differentiation (Figure 3-4).

Figure 3-4 Cholesterol metabolism during RD muscle cell differentiation

RD cells were grown to confluence and then induced to differentiate by a change to low-serum differentiation medium on day 0. Expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGCR*) and low-density lipoprotein receptor (*LDLR*) was measured by quantitative RT-PCR (TaqMan). Values plotted represent mean $\pm$ standard deviation (SD), from a single experiment where N=4. Statistical significance determined by one-way ANOVA followed by Bonferroni post hoc test: * = $P < 0.05$ compared to day 0.

However, both showed a significant decrease in expression following 10 days of differentiation, with approximately a 4-fold decrease in *LDLR* expression ($P < 0.05$ when compared to days 0 and 2; Bonferroni post hoc test) and a 2.5 fold-

decrease in *HMGC*R expression ($P < 0.05$ when compared with days 2, 4 and 6; Bonferroni post hoc test).

3.2.3 Clock Gene Expression

The expression of several key clock genes was also determined in order to both verify that RD cells express components of the molecular clock, since circadian rhythmicity has not previously been studied in this cell line, and to examine the effect of differentiation on these expression levels. TaqMan analysis confirmed the expression of brain and muscle arnt-like protein-1 (*BMAL1*), period 2 (*PER2*), cryptochrome 1 (*CRY1*) and D site of albumin promoter (albumin D-box) binding protein (*DBP*) in RD cells (Figure 3-5).

Figure 3-5 Expression of clock genes during RD muscle cell differentiation

RD cells were grown to confluence and then induced to differentiate by a change to low-serum differentiation medium on day 0. Expression of the clock genes brain and muscle arnt-like protein-1 (*BMAL1*), period 2 (*PER2*), cryptochrome 1 (*CRY1*) and D site of albumin promoter (albumin D-box) binding protein (*DBP*) was measured by quantitative RT-PCR (TaqMan). Values plotted represent mean $\pm$ standard deviation (SD), from a single experiment where $N=4$. Statistical significance determined by one-way ANOVA followed by Bonferroni post hoc test: ** = $P < 0.01$ compared to day 0.

Levels of *BMAL1*, *PER2* and *CRY1* expression did not significantly change during the 10 days post-differentiation; however a 4.5-fold increase was observed between days 0 and 10 in the level of expression of *DBP* ($P < 0.01$, Bonferroni post hoc test). This confirmation of clock gene expression suggests that there could be a functional molecular clock in these cells.

Taken together, these results show that RD cells can be induced to differentiate in low serum medium, and that this differentiation has occurred after 4 days. However, for many of the genes examined there is a significant decrease in expression following 8 or 10 days of differentiation. Therefore, for all subsequent experiments a differentiation time of 5 days was selected, since cells will have differentiated and this will allow an additional 48 hours for experiments prior to any significant decrease in gene expression.

3.3 Characterisation of Circadian Rhythms

Having confirmed the expression of several key genes in the RD cell line and optimised cell differentiation, the next step was to study temporal gene expression in RD muscle cells. It is currently thought that there is an endogenous molecular clock in every individual cell; however, in the absence of external cues the circadian rhythms across a population of cells in culture are desynchronised (Nagoshi et al. 2004; Welsh et al. 2004). The application of a stimulus, such as a serum shock with 50% horse serum, has been shown to synchronise the rhythms of cells in culture, therefore enabling the analysis of circadian gene expression (Balsalobre et al. 1998). This method for synchronising cell rhythms is known as the serum pulse technique (section 2.2.1.10).

3.3.1 Pilot Experiment

Prior to studying circadian gene expression in RD cells, a pilot experiment was first carried out using 3 cell lines that are known to have previously shown circadian rhythmicity. As already described, rhythmic gene expression has previously been demonstrated in the HepG2 human liver cell line (Koyanagi et al. 2006; Matsunaga et al. 2008; Takiguchi et al. 2007). Rhythmic gene expression has been less well studied in the Huh7 human liver cell line, but nevertheless circadian rhythmicity has been demonstrated in the core clock gene *PER1*, the deadenylase nocturnin (Li et al. 2008), and the cytokine thrombopoietin (Tracey et al. 2012). The 3T3-L1 murine preadipocyte cell line has also previously shown rhythmic expression of key clock genes (Otway et al. 2009).

Following a serum pulse, RNA was extracted at 4 time points to allow monitoring of gene expression over 36 hours, with time points selected for each cell line to match rhythmic expression profiles previously reported in the published literature (Koyanagi et al. 2006; Li et al. 2008; Otway et al. 2009). The time points were carefully chosen to enable observation of an expected peak of expression after 24-28 hours, as well as troughs after 12-16 and 36 hours, in 3T3-L1 and HepG2 cells but a peak after 16-20 as well as troughs after 8-12 and 24-32 hours in Huh7 cells. Expression of the representative core

clock gene, *Per2*, and clock-controlled gene, *Dbp*, was analysed (Figure 3-6) since these genes often show the most robust rhythmic expression patterns in cultured cells (Otway et al. 2009).

Figure 3-6 Temporal expression of *PER2* and *DBP* in 3 different cell lines
 3T3-L1, HepG2 and Huh7 cells were synchronised using a serum pulse (at T=0). RNA was extracted from 3T3-L1 and HepG2 cells at T=0, 14, 26, 36 and from Huh7 cells at T=0, 12, 18 and 26. Temporal gene expression of period 2 (*PER2*) and D site of albumin promoter (albumin D-box) binding protein (*DBP*) was measured by quantitative RT-PCR (TaqMan). Unnormalised data are presented as mean ± standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

TaqMan analysis revealed statistically significant rhythmic expression of both *Per2* (P = 0.0072, one-way analysis of variance (ANOVA)) and *Dbp* (P < 0.0001, one-way ANOVA) in 3T3-L1 cells, with the expected peak at 26 hours and trough at 14 hours.

There were no statistically significant changes observed in expression of *PER2* and *DBP* in either HepG2 cells ($P = 0.9851$ and 0.3527 respectively, one-way ANOVA) or Huh7 cells ($P = 0.4590$ and 0.1793 respectively, one-way ANOVA).

Normalisation with the housekeeping genes *18S*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) or β -actin did not significantly alter expression patterns for either *PER2* or *DBP* in any of the 3 cell lines used (two-way ANOVA; appendix 8.1).

The fact that rhythmic gene expression was detected in the 3T3-L1 cells indicated that the circadian rhythms of individual cells were being synchronised here by the serum pulse. It is possible that the lack of clear observation of rhythmic gene expression in the liver cell lines could be due to the small number of time points used in this experiment; therefore a more comprehensive analysis was carried out, using only 1 of the liver cell lines, the Huh7 cell line, alongside the RD cell line which has previously not been examined for circadian rhythmicity.

3.3.2 Circadian Analysis in Huh7 and RD Cells

A serum-pulse was used to synchronise both Huh7 (Figure 3-7) and RD-D (Figure 3-8) cells, and TaqMan analysis was used to examine gene expression over a longer time course of 56 hours, to enable the observation of 2 full 24-hour cycles after cell synchronisation.

3.3.2.1 Analysis of Temporal Gene Expression in Huh7 Cells

In Huh7 liver cells (Figure 3-7), no statistically significant effect of time was seen on the expression of the core clock gene *PER1* ($P = 0.6926$, one-way ANOVA). This is the only core clock gene that has previously been analysed for rhythmic gene expression in this cell line (Li et al. 2008), with an observed peak in expression after 16-20 hours as well as troughs after 8-12 and 24-32 hours in both Huh7 and a variety of other cells types (Chalmers et al. 2008; Li et al. 2008; Wu et al. 2007). This rhythmic expression profile would also have been expected here.

Figure 3-7 Temporal gene expression in Huh7 liver cells

Huh7 cells were synchronised using a serum pulse (at T=0). RNA was extracted every 4 hours, for 56 hours, and temporal gene expression was measured by quantitative RT-PCR (TaqMan). Unnormalised data are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4. Statistical significance determined by one-way ANOVA followed by Bonferroni post hoc test: * = $P < 0.05$.

Statistical significance for an effect of time on expression of the core clock gene *PER2* was shown only between data points at time 0 and 40 hours after the serum pulse ($P < 0.05$, Bonferroni post hoc test). However, since large changes in gene expression are often seen immediately after a serum pulse and prior to the observation of a circadian expression profile, this had no clear relevance for determination of rhythmic expression. Likewise, the only

statistically significant changes in expression of the transcription factor reverse erythroblastosis virus α (*REV-ERB α*) were those based on data from time points immediately following the serum pulse (0 - 8 hours) or on the 56 hour time point, when gene expression was starting to decrease at the end of the experiment.

HMGCR was included in this analysis since diurnal variation has been previously shown in both its transcription and enzyme activity in other models, and these variations potentially impact upon both statin efficacy and toxicity. However, there was no statistically significant effect of time on HMGCR gene expression in Huh7 cells in this analysis ($P = 0.1192$, one-way ANOVA).

Only *DBP*, a transcription factor that regulates the expression of many clock-controlled genes, showed an indication of rhythmic variation in gene expression with peaks of expression at 24 and 44 hours. There was a statistically significant difference between expression at 16 and 44 hours ($P < 0.05$, Bonferroni post hoc test), where these time points represent a trough and a peak respectively, and these observed peaks would be consistent with *DBP* rhythmic expression profiles reported elsewhere (Otway et al. 2009).

3.3.2.2 Analysis of Temporal Gene Expression in RD-D Cells

It has been shown that circadian gene expression profiles of key clock genes in skeletal muscle are frequently similar to those seen in liver (Miyazaki et al. 2011), and therefore the expectations for rhythmic variation of these genes in RD cells would be similar to those for Huh7 cells. However, in RD-D muscle cells (Figure 3-8) there was no statistically significant effect of time on the expression of the core clock genes *CRY1*, *PER1*, *PER2*, transcription factors *REV-ERB α* and *DBP*, or *HMGCR*. This is after taking into account the large changes in expression, particularly in *CRY1*, *PER1* and *PER2*, immediately following the serum pulse.

Figure 3-8 Temporal gene expression in RD-D muscle cells

RD-D cells were synchronised using a serum pulse (at T=0). RNA was extracted every 4 hours, over a time of 56 hours, and temporal gene expression was measured by quantitative RT-PCR (TaqMan). Unnormalised data are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4. Statistical significance determined by one-way ANOVA followed by Bonferroni post hoc test: * = $P < 0.05$.

There was also no statistically significant effect of time on the expression of muscle-specific transcription factors *MYOD1* and *MYOG*. This is surprising, as an effect might have been expected since *MyoD1* has shown circadian expression of RNA in rat skeletal muscle (Miyazaki et al. 2011) and circadian expression of both RNA and protein in mice (Andrews et al. 2010; Zhang et al. 2012).

The core clock gene *BMAL1* has previously shown rhythmic gene expression in cultured cells, with expression peaks at 12-20 hours and 32-40 hours as well as troughs at 20-28 hours in both adipose-derived stem cells (Wu et al. 2007) and Movas-1 mouse smooth muscle cells (Chalmers et al. 2008). In this analysis with RD-D cells, statistical significance was found between *BMAL1* gene expression at 16 hours, a potential peak, and gene expression at 32 and 48 hours, 2 potential troughs ($P < 0.05$, one-way ANOVA).

3.3.2.3 Normalisation of Gene Expression Data

The gene expression data presented in Figure 3-7 and Figure 3-8 is not normalised; however, in quantitative PCR (qPCR) studies it is common for data to be normalised to the expression of internal control genes (often referred to as “housekeeping genes”). The use of internal control genes for the normalisation of such data is primarily to minimise any error that may occur from discrepancies in the initial cDNA input, which may arise from differing efficiencies of the cDNA synthesis reactions. These genes should not vary in expression in the tissue(s) or cell line(s) being investigated, or as a result of the experimental treatment (Vandesompele et al. 2002).

For this analysis, 18S ribosomal RNA (rRNA) and β -actin were chosen as housekeeping genes. 18S rRNA has commonly been used for normalisation, and hence was also tried here. β -actin has been shown to exhibit very little circadian variation in several tissues when compared to other housekeeping genes (Kamphuis et al. 2005) and it has been used previously as an internal control for experiments in RD cells (Phillips et al. 2007; Yang et al. 2009). TaqMan analysis was therefore carried out, but revealed large variations in the expression of 18S and β -actin in both Huh7 and RD-D cells (Figure 3-9).

Figure 3-9 Temporal gene expression of two different housekeeping genes in Huh7 and RD-D cells

Huh7 and RD-D cells were synchronised using a serum pulse (at T=0). Temporal gene expression of housekeeping genes 18S and ACTB was measured by quantitative RT-PCR. Data are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

Temporal gene expression data from both Huh7 (Figure 3-10) and RD-D (Figure 3-11) cells was normalised to either *18S* or β -actin. Initially data for the expression of *PER2* and *DBP* was used since these were the two genes analysed previously in the pilot experiment.

Figure 3-10 Effect of normalisation of Huh7 TaqMan data using two different housekeeping genes

Huh7 cells were synchronised using a serum pulse (at T=0) and temporal gene expression of *PER2* and *DBP* was measured by quantitative RT-PCR. Data are shown unnormalised and normalised to *18S* or *ACTB* expression, and are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

Normalisation of data from Huh7 cells introduced new, large peaks in the expression profiles of *PER2* and *DBP*, as well as greater overall variability within the dataset, as seen particularly in data points from 0 - 16 hours when data was normalised to *18S*. This was a direct reflection of the variation seen in the *18S* TaqMan data, which could have arisen due to a genuinely large variation in the efficiencies of the individual reactions used to synthesize the cDNA used for TaqMan analysis or an actual variation in *18S* gene expression. Since the normalised data profile seemed to relate directly to the housekeeping gene expression, it would suggest that the latter may play a more important role and therefore data normalisation may have the potential to mask any genuine temporal effects.

Bearing this in mind, when time points 0 – 16 were removed from the analysis there was no statistically significant difference in gene expression profiles for either *PER2* ($P = 0.8231$, two-way ANOVA) or *DBP* ($P = 0.7657$, two-way ANOVA) between unnormalised data or data normalised to the housekeeping genes *18S* or β -actin.

Figure 3-11 Effect of normalisation of RD-D TaqMan data using two different housekeeping genes

RD-D cells were synchronised using a serum pulse (at T=0) and temporal gene expression of *PER2* and *DBP* was measured by quantitative RT-PCR. Data are shown unnormalised and normalised to *18S* or *ACTB* expression, and are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

The normalisation of data from RD-D cells, to housekeeping gene *18S* or β -actin, also had no statistically significant effect on gene profiles of *PER2* ($P = 0.7699$, two-way ANOVA) or *DBP* ($P = 0.9225$, two-way ANOVA). Similarly, normalisation of the other genes analysed had no significant effect on gene expression profiles and did not lead to any unmasking of any previously unseen

circadian effects (appendix 8.2). Therefore, the decision was made to leave TaqMan data unnormalised.

In summary, no clear circadian rhythms of gene expression have been observed in either Huh7 (Figure 3-7) or RD-D (Figure 3-8) cells. Out of all of the genes examined, the only potentially significant temporal variations in gene expression were observed with the transcription factor *DBP* in Huh7 cells and the core clock gene *BMAL1* in RD-D cells.

3.3.2.4 Relative Abundance of Clock Genes

Data obtained from the TaqMan analysis of temporal gene expression was used to compare the abundance of the different gene transcripts between Huh7 and RD-D cells (Figure 3-12). The data used was from the T = 0 time point of the analysis, meaning that this represents transcript abundance prior to cells receiving a serum pulse.

Figure 3-12 Comparison of gene expression in Huh7 liver and RD-D muscle cells

RT-PCR (TaqMan) data from T=0 time point of serum pulse experiments in Huh7 liver (Figure 3-7) and RD-D muscle (Figure 3-8) was used to compare the abundance of different genes between cell lines. Gene expression of *BMAL1* and *CRY1* was not determined (ND) in Huh7 liver cells. Data represent mean $\pm$ standard deviation (SD), from a single experiment where N=4

From this comparison, it seems that there is greater expression of the core clock gene *PER1* in RD-D than in Huh7 cells ($P = 0.0930$, unpaired two-tailed t-test), whereas *PER2* expression may be greater in Huh7 liver cells than in RD-D ($P = 0.1755$, unpaired t-test). *REV-ERB α* expression is similar in both Huh7 and RD-D cells ($P = 0.1981$, unpaired t-test), but there is significantly greater expression of the clock controlled transcription factor *DBP* in RD-D than Huh7 ($P = 0.0058$, unpaired t-test). Expression levels of *CRY1* and *BMAL1* were only analysed in RD-D cells. The level of *CRY1* expression in RD-D cells was not significantly different from that of the other clock genes, apart from *BMAL1* (one-way ANOVA). The core clock gene *BMAL1* had the lowest expression, with a transcript level that was almost 3 times lower than the next lowest expressed transcript in RD-D cells, *REV-ERB α* . This is consistent with what was also observed during RD cell differentiation (Figure 3-5).

3.4 Discussion

In order to study the circadian aspects of statin-induced myopathy it is important to establish a robust *in vitro* model. For this reason the first stage of this project was to characterise RD muscle cell differentiation, by looking at the morphological and molecular changes that occurred during this process, followed by characterisation of circadian rhythms in this same cell line (objectives 1 and 2; section 1.5).

3.4.1 RD Cell Differentiation

Muscle cell differentiation is a highly ordered, multi-step process in which, on mitogen withdrawal, proliferating myoblasts differentiate into myocytes, which exit from the cell cycle and fuse to form multinucleated myotubes and myofibres that express a number of structural and enzymatic muscle-specific proteins (Andres and Walsh 1996). This process is regulated by four myogenic regulatory factors (MRFs): MYOD1, MYF5, MYOG, and MRF4, which are muscle-specific basic helix-loop-helix transcription factors (Moran et al. 2002) that are activated at differing stages of the differentiation process (Figure 3-13). MYOD1, a “primary MRF”, is expressed early in the differentiation process. It is expressed both before and after differentiation *in vitro* and is thought to be important for myoblast determination *in vivo* (Megeney and Rudnicki 1995).

MYOG, a so-called “secondary MRF”, is expressed upon differentiation and then plays a role in terminal differentiation (Megeney and Rudnicki 1995). The results obtained here, both in terms of the observation of an increasing number of myotubes in the cell culture and the observed changes in gene expression, are consistent with the occurrence of RD cell differentiation.

Figure 3-13 Regulation of skeletal myogenesis

Myogenesis is regulated by the myogenic regulatory factors (MRFs): MYF5, MYOD1, MYOG and MRF4, which are transcription factors that activate expression of muscle-specific genes, such as α -actin, myosin heavy chain (MHC) and muscle creatine kinase (MCK). On differentiation, the inhibitor of differentiation protein (ID) is downregulated, allowing MRFs to form functionally active heterodimers with the ubiquitously expressed E proteins. Each MRF plays a role at a different stage of differentiation, as shown. MYOD1 and MYF5 are expressed early during muscle development and play a role in myoblast determination, whereas MYOG and MRF4 are expressed later in the differentiation process. Figure adapted from (Lluis et al. 2006).

MYOD1 was seen to be expressed prior to, and throughout, differentiation. The former could suggest that even before RD cells are stimulated to fully differentiate they are committed to the skeletal-muscle cell lineage. A large increase in *MYOG* expression was noted 2 days after the change to low-serum medium, which would be consistent with the onset of early differentiation and the formation of myocytes. It is interesting to note that upon differentiation there is a difference between the extent of *MYOG* RNA induction and protein induction (Figure 3-2 and Figure 3-3). This indicates that there may be posttranscriptional regulation of myogenic regulatory factors, and shows the importance of examining changes in protein expression as well as in gene expression.

Myosin plays an important role in muscle contraction (Bandman 1992). It is a marker of terminal differentiation (Bennett and Tonks 1997) and myosin heavy chain (MHC) has previously been used to monitor the differentiation status of RD cells (Ma et al. 2003). *MYH3* is an embryonic skeletal muscle MHC isoform (Moran et al. 2002) and so its expression was examined here as the RD cell line originates from an embryonic rhabdomyosarcoma (Knudsen et al. 1998) and is known to express this MHC isoform (Rubin et al. 2011; Schiaffino et al. 1986; Yang et al. 2009). The large increase in *MYH3* expression by day 4 of RD cell differentiation is similar to what has previously been observed during differentiation of the C2C12 mouse myoblast cell line (Bennett and Tonks 1997; Moran et al. 2002) and further supports the conclusion that terminal RD cell differentiation has been observed here.

The TaqMan analysis that has been carried out also verifies that this cell line expresses a number of cholesterol metabolism genes (*HMGCR* and *LDLR*) and clock genes (*BMAL1*, *PER2*, *CRY1*, *DBP*). Expression levels for most of these genes seem to remain unchanged during the differentiation process. There is, however, a significant decrease in expression levels of *HMGCR* and *LDLR* by day 10, which is similar to the observed decrease in *MYOD1* and *MYOG* expression levels, and could be due to the continued proliferation of these particular cells throughout differentiation (Figure 3-1); by day 10 the cells are becoming overgrown and therefore perhaps starting to die by apoptosis.

However, in contrast there is no similar decrease observed in expression of *MYH3* or *DBP*. This implies that the decrease could also be due to changes in cell requirements upon differentiation, where differentiated cells need less *MYOD1* and *MYOG* and have a reduced requirement for cholesterol synthesis. The reason for the continued steady increase in *DBP* expression is as yet unknown but it could be possible that, as a transcription factor, as well as playing a role in the output of the core clock *DBP* may play an as yet unidentified role in the process of myogenic differentiation.

It is important to recognise that the referred-to differentiation of a population of RD cells will always be limited. As was previously described (section 3.2.1), rhabdomyosarcoma cells are deficient in their ability to exit the cell cycle and complete terminal differentiation, despite their commitment to the muscle cell lineage and expression of the myogenic regulatory factors *MYOD1* and *MYOG*. Several possible explanations for this deficiency have been proposed (Aguanno et al. 1990; Knudsen et al. 1998; Tapscott et al. 1993), including the presence of inactive *MYOD1* that is still able to bind DNA but not activate transcription (Tapscott et al. 1993). Several research groups have been able to partially rescue the phenotype of limited RD cell differentiation, demonstrating growth inhibition as well as induction of both partial differentiation and the expression of muscle-specific proteins (Aguanno et al. 1990; Bouche et al. 2000; Wang et al. 2010b), or the restoration of *MYOD1* activity (Guo et al. 2003; Ostrovsky et al. 2002; Puri et al. 2000). However, despite all of these treatments and modifications, it is clear that the rescue remains only partial and that resulting cell populations remain mixed, containing RD cells at varying stages of differentiation, making it unclear how beneficial these additional steps are. Within the experimental paradigm used here, it is clear that reliable cell differentiation is being observed, as demonstrated by expression of *MYH3*, even if this differentiation is not complete. Therefore, since any additional approaches have the potential for generating further experimental variability, I will continue to use this cell line, using serum deprivation to initiate cell differentiation, as a starting point for generating the *in vitro* model for studying statin administration.

One main reason for the characterisation of the differentiation process was to find the most suitable length of time to use for muscle cell differentiation prior to future experiments in this particular project. RD cells used by other research groups have been left in low-serum differentiation medium for varying lengths of time prior to further experiments, including for 4-5 days (Knudsen et al. 1998), 6 days (Vachon et al. 1996) or 7 days (Nishimoto et al. 2003). From the results obtained, it was decided that cells should be left in low serum differentiation medium for 5 days prior to future experiments. After this time the muscle regulatory factors *MYOD1* and *MYOG* are being expressed near their highest level, there is a high level of *MYH3* expression indicating that there are differentiated cells, and expression levels of the cholesterol metabolism genes and clock genes remain stable. Using 5 days for differentiation will also allow enough time to carry out any required time courses before the cells get too overgrown and gene expression levels start to decrease.

3.4.2 Analysis of Circadian Rhythms

Having observed these changes in gene expression during RD muscle differentiation, temporal gene expression was then studied in this cell line following a serum pulse. Circadian rhythms have now been observed in most peripheral tissues and also in many human cell lines (Ko and Takahashi 2006), but there have, to date, been no reported studies into circadian rhythmicity in this particular human muscle cell line.

TaqMan analysis of temporal gene expression in both Huh7 (Figure 3-7) and differentiated RD (Figure 3-8) cells indicated that the genes examined were not rhythmic in these cell populations. The lack of observed rhythmicity is of some surprise, especially in light of the fact that rhythms have to date been observed in a large range of different cell lines, using this same serum pulse protocol to synchronise the rhythms of individual cells within a population.

There is also increasing evidence regarding circadian rhythmicity in skeletal muscle (Andrews et al. 2010) and so it might be expected that RD skeletal muscle cells would contain molecular clocks which could be synchronised in culture. On the other hand, it could perhaps be that disruption of circadian

rhythmicity is a phenotype of the rhabdomyosarcoma tumour from which this cell line is derived. There is currently no evidence for this, and these cells clearly do express a variety of core clock genes; however it is interesting to note that expression of *BMAL1* is very low in RD cells, compared to the expression of other clock genes (Figure 3-12). It is unclear from the literature whether *BMAL1* is always expressed at a low level in cultured cells or whether this is specific to RD cells. If it is the latter then perhaps this could partially explain the lack of observed rhythmicity, since it is known that deficiency in *BMAL1* can lead to arrhythmicity (Bunger et al. 2000). It would also be interesting to know whether or not this low expression is mirrored in the expression of the dimerisation partner of *BMAL1*, circadian locomotor output cycles kaput (*CLOCK*).

However, lack of a functional molecular clock in RD cells would not explain the lack of rhythmic gene expression also observed in Huh7 cells (Figure 3-7). Unlike for RD cells, circadian rhythmicity has previously been demonstrated in this cell line (Li et al. 2008) and so it could somehow be that there was a problem with the protocol that was used here. This would be supported by the fact that, in the pilot experiment, although rhythmic expression of *PER2* and *DBP* was observed in the 3T3-L1 cell line (Figure 3-6), rhythmic gene expression was not clearly observed in human HepG2 and Huh7 cells. In further support of this, although there were also no clear rhythms in temporal gene expression observed in Huh7 or RD-D cells in the more comprehensive analysis, there were indications of slight rhythmic variations in the expression of *DBP* in Huh7 cells and *BMAL1* in RD-D cells. In Huh7 cells, there was a statistically significant difference between expression of *DBP* at 16 hours (a potential trough) and 44 hours (a potential peak), and in RD-D cells statistical significance was found between *BMAL1* gene expression at 16 hours (a potential peak) and at 32 or 48 hours (2 potential troughs). These temporal variations match the expression profiles seen previously in 3T3-L1 cells (Otway et al. 2009) and HepG2 cells (Koyanagi et al. 2006). In addition, the errors on the data from TaqMan analysis were quite large. All of this could indicate that the serum pulse was only partially achieving its intended goal of cell synchronisation. In order to investigate this possibility, it would have perhaps

been interesting to see whether either serum starvation prior to serum shock (Tracey et al. 2012) or cell synchronisation using the glucocorticoid hormone analogue dexamethasone rather than horse serum (Balsalobre et al. 2000) would have had any impact on the observation of circadian rhythmicity.

It has been demonstrated that the housekeeping genes that are most commonly used for data normalisation are not always the most appropriate genes (Kamphuis et al. 2005), and that the decision of housekeeping gene should be made in light of each individual experimental situation (Vandesompele et al. 2002). This is perhaps especially important to bear in mind in circadian experiments when a large number of genes, other than the genes of interest, are likely to also exhibit circadian variation. In this particular case, any control gene used would need to be expressed in both liver and muscle, to not vary in a circadian manner and not be affected by the statin treatment which would be a part of later experiments. In this case it was clear that there was large variation in the expression of each of the housekeeping genes used (Figure 3-9), which could potentially complicate the interpretation of data when normalised and so the decision was taken to leave data unnormalised. In addition there was no significant difference to the observation of circadian rhythmicity between unnormalised data or data normalised to either of the two housekeeping genes 18S or β -actin (Figure 3-10). Therefore, it seems unlikely that the choice of an incorrect housekeeping gene was responsible for the observation of arrhythmicity in this particular case.

It remains possible that circadian rhythmicity may be observable in undifferentiated RD cells where no rhythms were observed here in differentiated cells (Figure 3-8). Alternatively, perhaps protein expression may have shown temporal variation even where RNA did not, since changes in functional protein are not always directly related to changes at the RNA level (Brockmann et al. 2007; Greenbaum et al. 2003; Griffin et al. 2002). However, given the lack of evidence for rhythmicity in gene expression that has been observed so far, a decision was made not to commit further resources to continue investigating this line of research.

3.4.3 New Aims and Objectives:

Therefore, having characterised the differentiation process of RD muscle cells (objective 1; section 1.5) but having been unable to verify the ability of this cell line to establish and maintain a circadian cycle *in vitro* (objective 2, section 1.5), a new set of objectives needed to be decided upon. Having established human liver and muscle models, I shall continue to compare the responses of liver and muscle cells to statin treatment, now focusing on investigating differences in the effects on cell viability, amount of cellular cholesterol and on prenylation, which may give us further clues into the mechanism of statin-induced muscle toxicity.

The new aim is to use liver and muscle cells in culture as a model system for studying the effects of statin administration on both cholesterol and isoprenoid biosynthesis, via the following objectives:

Objective 2: Examine the effects of statins on cell viability to determine whether there are differences between liver and muscle.

Objective 3: Assess the relative expression of HMGCR, and changes in cellular cholesterol in response to statin treatment, in liver and muscle.

Objective 4: Assess whether differences measured in objectives 2 and 3 correspond to effects of statin treatment on protein prenylation.

3.5 Summary

Circadian rhythms have been shown to impact upon the toxicity of xenobiotics. To examine whether differences exist between the circadian aspects of statin efficacy and toxicity the temporal affects of statins can be studied using an *in vitro* model. In order to compare the responses of liver (the target tissue) and skeletal muscle (the site of toxicity) to temporal statin administration two human cell lines were chosen: RD human embryonic rhabdomyosarcoma cells, which express a number of muscle-specific genes following differentiation, and HepG2 human hepatocellular carcinoma cells.

The morphological and molecular changes that occurred during RD muscle cell differentiation were characterised. RD differentiation was observed in response to a change to low-serum medium, as determined by large increases in the expression of muscle-specific transcription factor *MYOG* and the marker of terminal differentiation *MYH3*. Levels of *MYH3* rose rapidly after 4 days in low serum medium and remained high throughout the remainder of the 10-day experiment, whereas levels of *MYOG* began to decline after 8-10 days. As a result of this analysis, it was decided to use a time of 5 days for the differentiation of RD cells in future experiments.

RD cells were also observed to express a number of important cholesterol-related and clock genes. However, significant rhythmic gene expression was not observed in response to a serum pulse, in either liver or muscle cells, but was observed in the positive control 3T3-L1 cell line. Therefore, a new set of objectives were defined, whereby the focus of future experiments would remain on examining the differences in the response of liver and muscle to statin administration, but without the circadian aspect.

4 Differential Sensitivity of Liver and Muscle Cell Lines to Statin Treatment

4.1 Introduction

Statins act by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the enzyme that catalyzes the rate-limiting step in cholesterol synthesis (Shitara and Sugiyama 2006). Decreased *de novo* cholesterol synthesis leads to an upregulation of the LDL receptor, increased uptake of low-density lipoprotein (LDL) from the bloodstream and an overall reduction in patient plasma levels of cholesterol in the form of LDL (Brown and Goldstein 2004). As patient plasma LDL-cholesterol is a direct marker of cardiovascular risk, the action of the statins reduces risk of cardiovascular disease in patients (Mensink et al. 2003). The extent of the reduction in LDL is dependent on the specific statin used in treatment and on the dose (Chong et al. 2001).

There are a number of different statins currently clinically available, including simvastatin, lovastatin, fluvastatin, atorvastatin, rosuvastatin and pravastatin. All statins exert their effects via the same mechanism (Chong et al. 2001); however, they have differences in their physicochemical properties (Table 1-3). These include differences in their chemical structure (Figure 1-4), their bioavailability, half-life, potency, lipophilicity, metabolism and transport (Chong et al. 2001; Shitara and Sugiyama 2006; Vaklavas et al. 2009). In addition, both simvastatin and lovastatin are administered as a prodrug, containing an inactive lactone ring, which is converted into the biologically active open acid form in the body. All other statins are given in the open acid form (Chong et al. 2001; Shitara and Sugiyama 2006).

Myopathy, both in general and in its severest form as rhabdomyolysis, has been observed as an adverse effect with all statins (Bolego et al. 2002), although there are marked differences in myopathy incidence rates (Staffa et al. 2002). The fact that cerivastatin was withdrawn from the worldwide market in 2001 due to its high incidence of cases of fatal rhabdomyolysis (Bellingham 2001) highlights the significance of the problem of statin-induced muscle toxicity.

However, the exact mechanism of this greater muscle sensitivity has not been fully elucidated (Baker 2005).

In the previous chapter, the muscle cell line RD was characterised in terms of its differentiation, for use together with the control liver cell line Huh7 as part of a model system. The next stage was to begin characterising the responses of these liver and muscle cell lines to statin treatment, in terms of overall cell toxicity and cholesterol metabolism. Cell toxicity assays have been carried out, in order to test the hypothesis that greater toxicity would be observed in the RD muscle cells than in Huh liver cells, since muscle toxicity is an important adverse effect of statins in the clinic. This study represents the first comprehensive comparative analysis of statin toxicity in both liver and muscle cell lines.

In addition, the expression of HMGCR has been compared between cell lines, and the effect of statin treatment on the level of total cellular cholesterol examined, in order to investigate whether any differences observed in toxicity translate to differences in statin-induced cholesterol reduction.

4.2 Characterisation of Statin-Mediated Cell Toxicity

In order to begin characterising the effects of statin treatment in RD and Huh7 cells, MTT assays were carried out to assess metabolic and cellular viability after cells were exposed to different concentrations of statin for 48 hours (section 2.2.1.12).

Huh7 cells have been used previously for MTT assays in this research group, using a period of 150 minutes for the incubation of cells with MTT reagent (Mary Hong, personal communication, 10 November 2009). However, RD cells have not previously been used by this group, and therefore a preliminary experiment was carried out using untreated cells in order to determine optimum assay conditions prior to statin treatment (Figure 4-1).

Figure 4-1 Optimisation of time of incubation with MTT reagent

MTT reagent was added to untreated RD-U and RD-D cells in 6-well plates (T = 0) and absorbance was read (section 2.2.1.12) after incubation for 90, 150 and 240 minutes. Different wells of cells were used for each time point. Values plotted represent mean $\pm$ standard deviation (SD), from a single experiment where N=3.

One hundred and fifty minutes was found to be the minimum time at which absorbance no longer increased linearly with incubation time, using both undifferentiated RD (RD-U) and differentiated RD (RD-D) cells. This would therefore allow sufficient time for the conversion of MTT reagent by viable cells, and was chosen for incubation of all cells with MTT reagent in following assays.

Subsequently, MTT assays were carried out using the full range of available statins: simvastatin (acid and lactone), lovastatin (acid and lactone), cerivastatin, fluvastatin, atorvastatin, rosuvastatin and pravastatin (Figure 4-2).

Figure 4-2 Cell viability in response to statin treatment

Huh7 (shown in red), RD-U (blue) and RD-D (black) cells were treated with statin for 48 hours and MTT assays carried out to measure cell viability. Graphs are shown in decreasing order of statin lipophilicity. Final absorbance is expressed as a percentage of the control (defined as 100%). At least three independent experiments were carried out, using 6 replicate wells in each. Mean values were generated from each experiment, and combined to produce the data shown here. Error bars represent standard error of the mean (SEM). Data were analysed by two-way analysis of variance (ANOVA) and P values are shown where the difference between responses of different cell lines was determined to be statistically significant ($P < 0.05$).

There was a significant dose-dependent reduction in cell viability, in all cell types, on treatment with all statins ($P = 0.0041$, atorvastatin; $P < 0.0001$, all other statins, two-way analysis of variance (ANOVA)). The largest reductions in cell viability were observed on treatment with lipophilic simvastatin (acid) and cerivastatin, whereas loss of cell viability was only observed with very high concentrations of the hydrophilic pravastatin (1mM and greater).

It was not possible to determine robust IC50 values for all of the statins in all cell types, since full dose-response curves could not always be obtained, due to limits in statin solubility and the need to not exceed 0.1% DMSO in cell medium. However, where IC50 values could be determined, they were consistent with other values found in the literature (Table 4-1).

	IC50 (µM)			
Statin	Huh7	RD-U	(Kobayashi 2008)	RD-D
Simvastatin (acid)	102.2	2.36 ± 0.39	3.99	0.296 ± 0.20
Simvastatin (lactone)	0.27 ± 0.10	3.60 ± 5.27	-	
Lovastatin (acid)			52.2	
Lovastatin (lactone)	15.98		-	
Cerivastatin		2.06 ± 0.48	1.77	4.437 ± 1.90
Fluvastatin	8.51 ± 5.49	6.02 ± 1.83	8.34	2.624 ± 2.56
Atorvastatin			9.26	
Rosuvastatin		160.9	>100	
Pravastatin			4890	

Table 4-1 IC50 values for the reduction in cellular viability on statin treatment

Values are shown as mean ± SEM, where possible, or otherwise as a single value, determined from the relevant graph (shown in Figure 4-2) using GraphPad Prism version 5 (section 2.2.5). Not all IC50 values could be determined from this data. IC50 values taken from Kobayashi et al. 2008, as determined by MTT assay using RD-U cells, are also given for comparison. Kobayashi et al. 2008 used only the acid forms of simvastatin and lovastatin, as prepared from their lactone forms prior to treatment.

In general, greater cell toxicity for a given concentration of statin was observed in the RD cells, either differentiated or undifferentiated, compared to the Huh7 cells. The extent of this difference was dependent on the statin and the overall difference in responses between cell lines was only determined to be statistically significant with simvastatin ($P < 0.0001$, acid form and $P = 0.0049$, lactone, two-way ANOVA), cerivastatin ($P < 0.0001$), atorvastatin ($P = 0.0044$) and rosuvastatin ($P = 0.0144$) (Table 4-2). The difference between toxicity in the different cell types was found to be of greater significance with lipophilic simvastatin and cerivastatin than with hydrophilic rosuvastatin.

Statin	P value summary	P value
Simvastatin (acid)	****	<0.0001
Simvastatin (lactone)	**	0.0049
Lovastatin (acid)	ns	0.1137
Lovastatin (lactone)	ns	0.1128
Cerivastatin	****	<0.0001
Fluvastatin	ns	0.3394
Atorvastatin	**	0.0044
Rosuvastatin	*	0.0144
Pravastatin	ns	0.6118

Table 4-2 Significance of cell type in determining response to statin treatment

Data from MTT assays (Figure 4-2) were analysed by two-way analysis of variance (ANOVA) to determine whether there was a significant difference between the responses of different cell types to statin treatment. Ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, **** = $P < 0.0001$.

It is also interesting to note that there appears to be a difference in the cell viability responses of RD-U and RD-D cells to statin treatment. Not all of the relevant IC50 values could be determined, making a quantitative comparison difficult. However, Bonferroni post hoc analysis suggested that these differences were not statistically significant ($P > 0.05$).

In summary, these data demonstrate effects of cell type and of different statins on cell toxicity in response to statin treatment.

4.3 Analysis of Cholesterol Metabolism in Liver and Muscle Cells

Having characterised cell viability in response to statin treatment it was important to also examine cholesterol metabolism in these different cell lines, to see whether there were any differences that could account for the differences in cell toxicity.

4.3.1 HMGCR Expression

Since HMGCR, the enzyme that catalyses the rate-limiting step in cholesterol biosynthesis, is the target of the statins (section 4.1), an initial step in characterising cholesterol metabolism in RD and Huh7 cells was to examine the relative abundance of this enzyme.

Analysis of HMGCR gene expression in cells growing in normal conditions revealed a statistically significant difference between transcript levels of HMGCR in RD and Huh7 cells (Figure 4-3). HMGCR transcript levels were 1.5 fold lower in RD-U cells than Huh7 cells ($P < 0.05$, Bonferroni post hoc), and 3-fold lower in RD-D cells than Huh7 cells ($P < 0.001$).

Figure 4-3 HMGCR gene expression in different cell types

Total RNA was extracted from Huh7, RD-U and RD-D cells in standard growth conditions and expression of HMGCR was measured by quantitative RT-PCR (TaqMan). Data are presented as mean $\pm$ standard deviation (SD), from a single experiment where $N=4$. Statistical significance was determined by one-way ANOVA followed by Bonferroni post hoc test, * = $P < 0.05$, *** = $P < 0.001$ compared to Huh7 expression.

Western blot analysis of total protein showed that HMGCR protein expression was similarly found to be considerably lower in RD-U than Huh7 cells (Figure 4-4). HMGCR protein levels were even lower in RD-D cells, as evidenced by the need to increase the exposure time in order to detect HMGCR in this cell type. Densitometry suggested that there was a 6-fold difference in protein expression between Huh7 and RD-U cells however densitometry was not possible on protein levels in RD-D cells due to the large difference in signal intensity between the cell types.

Figure 4-4 HMGCR protein expression in different cell types

Total protein was extracted from Huh7, RD-U and RD-D cells in standard growth conditions and 15µg was separated by SDS-PAGE. The level of HMGCR (90kDa) was detected by western blot and β-actin was used as a loading control. Two different exposure times were used to allow detection of HMGCR in all cell types. N = 2.

These data demonstrate that HMGCR is expressed in all cell types of interest: Huh7, RD-U and RD-D cells. At both the transcript and the protein level, there is a much greater expression of this enzyme in Huh7 liver cells than in RD muscle cells. Interestingly, a difference was also observed between HMGCR expression in RD-U and RD-D cells, with lower expression in RD-D cells, particularly at the protein level. The different levels of HMGCR expression between the cell types may need to be taken into account when interpreting results from future experiments.

4.3.2 Total Cellular Cholesterol

Having verified the presence of HMGCR in all cell types, the next stage was to determine what effect statin treatment would have on total cellular cholesterol content in these cells, since reduction of cholesterol is the main aim of statin use in the clinic.

Assays were carried out in which cells were treated with statin for a 48 hour period, after which cholesterol was extracted and quantified (section 2.2.4). This time period was chosen based on those reported in the literature (Kobayashi et al. 2008; Mullen et al. 2010), and to remain consistent with the conditions used for the previous MTT assays (Figure 4-2). Simvastatin (acid), cerivastatin and pravastatin were chosen for use in this analysis since both simvastatin and cerivastatin showed a statistically significant difference in toxicity between RD muscle and Huh7 liver cells (Figure 4-2). In contrast, pravastatin treatment resulted in toxicity only at high concentrations and showed no statistically significant difference in toxicity between cell lines, and was included as a relatively non-toxic comparator.

Initially, cells were treated with 1 μ M of each of the chosen statins. This concentration was chosen based on the work of Kobayashi and colleagues (Kobayashi et al. 2008), and results from the MTT assays: In these MTT assays, treatment with 1 μ M simvastatin and cerivastatin resulted in differences between the responses of RD muscle and Huh7 liver cells without causing an excess of cell death that could complicate extracting sufficient cholesterol for quantification (Figure 4-2). Where appropriate, the statistical significance of statin treatment on total cellular cholesterol in individual cell lines was determined using unpaired two-tailed t-test.

There appeared to be a decrease in total cellular cholesterol in Huh7, RD-U and RD-D cells upon 1µM simvastatin treatment, and in Huh7 and RD-D cells on 1µM cerivastatin treatment (Figure 4-5). However, where statistical analysis was appropriate, these changes were not found to be significant using unpaired t-tests; (P = 0.2262 (Huh7) and 0.0574 (RD-U) on treatment with 1µM simvastatin.) There appeared to be no change in cholesterol level upon RD-U treatment with cerivastatin or in any of the cell types on pravastatin treatment.

Figure 4-5 Effect of 1µM statin treatment on total cellular cholesterol

Huh7, RD-U and RD-D cells were treated with 1µM simvastatin (acid), cerivastatin or pravastatin for 48 hours, after which membrane cholesterol was extracted using chloroform: methanol, quantified using the Amplex Red Cholesterol Assay Kit and normalised to total protein. DMSO was used as a vehicle control for simvastatin and water for cerivastatin and pravastatin. Values plotted represent mean ± SEM from two (cerivastatin and pravastatin) or three (simvastatin) independent experiments, where there were 3 replicates in each experiment.

Since no change in cholesterol was observed on treatment with 1µM pravastatin, and changes with simvastatin treatment were not found to be statistically significant, the same analysis was then carried out after treating cells with 10µM simvastatin and pravastatin. Data is not shown for the use of 10µM cerivastatin since at this concentration the amount of cell death, particularly in the RD-U cells, was so great that it was not possible to extract sufficient cholesterol from the remaining cells as to enable quantification.

Treatment with 10µM of both simvastatin and pravastatin seemed to result in a decrease in total cellular cholesterol in Huh7 cells ($P = 0.00535$ and 0.2144 respectively). A statistically significant cholesterol decrease was observed in RD-D cells on treatment with both simvastatin ($P = 0.0027$) and pravastatin ($P = 0.0023$) (Figure 4-6). Therefore, since these are the concentrations that resulted in a statistically significant cholesterol decrease 10µM simvastatin and pravastatin were chosen for use, alongside 1µM cerivastatin, in future experiments.

Figure 4-6 Effect of 10µM statin treatment on total cellular cholesterol

Huh7, RD-U and RD-D cells were treated with 10µM simvastatin (acid) or pravastatin for 48 hours, after which membrane cholesterol was extracted, quantified and normalised to total protein. Values plotted represent median $\pm$ range; from a single experiment where $N=3$. Statistical significance determined by unpaired t-test, ** = $P < 0.01$ compared to relevant control.

There appeared to be some variability with this method of cholesterol extraction and quantification. This is particularly apparent when comparing the level of cholesterol between control treatments, for example cellular cholesterol ranged from 10.6 - 20.1µg/mg protein in control Huh7 cells and from 3.6 - 9.3 µg/mg

protein in control RD-U cells (Figure 4-5 and Figure 4-6). Therefore, experiments were carried out in order to investigate the source of this variation.

In order to assess whether the vehicle control used could have had an effect on level of cholesterol measured Huh7 cells were seeded into 6-well plates and T25 flasks and treated with a range of different controls (Figure 4-7). Controls used were water (control for cerivastatin and pravastatin), 0.1% DMSO (simvastatin control) and 1% DMSO. A medium-only control was not included here since the water-containing control was considered equivalent to this. The effect of exclusion of non-essential amino acids (NEAA) from Huh7 cell medium was also examined. No statistically significant difference was found between control treatments ($P = 0.2838$ (6-well plates), $P = 0.1447$ (T25 flasks), one-way ANOVA).

Figure 4-7 Effect of control treatment on total cellular cholesterol in Huh7 cells

Huh7 cells in 6-well plates (A) or T25 flasks (B) were treated with 0.1% DMSO, water, 1% DMSO, or serum-free media without the addition of non-essential amino acids (NEAA) for 48 hours, after which membrane cholesterol was extracted, quantified and normalised to total protein. Values plotted represent median $\pm$ range; from a single experiment where $N=3$.

In previous experiments, cholesterol content was normalised to total cellular protein, to take cell number into account, but this may not allow for all effects of variation in cell density. However, no significant difference was observed between cholesterol content of Huh7 cells seeded in T25 flasks at two different densities ($P = 0.9442$, unpaired t-test). A 3rd, lower, seeding density appeared to lead to similar normalised cholesterol content however statistical analysis could not be carried out due to insufficiency of data (Figure 4-8).

Figure 4-8 Effect of seeding density on total cellular cholesterol in Huh7 cells

Huh7 cells were seeded in T25 flasks using a range of seeding densities (as shown) and treated with 0.1% DMSO for 48 hours, after which membrane cholesterol was extracted, quantified and normalised to total protein. Values plotted represent median $\pm$ range from a single experiment where $N=3$ (medium and high densities), or a single value (low density) where sufficient values could not be obtained.

In summary, these data clearly show that there is a cholesterol pool in both of our model cell lines, Huh7 and RD, and that statin treatment appears to decrease the total cellular cholesterol level. However, variation with the method of cholesterol quantification prevented statistical significance being achieved, with the cause of this variability undetermined.

4.4 Discussion

Myopathy is a well known adverse effect of statin treatment in some patients, with effects ranging from mild to fatal. Importantly, the mechanism of this muscle toxicity remains unclear, making it difficult to minimise the risk of toxicity. In order to gain further understanding, this study first compared statin-mediated cell toxicity in Huh7 liver and RD muscle cells, to test the hypothesis that RD muscle cells would show greater susceptibility to the toxic effects of statins. This represented the first comprehensive comparative analysis of statin toxicity in both liver and muscle cell lines. Subsequently, since HMGCR is the enzyme inhibited by the statins, expression of this enzyme was compared between cell lines at both transcript and protein levels. Finally, cellular cholesterol content in response to statin treatment was also quantified, in order to further examine differences in the responses of these two cell lines.

4.4.1 Cell Viability

MTT assays are widely used for assessing the viability of cells (Saad et al. 2006). They rely on the reduction of a tetrazolium bromide salt to a water-insoluble formazan salt by dehydrogenase enzymes in active mitochondria of living cells (Mosmann 1983). The assay therefore provides a measure of the metabolic activity of cells and does not assess other parameters of cell growth, such as cell division. However, they remain very useful for giving an indication of cell viability in response to xenobiotics and they have been used here to assess cell viability in response to treatment of both Huh7 and RD cells with a range of statins (section 4.2).

4.4.1.1 Differences between Liver and Muscle

The results obtained here showed that there was greater toxicity in RD muscle than Huh7 liver cells in response to treatment with all statins, with statistical significance between the responses on treatment with simvastatin, cerivastatin, atorvastatin and rosuvastatin. This is consistent with the observation of skeletal muscle toxicity as a well known adverse effect of the statins in the clinic (Hedenmalm et al. 2010), and confirms the hypothesis as well as further supporting the relevance of this model system for the study of the effects of statins.

In addition to a difference between sensitivity of liver- and muscle-derived cells to the toxic effects of statin exposure, treatment appeared to result in greater cell toxicity in RD-U compared to RD-D cells, with statins other than atorvastatin, rosuvastatin and pravastatin. These data are consistent with the recent finding that differentiated C2C12 mouse skeletal muscle cells are more resistance to cell death by apoptosis than undifferentiated cells, due to an elevation of anti-apoptotic proteins (Xiao et al. 2011). From the data in this study not all IC50 values could be obtained, due to technological considerations, and this prevented a quantitative comparison in some cases. However, Bonferroni post hoc analysis suggests that the differences between RD-U and RD-D responses were not statistically significant, but further work is required to fully examine this finding in a robust manner.

4.4.1.2 The Effect of Different Statins and the Role of Statin Exposure

The results of these MTT assays reveal a difference in toxicity between not only liver and muscle cells, but also between treatment with different statins, with more lipophilic statins such as simvastatin, lovastatin and cerivastatin showing the greatest toxicity, particularly in muscle cells. This is consistent with the observation of the highest incidence of myopathy with simvastatin, lovastatin and cerivastatin treatment in patients (Staffa et al. 2002).

In general, the exposure is thought to play a major role in determining the toxicity of a drug (Caldwell et al. 1995; Hedenmalm et al. 2010), with systemic exposure to xenobiotics determined by a complex interaction of absorption, distribution, metabolism and excretion (ADME) (Caldwell et al. 1995). With the statins, this role of drug exposure in toxicity is supported by the fact that pharmacokinetic interactions that increase systemic and skeletal muscle statin exposure, such as concomitant treatment of a patient with a statin and a xenobiotic that affects metabolism by cytochrome P450 (CYP) enzymes, have been shown to increase myopathy incidence (Bolego et al. 2002; Law and Rudnicka 2006; Mastaglia and Needham 2012; Sidaway et al. 2009). In addition, genetic polymorphisms in the gene encoding organic anion transporting polypeptide 1B1 (OATP1B1), a transporter involved in the hepatic uptake of many of the statins, has been associated with a higher risk of

myopathy (Link et al. 2008). A correlation has previously been shown between accumulation of statins and cytotoxicity *in vitro* in RD-U cells, with greater sub-cellular accumulation of the lipophilic statins being reported (Kobayashi et al. 2008).

The liver is the target organ of the statins, and it has been suggested that non-specific distribution into other tissues, via passive diffusion, by the more lipophilic statins could explain their increased potential for statin-related muscle problems (Vaklavas et al. 2009). However, myopathy has also been reported with hydrophilic pravastatin, which is not subject to significant passive diffusion, arguing against this hypothesis. It is now known that there are a number of transporters which are expressed in muscle tissue and recognise statins as substrates, and these are more likely to be responsible for uptake of statins into muscle cells (Shitara and Sugiyama 2006). Examples include organic anion transporter 3 (OAT3) (Takeda et al. 2004) and OATP1A4 (Sakamoto et al. 2008) which have been shown to mediate pravastatin transport, and OATP2B1, which is expressed in both liver and muscle and can transport atorvastatin and rosuvastatin (Knauer et al. 2010) as well as pravastatin (Sakamoto et al. 2008).

It would be of interest to determine the transporter profile of the RD cell line and how this compares to skeletal muscle tissue *in vivo*. It has been shown that the level of expression of transporters, as well as drug metabolising enzymes, can at times be different between cultured cell lines and native tissue (Hilgendorf et al. 2007) and this could have implications for drug distribution. It has also been suggested that differences in transporter profile could partially explain why pravastatin has been shown to have limited effects in some *in vitro* systems of rat origin, when it clearly can lead to muscle toxicity in human patients (Sakamoto et al. 2008). However, the transporter profile in the human RD muscle cell line is not currently known and so it cannot be certain whether this is also the case in this model system. It is true that the MTT assays did reveal a lesser toxic effect with pravastatin than many of the other statins, but, as already discussed, this does also reflect differences in myopathy incidence observed between statins in the clinic and so the use of pravastatin in this study will be continued.

Differences in statin cellular exposure are likely to play some role in statin-induced muscle toxicity, however they probably do not completely account for differences in the sensitivity between tissues. One recent rat study demonstrated that *in vivo* there was no difference in systemic or skeletal muscle statin exposure before or during myopathy, and that there was no difference in exposure between different animals and different muscle types that exhibited different sensitivity to muscle toxicity (Sidaway et al. 2009). Since responses of RD and Huh7 cells to statin treatment have been characterised here in terms of gross changes in cellular viability, it is now important to look in more detail at molecular changes occurring in these cell lines in response to statins for further elucidation.

4.4.2 Cholesterol Metabolism

4.4.2.1 Differences in HMGCR Expression

These analyses have shown that HMGCR, the enzyme that catalyses the rate-limiting step in cholesterol biosynthesis and is inhibited by the statins (Shitara and Sugiyama 2006), is present in all cell types. This was important to establish prior to further investigations into the mechanisms of statin-induced muscle toxicity, using these cell lines.

Higher levels of HMGCR were observed, at both the transcript and protein level, in Huh7 cells compared to RD muscle cells. Although cholesterol synthesis occurs in almost all cells, the liver is the main site of cholesterol biosynthesis (Catapano 2007; Shitara and Sugiyama 2006). Therefore, the presence of a higher level of HMGCR in Huh7 liver cells is not entirely unexpected, although such a large difference, particularly in the protein levels, was surprising. It is perhaps interesting to note that there is a larger fold-difference in HMGCR expression levels between liver and muscle cells than observed in cholesterol levels. This difference might reflect altered rates of efflux of cholesterol from these cells, or that liver cells have a larger reserve of metabolic capacity for the synthesis of cholesterol compared to muscle cells.

A difference was also found between HMGCR expression in RD-U and RD-D cells, with much lower levels of expression in RD-D cells. One potential explanation could be a greater need of RD-U cells to produce cholesterol for the synthesis of new membranes, which is reduced as RD cells differentiate. It has also been suggested that HMGCR could play an important role in the induction of differentiation, in particular due to the production of isoprenoids for protein prenylation. In rat L6 skeletal muscle cells HMGCR was found to be upregulated at a very early stage of differentiation (6 hours), followed by a subsequent downregulation to allow differentiation to occur (Martini et al. 2009). Since RD-D cells have been allowed to differentiate for 5 days prior to experiments (section 2.2.1.9), it is possible that this downregulation is what is being observed here.

It would be interesting to know whether these effects are observed only in this rate-limiting enzyme, or whether this is also the case for other enzymes in the cholesterol synthesis pathways and for the total flux through the biosynthetic pathway in these cell lines. The different levels of HMGCR expression between the cell types may need to be taken into account when interpreting results from future experiments.

4.4.2.2 The Effect of Statin Treatment on Total Cellular Cholesterol

Having shown the presence of HMGCR in all cell types, subsequent data demonstrated that cholesterol was also present in all cell types of interest and decreases in total cellular cholesterol on statin treatment were observed.

In this study, a period of 48 hours was used for statin treatment prior to cholesterol quantification. This remained consistent with the conditions used for MTT assays as well as taking studies from the literature into account. Previously one research group reported seeing no effect on total cellular cholesterol after C2C12 mouse myoblasts and HepG2 human liver cells were treated with 10 μ M simvastatin for 6 or 18 hours (Mullen et al. 2010). However, another research group was able to observe a decrease in intracellular cholesterol in HepG2 cells after 24 and 48 hour treatment with a 1 μ M concentration of a range of statins (Kobayashi et al. 2008). Given that

cholesterol synthesized within a cell will have many fates, including sub-cellular sequestration, it is possible that cells are buffered against acute exposure to statins due to sub-cellular pharmacokinetic considerations. Chronic exposure may have a more unequivocal effect, but this must be balanced with the increased cell toxicity associated with such prolonged exposures.

Three statins were chosen for use in this cholesterol analysis: simvastatin (acid), cerivastatin and pravastatin. Simvastatin remains one of the most frequently prescribed statins in the UK (Bakhai et al. 2012; Jick et al. 2012) and, together with cerivastatin, showed the greatest difference in toxicity between RD muscle and Huh7 liver cells (Figure 4-2). The open acid form of simvastatin was used here since this is the form that is biologically active, without need for prior metabolism (Shitara and Sugiyama 2006). Cerivastatin is also of interest in this study due to its high incidence of myopathy in patients (Bolego et al. 2002). In contrast, pravastatin has shown much lower levels of toxicity both in MTT assays (Figure 4-2) and in the clinic (Staffa et al. 2002), as well as having shown less of a difference in toxicity between RD muscle and Huh7 cells compared to simvastatin or cerivastatin (Figure 4-2). It can therefore be used for comparison.

There appeared to be decreases in cholesterol content of Huh7 and RD-D cells in response to 1 μ M cerivastatin treatment, and in Huh7 cells in response to 10 μ M simvastatin and pravastatin treatment. There were statistically significant decreases in cholesterol in RD-D cells with 10 μ M simvastatin ($P = 0.0027$) and pravastatin ($P = 0.0023$).

There was a large degree of variability in the data obtained, which could explain why some of the observed decreases in cholesterol were not found to be statistically significant. This variability was both between replicates within an individual experiment and between experiments. This was particularly evident in the variable levels of cholesterol in control cells. Due to this variability, statistical analysis was not carried out to examine differences in levels of cholesterol and cholesterol reduction on statin treatment between cell lines. However, it appeared that there may be slightly lower levels of cholesterol in RD

muscle compared to Huh7 liver cells, which could be explained by the lower level of HMGCR in these cells. There also seemed to be no obvious differences in cholesterol reduction in response to statin treatment that could account for the differences in toxicity between the liver and muscle cell lines.

In order to investigate the cause of the variability, experiments were carried out to examine the effect of several variables on the level of cholesterol measured. Since statin stock solutions were made using different solvents, depending on the solubility of the statin, an experiment was carried out to determine whether this could account for the variability in control cholesterol levels between statin treatments. Simvastatin was dissolved in DMSO and, since high levels of DMSO can be toxic to cells, stock was always diluted by 1000-fold to ensure that the final percentage of DMSO in cell medium did not exceed 0.1%. Cerivastatin and pravastatin were both dissolved in water. No significant difference was found in cholesterol levels between Huh7 cells in either 6-well plates or T25 flasks treated with 0.1% DMSO, water or 1% DMSO.

Another potential cause of discrepancy between experiments is cell density. Although cells are seeded at the same initial density, a variety of factors, such as cell passage number, can lead to slight differences in cell growth rate and, ultimately, in cell density. However, no difference was observed in normalised cholesterol levels between Huh7 cells seeded in T25 flasks at 3 different seeding densities.

These two experiments using untreated Huh7 cells were therefore unable to determine the cause of the variation in the data. Nevertheless, despite this variation, the experiments gave us sufficient information to progress as they clearly showed that there was a cholesterol pool in both of our model cell lines, Huh7 and RD, and that statin treatment did result in a decrease in total cellular cholesterol level.

4.4.3 Choice of Statin Concentration

With the use of xenobiotics in *in vitro* models there is always the issue of choosing therapeutically relevant concentrations (Bjorkhem-Bergman et al. 2011). With simvastatin, concentrations of up to 1 μ M have been observed in the plasma of patients (Mullen et al. 2010), meaning that the concentrations used for the cholesterol analysis here are not too dissimilar from the physiological range.

However it has also been suggested that localised concentrations in some tissues could be higher than this, due to the activity of transporters, and that this could be variable between individuals (Sirvent et al. 2005). Indeed in treated rats simvastatin concentration was found to be 4-fold higher in the liver than in the plasma, with an even greater difference for cerivastatin (Sidaway et al. 2009). Statin concentrations have been found to be a third lower in rat skeletal muscle than in plasma for simvastatin, with an even greater difference with cerivastatin (a sixth lower) and rosuvastatin (1/16) (Sidaway et al. 2009). Similar findings have been reported in guinea pig skeletal muscle for cerivastatin, rosuvastatin and atorvastatin (Madsen et al. 2008). The plasma-to-muscle ratios are not currently known for humans (Knauer et al. 2010) and localised concentrations in muscle may be increased in patients when statin treatment is given in combination with a fibrate or a CYP inhibitor (Mullen et al. 2010).

The biological effect of statins is a decrease in cholesterol (Chong et al. 2001) and it has been shown here that decreases in cholesterol can be observed on treatment with 10 μ M simvastatin and pravastatin, and 1 μ M cerivastatin (section 4.3.2). Therefore, since these are the statin concentrations which reproduce the expected biological effect of the statins, and are concentrations at which RD muscle cell toxicity can be observed with simvastatin and cerivastatin, these are the concentrations that were used in future experiments.

4.5 Summary

Responses of Huh7 liver and RD muscle cells to statin treatment have been characterised in terms of both cellular viability and cholesterol metabolism.

Using MTT assays, significant reductions in cell viability were observed for all three cell types (Huh7, RD-U and RD-D) on treatment with simvastatin (acid and lactone), lovastatin (acid and lactone), cerivastatin, fluvastatin, atorvastatin, rosuvastatin and pravastatin in a dose-dependent manner. The largest reductions in cell viability were observed with the more lipophilic statins: simvastatin (acid), lovastatin (acid) and cerivastatin, whereas loss of cell viability was only observed with very high concentrations of the hydrophilic pravastatin (1mM and greater). Higher cell toxicity was also observed for all statins in the RD cells, either differentiated or undifferentiated, compared to the Huh7 cells, where this difference was statistically significant with simvastatin, cerivastatin, atorvastatin and rosuvastatin. Taken together, these data represent the first comprehensive comparative analysis of statin toxicity in human liver and muscle cell lines.

The level of HMGCR, the rate-limiting enzyme in cholesterol biosynthesis and the enzyme inhibited by the statins, was analysed. HMGCR was found to be expressed at both the transcript and protein level in all cell types, although to a lesser extent in RD muscle cells (particularly when differentiated). Decreases in total cellular cholesterol were observed on treatment with 10 μ M simvastatin and pravastatin, and 1 μ M of cerivastatin. Since these are the concentrations that replicate the biological effect of the statins (a decrease in cholesterol), these are the concentrations that were then used in future experiments, examining other molecular changes that occur in these cells on statin treatment.

5 The Effects of Statins on Prenylation in Liver and Muscle Cells

5.1 Introduction

Due to the large number of intermediates in the mevalonate pathway (Figure 1-3), inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) by statins not only inhibits cholesterol synthesis but also impacts upon a number of other cellular processes, including protein prenylation (Vaklavas et al. 2009).

Farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) are intermediates of the mevalonate pathway. In protein prenylation, these isoprenoids donate farnesyl (15-carbon) or geranylgeranyl (20-carbon) groups, respectively, for post-translational covalent addition to cysteine residues at or near the C-terminus of proteins. This addition is thought to be essential for the biological function of many proteins by enabling them to anchor to cell membranes, specifying cellular localisation (McTaggart 2006). There is increasing evidence that disruption of various prenylation targets could lead to myopathy (Blanco-Colio et al. 2002; Matzno et al. 2005; Sakamoto et al. 2011).

The three distinct protein prenyltransferase enzymes that are responsible for the addition of prenyl groups to proteins are farnesyltransferase (FT), geranylgeranyltransferase-I (GGT-I) and geranylgeranyltransferase-II (GGT-II). Other than a small number of specific cases of overlap (sections 1.3.5.2i and ii), each enzyme is thought to have a distinct subset of substrates (Figure 5-1). Substrates for FT include Ras guanine nucleotide-binding proteins (GTPases) and the nuclear lamins; GGT-I adds geranylgeranyl groups to Ras-related GTPases, including of the Ras and Rho subfamilies, and the γ subunits of heterotrimeric G-proteins; GGT-II is responsible for the prenylation of Ras-related GTPases in the Rab subfamily (Casey and Seabra 1996).

Figure 5-1 Protein prenylation

Farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GPP), isoprenoid products of the mevalonate pathway, donate farnesyl or geranylgeranyl groups respectively for the post-translational prenylation of proteins. The 3 prenyltransferase enzymes which catalyse this step are farnesyltransferase (FT), geranylgeranyltransferase-I (GGT-I) and geranylgeranyltransferase-II (GGT-II). Each enzyme has a distinct group of substrates.

Work discussed in previous chapters has established human liver and muscle cell-based models and characterised their response to statin treatment, in terms of both cell viability and changes in cholesterol levels. In this chapter, the effect of statin treatment on the cellular process of protein prenylation, by each of the three prenyltransferase enzymes, was examined in these liver and muscle models, with the hypothesis that statins would preferentially disrupt prenylation within muscle, the site of observed human toxicity.

5.2 Rationale for Experimental Design

Protein was extracted from Huh7, RD-U and RD-D cells treated with vehicle control, statin or mevalonic acid in addition to statin, and then subjected to analysis by western blot (section 2.2.3) to detect any changes in the prenylation status of selected target proteins as well as to examine the expression of the prenyltransferase enzymes.

Simvastatin (acid), cerivastatin and pravastatin, were used in this analysis as these are the statins used previously to examine the effects of statin treatment on total cellular cholesterol (section 4.3.2). A decrease in cholesterol, the expected biological effect of the statins, was observed on treatment with 10µM simvastatin and pravastatin, and 1µM cerivastatin, and so these were the initial

concentrations also used here. Both simvastatin and cerivastatin treatments were previously shown to result in greater toxicity in RD muscle cells than Huh7 liver cells (section 4.2). In contrast, pravastatin treatment resulted in toxicity only at high concentrations and showed no statistically significant difference in toxicity between cell lines, therefore it can be used for comparison.

Mevalonic acid (mevalonate) is an intermediate in the mevalonate pathway that is just downstream of the site of statin inhibition. Its addition has been shown to compensate for the statin-induced block of the mevalonate pathway, thereby indicating whether any effects that are observed are due to the specific inhibition of HMGCR or to non-specific effects.

Cells were also treated with 3 inhibitors of prenylation: FTI-277, GGTI-2133 and perillyl alcohol. FTI-277 is a trifluoroacetate salt that inhibits FT by acting as a highly potent Ras CAAX peptidomimetic, mimicking the Ras C-terminal CAAX FT recognition motif and site of prenylation (Lerner et al. 1995; Sigma-Aldrich 2011a). GGTI-2133 is a non-thiol peptidomimetic that inhibits GGT-I with an 140-fold selectivity over FT (Sigma-Aldrich 2011b). There are only a limited number of specific inhibitors of GGT-II available (Leung et al. 2006), none of which were commercially obtainable. Therefore perillyl alcohol was used, which is a monoterpene that inhibits both GGT-I and II, with 2-fold greater potency for the inhibition of GGT-II (Ren et al. 1997; Tanaka et al. 2010).

5.3 The Effects of Statins on Prenylation

The small GTPases represent the largest group of proteins where prenylation is vital for their membrane localisation and function (McTaggart 2006). In this analysis, the prenylation of several small GTPases in response to statin treatment was examined. Rap1A was chosen as a representative small GTPase prenylated by GGT-I, with H-Ras as a substrate of FT and with Rab1A as a substrate of GGT-II.

5.3.1 *Rap1A*

Rap1A is a 22kDa Ras-related GTPase which is prenylated by GGT-I and is thought to play roles in cell adhesion, the establishment of cell polarity and the regulation of cell-cell contacts (Frische and Zwartkruis 2010). It is one of two very closely related isoforms of Rap1 (Bos 1997; Frische and Zwartkruis 2010) and it has previously been shown that levels of unprenylated Rap1A increase on treatment with simvastatin in both HepG2 liver cells and C2C12 mouse myotubes (Mullen et al. 2010). It was important to verify whether this effect is also observed in human cell lines and whether there is a difference between the effects of different statins.

In order to investigate the geranylgeranylation state of Rap1A two antibodies were used. One antibody (referred to as Rap1A) recognises only the unprenylated form of the protein (Antoine et al. 2010; Reszka et al. 2001) and the other (referred to as Rap1) recognises both Rap1 isoforms whether prenylated or unprenylated (Reszka et al. 2001), thereby giving an indication of total Rap1 protein expression. This meant that it was possible to use total protein extracts in this case (section 2.2.3.1).

There was no unprenylated Rap1A detected in any of the control samples, suggesting that this prenylation step is highly efficient. Unprenylated Rap1A was observed in all three cell types following treatment with 10 μ M simvastatin or 1 μ M cerivastatin, but not 10 μ M pravastatin (Figure 5-2). In all cases, this was largely rescued on co-addition of mevalonic acid, showing that the increase in unprenylated Rap1A was due to statin inhibition of HMGCR rather than a non-specific effect.

Figure 5-2 Effect of statin treatment on Rap1A prenylation

Total protein was extracted from cells treated for 48 hours with vehicle control, statin, or mevalonic acid in addition to statin. Simvastatin (simva) and pravastatin (prava) were used at a concentration of 10 μ M and cerivastatin (ceriva) at 1 μ M. DMSO was used as a vehicle control for simvastatin and water for cerivastatin and pravastatin. Mevalonic acid was used in excess, at 100 μ M. Protein concentration was determined using the Lowry method and 30 μ g was separated by SDS-PAGE and transferred to PVDF membrane. The level of unprenylated Rap1A (21kDa) was detected in Huh7 (A), undifferentiated RD (RD-U) (B) and differentiated RD (RD-D) (C) cells using a specific antibody, and compared to the levels of total Rap1 and of the loading control, β -actin. Protein size was determined by comparison to a coloured protein marker. Western blots are representative of three independent experiments.

β -actin was used as a loading control, as its expression is unchanged on statin treatment and therefore any variation detected can be attributed to uneven protein loading. The only changes observed in total Rap1, in samples from Huh7 cells (Figure 5-2A), corresponded to similar differences in β -actin. Therefore, the observed increase in the unprenylated form of Rap1A was not due to an increase in protein expression upon statin treatment. There were also no changes in β -actin that would allow uneven loading to account for the changes observed in unprenylated Rap1A.

In order to investigate whether this detection of unprenylated Rap1A was due to a specific effect of the statins on the process of prenylation, cells were treated with the prenyltransferase inhibitors described in section 5.2. Treatment with the GGT-I inhibitor GGTI-2133 resulted in observation of unprenylated Rap1A in all cell types (Figure 5-3).

Figure 5-3 Effect of prenylation inhibitors on Rap1A prenylation

Total protein was extracted from Huh7, RD-U and RD-D cells treated for 48 hours with10μM farnesyltransferase inhibitor FTI-277 (F), 10μM geranylgeranyltransferase-I inhibitor GGTI-2133 (GI) or 100μM perillyl alcohol (GII), and 30μg was separated by SDS-PAGE. Western blots are representative of three independent experiments.

This was similar to the effect seen with simvastatin and cerivastatin treatment. However, treatment with neither the FT inhibitor FTI-277 nor perillyl alcohol resulted in any comparable detection of unprenylated Rap1A. Since specifically inhibiting GGT-I has the same effect as statin treatment, this supports the idea that statins are having an inhibitory effect on protein prenylation and confirms that Rap1A is a GGT-I substrate.

5.3.1.1 Dose-Dependent Effects of Statin Treatment on Rap1A Prenylation

Since treatment with 10μM simvastatin and 1μM cerivastatin treatment resulted in a similar increase in unprenylated Rap1A in all cell types, the dose-dependent effects of simvastatin and cerivastatin on Rap1A prenylation were examined. For this, concentrations were chosen from across the range of the dose-response curves (Figure 4-2). The greatest effects on treatment with 30μM of these statins, the highest concentration used in this analysis, previously led to the reduction of cell viability to 60% in Huh7 cells, 23% in RD-U cells and 39% in RD-D cells; where 100% was defined as cell viability on treatment with vehicle control.

On simvastatin treatment, there was a dose-dependent increase in the inhibition of Rap1A prenylation in all cell types (Figure 5-4), with unprenylated Rap1A observed at all concentrations equal to or greater than 10μM simvastatin in Huh7 cells (Figure 5-4A); in both RD-U and RD-D cells unprenylated Rap1A was evident for all concentrations equal to or greater than 1μM (Figure 5-4B and C). Hence, in this model, disruption of Rap1A prenylation occurs at a 10-fold lower concentration in muscle than in liver following simvastatin treatment.

Figure 5-4 Effect of simvastatin treatment on Rap1A prenylation
Total protein was extracted from cells treated for 48 hours with vehicle control (DMSO) or a range of simvastatin concentrations and 15μg was separated by SDS-PAGE. The level of unprenylated Rap1A (21kDa) was detected in Huh7 (A), RD-U (B) and RD-D (C) cells using a specific antibody, and compared to the levels of total Rap1 and of the loading control, β-actin. Protein size was determined by comparison to a coloured protein marker.

On cerivastatin treatment, there was likewise a dose-dependent decrease in Rap1A prenylation (Figure 5-5). Prenylation of Rap1A was also inhibited at 10-fold lower concentrations of cerivastatin than simvastatin, with some unprenylated Rap1A being observed even with concentrations of cerivastatin as low as 0.1μM in all cell types. Cerivastatin seemed to also have a greater effect on the disruption of prenylation in muscle compared to liver, although the difference was only 3-fold compared to the previously observed 10-fold difference in sensitivity to simvastatin.

Figure 5-5: Effect of cerivastatin treatment on Rap1A prenylation

Total protein was extracted from cells treated for 48 hours with vehicle control (DMSO) or a range of cerivastatin concentrations and 15μg was separated by SDS-PAGE. The level of unprenylated Rap1A (21kDa) was detected in Huh7 (A), RD-U (B) and RD-D (C) cells using a specific antibody, and compared to the levels of total Rap1 and of the loading control, β-actin.

Overall, both simvastatin and cerivastatin showed a dose-dependent inhibition of Rap1A prenylation. However, cerivastatin showed effects at lower concentrations than simvastatin, and prenylation was inhibited at lower concentrations in muscle compared to liver, in this model. The observation of

greater sensitivity to disruption of prenylation in muscle compared to liver further supports a mechanism where disruption of intermediates in the mevalonate pathway is indeed a cause of statin-induced muscle toxicity.

5.3.2 *H-Ras*

In order to examine whether statin treatment had similar effects on the prenylation of other small GTPases, further western blot analysis was undertaken. These subsequent analyses were carried out using membrane and cytosolic protein extracts (section 2.2.3.2), rather than total protein extracts as, unlike the Rap1A antibody used, the other antibodies did not directly distinguish between prenylated and unprenylated forms of the protein. Since, under normal conditions, GTPases are anchored to cell membranes by their added prenyl groups (Jin et al. 1996), a reduction in prenylation will result in less protein able to remain in the membrane and will lead to possible detection in cytosolic fractions. Therefore, observing the change in subcellular localisation of the protein may be used as an indirect measure of the inhibition of prenylation by statins (Blanco-Colio et al. 2002; Casey et al. 1989; Coxon et al. 2005).

H-Ras is one of four classical Ras proteins which, along with N-Ras, K-Ras4A and K-Ras4B, was identified originally as an oncogene (Guo et al. 2005). H-Ras acts as a molecular switch; The binding of GTP induces a conformational change in the protein, allowing it to then bind to downstream effectors (Castro et al. 2005) and initiate protein kinase cascades, such as the MAPK (mitogen-activated protein kinase) cascade (Matozaki et al. 2000; Vojtek and Der 1998), in order to exert its effects on gene transcription, cellular growth and differentiation (Keduka et al. 2009). The addition of a farnesyl group to H-Ras by FT is essential for its membrane anchoring, and is thereby also essential for allowing it to carry out its role in cellular signalling (Dirks and Jones 2006).

In agreement with this, H-Ras was found almost exclusively in the membrane fraction of control cells (Figure 5-6). Cerivastatin treatment resulted in a reduction in the amount of H-Ras in the membrane fraction, indicating decreased prenylation. This reduction was seen in all cell types, with H-Ras being detected in the corresponding cytosolic fractions in Huh7 and RD-D cells. This effect of cerivastatin on H-Ras membrane association was largely rescued by mevalonic acid co-treatment, demonstrating this to be a specific impact on the mevalonate pathway.

Figure 5-6 Effect of statin treatment on membrane association of H-Ras

Fractions enriched with either cytosolic or membrane protein were extracted from cells treated for 48 hours with vehicle control, statin, or mevalonic acid in addition to statin, and 4µg was separated by SDS-PAGE. Simvastatin and pravastatin were used at a concentration of 10µM and cerivastatin at 1µM. DMSO was used as a vehicle control for simvastatin and water for cerivastatin (and pravastatin). Mevalonic acid was used in excess, at 100µM. The level of H-Ras (21kDa) in each fraction was detected in Huh7 (A), RD-U (B) and RD-D (C) cells using a specific antibody. Protein size was determined by comparison to a coloured protein marker.

In contrast, treatment with pravastatin had no effect on the subcellular localisation of H-Ras in any of the cell types, and the only apparent reduction of membrane-associated H-Ras due to simvastatin treatment was in Huh7 cells co-treated with mevalonic acid. The latter was surprising, since previously the addition of mevalonic acid rescued the effect of statins; it is possible that this

could be an artefact arising from a problem with this section of the original gel or membrane, which may be clarified by further analysis of this membrane.

Since treatment with cerivastatin resulted in a change in H-Ras localisation in all cells, cells were treated with prenyltransferase inhibitors to see if this would lead to similar changes. As expected, there was a reduction in H-Ras observed in the membrane fraction of RD-U and RD-D cells treated with the FT inhibitor FTI-277 (Figure 5-7), providing evidence that the changes in H-Ras localisation observed in muscle cells on statin treatment are due to inhibition of farnesylation. Interestingly, in Huh7 liver cells a reduction in membrane associated H-Ras was observed on treatment with geranylgeranyltransferase inhibitor GGTI-2133 and not with FTI-277, as in RD cells.

Figure 5-7 Effect of prenylation inhibitors on membrane association of H-Ras

Fractions enriched with either cytosolic or membrane protein were extracted from cells treated for 48 hours with 10µM farnesyltransferase inhibitor FTI-277 (F), 10µM geranylgeranyltransferase-I inhibitor GGTI-2133 (GI) or 100µM perillyl alcohol (GII) and 4µg was separated by SDS-PAGE. The level of H-Ras (21kDa) in each fraction was detected using a specific antibody.

In general, inhibition of prenylation resulted in a decrease of membrane-associated H-Ras, an effect also observed in all cell types upon cerivastatin treatment. Therefore, it can be inferred that cerivastatin has an effect on the prenylation of the small GTPase H-Ras in addition to that of Rap1A.

5.3.3 *M-Ras*

M-Ras (also known as R-Ras3 due to its similarities to R-Ras and R-Ras2 (Ehrhardt et al. 1999)) is one of the more recently described members of the Ras subfamily of GTPases, having been originally cloned from the cDNA libraries of mouse skeletal muscle and rat brain. It was initially thought that the expression of this 29kDa protein was restricted to brain, muscle and heart (Kimmelman et al. 1997; Matsumoto et al. 1997). However, it has since been shown that its expression is actually much more widespread, including spleen, thymus, liver, lung, pancreas, placenta, skin, kidney, fibroblasts, hematopoietic and epithelial cells (Ehrhardt et al. 1999) as well as MCF-7 breast, HeLa cervical and LNCaP prostate cancer cell lines (Castro et al. 2011). M-Ras shows 47% identity with H-Ras (Ehrhardt et al. 1999) and they share many upstream regulators and downstream effectors. Nevertheless, M-Ras has several distinctive effectors, for example RA-GEF-2 and MR-GEF (Guo et al. 2005), and M-Ras is thought to have evolved independently of H-Ras and other members of the Ras family (Keduka et al. 2009). It is thought that M-Ras, unlike H-Ras, is a substrate of GGT-I (Kimmelman et al. 1997; Matsumoto et al. 1997).

The western blot analysis of M-Ras is not as clear as those western blots looking at H-Ras expression, due to the quality of the available antibody. However, it is still evident that M-Ras is found in the prenylated, membrane-bound form under normal conditions (Figure 5-8). It is also clear that there is a decrease in the amount of M-Ras in the membrane of RD-U cells (Figure 5-8B), along with a concomitant increase in M-Ras in the cytosol of RD-D (Figure 5-8C) on cerivastatin treatment, as is also the case with H-Ras (Figure 5-6). These effects were rescued on co-treatment with mevalonic acid. It is unclear as to whether this occurs in Huh7 cells; however a repeat experiment, perhaps with an alternative antibody, would clarify this.

Figure 5-8 Effect of statin treatment on membrane association of M-Ras

Fractions enriched with either cytosolic or membrane protein were extracted from cells treated for 48 hours with vehicle control, statin, or mevalonic acid in addition to statin, and 4µg was separated by SDS-PAGE. Simvastatin and pravastatin were used at a concentration of 10µM and cerivastatin at 1µM. DMSO was used as a vehicle control for simvastatin and water for cerivastatin and pravastatin. Mevalonic acid was used in excess, at 100µM. The level of M-Ras (29kDa) in each fraction was detected in Huh7 (A), RD-U (B) and RD-D (C) cells using a specific antibody. Protein size was determined by comparison to a coloured protein marker.

There also appears to be an increase in M-Ras observed in the cytosol of all cells on treatment with the GGT-I inhibitor GGTI-2133 but not with the FTI inhibitor FTI-277 (Figure 5-9). This would seem to confirm the predictions that M-Ras is a substrate of GGT-I and again shows statin treatment to have a similar effect to direct inhibition of protein prenylation.

Figure 5-9 Effect of prenylation inhibitors on membrane association of M-Ras

Fractions enriched with either cytosolic or membrane protein were extracted from cells treated for 48 hours with 10µM simvastatin, 10µM farnesyltransferase inhibitor FTI-277 (F) or 10µM geranylgeranyltransferase-I inhibitor GGTI-2133 (GI), and 4µg was separated by SDS-PAGE. The level of M-Ras (29kDa) in each fraction was detected using a specific antibody.

5.3.4 Rab1A

Rab1 is a 25kDa small GTPase important in the secretory pathway for regulating vesicle traffic from the endoplasmic reticulum (ER) to the Golgi apparatus, as well as intra-Golgi traffic. Rab1 is found in all cell types (Leung et al. 2006), including the liver (Jin et al. 1996) and skeletal muscle (Bao et al. 1998). It has been reported that Rab1 is one of the forms of Rab most susceptible to depletion of GGPP (Tanaka et al. 2010) and recently it has been shown that inactivation of Rab1 may play an important role in fluvastatin toxicity in rat myofibres (Sakamoto et al. 2011; Tanaka et al. 2010). There are two different isoforms of Rab1, Rab1A and Rab1B, which share 92% identity and are thought to be functionally interchangeable (Nuoffer et al. 1994). Rab1A is the predominant isoform in human skeletal muscle, and so was chosen here as a representative GGT-II substrate.

Western blot analysis showed two distinct bands in the cytosolic fractions of Huh7 and RD-D cells and more faintly in all membrane fractions (Figure 5-10). It could be that these correspond to differently processed forms of the Rab1A protein, with resulting differences in molecular weight, or it could be that the higher molecular weight band represents non-specific binding of the antibody, with Rab1A being represented solely by the lower molecular weight band in these samples.

Figure 5-10 Effect of statin treatment on membrane association of Rab1A

Fractions enriched with either cytosolic or membrane protein were extracted from cells treated for 48 hours with vehicle control, statin, or mevalonic acid in addition to statin, and 4 μ g was separated by SDS-PAGE. Simvastatin and pravastatin were used at a concentration of 10 μ M and cerivastatin at 1 μ M. DMSO was used as a vehicle control for simvastatin and water for cerivastatin and pravastatin. Mevalonic acid was used in excess, at 100 μ M. The level of Rab1A (25kDa) in each fraction was detected in Huh7 (A), RD-U (B) and RD-D (C) cells using a specific antibody. Approximate location of the relevant section of the coloured protein marker is shown.

Treatment with cerivastatin, but not simvastatin or pravastatin, resulted in a decrease in the amount of the lower molecular weight protein observed in the membrane fractions of all cells (Figure 5-10), which is similar to the effects of the statin on membrane association of H-Ras (Figure 5-6) and M-Ras (Figure 5-8). This lower molecular weight protein was detected in cytosolic fractions only from Huh7 (Figure 5-10A) and RD-D (Figure 5-10C) cells treated with cerivastatin and from RD-D (Figure 5-10C) cells treated with simvastatin.

There also seems to be less Rab1A in the membrane fraction from Huh7 cells treated with simvastatin and mevalonic acid. This effect is the same as that seen when these same PVDF membranes were analysed for H-Ras expression (Figure 5-6). This is further indication that there may have been problems with the original protein loading or with the processing of this particular area of the PVDF membrane.

Treatment with perillyl alcohol, to inhibit both GGT-I and GGT-II, resulted in a lower amount of Rab1A observed in the membrane fraction from RD-U cells (Figure 5-11). However, it seems that this effect was observed to an even greater extent on inhibition of FT in RD-U and RD-D cells, and on inhibition of GGT-I in Huh7 cells. This pattern of reduced membrane-association seems to be the same as when the same samples were analysed for localisation of H-Ras on treatment with prenyltransferase inhibitors (Figure 5-7), despite the fact that H-Ras and Rab1A are reported to be prenylated by different prenyltransferase enzymes. This is surprising, and could indicate that either the prenyltransferase enzymes, or these prenyltransferase inhibitors, are not as specific under all conditions as traditionally thought.

Figure 5-11 Effect of prenylation inhibitors on membrane association of Rab1A

Fractions enriched with either cytosolic or membrane protein were extracted from cells treated for 48 hours with 10µM farnesyltransferase inhibitor FTI-277 (F), 10µM geranylgeranyltransferase-I inhibitor GGTI-2133 (GI) or 100µM perillyl alcohol (GII), and 4µg was separated by SDS-PAGE. The level of Rab1A (25kDa) in each fraction was detected. Approximate location of the relevant section of the coloured protein marker is shown.

Another interesting point to note is that neither of the two molecular weight bands can be observed in the cytosolic fractions of cells treated with the prenyltransferase inhibitors, and only the lower molecular weight band can be observed in membrane fractions. The PVDF membranes with these samples were prepared at a later time than the membranes with the samples from statin treated cells. It could therefore be that these later western blot analyses are showing greater Rab1A specificity.

In summary, treatment with both cerivastatin and prenyltransferase inhibitors led to a reduction in membrane-associated Rab1A in both muscle and liver cells. However, this analysis was complicated by the detection of several protein bands, in both the cytosolic and membrane fractions, which could be due to non-specific antibody binding.

5.3.5 Lactate Dehydrogenase (LDH)

In order to assess the quality of the membrane and cytosolic extracts from Huh7, RD-U and RD-D cells, the localisation of the cytosolic protein lactate dehydrogenase (LDH) was examined by western blot (Figure 5-12 and Figure 5-13).

Figure 5-12 Localisation of lactate dehydrogenase (LDH), in response to statin treatment

Fractions enriched with either cytosolic or membrane protein were extracted from cells treated for 48 hours with vehicle control, statin, or mevalonic acid in addition to statin, and 4µg was separated by SDS-PAGE. Simvastatin and pravastatin were used at a concentration of 10µM and cerivastatin at 1µM. DMSO was used as a vehicle control for simvastatin and water for cerivastatin and pravastatin. Mevalonic acid was used in excess, at 100µM. The level of LDH (35kDa) in each fraction was detected in Huh7 (A), RD-U (B) and RD-D (C) cells.

* Protein sample precipitated and resuspended prior to SDS-PAGE.

Initially there were problems with the antibody that meant that it was not possible to examine LDH using the same membranes as used for previous analysis of H-Ras (Figure 5-6 and Figure 5-7), M-Ras (Figure 5-8 and Figure 5-9) and Rab1A localisation (Figure 5-10 and Figure 5-11). Therefore, a second set of protein extracts were generated, separated by SDS-PAGE and transferred to PVDF membrane for use in LDH analysis, with the aim of subsequently also blotting these membranes for H-Ras, M-Ras and Rab1A as a repeat experiment.

In extracts from all cell types, on control treatment and treatment with both statins (Figure 5-12) and prenylation inhibitors (Figure 5-13) a much greater amount of LDH was observed in the cytosolic fraction than in the corresponding membrane fraction. A small amount of LDH remained in the majority of membrane fractions, indicating that the separation of cytosolic and membrane proteins achieved by this extraction method was not 100%. This could be further examined by analysing the same samples for the presence of proteins known to be exclusively found in the membrane, for example membrane transporters, but unfortunately time constraints did not allow this in this instance.

Figure 5-13 Localisation of LDH, in response to treatment with prenyltransferase inhibitors

Fractions enriched with either cytosolic or membrane protein were extracted from cells treated for 48 hours with 10µM farnesyltransferase inhibitor FTI-277 (F), 10µM geranylgeranyltransferase-I inhibitor GGTI-2133 (GI) or 100µM perillyl alcohol (GII), and 4µg was separated by SDS-PAGE. The level of LDH (35kDa) in each fraction was detected using a specific antibody. * Protein sample precipitated and resuspended prior to SDS-PAGE.

There appears to be some variation in the amount of LDH observed between protein samples. This could possibly be explained by the fact that some of the RD-U and RD-D samples (denoted * on Figure 5-12 and Figure 5-13)

underwent protein precipitation and resuspension (section 2.2.3.4) due to the extracted protein not being concentrated enough for SDS-PAGE analysis. The samples that were not pre-treated in this way seem to show detection of greater amounts of LDH.

The difference between the amount of LDH in the cytosolic and membrane fractions is enough that any gross changes in subcellular localisation caused by reduced prenylation should still be observed using protein extracted using this method. However, due to the variability between samples, further analysis of H-Ras, M-Ras and Rab1A was not carried out using these repeat membranes.

5.4 The Effect of Statins on Prenyltransferase Expression

The differences in prenylation observed on statin treatment could be due to either a change in the isoprenoids themselves, or in the expression of the prenyltransferase enzymes. In addition, it has been suggested that levels of prenyltransferase enzymes could vary between cell types, and that this could impact upon results observed (Ali et al. 2010). In order to rule out these possibilities, expression of each of the three prenyltransferase enzymes was measured, by western blot analysis, before and after treatment with statin or prenylation inhibitor.

FT and GGT-I are heterodimers that share a 48kDa α subunit but have different β subunits (46kDa and 42kDa respectively). GGT-II consists of distinct α (60kDa) and β (40kDa) subunits. It is thought that the β subunits contain the substrate binding sites and that it is these different β subunits which provide the different substrate specificities of these enzymes (Casey and Seabra 1996; McTaggart 2006). For this reason, it is necessary to use antibodies specific to the β subunits rather than the alpha subunits.

Expression of all three of these enzymes was confirmed in Huh7, RD-U and RD-D cells. There was no significant change in expression of FT, GGT-I or GGT-II in any of the cell types on treatment with statin alone or in combination with mevalonic acid (Figure 5-14 and Figure 5-15). The apparent decrease in GGT-II β in RD-U cells on treatment with cerivastatin (Figure 5-15B) corresponds to a lower level also of β -actin in this sample, and so is not a real effect of cerivastatin. Therefore, the changes in prenylation state of Rap1A and the change in membrane association of H-Ras, M-Ras or Rab1A in response to statin treatment cannot be explained by alteration in the expression level of the prenyltransferase enzymes.

Figure 5-14 Effect of statin treatment on expression of farnesyltransferase and geranylgeranyltransferase-I

Total protein was extracted from cells treated for 48 hours with vehicle control, statin, or mevalonic acid in addition to statin, and 30 μ g was separated by SDS-PAGE. Simvastatin and pravastatin were used at a concentration of 10 μ M and cerivastatin at 1 μ M. DMSO was used as a vehicle control for simvastatin and water for cerivastatin and pravastatin. Mevalonic acid was used in excess, at 100 μ M. Expression of farnesyltransferase (46kDa) and geranylgeranyltransferase-I (42kDa) was detected in Huh7 (A), RD-U (B) and RD-D (C) cells using specific antibodies, and compared to the level of the loading control, β -actin.

Figure 5-15 Effect of statin treatment on expression of geranylgeranyltransferase-II

Total protein was extracted from cells treated for 48 hours with vehicle control, statin, or mevalonic acid in addition to statin, and 30μg was separated by SDS-PAGE. Simvastatin and pravastatin were used at a concentration of 10μM and cerivastatin at 1μM. DMSO was used as a vehicle control for simvastatin and water for cerivastatin (and pravastatin). Mevalonic acid was used in excess, at 100μM. Expression of geranylgeranyltransferase-II (43kDa) was detected in Huh7 (A), RD-U (B) and RD-D (C) cells using a specific antibody, and compared to the level of the loading control, β-actin.

Similarly, treatment with prenyltransferase inhibitors also did not alter expression of FT, GGT-I or GGT-II (Figure 5-16). In addition, this analysis shows that there may be slightly higher levels of prenyltransferase expression in muscle compared to liver cells, although densitometry would be needed to confirm this: Regardless, any differences would not be enough to explain the differences in sensitivity to the disruption of prenylation between muscle and liver cells observed previously (section 5.3.1.1). To my knowledge, this is the first time that the expression levels of these enzymes have been compared between liver and muscle.

Figure 5-16 Effect of prenylation inhibitors on expression of farnesyltransferase, geranylgeranyltransferase-I and geranylgeranyltransferase-II

Total protein was extracted from Huh7, RD-U and RD-D cells treated for 48 hours with 10μM farnesyltransferase inhibitor FTI-277 (F), 10μM geranylgeranyltransferase-I inhibitor GGTI-2133 (GI) or 100μM perillyl alcohol (GII), and 30μg was separated by SDS-PAGE. Expression of farnesyltransferase (46kDa), geranylgeranyltransferase-I (42kDa) and geranylgeranyltransferase-II (43kDa) was detected using specific antibodies, and compared to the level of the loading control β-actin.

5.5 Discussion

5.5.1 *The Significance of Prenylation*

The post-translational modification of proteins by prenylation is of great physiological importance, with at least 100 proteins known to require this modification in order to carry out their role in a range of cellular processes (Lane and Beese 2006). This importance has been highlighted by the abnormalities observed in recently generated conditional knockout mice for the β subunits of FT (Fntb) and GGT-I (Pggt1b), which therefore lack functional FT or GGT-I. Keratinocyte-specific Fntb knockout mice survived but developed severe hair loss, and cells from these mice could not proliferate in culture, whereas keratinocyte-specific Pggt1b knockout mice died shortly after birth (Lee et al. 2010).

The small GTPases represent the largest group of prenylated proteins. They are a large class of cell-signalling proteins that have important roles in the regulation of cellular proliferation, gene expression, cytoskeleton organisation, cell motility and intracellular vesicle trafficking (Cordle et al. 2005; Matozaki et al. 2000). Therefore, disruption of the prenylation of small GTPases can have wide-ranging physiological consequences, and has been postulated to play a role in statin-induced myopathy.

5.5.2 *Prenylation and the Statins*

There is a growing body of evidence supporting the idea that statins can disrupt prenylation of small GTPases, resulting in the disruption of normal cellular signalling. Such evidence includes the observation that inhibition of GGT-I and II, with perillyl alcohol treatment, has been shown to mimic the effect of fluvastatin on isolated rat skeletal myofibres, with these effects being rescued by co-addition of GGPP (Sakamoto et al. 2007). Treatment of rat skeletal myoblasts with cerivastatin has been shown to result in the activation and detachment of membrane-bound Ras, leading to apoptosis (Matzno et al. 2005). Statins have been shown to induce apoptosis *in vitro* in a range of different cells (Dirks and Jones 2006); for example, treatment of rat vascular smooth muscle cells with atorvastatin or simvastatin *in vitro* resulted in

apoptosis that could only be rescued by addition of mevalonate, FPP or GGPP (Blanco-Colio et al. 2002).

However, much of the previous research has been carried out only in muscle and often in non-human tissue. It is therefore important to ensure that these effects are similarly observed in human cells. The analysis here also aimed to compare the effects in liver (the target tissue) with muscle (the main site of toxicity) across a range of statins, as it is thought that any differences observed between the responses of muscle and liver may give us further insight into the mechanism of statin muscle toxicity (Mullen et al. 2010). The hypothesis to be tested was that prenylation, postulated to play a role in statin-induced myopathy, would show preferential disruption in muscle compared to liver.

5.5.2.1 Membrane Association of Small GTPases

The results described in this chapter confirm that statin treatment does have an effect on the prenylation of small GTPases in both human liver and muscle cells. On cerivastatin treatment, detection of the FT substrate H-Ras, GGT-I substrate M-Ras and the GGT-II substrate Rab1A in the membrane protein fraction was greatly reduced in both human muscle and liver cells. Since prenylation is vital for the membrane association of small GTPases, this change in subcellular localisation indicated a reduction in prenylation.

In some cases, the reduced membrane-association was accompanied by detection of the protein in the corresponding cytosolic fractions, which could sometimes also be seen with simvastatin treatment. It is interesting that this corresponding cytosolic increase was not observed in all instances. It could perhaps be that the unprenylated protein is targeted for degradation, which would explain why it could not always be detected.

It is also interesting to note that these effects on prenylation were only observed on treatment with lipophilic cerivastatin or simvastatin. Treatment with hydrophilic pravastatin, which was previously shown to elicit lower levels of toxicity (section 4.2), did not lead to a reduction in membrane-association under the conditions used.

Since the reduction in membrane-associated (i.e. prenylated) proteins was observed both in liver and muscle cells, it would be beneficial to further dissect this inhibition of H-Ras, M-Ras and Rab1A prenylation. This could be achieved through the extraction of cytosolic and membrane protein from cells treated with a range of simvastatin and cerivastatin doses, as was previously carried out during the examination of Rap1A prenylation using total protein extracts (section 5.3.1.1).

A limitation of this data is the lack of complete confidence in the quality of cytosolic and membrane separation in those samples used to look at H-Ras, M-Ras and Rab1A localisation, in terms of the degree of separation obtained. LDH is exclusively a cytosolic protein (Saad et al. 2006), and so the amount of the protein present in cytosolic, compared to membrane, extracts was examined to act as a control and give an indication of the degree of separation achieved by the extraction process used. However, the results obtained were inconclusive due to variations within the particular set of protein samples used in this analysis (Figure 5-12 and Figure 5-13). This was likely the result of the protein concentration, by precipitation and resuspension in a smaller amount of solvent, in a number of these samples prior to analysis. The protein was resuspended in sodium hydroxide, which allowed for the determination of final protein concentration using the Lowry method but was later found to interfere with SDS-PAGE. This meant that the sodium hydroxide needed to be neutralised prior to analysis (section 2.2.3.4), which may have affected the subsequent separation of proteins by gel electrophoresis. A repeat analysis of LDH localisation, using samples that had not been pre-treated in this way or had been resuspended in a solvent other than sodium hydroxide, may rectify this. Time did not allow in this case, however it would be beneficial to also analyse the localisation of a membrane protein in the different protein fractions. This could include the analysis of a membrane-specific protein, such as calnexin (Spindler et al. 2012), which has been shown not to be affected by statin treatment (Ostrowski et al. 2007). Finally, measurement of the expression level of the membrane transporter OATP2B1 would be beneficial, as it is known to be expressed in both liver and skeletal muscle (Knauer et al.

2010) and is responsible for the uptake of hydrophilic statins into cells (section 1.1.4).

5.5.2.2 Differential Disruption of Prenylation

In addition to the effect on small GTPase localisation, a direct effect was observed on the prenylation of the Ras-related GTPase Rap1A through the use of two antibodies able to detect the unprenylated form of the protein or total protein expression respectively. Unprenylated Rap1A was detected in both liver and muscle following simvastatin and cerivastatin treatment. This inhibition of Rap1A prenylation was dose-dependent in both cases with all cells showing much greater sensitivity to cerivastatin than simvastatin, with lower cerivastatin concentrations required to produce detectable levels of unprenylated Rap1A.

The differences between the responses to simvastatin and cerivastatin treatment are unlikely to be dependent on differential uptake of the two statins. These are two of the most lipophilic statins, and they both show distribution to non-target tissues via passive diffusion. They are also both substrates of OATP1B1 (section 1.1.4), a transporter predominantly found in the liver which plays an important role in the hepatic uptake of statins (Shitara and Sugiyama 2006).

RD muscle cells were more sensitive to treatment with both simvastatin and cerivastatin compared to Huh7 liver cells, with a much lower statin concentration needed to detect unprenylated Rap1A in muscle. This finding supports the hypothesis, that statin treatment does lead to both greater toxicity and preferential disruption of protein prenylation within muscle cells.

It is unlikely that this difference between muscle and liver is due to increased statin metabolism in Huh7 liver cells. The liver is the main site of xenobiotic metabolism *in vivo* (Caldwell et al. 1995), however it has been shown that *in vitro* Huh7 liver cells express very low levels of drug metabolising enzymes (Phillips et al. 2005). This could mean that the differential effect seen between muscle and liver *in vivo* may be even greater.

5.5.2.3 Prenyltransferase Inhibitors

Treatment of cells with prenyltransferase inhibitors resulted in disruption of protein prenylation, as observed through detection of unprenylated Rap1A and reduction of H-Ras, M-Ras and Rab1A found in membrane protein fractions. This was similar to effects seen with simvastatin and cerivastatin treatment in these experiments, and agrees with previous findings of similar effects of statin and prenylation inhibitor treatment in other models (Cao et al. 2009; Sakamoto et al. 2007; Wagner et al. 2011). This supports the idea that statins are affecting the process of protein prenylation.

Treatment with the GGT-I inhibitor GGTI-2133 resulted in observation of unprenylated GGT-I substrate Rap1A in all cell types (Figure 5-3). However, it is surprising that although H-Ras and Rab1A are reported to be substrates of different prenyltransferase enzymes (FT and GGT-II respectively), on treatment with prenyltransferase inhibitors the pattern of their reduced membrane-association was similar. For both small GTPases, treatment with GGTI-2133 resulted in loss of membrane association in Huh7 liver cells, but in RD muscle cells this was observed on treatment with the FT inhibitor FTI-277.

Under normal conditions H-Ras is reputed to undergo farnesylation by FT. However, it is known that K- and N-Ras proteins are alternately prenylated by GGT-I in the human colon carcinoma cell line DLD-1 on treatment with FT inhibitors (Whyte et al. 1997). It is perhaps possible that when FT is inhibited *in vitro* in liver cells then H-Ras can similarly be alternately prenylated by GGT-I. This would explain why treatment of Huh7 cells with FTI-277 did not result in a decrease in membrane-associated H-Ras. In addition, GGTI-2133 can also inhibit FT, as described previously (section 5.2), but with lower potency than for GGT-I inhibition. The IC_{50} for GTI-2133 inhibition of FT is reported to be 5.4 μ M (Sigma-Aldrich 2011b), and hence the 10 μ M inhibitor concentration used herein should result in approximately 65% inhibition of activity. Therefore, the observed reduction of membrane-associated H-Ras on treatment of Huh7 cells with 10 μ M GTI-2133 is likely to be due to its inhibition of FT at this concentration, although this does not explain why this effect is seen in liver cells

and not also in muscle cells. Another possibility is that the FT inhibitor used here is inhibiting GGT-II in the RD cell line, as has been previously observed in *Caenorhabditis elegans* (Lackner et al. 2005; McTaggart 2006). This would explain the reduction in Rab1A membrane-association in RD cells treated with FT inhibitor. Perhaps the similar expression profiles of H-Ras and Rab1A indicate that either the prenyltransferase enzymes, or these prenyltransferase inhibitors, are not as specific under all conditions as traditionally thought.

Future repeat experiments, perhaps with a range of lower inhibitor concentrations, would help to investigate these discrepancies and also help to rule out any possibility of having mixed up samples. Lower concentrations of GGTI-2133 will be less likely to have a possible effect on prenylation by FT, (IC_{50} 5.4 μ M) but will still inhibit GGT-I (IC_{50} 38nM) (Sigma-Aldrich 2011b). Similarly, the use of lower concentrations of FTI-277 may help to clarify whether or not FTI-277 inhibition of GGT-II was the reason for observing loss of membrane association of Rab1A in muscle on treatment with this inhibitor.

5.5.3 Prenyltransferase Expression

No change in the expression levels of any of the 3 prenyltransferase enzymes was observed, in either liver or muscle cells, in response to treatment with statins or prenyltransferase inhibitors. If any of these enzymes are upregulated or downregulated in response to statin inhibition of prenylation, then it would be expected to have occurred after the given treatment time of 48hours. The fact that no change was observed after this time excludes this as an explanation of the effects of statin treatment on prenylation or as an explanation of the differences between the responses to simvastatin and cerivastatin. There were also no differences in enzyme levels between the cell types that would account for the different sensitivities of liver and muscle to disruption of protein prenylation. Therefore, several alternative explanations for this differential sensitivity will be discussed in the final chapter.

5.6 Summary

The post-translational modification of small GTPases by prenylation plays a vital role in enabling their membrane localisation in order to carry out their varied roles in cellular signalling. It is thought that statins inhibit not only the production of cholesterol but also the production of the isoprenoids FPP and GGPP which are used to prenylate proteins. It has therefore been suggested that statins inhibit the prenylation of proteins and that this is an important contributing factor in the development of statin-induced muscle toxicity.

The results presented here confirm that treatment with both simvastatin and cerivastatin does inhibit the prenylation of a range of small GTPases in both human liver and muscle in a similar way to the inhibition of prenylation observed on treatment with prenyltransferase inhibitors.

The inhibition of prenylation of Rap1A, a Ras-related GTPase, was more sensitive to treatment with cerivastatin than simvastatin in both muscle and liver. Inhibition of Rap1A prenylation was also more sensitive to statin treatment in muscle (the tissue where the most toxicity is observed) than in liver (the target tissue). These effects could not be explained by changes in total protein expression. This supports my hypothesis, that statin treatment does lead to both greater toxicity and preferential disruption of protein prenylation within muscle cells.

There was no change observed in expression of any of the three prenyltransferase enzymes in liver or muscle in response to treatment with statins or prenylation inhibitors. The changes in prenylation status of Rap1A, or any of the other small GTPases examined, therefore cannot be explained by changes in enzyme expression.

6 Discussion

The physiological consequences of xenobiotic intervention are often complex. A drug frequently has many effects in addition to the intended ones. These can either benefit the patient or can lead to adverse drug reactions, depending on the treatment context and on the individual patient. This highlights the ever increasing need to understand the in-depth mechanisms of drug action, in order to improve patient treatment both through enabling the best use of established medication as well as directing the future design of new drugs.

The statins are an important class of drugs used to treat hypercholesterolaemia for the primary and secondary prevention of cardiovascular disease, currently the leading cause of death worldwide. They are widely used, with their use continually increasing, however a small percentage of patients experience drug side effects; the most significant of these is myopathy, which can range from the muscle aches or weakness of myalgia to the more serious condition of rhabdomyolysis (Harper and Jacobson 2007). In addition to the obvious adverse effects for the patients myopathy is also considered a leading cause of the high statin discontinuation rates (Bakhai et al. 2012), and therefore it is important to understand the mechanism of this myopathy to enable better management of statin treatment. It is currently thought that myopathy occurs due to the secondary effects resulting from statin inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate limiting enzyme in cholesterol biosynthesis and the mevalonate pathway (Shitara and Sugiyama 2006). By inhibiting this enzyme, statin treatment lowers cholesterol in the form of low-density lipoprotein (LDL) but can also disrupt other aspects of the mevalonate pathway, such as protein prenylation. It is this disruption of protein prenylation that has been postulated to play a particular role in statin-induced myopathy, however the mechanism has not yet been fully elucidated (Vaklavas et al. 2009).

The aim of this study was to use two human cell lines as an *in vitro* model for comparing the effects of statins on both cholesterol synthesis and protein

prenylation between the liver, the intended target tissue for therapeutic benefit, and skeletal muscle, the major site of toxicity.

6.1 *In Vitro* Modelling of Statin-Induced Toxicity

Initially, the suitability of the RD rhabdomyosarcoma skeletal muscle cell line for use in this model system was determined (section 3.2). The morphological and molecular changes that occur during the differentiation process of these cells were examined. RD cells were found to express a number of muscle-specific markers, including the muscle-specific transcription factor myogenin and myosin heavy chain 3, as well as a number of other genes of interest such as HMGCR and the LDL receptor. On the basis of this investigation, a cell differentiation period of 5 days was chosen for use in later experiments.

The original hypothesis to then be tested was that the process of protein prenylation exhibits circadian variation, as has already been shown for cholesterol biosynthesis, and that this might enable temporal separation between the cholesterol-lowering efficacy and the toxicity of statins. However, being unable to verify the ability of the RD cell line to establish and maintain a circadian cycle *in vitro* (section 3.3), the project took a slightly different direction, whilst still focusing on examining the differences between the responses of liver and muscle to statin treatment.

In order to investigate any such differences, a number of parameters were examined. MTT assays were carried out in order to examine the cell toxicity response of Huh7 liver and RD muscle cells to treatment with a range of statins (section 4.2). Significant reductions in cell viability were observed in all cells on statin treatment, with greater toxicity observed in response to treatment with more lipophilic statins, such as simvastatin, lovastatin and cerivastatin. Where it was possible to determine IC₅₀ values, these were consistent with those previously reported by Kobayashi and colleagues using RD cells (Kobayashi et al. 2008). Greater toxicity was also observed in RD muscle cells compared to Huh7 liver cells on treatment with all statins, with this difference being statistically significant for treatment with simvastatin, cerivastatin, atorvastatin and rosuvastatin. Previous studies examining the toxicity of the statins have

tended to either compare the toxicity of a large range of statins in only one tissue, such as muscle (Kobayashi et al. 2008), or to compare the responses between liver and muscle using only a small number of statins (Mullen et al. 2010; Mullen et al. 2011). Therefore, since this study both compared responses between liver and muscle cell lines and used a large range of different statins, it represents the first comprehensive comparative analysis of statin toxicity.

The process of cholesterol synthesis was then investigated in these cells (section 4.3). Much lower expression of HMGCR was found, at both the transcript and protein level, in RD cells compared to Huh7 cells. This difference was particularly evident in differentiated RD cells. Cholesterol was found to be present in both Huh7 and RD cells, with 10 μ M simvastatin and pravastatin treatment, and 1 μ M cerivastatin treatment, seeming to lead to a decrease in total cellular cholesterol in these cells. This cholesterol decrease was observed after 48 hour statin treatment, which agrees with previous observations of a cholesterol decrease after 24 and 48 hour treatment of HepG2 cells (Kobayashi et al. 2008) but not after 6 or 18 hour treatment of HepG2 cells and C2C12 mouse myoblasts (Mullen et al. 2010).

Having characterised the response of Huh7 and RD cells to statin treatment, in terms of cell viability and changes in cholesterol levels, experiments were designed to test the hypothesis that statins preferentially disrupt the process of protein prenylation within RD muscle cells (chapter 5), the site of greater toxicity. Huh7 and RD cells were treated with simvastatin, cerivastatin and pravastatin, mevalonic acid in addition to statin, or prenylation inhibitors. Protein was extracted and then subjected to analysis by western blot to detect any changes in the prenylation status of selected small GTPases. Inhibition of prenylation was found by directly analysing changes in the unprenylated form of Rap1A, using a specific antibody, and by examining the reduction in membrane association of H-Ras, M-Ras and Rab1A. Treatment with simvastatin and cerivastatin inhibited the prenylation of Rap1A, and frequently seemed to also affect the membrane association of the small GTPases H-Ras, M-Ras and Rab1A. These effects could not be explained by changes in total protein expression or changes in the expression of the prenyltransferase enzymes.

In both muscle and liver the inhibition of prenylation of Rap1A, a Ras-related GTPase, was more sensitive to treatment with cerivastatin, the statin that was withdrawn from the market in 2001 due to high incidence of rhabdomyolysis (Bellingham 2001), than simvastatin. Inhibition of Rap1A prenylation in muscle cells was also more sensitive to simvastatin and cerivastatin treatment than in liver cells (section 5.3.1.1). This supports my hypothesis, that statin treatment does lead to both greater toxicity and preferential disruption of protein prenylation within muscle cells and further supports a mechanism where disrupting production of intermediates in the mevalonate pathway is indeed a cause of statin-induced muscle toxicity.

A question arising from this conclusion is that of why there is this differential sensitivity between liver and muscle cells to the inhibition of prenylation by statins. One possible explanation could be that there is a lower background level of prenylation occurring in muscle compared to liver. This is unlikely to be due to differences in the levels of the prenyltransferase enzymes, since no large differences in expression were observed between the cell types (Figure 5-16); although this analysis only examined protein expression and did not investigate whether there were differences in prenyltransferase enzyme activity. However, the results did reveal a considerable difference in HMGCR expression, at both the transcript and the protein level, between Huh7 liver and RD muscle cells (section 4.3.1). This implies that there may be a much lower throughput along the mevalonate pathway in muscle when compared to liver, the main site of cholesterol synthesis (Catapano 2007). In the absence of statin treatment, there are sufficient isoprenoids in both liver and muscle cells to ensure complete prenylation of Rap1A, as far as can be detected by the western blot analysis; but a lower capacity of the mevalonate pathway could lead to a smaller resting pool of the isoprenoids FPP and GGPP within muscle and would mean that less inhibition of this pathway would be required before the effects on prenylation would be seen in this tissue. In order to examine this as a possibility, it would be interesting to measure and compare the levels of FPP and GGPP, under resting conditions, in both liver and muscle, initially in the cell lines that were used here. This could be achieved by using a combination of HPLC (high performance liquid chromatography) and LC-MS (liquid

chromatography – mass spectrometry)-based techniques (Tong et al. 2005). This method could also be used for looking at the rate at which a range of statins decrease this isoprenoid pool in both liver and muscle, both over time and on treatment with varying statin concentrations.

6.2 Prenylation and Muscle Physiology

A second question is how this differential sensitivity of muscle to disruption of protein prenylation could lead to the toxicity seen in muscle. There is the possibility that prenylation, or at least the prenylation of certain proteins, may be more obligate in muscle than in liver. Perhaps there are certain prenylated proteins that are more highly expressed in muscle than in liver or are particularly crucial for correct muscle function. M-Ras, initially thought to be such a target, is now known to be of more widespread importance, and so cannot now be included in this category (Ehrhardt et al. 1999). However if such targets do exist, and their prenylation status is affected by statin treatment, then they could play an important role in statin muscle toxicity.

In the past, knockout models have frequently been used to elucidate the physiological importance of a target protein or pathway; however knowledge gained in this way regarding protein prenylation remains limited. Recently, several conditional knockouts of prenyltransferase enzymes have been generated and studied, which is starting to fill this gap in knowledge: Conditional knockout of the β subunit of FT in mouse keratinocytes led to development of severe hair loss and an inability of these cells to proliferate in culture, whereas knockout of the GGT-I β subunit led to the death of mice shortly after birth (Lee et al. 2010), highlighting the essential role of protein prenylation. Similar deficiencies in either FT or GGT-I in hepatocytes led to elevated plasma transaminases and abnormal histology of livers from the knockout mice, with FT knockout mice also growing slower than wild-type mice and GGT-I knockout mice showing reduced survival (Yang et al. 2012). In addition, conditional knockout of GGT-I has been shown to lead to hyperactivated mouse macrophages, which secreted inflammatory cytokines and induced an inflammatory response (Khan et al. 2011; Philips 2011). Other recently generated models include the conditional knockout of GGT-I in mouse

haematopoietic cells (Sjogren et al. 2011), knockdown of prenyltransferase enzymes in zebrafish embryos (Cao et al. 2009), knockout mouse models of several Rho family GTPases (Heasman and Ridley 2008; Ueda et al. 2010) and mouse knockout of the skeletal muscle-specific G protein α subunit (Chen et al. 2009a). Further study, particularly of these latter models, as well as the generation of muscle-specific conditional prenyltransferase knockout or knockdown models, may assist in the elucidation of the specific role of protein prenylation in muscle.

Meanwhile, in order to link the molecular disruption caused by statins to muscle toxicity we can gain clues through alternative *in vitro* studies, in many of which statin treatment has been linked with the apoptosis of muscle fibres, and through the identification of syndromes which are associated with muscle disease (reviewed by (Baker 2005)). Many such studies have been described in previous chapters but some of these findings, and the resulting proposed links between statin treatment and muscular effects, are now summarised in Figure 6-1.

Statin treatment has been shown to affect many aspects of the mevalonate pathway *in vitro*, which in turn has the potential to affect a wide range of cellular processes (Figure 6-1). Statins have been shown to cause a reduction in isopentenylation of sec tRNA and therefore reduce selenoprotein synthesis (Warner et al. 2000). There are approximately 25 human selenoproteins, but out of these selenoprotein N (Jurynek et al. 2008; Petit et al. 2003) and glutathione peroxidase (Kromer and Moosmann 2009) have been identified as having a potential role in linking the statins to effects on muscle physiology. Statin treatment has reduced synthesis of dolichol and thereby disrupted N-linked glycosylation (Mullen et al. 2010; Siddals et al. 2004) of proteins known to have an important role in normal muscle function (Barton et al. 2012). Statins have also been shown to disrupt the process of protein prenylation (Sakamoto et al. 2011), through either farnesylation or geranylgeranylation, which is known to affect cellular signalling via small GTPases and lead to muscle cell apoptosis (Blanco-Colio et al. 2002; Matzno et al. 2005).

Figure 6-1 Linking the molecular effects of the statins with muscle physiology and myopathy

Statin treatment leads to not only reduced synthesis of cholesterol, but also to reduced protein prenylation, reduced N-linked glycosylation and reduced selenoprotein synthesis. The reduction of each of these processes (blue boxes) has been linked to a variety of effects in muscle (brown boxes), including muscle fibre apoptosis, decreased protein synthesis and increased degradation of muscle proteins, as well as effects on myogenesis, myoregeneration and muscular oxidative stress.

? = a link that has been postulated but direct evidence, of causation specifically by the statins, is lacking. * = syndromes that have myopathy as a symptom: Mutations in the gene coding for lamins A and C (Wilson 2000); Mutations leading to altered RAS signalling (Tidyman et al. 2011); Selenium deficiency (Moosmann and Behl 2004); Selenoprotein N deficiency (Juryneć et al. 2008; Petit et al. 2003); Defects in dystroglycan processing (Martin-Rendon and Blake 2003; Muntoni et al. 2004).

Figure produced using the following references: Prenylation: Lamins (Beck et al. 1990; Lammerding et al. 2004; Lutz et al. 1992; Sinensky et al. 1994); Ras (Dirks and Jones 2006; Matzno et al. 2005; Mullen et al. 2010; Tidyman et al. 2011); Rho (Bifulco et al. 1993; Blanco-Colio et al. 2002; Liao 2002; Matzno et al. 2005; Meske et al. 2003); Rab (Sakamoto et al. 2007; Sakamoto et al. 2011; Wagner et al. 2011); Rap (Cao et al. 2009; Mullen et al. 2010); Dolichol (Mullen et al. 2010; Siddals et al. 2004); Insulin-like growth factor (IGF) receptor (Barton et al. 2012; Siddals et al. 2004); Forkhead box O3 (Hers et al. 2011; Sandri et al. 2004); Atrogin-1 (Cao et al. 2009; Wang et al. 2010a); Dystroglycan (Cohn et al. 2002; Martin-Rendon and Blake 2003); Selenoproteins (Moosmann and Behl 2004; Warner et al. 2000); Selenoprotein N (Juryneć et al. 2008; Petit et al. 2003); Glutathione peroxidase (Kromer and Moosmann 2009).

There are therefore many potential explanations for statin myopathy; however one pathway that has been emerging in recent years as having a particularly important role is the phosphatidylinositol 3-kinase (PI3K) - Akt signalling pathway. Insulin-like growth factor 1 (IGF-1) signalling, via this pathway, is known to have an important role in the regulation of skeletal muscle growth (Schiaffino and Mammucari 2011) but this signalling can be suppressed by the statins (Siddals et al. 2004). In addition, simvastatin treatment has been shown to lead to dephosphorylation of Akt in C2C12 mouse myotubes, but not in HepG2 liver cells, and co-treatment with simvastatin and IGF-1 has been able to rescue C2C12 myotubes from apoptosis (Mullen et al. 2011). Suppression of normal signalling through the PI3K-Akt pathway leads to the expression of the muscle specific Skp1-Cul1-F-Box protein (SCF) ubiquitin E3 ligase atrogin-1 (Sacheck et al. 2004) through the dephosphorylation, nuclear translocation and activation of the transcription factor FOXO3 (Sandri et al. 2004); it has now been shown that statin treatment can lead to the activation of atrogin-1 through this same pathway of FOXO3 activation (Cao et al. 2009).

It has been suggested that statins suppress IGF-1 signalling through two mechanisms. Firstly, statins have been shown to affect the N-linked glycosylation and processing of IGF receptors (Siddals et al. 2004). Incorrectly processed IGF receptor could lead to impaired response to IGF growth factors in muscle cells.

Secondly, the inhibition of geranylgeranylation by statins has been shown to promote both upregulation of atrogin-1 and muscle damage in mouse myotubes and zebrafish, with myoblasts from atrogin-1 knockout mice proving resistant to this statin-induced muscle damage. This has led to the suggestion that there may be small GTPases that couple statin disruption of prenylation to the upregulation of atrogin-1 and muscle damage (Cao et al. 2009). The small GTPase Rap1, which is prenylated by geranylgeranyltransferase-I (GGT-I), has been suggested as an attractive candidate for this link (Cao et al. 2009; Mullen et al. 2011). This is because it is thought to play a role in IGF-1 signalling, as it can be activated by

IGF-1 (Romano et al. 2006) and can then activate PI3K and Akt (Stork 2003; Van Kolen et al. 2006; Wang et al. 2001), and its prenylation can also be disrupted by the statins. This is further supported by the recent finding that transient transfection of constitutively active Rap1 into C2C12 myotubes was able to partially rescue simvastatin-induced inhibition of mitochondrial respiration (Mullen et al. 2011). Rap1A, one of two very closely related isoforms of Rap1 (Bos 1997; Frische and Zwartkruis 2010), showed greater sensitivity to disruption of its prenylation in muscle compared to liver cells in my study (section 5.3.1.1). Therefore, the inhibition of Rap1 prenylation as a mechanism by which statins can suppress IGF-1 signalling would offer a potential interpretation for the relevance of my findings to statin-induced myopathy, however further investigation would be required to clarify this.

The suppression of IGF-1 signalling by both of these mechanisms ultimately results in the upregulation of atrogin-1, which is known to play an important role in muscle atrophy. The importance of atrogin-1 in muscle atrophy was first identified through the use of microarray experiments when its expression was found to be strongly upregulated in atrophying muscle, with this upregulation being prior to detection of muscle weight loss (Gomes et al. 2001). It has since been discovered to both stimulate degradation of muscle proteins and inhibit protein synthesis (Wang et al. 2010a). Potential degradation targets of atrogin-1 in muscle include myogenic differentiation 1 (*MyoD1*), the calcium-dependent phosphatase calcineurin, the translation initiation factor eIF3-f, and intermediate filament components desmin and vimentin (Cao et al. 2009; Lokireddy et al. 2012). Degradation of these proteins would affect multiple processes that are essential for muscle cell function; such as differentiation of muscle satellite cells and thereby myoregeneration (*MyoD1*, calcineurin) (da Costa et al. 2007; Friday et al. 2000; Megeney and Rudnicki 1995); regulation of muscle fibre remodelling (calcineurin) (Bassel-Duby and Olson 2006; da Costa et al. 2007); synthesis of new proteins (eIF3-f) (Csibi et al. 2008); and the integrity of the muscle cytoskeleton (desmin, vimentin) (Favre et al. 2011).

6.3 Additional Future Work

In this study, only the prenylation of small GTPases in liver and muscle has been examined in response to statin treatment; however, there are a number of other proteins that have also been proposed to have a role in mediating statin-induced muscle toxicity, indicating that multiple pathways may yet be important for this adverse effect. Therefore, future work could include similar western blot analysis of non-GTPase proteins in liver and muscle that are known to be prenylated and whose dysfunction has been implicated in muscular dystrophy, indicating their importance in muscle; such proteins would include the nuclear lamins (Lutz et al. 1992; Wilson 2000). Additional mechanistic insight could be gained through further use of enzyme inhibitors and through the use of inhibitory RNA, to clarify whether changes in the extent of prenylation, as measured by western blot analysis, translate to an impact upon muscle cell integrity.

It would also be of interest to examine the differences between liver and muscle in a wider range of cellular processes, in response to a range of statins. For example, defective glycosylation has also been linked to muscular dystrophy (Muntoni et al. 2004), and it has previously been shown that simvastatin treatment affects N-linked glycosylation in C2C12 mouse myoblasts but not in human HepG2 liver cells (Mullen et al. 2010). However, it is not known if this would be the case in human muscle cells, such as the RD cell line used here, and whether there would be any differences between the responses to different statins. There are also many specific proteins that have been postulated to play a role in the mechanism of muscle toxicity where direct evidence is lacking. Such proteins include the glycoprotein dystroglycan and selenoprotein N and further investigation of their possible involvement, including differences between liver and muscle, may help to further clarify the mechanism of statin-induced muscle toxicity.

Finally, the issue of the contribution of circadian rhythms towards the development of statin-induced toxicity, as per my original hypothesis and objectives (section 1.5), remains an interesting question, albeit one which I was unable to fully

investigate here due to the limitations of the model system used. Additional future work in this area of muscle toxicity could therefore include a return to this initial hypothesis using a different model system, such as the mouse C2C12 myoblast cell line which, unlike the RD cell line, has previously been used for the analysis of the molecular clock in skeletal muscle (Zhang et al. 2012). Furthermore, since animal models have previously been used to analyse circadian variations in both liver (Akhtar et al. 2002) and skeletal muscle (Almon et al. 2008; McCarthy et al. 2007) *in vivo*, they could also be used as a starting point to examine the rhythmicity of protein prenylation in these tissues. The ubiquitous nature of circadian regulation, and the implication for xenobiotic toxicity, is increasingly being recognised as important (Gachon and Firsov 2011; Ohdo 2010) and therefore it is vital that we continue to develop model systems which can be used to investigate the potential applications for specific drugs, such as the statins.

6.4 Summary

In summary, this work has shown that, compared to Huh7 liver cells, RD muscle cells show both greater sensitivity to the toxic effects of statins and preferential disruption of protein prenylation. This further supports a mechanism whereby disrupting production of the intermediates in the cholesterol synthesis pathway, including the isoprenoids used in protein prenylation, is a significant cause of statin-induced muscle toxicity. The difference between muscle and liver cell responses is not due to a difference in the protein expression levels of the prenyltransferase enzymes but may perhaps be due to differences in isoprenoid levels, resulting from differences in the protein expression levels of HMGCR between the two. Differences in isoprenoid levels are likely to affect a number of processes, including protein prenylation. In particular, the small GTPase Rap1A showed differential disruption of its prenylation between muscle and liver in this study, which may prove important in the mechanism of statin-induced muscle toxicity. This study highlights the importance of examining the effects of drugs at both the target site of action and at the site of toxicity, rather than only studying one site in isolation, in order to gain insight into the mechanism of adverse drug effects.

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8 Appendices

8.1 Normalisation of Temporal Gene Expression Data from Pilot Serum Pulse Experiment

8.1.1 3T3-L1 Murine Preadipocyte Cell Line

Figure 8-1 Temporal gene expression of two different housekeeping genes in 3T3-L1 cells
3T3-L1 cells were synchronised using a serum pulse (at T=0) and RNA was extracted at T=0, 14, 26 and 36. Temporal gene expression of housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and *18S* was measured by quantitative RT-PCR (TaqMan). Data are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

Figure 8-2 Effect of normalisation of 3T3-L1 TaqMan data using two different housekeeping genes

3T3-L1 cells were synchronised using a serum pulse (at T=0) and temporal gene expression of *PER2* and *DBP* was measured by quantitative RT-PCR (TaqMan). Data are shown unnormalised and normalised to *GAPDH* or *18S* expression, and are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

8.1.2 HepG2 and Huh7 Human Liver Cell Lines

Figure 8-3 Temporal gene expression of three different housekeeping genes in HepG2 and Huh7 cells

HepG2 and Huh7 cells were synchronised using a serum pulse (at T=0). RNA was extracted from HepG2 cells at T=0, 14, 26, 36 and from Huh7 cells at T=0, 12, 18 and 26. Temporal gene expression of housekeeping genes *GAPDH*, *18S* and *ACTB* was measured by quantitative RT-PCR (TaqMan). Data are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

Figure 8-4 Effect of normalisation of HepG2 TaqMan data using three different housekeeping genes

HepG2 cells were synchronised using a serum pulse (at T=0) and temporal gene expression of *PER2* and *DBP* was measured by quantitative RT-PCR (TaqMan). Data are shown unnormalised and normalised to *GAPDH*, *18S* or *ACTB* expression, and are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

Figure 8-5 Effect of normalisation of Huh7 TaqMan data using three different housekeeping genes

Huh7 cells were synchronised using a serum pulse (at T=0) and temporal gene expression of *PER2* and *DBP* was measured by quantitative RT-PCR (TaqMan). Data are shown unnormalised and normalised to *GAPDH*, *18S* or *ACTB* expression, and are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

8.2 Additional Normalisations of Temporal Gene Expression Data from Circadian Analysis in Huh7 and RD Cells

8.2.1 Normalisation of Temporal Gene Expression Data from Huh7 Cells

Figure 8-6 Effect of normalisation of Huh7 TaqMan data (*PER1* and *REV-ERB α*) using two different housekeeping genes

Huh7 cells were synchronised using a serum pulse (at T=0) and temporal gene expression of *PER1* and *REV-ERB α* was measured by quantitative RT-PCR (TaqMan). Data are shown unnormalised and normalised to *18S* or *ACTB* expression, and are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

Figure 8-7 Effect of normalisation of Huh7 TaqMan data (*HMGCR*) using two different housekeeping genes

Huh7 cells were synchronised using a serum pulse (at T=0) and temporal gene expression of *HMGCR* was measured by quantitative RT-PCR (TaqMan). Data are shown unnormalised and normalised to *18S* or *ACTB* expression, and are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

8.2.2 Normalisation of Temporal Gene Expression Data from RD-D Cells

Figure 8-8 Effect of normalisation of RD-D TaqMan data (*BMAL1* and *CRY1*) using two different housekeeping genes

RD-D cells were synchronised using a serum pulse (at T=0) and temporal gene expression of *BMAL1* and *CRY1* was measured by quantitative RT-PCR (TaqMan). Data are shown unnormalised and normalised to *18S* or *ACTB* expression, and are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4. Statistical significance determined by one-way ANOVA followed by Bonferroni post hoc test: * = $P < 0.05$.

Figure 8-9 Effect of normalisation of RD-D TaqMan data (*PER1* and *REV-ERBα*) using two different housekeeping genes

RD-D cells were synchronised using a serum pulse (at T=0) and temporal gene expression of *PER1* and *REV-ERBα* was measured by quantitative RT-PCR (TaqMan). Data are shown unnormalised and normalised to *18S* or *ACTB* expression, and are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

Figure 8-10 Effect of normalisation of RD-D TaqMan data (*MYOD1* and *MYOG*) using two different housekeeping genes

RD-D cells were synchronised using a serum pulse (at T=0) and temporal gene expression of *MYOD1* and *MYOG* was measured by quantitative RT-PCR (TaqMan). Data are shown unnormalised and normalised to *18S* or *ACTB* expression, and are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

Figure 8-11 Effect of normalisation of RD-D TaqMan data (*HMGCR*) using two different housekeeping genes

RD-D cells were synchronised using a serum pulse (at T=0) and temporal gene expression of *HMGCR* was measured by quantitative RT-PCR (TaqMan). Data are shown unnormalised and normalised to *18S* or *ACTB* expression, and are presented as mean $\pm$ standard deviation (SD), relative to expression at T=0, from a single experiment where N=4.

8.3 Conference Presentations

- 2009 - University of Surrey FHMS Annual Festival of Research (poster)

- 2010 - British Toxicology Society (BTS) Annual Congress 2010 (poster)
 - University of Surrey FHMS Annual Festival of Research (poster)

- 2011 - BTS Annual Congress (poster, commended)
 - University of Surrey FHMS Annual Festival of Research (poster)

- 2012 - BTS Annual Congress (oral presentation)
 - Joint 19th International Symposium on Microsomes and Drug Oxidations (MDO) and 12th European meeting of the International Society for the Study of Xenobiotics (ISSX) (poster)
 - University of Surrey FHMS Annual Festival of Research (poster)

University of Surrey FHMS Annual Festival of Research 2009 - abstract

Title	THE EFFECTS OF TIME OF TREATMENT ON STATIN MUSCLE TOXICITY: INITIAL CHARACTERIZATION OF A HUMAN MUSCLE CELL LINE
Authors	<u>ROWENA GEE</u>, NICK PLANT, JONATHAN JOHNSTON, KATE PLANT
Group	Biochemical Sciences Division: Pharmacology & Toxicology and Sleep & Chronobiology Themes
Abstract	<p>Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. They are the first line of treatment for hypercholesterolemia, a known risk factor for the development of cardiovascular disease. Although statin therapy is generally considered to be safe, some patients experience muscle toxicity (myopathy), which is the most notable adverse effect of the statins and in the most severe cases can prove fatal. It is thought that myopathy most likely occurs due to the secondary effects of inhibiting HMG-CoA reductase, for example the effects on synthesis of other isoprenoids and the resulting disruption of protein prenylation.</p> <p>Daily circadian rhythms are known to regulate a multitude of biological processes, and many aspects of cholesterol synthesis and metabolism have been shown to be under circadian control. To date, most studies looking into the optimum time for statin dosing focus on the effects of time on their cholesterol-lowering efficacy; however it is not currently known how temporal dosing of statins affects muscle toxicity. We propose that, like many aspects of cholesterol synthesis and metabolism, protein prenylation is also under circadian control. By looking into the effects of temporal dosing on cholesterol synthesis and protein prenylation, it may prove possible to achieve greater separation between the cholesterol-lowering effects of statins and unwanted toxicity. For this work we will use human liver and muscle cells in culture as a model system.</p> <p>In the first phase of this project we have identified a human undifferentiated muscle cell line, the RD human rhabdomyosarcoma cell line which has previously been used for studying the myotoxic effects of statins. We have studied differentiating RD cells to characterise the morphological and molecular changes which occur during this process and to confirm the expression of genes involved in cholesterol metabolism and circadian rhythmicity. Current work is employing real-time PCR to investigate temporal changes of gene expression.</p>

THE EFFECTS OF TIME OF TREATMENT ON STATIN MUSCLE TOXICITY: INITIAL CHARACTERIZATION OF A HUMAN MUSCLE CELL LINE

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1. INTRODUCTION

- Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis.
- They are used to treat hypercholesterolemia, a risk factor for cardiovascular disease.
- They are generally considered safe, but a small number of patients experience muscle toxicity; this is thought to occur due to the secondary effects of inhibiting HMGCR (Baker 2005).
- Studies so far have looked at the effect of temporal dosing on efficacy but not on muscle toxicity.
- We propose that by altering the time of day statins are taken, it may prove possible to improve separation between cholesterol lowering and unwanted muscle side effects.
- For this we have identified a human muscle cell line, the RD human rhabdomyosarcoma cell line, to use as a model. This cell line has previously been used for studying the myotoxic effects of statins (Kobayashi 2008).

2. AIMS

- To characterise the morphological and molecular changes that occur in RD muscle cells during the process of cell differentiation, so that we might then use them as a model.

3. METHODS

Figure 1: Differentiation protocol.

- At 2 day intervals cells were photographed and total RNA was extracted for analysis by quantitative RT-PCR (Tagman).

4. RESULTS

I. Muscle-Specific Markers

- Over time, there are an increasing number of myotubes (long, more muscle-like structures).
- Arrows indicate example myotubes; scale bar is approximately 50µm (Vachon et al. 1996).

- One of the four myogenic regulatory factors (MRFs), muscle-specific transcription factors that play an important role in myogenic differentiation.
- The 4-fold increase in MYOG expression observed between days 0 and 2 confirms that we are observing RD cell differentiation.

- Like myogenin, MYOD1 is an MRF which plays an important role in myogenic differentiation.
- However, unlike for MYOG no significant increase in transcript level is seen early on in the differentiation process.

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- Receptor involved in uptake of cholesterol in the form of LDL into cells.

II. Cholesterol Metabolism

- Catalyses the conversion of HMG-CoA to mevalonate, the rate limiting step in cholesterol biosynthesis.

III. Clock gene expression

- One of the key basic helix-loop-helix-PAS transcription factors involved in the mechanism of the core circadian clock.
- Present at a much lower level than the other transcripts.

- The Per genes are important components of the negative-feedback loop of the core circadian clock.

- The Cry genes, like the Per genes, are important components of the core clock.

- A transcription factor that may be directly regulated by the core circadian clock and modulates important clock output genes.

Figure 2: Differentiation of RD cells in culture: photographs showing morphology changes over the 10 day period, and changes in transcript levels as measured by quantitative RT-PCR (Tagman). Statistical significance determined by 1-way ANOVA followed by Bonferroni post hoc test: N = 4, error bars represent standard error of the mean. * = P < 0.05, ** = P < 0.01, *** = P < 0.001 compared to day 0

5. DISCUSSION & FUTURE WORK

- We have confirmed the expression of genes involved cholesterol metabolism and circadian rhythmicity, as well as a number of muscle-specific genes, in the RD muscle cell line.
- The expression of many of these genes does not significantly change during the differentiation process.
- However, a significant increase in MYOG expression was observed between days 0 and 2, confirming that cell differentiation was occurring.
- Other changes, such as the decrease in HMGCR and LDLR and increase in DBP expression after the full 10 days, may be explained by the continued proliferation of the cells after the change to differentiation media.
- We have characterised the changes that occur during RD cell differentiation and confirmed that these cells should be allowed to differentiate for 5-6 days in future experiments.

Future Work

- Western blotting to confirm these results at the protein level. In particular looking at MyoD1, for which gene expression does not appear to change as significantly as for MyoG.
- Further qPCR to look at changes in the expression of a late-differentiation gene, such as the myosin heavy chain.
- Using the serum pulse technique and further qPCR to look at temporal gene expression in RD cells and in the liver cell line, HepG2.

British Toxicology Society (BTS) Annual Congress 2010 – abstract

The Effects Of Time Of Treatment On Statin Muscle Toxicity: Preliminary Characterization Of A Human Muscle Cell Line

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Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis, and are used to treat hypercholesterolemia, a risk factor for cardiovascular disease. They are generally considered safe, but a small number of patients experience muscle toxicity; which is thought to occur due to the secondary effects of inhibiting HMGCR, for example the disruption of protein prenylation (Baker 2005). Studies so far have looked at the effect of temporal dosing on efficacy but not on muscle toxicity. Since muscle is known to also show circadian changes in gene expression in mice (McCarthy et al. 2007), we hypothesise that by altering the time of day statins are taken, it may prove possible to improve separation between cholesterol lowering and unwanted muscle side effects.

The RD human rhabdomyosarcoma cell line has previously been used for studying the myotoxic effects of statins (Kobayashi et al. 2008). In the current work, we first characterised the morphological and molecular changes that occur during RD cell differentiation. We have also begun to characterise the gene expression changes that occur across circadian time, initially focussing on clock genes (*Per2*, *BMAL1* and *CRY1*) and *HMGCR* and the LDL receptor. Preliminary statin dosing experiments were also carried out in order to determine the optimum statin dose for future experiments.

RD cells were grown to confluence in standard growth medium (DMEM containing 10% fetal bovine serum), before the initiation of differentiation by changing to low-serum medium (1%). At 48 hour intervals cells were imaged and total RNA extracted for analysis by quantitative RT-PCR (Taqman). Statistical significance was determined by one-way ANOVA followed by Bonferroni post hoc test.

Over differentiation time an increased number of long myotubes was observed in culture. This was accompanied by an observed 4-fold increase in the expression of the muscle regulatory factor myogenin, which is consistent with the occurrence of cell differentiation; this peaked at day five before decreasing. The expression of key cholesterol metabolism-related and clock genes in the RD cell line was also examined, showing steady expression of *HMGCR*, the LDL receptor (*LDLR*), and clock genes *PER2*, *BMAL1* and *CRY1*. In light of the results, it was decided to use a period of 5 days for RD cell differentiation prior to future experiments.

3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assays were also carried out, in order to assess the effect of simvastatin on cell viability. RD cells were seeded at a density of 2×10^3 cells per well on 96-well plates and left to differentiate for 5 days in low-serum medium. Various concentrations of simvastatin were then added, in a total of 100µl serum-free medium per well. For the final 2.5 hours of the 48 hour dosing period 10µl of PBS-containing MTT solution (5mg/mL) was added to the medium. The MTT medium was then replaced with 100µl dimethylsulfoxide and the absorbance read at 540nm. Absorbance measured was expressed as a percentage of the control.

The observed IC₅₀ value of simvastatin on the growth of differentiating RD muscle cells (0.58 µM)

was in a similar range to values seen in the literature (Kobayashi et al. 2008). However, further assays using a larger range of concentrations are needed to confirm this.

References: Baker, S.K., 2005 Muscle Nerve 31, 572-580
Kobayashi, M. *et al.* 2008 Life Sci. 82, 969-975
McCarthy, J.J. *et al.* 2007 Physiol Genomics 31, 86-95

Funding from The British Toxicology Society and The University of Surrey’s Centre for Toxicology.

University of Surrey FHMS Annual Festival of Research 2010 - abstract

Title THE EFFECTS OF TIME OF TREATMENT ON STATIN MUSCLE TOXICITY: PRELIMINARY CHARACTERIZATION OF A HUMAN MUSCLE CELL LINE
Authors Rowena Gee, Nick Plant, Jonathan Johnston, Kate Plant
Group Biochemical Sciences Division: Drug Design, Cancer & Toxicity and Sleep, Chronobiology & Neurodisorders themes.

Abstract Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis, and are used to treat hypercholesterolemia, a known risk factor for cardiovascular disease. They are generally considered safe, but a small number of patients experience muscle toxicity; which is thought to occur due to the secondary effects of inhibiting HMGCR, for example the disruption of protein prenylation.

Daily circadian rhythms are known to regulate a multitude of biological processes, and many aspects of cholesterol synthesis and metabolism have been shown to be under circadian control. Studies so far have looked at the effect of temporal dosing on statin efficacy but not on muscle toxicity. Since muscle is known to show circadian changes in gene expression in mice, we hypothesise that by altering the time of day statins are taken, it may be possible to improve separation between cholesterol lowering and unwanted muscle side effects.

The RD human rhabdomyosarcoma cell line has previously been used for studying the myotoxic effects of statins. In the current work, we first characterised the morphological and molecular changes that occur during RD cell differentiation. We have also begun to characterise the gene expression changes that occur across circadian time, in both RD cells and the human hepatoma cell line Huh7, initially focusing on clock genes (*PER2*, *BMAL1* and *CRY1*), *HMGCR* and *LDLR*. Preliminary statin dosing experiments were also carried out in order to determine the optimum statin dose for future experiments.

THE EFFECTS OF TIME OF TREATMENT ON STATIN MUSCLE TOXICITY: PRELIMINARY CHARACTERIZATION OF A HUMAN MUSCLE CELL LINE

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1. INTRODUCTION

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- They are generally considered safe, but a small number of patients experience muscle toxicity; this is thought to occur due to the secondary effects of inhibiting HMGCR (Baker 2005).
- Studies so far have looked at the effect of temporal dosing on efficacy but not on muscle toxicity.
- We propose that by altering the time of day statins are taken, it may prove possible to improve separation between cholesterol lowering and unwanted muscle side effects.
- For this we have identified a human muscle cell line, the RD human rhabdomyosarcoma cell line, to use as a model. This cell line has previously been used for studying the myotoxic effects of statins (Nishimoto et al. 2003; Kobayashi et al. 2006).

2. AIMS

- To characterise the morphological and molecular changes that occur in RD muscle cells during the process of cell differentiation, so that we might then use them as a model.
- To carry out preliminary statin dosing experiments, to determine the optimum dose for future experiments.

3. METHODS

4. RESULTS

1) Characterization of Muscle Differentiation

i. Muscle-Specific Markers

REFERENCES

Baker SK (2005) Muscle Nerve 31:572-80
Kobayashi M et al. (2006) Life Sciences 82: 969-975
Nishimoto T et al. (2003) Biochem Pharmacol 66(11): 2133-2139
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II) Characterization of Statin Toxicity

5. DISCUSSION & FUTURE WORK

- We have confirmed the expression of genes involved cholesterol metabolism and circadian rhythmicity, as well as a number of muscle-specific genes, in the RD muscle cell line.
 - Significant increases were observed in the expression of MYOG (between days 0 and 2) and in MYH3 (between days 0 and 4), confirming that cell differentiation was occurring.
 - Other changes, such as the decrease in HMGCR and LDLR after the full 10 days, may be explained by the continued proliferation of the cells after the change to differentiation media.
 - The expression of BMAL1 and PER2 genes involved in the core circadian clock, does not significantly change during the differentiation process.
 - We have characterised the changes that occur during RD cell differentiation and based on these results we have determined that RD cells should be allowed to differentiate for 5-6 days in future experiments.
 - Using MTT assays we have also started to characterise statin toxicity in HepG2 and RD cells, with differentiated RD cells showing greater sensitivity to simvastatin than either undifferentiated RD cells or HepG2 cells.
- Future Work
- Western blotting to confirm these results at the protein level.
 - Finish characterization of statin toxicity in RD and HepG2 cells.
 - Using the serum pulse technique and further qPCR to look at temporal gene expression in RD cells and in the liver cell line, HepG2.

British Toxicology Society (BTS) Annual Congress 2011 - abstract

Differential Sensitivity to Toxicity of Statins: A Comparison Of Liver And Muscle Cell Lines

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Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis. They are widely used to treat hypercholesterolemia, a risk factor for cardiovascular disease. Whereas they are generally considered safe, muscular toxicity is a common side effect, thought to occur due to the secondary effects of inhibiting HMGCR, such as disruption of protein prenylation (Baker 2005).

Whereas previous studies have investigated the effect of statins on cholesterol metabolism in liver cells, few studies have investigated this effect in skeletal muscle, the major site of toxicity (Mullen 2010). Through the use of two human cell lines we have undertaken a comparison of the effects of a variety of statins on cholesterol metabolism in liver and muscle. We hypothesise that differences observed between these cell lines may help to further elucidate the mechanism for statin-induced muscle toxicity. First, the RD human rhabdomyosarcoma cell line was chosen for its skeletal muscle phenotype, and its previous use for studying the myotoxic effects of statins (Kobayashi et al. 2008); second, the Huh7 hepatocellular carcinoma cell line was used to represent human liver parenchyma.

Initially, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assays were carried out, assessing metabolic and cellular viability. RD_{diff} cells were seeded on 96-well plates and left to differentiate for 5 days in low-serum medium, while Huh7 and RD_{undiff} cells were seeded 24 hours prior to experiment. Cells were exposed to various statin concentrations for 48 hours in serum-free medium, with MTT-containing PBS added for the final 2.5 hours. Media was replaced with DMSO and MTT absorbance read at 540nm. Absorbance was expressed as a percentage of the control, and values plotted as mean $\pm$ SEM of three independent experiments, where N=6 in each experiment. Statistical significance of differences in observed IC50 values determined by unpaired t-test.

We observed statistically significant reductions in cell viability for all three cell types, Huh7; RD_{undiff} and RD_{diff}, on treatment with simvastatin (Na), simvastatin (lactone), lovastatin (Na), lovastatin (lactone), cerivastatin, fluvastatin, atorvastatin, rosuvastatin and pravastatin in a dose-dependent manner. The largest reductions in cell viability were observed with the most lipophilic statins: simvastatin (Na), lovastatin (Na) and cerivastatin, whereas significant loss of cell viability was only observed with very high concentrations of the hydrophilic pravastatin (1000 μ M and greater).

Higher cell toxicity was observed for all statins in the RD cells, either differentiated or undifferentiated, compared to the Huh7 cells. All observed IC50 values, that we have been able to determine, appear to be within a similar range of values as seen in the literature (Kobayashi et al. 2008). As one example, IC50 values in response to fluvastatin treatment were $8.51 \pm 5.49 \mu\text{M}$ (Huh7), $6.02 \pm 1.83 \mu\text{M}$ (RD_{undiff}) and $2.62 \pm 2.56 \mu\text{M}$ (RD_{diff}).

Taken together, these data represent the first comprehensive comparative analysis of statin toxicity in human liver and muscle cell lines. Such characterisation is an important validation step in the

characterisation of differences in the response of these cell lines to statins, and hence the elucidation of the full mechanism of toxicity.

References: Baker, S.K., 2005 Muscle Nerve 31, 572-580
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University of Surrey FHMS Annual Festival of Research 2011 - abstract

DIFFERENTIAL SENSITIVITY TO TOXICITY OF STATINS: A COMPARISON OF LIVER AND MUSCLE CELL LINES

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Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis. They are widely used to treat hypercholesterolemia, a risk factor for cardiovascular disease. Whereas they are generally considered safe, muscular toxicity is a common side effect, thought to occur due to the secondary effects of inhibiting HMGCR, such as disruption of protein prenylation.

Previous studies have investigated the effect of statins on cholesterol metabolism in liver cells, however few studies have investigated this effect in skeletal muscle, the major site of toxicity. Through the use of two human cell lines we have undertaken a comparison of the effects of a variety of statins on cholesterol metabolism in liver and muscle. We hypothesise that differences observed between these cell lines may help to further elucidate the mechanism for statin-induced muscle toxicity. The RD human rhabdomyosarcoma cell line was chosen for its skeletal muscle phenotype, and its previous use for studying the myotoxic effects of statins; the Huh7 hepatocellular carcinoma cell line was used to represent human liver.

Initially, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assays were carried out, assessing metabolic and cellular viability. We observed dose-dependent reductions in cell viability for all three cell types used, Huh7, RD_{undiff} and RD_{diff}, on treatment with simvastatin (Na), simvastatin (lactone), lovastatin (Na), lovastatin (lactone), cerivastatin, fluvastatin, atorvastatin, rosuvastatin and pravastatin. The largest reductions in cell viability were observed with the most lipophilic statins: simvastatin (Na), lovastatin (Na) and cerivastatin, whereas significant loss of cell viability was only observed with very high concentrations of the hydrophilic pravastatin (1000µM and greater). Higher cell toxicity was observed for all statins in the RD cells, either differentiated or undifferentiated, compared to the Huh7 cells. Taken together, these data represent the first comprehensive comparative analysis of statin toxicity in human liver and muscle cell lines.

Poster for BTS Annual Congress and FHMS Festival of Research 2011

DIFFERENTIAL SENSITIVITY TO TOXICITY OF STATINS: A COMPARISON OF LIVER AND MUSCLE CELL LINES

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1. INTRODUCTION

- Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis.
- They are used to treat hypercholesterolemia, a risk factor for cardiovascular disease.
- They are generally considered safe, but a small number of patients experience muscle toxicity; this is thought to occur due to the secondary effects of inhibiting HMGCR (Baker 2005).
- The majority of previous studies have looked at the effect of statins on cholesterol metabolism in liver, rather than also in skeletal muscle, the main site of toxicity (Mullen et al. 2010).
- Through the use of two human cell lines we have undertaken a comparison of the effects of a variety of statins on cholesterol metabolism in both liver and muscle. For this work we have chosen a human muscle cell line, the RD human rhabdomyosarcoma cell line, to use as a model. This cell line has a skeletal muscle phenotype and has previously been used for studying the myotoxic effects of statins (Nishimoto et al. 2003; Kobayashi et al. 2008). We have chosen the Huh7 human hepatocellular carcinoma cell line as our comparison liver cell line.
- Differences observed between the two cell lines may help to further elucidate the mechanism for statin-induced muscle toxicity.

3. METHODS

- MTT assays were carried out to assess the effects of a variety of statins on cell viability.
- The effect of statin treatment on total cellular cholesterol was measured to examine statin-mediated HMGCR inhibition. A concentration of 1 μ M was chosen based on the results of the MTT assays – at 1 μ M, cell death was not excessive but an effect could still be observed. Cholesterol was quantified using the Amplex Red Cholesterol Assay kit. See figure 1 for further details.

Figure 1: Experimental design for A) MTT assays and B) cholesterol assays

4. RESULTS

I) Characterization of Statin-Mediated Cell Toxicity

- Simvastatin and lovastatin are administered as inactive prodrugs (lactone form) which are then converted to the active open acid forms in the body. These are the most lipophilic of the statins, and therefore are thought to become most widely distributed non-specifically.
- Cerivastatin was withdrawn from the market in 2001, due to its high incidence of statin-induced muscle toxicity.
- Atorvastatin is the most commonly prescribed statin.
- Rosuvastatin and pravastatin are hydrophilic, and therefore the least likely to become distributed non-specifically.

Figure 2: MTT assays for cell viability. 48 hour statin treatment of Huh7 cells, undifferentiated RD cells (RD-U) and differentiated RD cells (RD-D). Graphs shown in decreasing order of statin lipophilicity. Final absorbance expressed as a percentage of the control (defined as 100%). Values plotted represent mean $\pm$ standard error of the mean (SEM) of at least three independent experiments, where N=6 in each experiment

II) Effect of Statins on Total Cellular Cholesterol

Figure 3: Effect of 1 μ M simvastatin (acid), cerivastatin or pravastatin on total cellular cholesterol content of Huh7 cells, undifferentiated RD cells (RD-U) and differentiated RD cells (RD-D) after exposure for 48 hours. Values plotted represent mean $\pm$ SEM from one experiment, where N=3. Statistical significance determined by unpaired t-test, * = $P < 0.05$, ** = $P < 0.01$ compared to relevant control.

5. DISCUSSION & FUTURE WORK

- This is the first comprehensive comparative analysis of statin toxicity in liver and muscle cell lines (figure 2).
- We observed a reduction in cell viability, in all three cell types, on treatment with all statins, in a dose-dependent manner.
- Greater toxicity was observed for all statins in the RD cells compared to Huh7 cells.
- The largest reductions in cell viability were observed on treatment with the most lipophilic statins: simvastatin (acid), lovastatin and cerivastatin.
- Significant loss of cell viability was only observed with very high concentrations of the hydrophilic pravastatin (1000 μ M and greater).
- We also observed decreases in total cholesterol on treatment of our cells with 1 μ M simvastatin and cerivastatin, but not with pravastatin (figure 3).

Future Work

- Measure total cellular cholesterol after exposure to higher concentrations of simvastatin (acid) and pravastatin, and using a number of other statins.
- Measure other cellular effects in response to statin exposure, including reduction in protein prenylation, to correlate this to the results from these analyses.

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British Toxicology Society (BTS) Annual Congress 2012
– abstract for oral presentation

Differential effects of statins on protein prenylation in liver and muscle cell lines

Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis, and are widely used to treat hypercholesterolemia, a risk factor for cardiovascular disease. Muscular toxicity is a side effect, thought to occur due to the secondary effects of inhibiting HMGCR, such as disruption of protein prenylation. Through the use of two human cell lines we have undertaken a comparison of the effects of statins on cell viability and protein prenylation in liver and muscle. We anticipate that differences observed may help to further elucidate the mechanism for statin-induced muscle toxicity.

Total protein extracted from cells treated with a range of simvastatin and cerivastatin concentrations was subjected to western blot analysis using the geranylgeranylated protein Rap1A as a marker of prenylation.

In both cell lines unprenylated Rap1A was observed following statin treatment; this was dose-dependent for both statins. Both cell lines were more sensitive to cerivastatin than simvastatin with a 10-fold difference in dose required to produce detectable levels of unprenylated Rap1A. On statin treatment, disruption of Rap1A prenylation occurred at a 10-fold lower concentration in muscle than in liver cells, both for cerivastatin (1 μ M and 10 μ M respectively) and simvastatin.

This data adds to our previous toxicity data, which showed that RD muscle cells were significantly more sensitive to both of these statins than Huh7 liver cells. This supports a mechanism whereby disruption in the intermediates in the cholesterol synthesis pathway, including the isoprenoids used in protein prenylation, is the primary cause of statin-induced muscle toxicity.

Joint 19th International Symposium on Microsomes and Drug Oxidations (MDO) and 12th European meeting of the International Society for the Study of Xenobiotics (ISSX) 2012 - abstract

The Muscle Cell Line RD Is More Sensitive To Statin-Mediated Disruption Of Protein Prenylation Than The Liver Cell Line Huh7: Implications for Statin-Mediated Toxicity

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Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis, and are widely used to treat hypercholesterolemia, a risk factor for cardiovascular disease. A common adverse effect associated with statin use is muscular aches, which can rarely progress to the potentially fatal rhabdomyolysis. The mechanism underlying this toxicity is still not fully understood, but secondary effects of HMGCR inhibition, such as disruption of protein prenylation, have been suggested as a likely mechanism. We have compared the effects of statins on both cell viability and protein prenylation in human liver and skeletal muscle cell lines, hypothesising that statins would preferentially disrupt prenylation within the muscle cells. Initially, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were carried out, in order to assess metabolic and cellular viability after cells were exposed to different statin concentrations. We observed significant reductions in cell viability for both cell lines, on treatment with simvastatin, cerivastatin and pravastatin in a dose-dependent manner. The largest reductions in cell viability were observed with the most lipophilic statins, simvastatin and cerivastatin, whereas significant loss of cell viability was only observed with very high concentrations of the hydrophilic pravastatin (1000 μ M and greater). Higher cell toxicity was observed for all statins in RD muscle cells compared to Huh7 liver cells. Subsequently, western blot analysis was used to examine the prenylation status of the geranylgeranylated protein Rap1A in total protein extracts from cells treated with a range of simvastatin and cerivastatin concentrations. Unprenylated Rap1A was observed in both cell lines, in a dose-dependent manner, following treatment with simvastatin and cerivastatin. This prenylation deficiency was a specific effect resulting from statin inhibition of HMGCR since co-treatment with mevalonate, a downstream intermediate in the metabolic pathway, rescued the phenotype. Both cell lines were more sensitive to cerivastatin than simvastatin, with a 10-fold difference in dose required to produce detectable levels of unprenylated Rap1A. In addition, statin treatment resulted in disruption of Rap1A prenylation at a 10-fold lower concentration in muscle than in liver cells, both for simvastatin (1 μ M and 10 μ M respectively) and cerivastatin (0.1 μ M and 1 μ M respectively). Together these data confirm that RD muscle cells, which are significantly more sensitive to statin treatment, do show preferential disruption of protein prenylation when compared to liver cells. This supports a mechanism whereby disruption in the intermediates in the cholesterol synthesis pathway, including the isoprenoids used in protein prenylation, is the primary cause of statin-induced muscle toxicity.

University of Surrey FHMS Annual Festival of Research 2012 – abstract

DIFFERENTIAL EFFECTS OF STATINS ON PROTEIN PRENYLATION IN LIVER AND MUSCLE CELL LINES

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Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis, and are widely used to treat hypercholesterolemia, a risk factor for cardiovascular disease. Muscular toxicity is a side effect, thought to occur due to the secondary effects of inhibiting HMGCR, such as disruption of protein prenylation. We have compared the effects of statins on both cell viability and protein prenylation in human liver and skeletal muscle cell lines, hypothesising that statins would preferentially disrupt prenylation within the muscle cells.

Total protein extracted from cells treated with a range of simvastatin and cerivastatin concentrations was subjected to western blot analysis using the geranylgeranylated protein Rap1A as a marker of prenylation.

In both cell lines unprenylated Rap1A was observed following statin treatment; this was dose-dependent for both statins. Both cell lines were more sensitive to cerivastatin than simvastatin with a 10-fold difference in dose required to produce detectable levels of unprenylated Rap1A. On statin treatment, disruption of Rap1A prenylation occurred at a 10-fold lower concentration in muscle than in liver cells, both for simvastatin (1 μ M and 10 μ M respectively) and cerivastatin (0.1 μ M and 1 μ M respectively).

These data add to our previous toxicity data, which showed that RD muscle cells were significantly more sensitive to both of these statins than Huh7 liver cells, and confirm that RD muscle cells do show preferential disruption of protein prenylation when compared to liver cells. This supports a mechanism whereby disruption in the intermediates in the cholesterol synthesis pathway, including the isoprenoids used in protein prenylation, is the primary cause of statin-induced muscle toxicity.

The Muscle Cell Line RD Is More Sensitive To Statin-Mediated Disruption Of Protein Prenylation Than The Liver Cell Line Huh7: Implications For Statin-Mediated Toxicity

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1. Introduction

- Statins are widely used to treat hypercholesterolemia, a risk factor for cardiovascular disease.
- They inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis.
- A common adverse effect associated with statin use is muscular aches, which can rarely progress to the potentially fatal rhabdomyolysis.
- The mechanism underlying this toxicity is still not fully understood, but secondary effects of HMGCR inhibition, such as disruption of protein prenylation, have been suggested as a likely mechanism.

2. Hypothesis and Objectives

Hypothesis: muscle cells show greater sensitivity to both statin toxicity and disruption of protein prenylation than liver cells.

Objectives:

- To use Huh7 human hepatocellular carcinoma cells and RD human rhabdomyosarcoma cells (undifferentiated and differentiated) in culture as a model system for comparing the effects of a variety of cholesterol-lowering statins on protein prenylation in both liver and skeletal muscle, where the liver is the target organ of the statins and skeletal muscle is the site of toxicity.
- MTT assays were carried out to assess the effects of a variety of statins on cell viability in both liver and muscle cells.
- Western blot analysis was used to examine the prenylation status of geranylgeranylated protein Rap1A on statin treatment, in both liver and muscle cells, and therefore to assess whether differences in prenylation could account for differences in toxicity.

3. Results

I) Characterization Of Statin-Mediated Cell Toxicity

- Simvastatin is one of the most lipophilic statins and is thought to become distributed to a wide variety of tissues via passive diffusion. Simvastatin also remains one of the most frequently prescribed statins in the UK
- Cerivastatin was withdrawn from the worldwide market in 2001, due to its high incidence of statin-induced muscle toxicity.
- Pravastatin is hydrophilic, and therefore the least likely to become widely distributed. Pravastatin has also shown much lower levels of muscle toxicity in patients in the clinic.

Figure 1: Cell viability in response to statin treatment
Huh7 cells, undifferentiated RD cells (RD-U) and differentiated RD cells (RD-D) were treated with statin for 48 hours and MTT assays were carried out to measure cell viability. Final absorbance is expressed as a percentage of the control (defined as 100%). Values plotted represent mean $\pm$ standard error of the mean (SEM) of at least three independent experiments, where 6 replicates were used in each experiment.

II) Effects of Statins On Prenylation

- **Rap1A** is a small GTPase that undergoes geranylgeranylation.
- Its prenylation status can be determined using a combination of antibodies.
Rap1A – detects the unprenylated protein
Rap1 – detects total protein
Beta-actin – used as a loading control

Figure 2: Effect of statin treatment on Rap1A prenylation
Total protein was extracted from Huh7, RD-U and RD-D cells treated for 48 hours with vehicle control, statin, or mevalonic acid in addition to statin. Simvastatin and pravastatin were used at a concentration of 10 μ M and cerivastatin at 1 μ M. DMSO was used as a vehicle control for simvastatin and water for cerivastatin (and pravastatin). Mevalonic acid was used in excess, at 100 μ M. Western blots are representative of three independent experiments.

Figure 3: Dose-dependent effects of statin treatment on Rap1A prenylation
Total protein was extracted from cells treated for 48 hours with vehicle control or a range of statin concentrations.

4. Conclusions

i) Statin treatment leads to differential cell toxicity

- Statin treatment led to a reduction in cell viability, in all cell types, in a dose-dependent manner (Figure 1).
- The largest reductions in cell viability were observed on treatment with lipophilic simvastatin and cerivastatin, with loss of cell viability observed only with very high concentrations of hydrophilic pravastatin (1000 μ M and greater).
- Greater toxicity was observed for all statins in RD muscle cells compared to Huh7 liver cells; this difference was statistically significant with simvastatin and cerivastatin treatment ($P < 0.0001$, two-way ANOVA). This is consistent with the observation of skeletal muscle toxicity as a well known adverse effect of the statins in the clinic.

ii) Statin treatment leads to differential disruption of protein prenylation

- Simvastatin and cerivastatin treatment led to detection of unprenylated Rap1A (Figure 2).
- This was not due to a change in levels of total Rap1 protein and co-treatment with mevalonate rescued this change in prenylation status.
- Muscle and liver cells were both more sensitive to cerivastatin than simvastatin (Figure 3).
- Lower concentrations of both simvastatin and cerivastatin were needed to disrupt protein prenylation in muscle compared to liver cells.

Together these data confirm that RD muscle cells, which are significantly more sensitive to statin treatment, do show preferential disruption of protein prenylation when compared to liver cells. This supports a mechanism whereby disruption in the intermediates in the cholesterol synthesis pathway, including the isoprenoids used in protein prenylation, is the primary cause of statin-induced muscle toxicity.