Université de Montréal

Inhibition of the lactic acid transporters MCT1 and MCT4 as an underlying mechanism for drug-induced myopathy

par Yat Hei (Henry) Leung

Faculté de pharmacie

Thèse présentée
en vue de l'obtention du grade de doctorat
en Sciences Pharmaceutiques
option Pharmacologie

Décembre, 2017

© Yat Hei Leung, 2017

Résumé

Les myopathies induites par les médicaments représentent un effet secondaire sérieux causé par plusieurs médicaments. Ces symptômes musculaires varient de myalgies légères avec ou sans élévation de créatine kinase, faiblesse musculaire, myosite, jusqu'à de rares rhabdomyolyses potentiellement mortelles. Bien que les myalgies légères soient tolérables, les myopathies chroniques affectent la qualité de vie des patients, requérant souvent la cessation d'une thérapie efficace.

Le mécanisme sous-jacent à ces myotoxicités causées par les médicaments est connu pour certains composés, mais demeure obscur pour plusieurs (ex. statines). Les statines constituent une thérapie efficace pour la diminution du cholestérol, mais elles sont reconnues pour causer ces effets secondaires. De nombreux facteurs augmentant les concentrations plasmatiques de statines (ex. doses élevées, interactions médicamenteuses, polymorphismes génétiques) semblent être liés à une fréquence de myotoxicité plus élevée. Conséquemment, le métabolisme et le transport des médicaments, contrôlant l'absorption globale, la distribution et l'élimination, peuvent devenir importants. Cependant, ces facteurs peuvent seulement expliquer partiellement les désordres musculaires observés.

Même si plusieurs mécanismes sont proposés pour les myotoxicités induites par les statines, le mécanisme exact responsable de cet effet est controversé, puisque les études rapportent des résultats contradictoires. Puisque l'exercice semble exacerber les douleurs musculaires chez les patients prenant des statines, l'hypothèse derrière ce projet est que le transport de l'acide L-lactique par les transporteurs de monocarboxylates serait impliqué dans le développement des myotoxicités. Puisque l'acide L-lactique est l'un des sous-produits

majeurs résultant de l'activité physique, son élimination efficace des cellules musculaires est essentielle. L'administration de médicaments inhibant compétitivement ces transporteurs pourrait mener à une perturbation de l'homéostasie de l'acide L-lactique et à des désordres musculaires.

L'objectif du premier volet de cette étude était d'évaluer le potentiel d'inhibition des médicaments acides sur le transport d'acide L-lactique en utilisant les lignées cellulaires cancéreuses Hs578T et MDA-MB-231 exprimant sélectivement MCT1 ou MCT4, respectivement. Ces lignées cellulaires ont permis la caractérisation des transporteurs avec la détermination de leurs paramètres cinétiques et d'inhibition. Le but principal du deuxième volet de cette étude était de confirmer le potentiel d'inhibition de l'atorvastatine, simvastatine, rosuvastatine et loratadine sur le transport d'acide L-lactique dans un cadre plus physiologique en utilisant des cellules musculaires squelettiques primaires humaines (SkMC). L'objectif global de ce projet de doctorat était de mieux comprendre les mécanismes derrière certaines myopathies induites par les médicaments, plus spécifiquement celles induites par les statines et la loratadine, en étudiant les transporteurs de monocarboxylates impliqués dans le transport d'acide L-lactique et l'homéostasie du pH dans le muscle.

La loratadine et l'atorvastatine ont démontré le meilleur potentiel d'inhibition de l'efflux d'acide L-lactique dans les lignées cellulaires, une observation confirmée dans les SkMC. Cette inhibition pourrait causer une accumulation intracellulaire d'acide L-lactique menant à une acidification et à des désordres musculaires.

De futures études dans des modèles *in vivo* sont requises pour confirmer l'impact physiologique de nos résultats dans un cadre clinique. Ces données permettraient une

meilleure compréhension des myopathies induites par les statines et la loratadine et permettront ainsi de prévenir leur occurrence en optimisant les stratégies thérapeutiques.

Mots-clés : Acide lactique, transporteurs de monocarboxylates, statines, transporteurs de médicaments, myopathie induite par les médicaments, muscle, effets indésirables, loratadine, cholestérol.

Abstract

Drug-induced myopathy is a serious side effect caused by various widely-administered medications. These muscle-related symptoms range from mild myalgia with or without creatine kinase increase, muscle weakness, myositis, to rare life-threatening rhabdomyolysis. While mild myalgias can be tolerable, chronic myopathies can affect the patients' quality of life, frequently requiring the cessation of an effective drug.

The underlying mechanism of these drug-induced myotoxicities is known for some drugs but remains unclear for most (*e.g.* statins). Statins constitute an effective cholesterol-lowering therapy, but they are known to cause these adverse drug reactions. Various factors increasing statin plasma levels (*e.g.* high doses, drug-drug interactions, genetic polymorphisms) seem to be linked with a higher occurrence of myotoxicity. Consequently, systemic drug metabolism and transport, controlling overall absorption, distribution and elimination, can become important. However, these factors only partly explain the observed muscular disorders.

Although there are several proposed mechanisms for statin-induced myotoxicity, the exact mechanism responsible for this effect is still debated with studies reporting conflicting results. Since exercise seems to exacerbate muscle pain in patients under statin treatment, the premise of this project is that L-lactic acid transport *via* the monocarboxylate transporters is involved in the development of drug-induced myopathy. Since lactic acid is one of the major byproducts resulting from physical activity, its efficient removal from the muscle cells is essential. Therefore, the administration of drugs competitively inhibiting those transporters may potentially lead to perturbation of L-lactic acid homeostasis and muscular disorders.

The aim of the first part of this study was to assess the inhibitory potential of acidic drugs on L-lactic acid transport using breast cancer cell lines Hs578T and MDA-MB-231, which selectively express MCT1 or MCT4, respectively. These cell lines allowed transporter characterization with the determination of their kinetic parameters and inhibition. The main objective of the second part of this study was to confirm the inhibitory potentials of atorvastatin, simvastatin, rosuvastatin and loratadine on L-lactic acid transport in a more physiological setting using primary human skeletal muscle cells (SkMC). The overall goal of this doctoral project was to better understand the mechanisms behind certain drug-induced myopathies, more specifically those induced by statins and loratadine, by studying monocarboxylate transporters involved in lactic acid transport and pH homeostasis in the muscle.

Loratadine and atorvastatin demonstrated the greatest potency for inhibition of L-lactic acid efflux first in cancer cell lines, an observation confirmed in SkMC. This inhibition may cause an accumulation of intracellular L-lactic acid leading to acidification and muscular disorders.

Further studies with *in vivo* models are required to confirm the physiological impact of our findings in a clinical setting. These data will help understand statin- and loratadine-induced myopathy and prevent its occurrence by optimizing treatment strategies.

Keywords: Lactic acid, monocarboxylate transporters, statins, drug transporters, drug-induced myopathy, muscle, adverse events, loratadine, cholesterol.

Table of contents

Résumé i

Abstract iv

Table of contents vi

List of tables ix

List of figures x

List of abbreviations xi

Acknowledgements xiv

Chapter 1 – Introduction 1

1. Introduction 2

1.1 Adverse drug reaction 3

1.1.1 Drug-induced myopathy 4

1.2 Drugs that can induce myopathy 5

1.2.1 Statins 6

1.2.1.1 Cardiovascular disease: risk factors, hypercholesterolemia and treatments 6

1.2.1.2 Statin characteristics 10

1.2.3 Other drugs that can induce myopathy 17

1.2.3.1 Loratadine 20

1.3 Mechanisms of drug-induced myotoxicities 22

1.3.1 Depletion of cholesterol, isoprenoids and coenzyme Q10 23

1.3.2 Disturbed calcium metabolism 24

1.3.3 Autoimmune 24

1.3.4 Drug-drug interactions 25

1.3.5 Genetic polymorphisms 26

1.3.6 Inhibition of lactic acid efflux by MCTs and drug-induced myopathy 26

1.3.6.1 Muscle and lactic acid 27

1.4 Drug transporters....30
1.4.1 ABC transporters....31
1.4.1.1 ABCB1....32
1.4.1.2 ABCC....33
1.4.1.3 ABCG2....33
1.4.2 SLC transporters....34
1.4.2.1 SLCO transporters....34
1.4.2.1.1 SLCO1B1....35
1.4.2.1.2 SLCO1B3....36
1.4.2.1.3 SLCO2B1....36
1.4.2.2 SLC22 transporters....37
1.4.2.3 Monocarboxylate transporters....37
1.4.2.3.1 MCT1....41
1.4.2.3.2 MCT4....44
1.4.2.3.3 Other MCT....45
1.4.3 Statin transporters....46
1.4.3.1 Intestinal and hepatic transporters....46
1.4.3.2 Muscular transporters....47
1.5 Rationale, hypothesis and objectives....48
Chapter 2- Articles....51
2.1 Article 1: Effects of a series of acidic drugs on L-lactic acid transport by the monocarboxylate transporters MCT1 and MCT4....52
2.1.1 Introduction....52
2.1.2 Objectives....53
2.1.3 Article....54
2.1.4 Discussion....87
2.1.5 Author contributions....88
2.2 Article 2: Statins and loratadine-induced muscle pain in human skeletal muscle cells....89
2.2.1 Introduction....89

2.2.2 Objectives 90
2.2.3 Article 91
2.2.4 Discussion 127
2.2.5 Author contributions 128
Chapter 3 - Discussion and Conclusion 129
3.1 General discussion 130
3.2 Conclusion 139
Bibliography i

List of tables

Table I. The pharmacodynamic properties of statins 13

Table II. The physicochemical and pharmacological properties of statins 14

Table III. Potential myopathy-inducing drugs 19

Table IV. Examples of H1-antihistamine from different generations 21

Table V. MCT1/4 substrates and inhibitors 40

Table VI. Kinetic parameters of human MCT 41

Table VII. MCT1 and MCT4 K_m values from different studies 133

Table VIII. IC50 values of statins for L-lactic acid MCT4 transport in different studies 134

List of figures

Figure 1. Bioabsorption pathway of cholesterol from diet 8
Figure 2. Cholesterol homeostasis 9
Figure 3. Biosynthesis pathway of cholesterol and its pharmaceutical inhibitors 12
Figure 4. The structures of the statins that have been evaluated 15
Figure 5. Loratadine structure 20
Figure 6. Glycolysis pathway. 29
Figure 7. Protein structure of MCT1 under two conformational states 39
Figure 8. ABC and SLC statin transporters in muscle cells. 48

List of abbreviations

ABC: ATP-binding cassette transporters

ACE: Angiotensin-converting enzyme

ACEi: Angiotensin-converting enzyme inhibitors

ADR: Adverse drug reaction

AP1/2: Activator protein 1/2

ARB: Angiotensin-II receptor blockers

ATP: Adenosine triphosphate

BCRP: Breast cancer resistance protein

CL_{int}: Intrinsic clearance

CNT: Concentrative nucleoside transporter

CVD: Cardiovascular disease

CK: Creatine phosphokinase

CYP450: Cytochrome P450

DNA: Deoxyribonucleic acid

ENT: Equilibrative nucleoside transporter

GI: Gastrointestinal

GWAS: Genome-wide association study

HDL: High-density lipoprotein

HDL-C: High-density lipoprotein cholesterol

HIF-1α: Hypoxia-inducible factor 1α

HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A

HMGCR: HMG-CoA reductase

HPLC: High performance liquid chromatography

IGF-I: Insulin-like growth factor I

K_i: Inhibition constant

K_m: Michaelis-Menten constant

LDL: Low-density lipoprotein

LDL-C: Low-density lipoprotein cholesterol

logD: Log_{10}(coefficient of distribution)

logP: Log_{10}(coefficient of partition)

MCT: Monocarboxylate transporter

MDR: Multidrug resistance protein

mRNA: Messenger ribonucleic acid

MRP: Multidrug resistance-associated protein

NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NSAID: Non-steroidal anti-inflammatory drug

OAT: Organic anion transporter

OATP: Organic anion transporter polypeptide

OCT: Organic cation transporter

OCTN: Organic cation/carnitine transporter

PEPT: Peptide transporter

P-gp: P-glycoprotein

RCT: Randomized controlled trial

RR: Relative risk

S.D.: Standard deviation

SkMC: Skeletal muscle cells

SLC: Solute carrier

SMCT: Sodium-coupled monocarboxylate transporter

SNP: Single nucleotide polymorphism

SP1: Stimulating protein 1

TC: Total cholesterol

TG: Triglycerides

ULN: Upper limit of normal

USF: Upstream stimulatory factors

UV: Ultraviolet

To my family and friends,

Whose support got me through the daily struggles

Acknowledgements

I would like to thank Dr Jacques Turgeon for accepting me in his lab for my masters' degree. At that point, I just wanted to get higher education to get a better quality of life. It was Dr Turgeon who convinced me that I can achieve more and be the best in whatever field of study I decided to pursue in. Thank you for giving me the opportunity to fast-track my project to a doctorate degree. To follow that, I would like to thank my co-director Dr Véronique Michaud for taking over the supervision of my project and supporting me, when Dr Turgeon had less time due to his administrative duty. Thank you to both of my directors for believing in me and having given me the opportunity to grow as an independent researcher.

I would like to thank my committee members for watching over my development as a scientist and giving me their advice and opinions on my project. I would like to thank the Faculty of Pharmacy for the financial and administrative support they provided throughout my studies.

Thank you to my evaluation committee for taking the time to read and give their comments on my thesis.

Thank you to the FRQS for their financial support during the majority of my studies, so that I can focus all my energy on my research without worrying too much about my finances.

Throughout my years in the lab, many people have not only helped me for my experiments, but also making my time there an overall good experience. The different lab members that I would like to acknowledge are, in order of appearance: Catherine Armstrong, François Bélanger, Jennifer Lu, Fleur Gaudette, Jade Huguet, Alexia Grangeon, Liliam Moya, Marie-Ève Papillon, Sophie Gravel, Nour Ghazal, Roxanne Pelletier, Sarah Maximos, Michel Chamoun, Charles Homsi, Valérie Clermont, and various summer students. I would also like to thank the other students of the 8th floor of CRCHUM who brought me some good laughs over the past few years.

Finally, I thank my family and friends for being there throughout this journey and supporting my career choice.

Chapter 1 – Introduction

1. Introduction

Muscle pain and weakness, defined as myopathy, can be associated with drug administration. Drug-induced myopathy is common with widely-used medications.[1] However, there was an increase of these drug-associated toxicities since the introduction of HMG-CoA reductase inhibitors (statins) for lipid management in patients suffering from cardiovascular disease.[2]

Statin treatment is highly prescribed for its effectiveness in decreasing low-density lipoprotein cholesterol (LDL-C) plasma levels.[3] Statins are well tolerated but the muscle symptoms associated with their use can limit adhesion to treatment or even lead to drug discontinuation.[4] The definitive mechanism of statin-induced muscle disorders is still not known. High doses and the presence of drug-drug interactions or genetic polymorphisms increasing statin plasma levels seem to be linked with a higher occurrence of myotoxicity.[5, 6] Consequently, systemic drug metabolism and transport, controlling overall absorption, distribution and elimination, can become important.[7] However, these factors only partly explain the observed muscular disorders, since only 10-15% of patients under statin treatment are affected by myotoxicity.

Therefore, it has been postulated that the tissue-specific local (muscle) drug absorption facilitated by membrane drug transporters is determinant for the occurrence of muscular events. Relevant drug transporters affecting systemic concentrations, and particularly those that can affect local concentrations, will be discussed below.

Exercise seems to exacerbate muscle pain in patients under statin treatment who are physically active.[8, 9] Since lactic acid is one of the major byproducts resulting from physical

activity, its efficient removal from the muscle cells is essential.[10] Thus, in this doctoral project, we will attempt to better understand the mechanisms behind certain drug-induced myopathies, more specifically those induced by statins and loratadine (over-the-counter antihistamine commonly used to relieve allergies) by studying monocarboxylate transporters (MCT) involved in lactic acid transport and pH homeostasis in the muscle. Our work will be focused on the interactions between different drugs and these MCT in different *in vitro* models.

1.1 Adverse drug reaction

Adverse drug reactions (ADR) are a substantial public health concern. In the 2014 report from the Center for Disease Control and Prevention, it was found that unintentional injuries were the 4th cause of death in the United States.[11] These included unintentional motor transport-related injuries, unintentional poisonings, firearms and falls. The ADR-related death rate has been increasing over the years, and in 2011, it has actually surpassed the motor vehicle traffic-related injuries.[11]

Results from a meta-analysis showed that adverse reactions (including non-serious, serious, and fatal reactions) related to a medication significantly affect around 15.1% of the hospitalized patients.[12] Since it is estimated that 6.5% of hospital admissions are related to ADR, it is important to comprehend the underlying mechanism of these adverse events.[13]

Many factors can contribute to this situation, such as the aging population, which is subject to comorbidities, polypharmarcy to treat these conditions, drug-drug interactions, and the interindividual genetic variability modulating the pharmacokinetics and pharmacodynamics of drugs inside the organism.[14, 15]

The subpopulation that is most affected by these adverse effects are the 10 million Canadians and the 107 million Americans suffering from cardiovascular diseases (CVD).[16] These pathologies are often associated with a great number of comorbidities which require the administration of a multitude of medications.[17] Treatments for CVD comprise various therapeutic approaches, including healthy diet and exercise, but they rely mainly on drugs that can effectively manage dyslipidemia.[4] The treatment of cardiovascular diseases and their comorbidities is associated with a significant interindividual variability, where some patients are resistant to the treatments, while others are over-sensitive to some drugs. Therefore, the management of therapeutics can become complex and would require various dose adjustments, increasing or decreasing depending on the medications. In 2001, there was raised awareness on the potential toxicity associated with the use of lipid-lowering agents, since cerivastatin was withdrawn from the market.[4] Cerivastatin was found to be associated with severe muscle toxicity and rhabdomyolysis causing 52 deaths.[4] Nevertheless, these cases were caused by higher doses or having it used concomitantly with gemfibrozil (same drug-metabolizing enzymes; CYP2C8), increasing plasma levels of cerivastatin.[18]

1.1.1 Drug-induced myopathy

Myopathy accounts for approximately 20% of common reasons for general practice consultations.[19, 20] Muscle pain is not uncommon with widely-used medications.[21] Since many drugs can cause musculoskeletal symptoms, patients presenting with these drug-induced myopathies should always be classified by differential diagnosis in order to separate them from the rest of the musculoskeletal disorders.[20] These muscle injuries can be produced in several different ways, such as direct toxicity (main cause), which is often dose-dependent, or indirect muscle-damaging effects such as electrolyte disturbances, excessive energy

requirements, inadequate delivery of energy or oxygen, or *via* immunological reaction.[20] Drug-induced myotoxicities can be classified by the absence or presence of muscle pain, including asymptomatic creatine phosphokinase (CK) elevation, mild to severe myalgias, cramps, exercise intolerance, muscle weakness, severe myositis, and rhabdomyolysis.[21]

While mild myalgias are relatively tolerable, chronic myopathies can affect quality of life. Therefore, an early recognition of these ADR is really important for patients as most of these effects are partially or completely reversible with a dose adjustment or drug substitution.[22] However, many of these drug-induced myopathies are observed in the context of a drug-drug interaction, which can complicate their diagnosis since they are not always related to one single agent.[22] Moreover, milder symptoms such as myalgia and muscular fatigue are not frequently reported by physicians, complicating the estimation of the actual incidence of these adverse events.[6]

Drug-induced myotoxicities are rare forms of ADR. The mechanisms by which they are caused are still relatively unknown. Some classes of drugs are more frequently associated with these adverse drug events, such as antifungal agents, antimalarials, antiviral agents, cardiovascular agents, corticosteroids, immunosuppressants and lipid-lowering agents (statins and fibrates).[1, 23, 24] In this doctoral thesis, we will be focusing mainly on the effects of statin, loratadine and other acidic drugs administration, to determine if myopathies observed with those drugs are generated through the same mechanisms.

1.2 Drugs that can induce myopathy

As previously stated, there are many drug classes that can cause myotoxicity, but this project will be focused on statin-induced myopathy.

1.2.1 Statins

1.2.1.1 Cardiovascular disease: risk factors, hypercholesterolemia and treatments

Coronary heart disease is the leading cause of death in the world.[11] About 610 000 Americans die of heart disease every year, which represents approximately 1 in 4 deaths. On top of having the highest mortality rate, CVD represents a major economic burden for the health care system, with an approximate $207 billion yearly expense (services, medication, loss of productivity).[11, 25]

High blood pressure, smoking and high levels of LDL-C constitute the three main risk factors for the development of CVD.[26, 27] In fact, about 33.5 million adults (16.2%) have high serum cholesterol levels (>240 mg/dL) in the United States.[28] It was also reported by the American Heart Association that less than half of the patients who should take lipid-lowering treatments actually adhere to their therapy.[28] Other contributing risk factors would be diabetes, obesity, poor diet, physical inactivity and excessive alcohol intake.[25] Even though CVD encompass a wide spectrum of diseases, this project will address mainly drugs used to manage LDL-C levels.

Cholesterol is either absorbed from the diet or synthesized endogenously in small quantities.[29] The average human consumes around 70-100 g of lipids every day, most of which are triglycerides. Pancreatic lipase is implicated in fat digestion in the small intestine and acts on the surface of lipid droplets.[29] Fat absorption is enhanced by its emulsification, which is obtained *via* mechanical disaggregation and emulsifying agents, such as phospholipids and biliary salts (amphipathic molecules derived from cholesterol).[29] Fatty acids and

monoglycerides are found in micelles and are absorbed by enterocytes.[29] However, those two compounds are used in cells to form triglycerides, maintaining a concentration gradient. Chylomicrons are formed inside of the cell and are composed of triglycerides, phospholipids, cholesterol and liposoluble vitamins.[29] Chylomicrons and very low-density lipoprotein (VLDL; produced from the liver) deliver triglycerides to cells throughout the body by having these triglycerides stripped by lipoprotein lipase. After the loss of these particles, VLDL becomes the denser LDL. LDL transports cholesterol to cells, which require cholesterol to function, by binding it with a specific LDL receptor. The high-density lipoprotein (HDL) precursor (synthesized in the liver and small intestine) collects excess cholesterol to form mature HDL and is brought back to the liver (reverse cholesterol transport).[29]

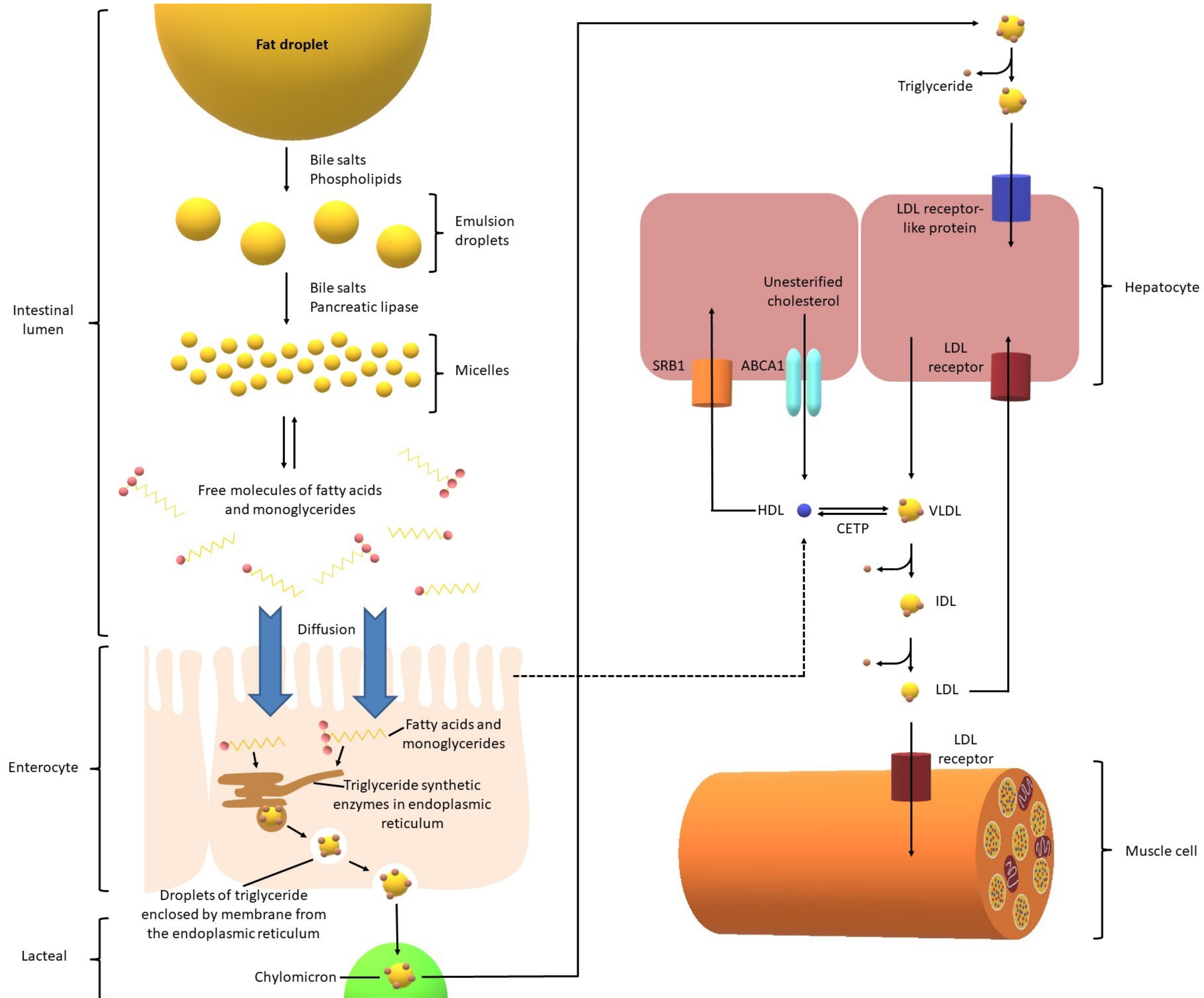


Figure 1. Bioabsorption pathway of cholesterol from diet
Figure adapted from Vander's Human Physiology- The Mechanisms of Body Function (2008)[29] and Charlton-Menys *et al*. (2008)[30]

Cholesterol is an important precursor in many biological structures, such as plasma membranes, biliary salts and steroid hormones.[29] However, high concentrations of cholesterol cause atherosclerosis, which leads to several CVD, such as myocardial infarction and cerebral vascular accidents.[29] Most cells use plasma cholesterol for their biological functions, whereas only hepatic and intestinal cells release cholesterol into the blood stream. Cholesterol is secreted in the bile by the liver, where part of it is reabsorbed as dietary cholesterol, and the other part is eliminated in the feces.[29] The liver also metabolizes a substantial amount of cholesterol into biliary salts, which are components of the bile helping with cholesterol absorption. Cholesterol levels are dictated by the liver, where high cholesterol concentrations inhibit the enzyme HMG-CoA reductase responsible for its own synthesis in a retrocontrolled manner. This inhibition is highly variable between individuals.[29]

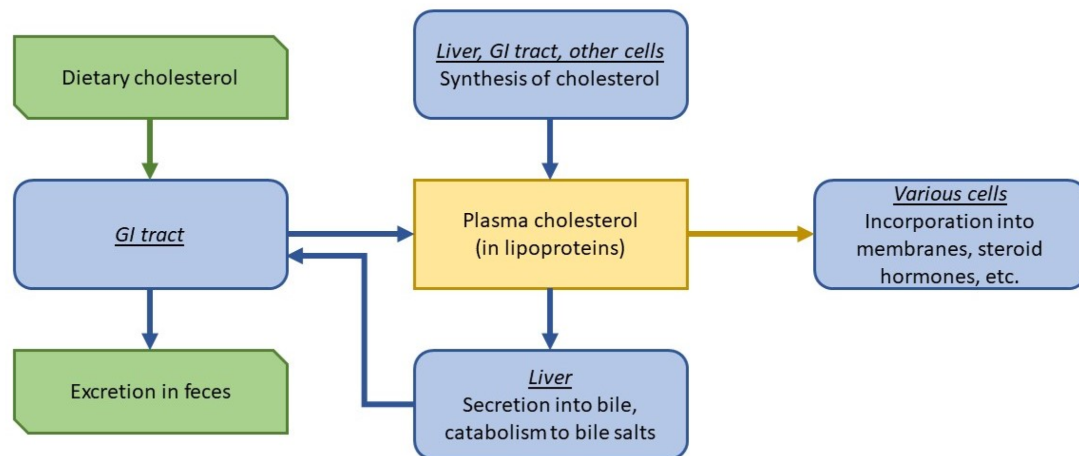


Figure 2. Cholesterol homeostasis
Figure adapted from Vander's Human Physiology- The Mechanisms of Body Function (2008)[29]

Recently, there is a novel drug class that targets the proprotein convertase subtilisin/kexin type 9 (PCSK9).[31] When PCSK9-bound LDL receptor binds to LDL, this

complex is ingested and degraded. Therefore, PCSK9 inhibition can block its ability to cause LDL receptor degradation and lower blood LDL-particle concentrations. However, most cholesterol lowering drugs target either biliary absorption (ezetimibe) or the hepatic cholesterol synthesis enzyme HMG-CoA reductase (statins).[29]

A meta-analysis regrouping 27 statin trials in patients at low risk of major vascular events found that the risk of these events decreased by 11 per 1000 over 5 years following every 1.0 mmol/L reduction in LDL-C levels.[32] This reduction of risk justifies the use of statins in lipid management. It was reported that patients using statins see their risk of heart attack decrease from 20 to 50%.[32]

An updated guideline from the US Preventive Services Task Force published in 2016 recommends the use of statins in patients from 40 to 75 years old who present at least one CVD risk factor without history of a previous cardiovascular event. Patients eligible for primary prevention statin therapy have a calculated 10-year CVD event of at least 10%, which is based on age, sex, race, cholesterol levels, systolic blood pressure, antihypertension treatment, diabetes and smoking status.[33] In the case of secondary prevention, patients already experienced a cardiovascular event and statins are prescribed to lower the risk of subsequent events.[34, 35]

1.2.1.2 Statin characteristics

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are the first line therapeutics for cardiovascular protection with well-demonstrated advantages.[4] Statins and other lipid-lowering drugs form the most prescribed class of drugs in the United States (255.4 million prescriptions in 2010) due to their capacity to lower the cardiovascular mortality and morbidity.[36] In 2012, there were 38 million statin prescriptions in Canada

alone.[37] The efficacy of statins is largely explained by their ability to diminish the plasma levels of LDL by inhibiting competitively the HMG-CoA reductase while increasing the level of HDL, and sequentially lowering the incidence of clinical cardiovascular endpoints.[38] LDL transports cholesterol to cells, whereas HDL extracts excess cholesterol from tissues.[29] The biosynthesis of cholesterol and where statins act (rate limiting step of the pathway) are presented in Figure 3. Statins also have other pleiotropic effects, like anti-inflammatory effects which can help treat the comorbidities associated with cardiovascular diseases, such as atherosclerosis and coronary heart disease.[39]

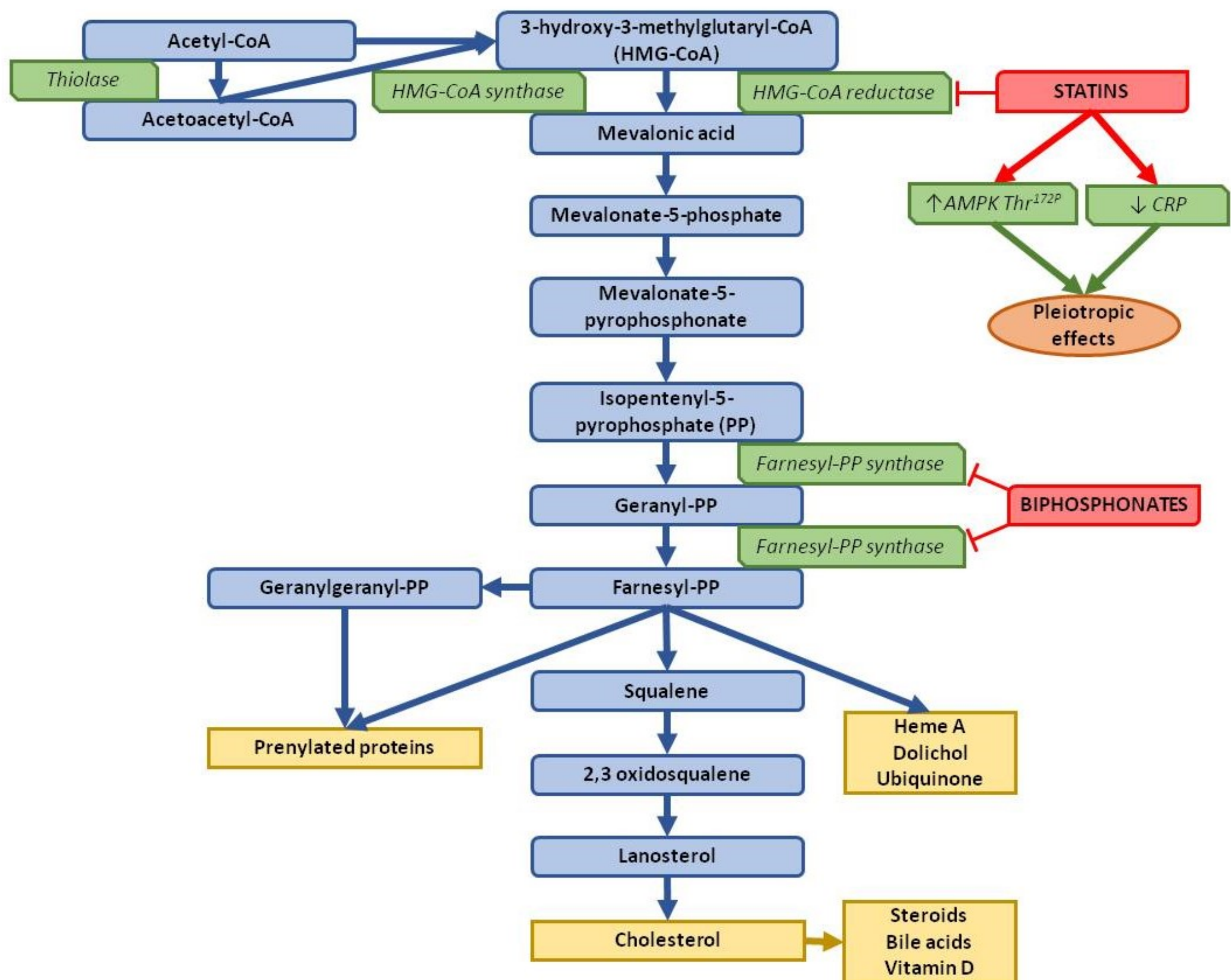


Figure 3. Biosynthesis pathway of cholesterol and its pharmaceutical inhibitors
Figure adapted from Du Souich *et al.* (2017)[40] and Mabuchi *et al.* (2005)[41]

Statins can inhibit HMG-CoA reductase by competing with HMG-CoA for the binding site. Crystallization studies have found that statins have the 3-hydroxy-3-methylglutaryl group in common, which is the part of the molecule interacting with the enzyme.[42]

Each statin has different physicochemical, pharmacokinetic and pharmacodynamic properties, as shown in Tables I and II. Cerivastatin and simvastatin are among the most lipophilic statins, while rosuvastatin and pravastatin represent the most hydrophilic. It was

found in the literature that there are fewer side effects associated with hydrophilic statins compared to lipophilic ones.[23, 43] The risk/benefit profile of each statin is determined by these differences in their characteristics.

Table I. The pharmacodynamic properties of statins

Compounds	Reduction in LDL-C (%)	Increase in HDL-C (%)	Reduction in TG (%)	Reduction in TC (%)	Dose (mg)
Atorvastatin	26-60	5-13	17-53	25-45	10-80
Cerivastatin*[44]	44	3	11	31	0.8
Fluvastatin	22-36	3-11	12-15	16-27	20-80
Lovastatin	21-42	2-10	6-27	16-34	10-40
Pravastatin	22-34	2-12	15-24	16-25	10-80
Rosuvastatin	45-63	8-14	10-35	33-46	5-40
Simvastatin	26-47	8-16	12-34	19-36	5-80

Table adapted from Vaughan *et al*., 2004[38]

* Pharmacodynamic properties of cerivastatin were found in a different source since it has been removed from the market. (Stein *et al*., 1999)[44]

Table II. The physicochemical and pharmacological properties of statins

Compounds	Bioavailability (%)	Metabolism	Half-life (h)	Protein binding (%)	Lipophilicity	Log D [Log P]
Atorvastatin	12	CYP3A4	13-30	98	Yes	1.00-1.25
Cerivastatin[45]	60	CYP2C8 and CYP3A4	2-3	99	Yes	1.50-1.75
Fluvastatin	19-29	CYP2C9	0.5-3.0	98	Yes	1.00-1.25
Lovastatin	5	CYP3A4	2-4	>95	Yes	[4.26] [46]
Pravastatin	18	Sulfation	2-3	43-67	No	-1.00-(-0.75)
Rosuvastatin	20	CYP2C9 and CYP2C19 (minor)	19	88	No	-0.50-(-0.25)
Simvastatin	5	CYP3A4	1-3	95-98	Yes	1.50-1.75

* Physicochemical and pharmacological properties of cerivastatin were found in a different source[45] since it has been removed from the market.[38, 47, 48]

The oral bioavailability of statins differs largely from one statin to the other due to their distribution coefficient (logD), their metabolism and their transporters.[3] The chemical structures of the different tested statins are presented in the figure below (Figure 4) Simvastatin and lovastatin have a low bioavailability of 5%, while it is elevated to 12-20% for atorvastatin, pravastatin and rosuvastatin, and 51% for pitavastatin.[49, 50] The CYP450 superfamily is responsible for the metabolism of most statins. CYP3A4 is the isoenzyme implicated in the metabolism of simvastatin, lovastatin, atorvastatin, and cerivastatin (which is also metabolized by CYP2C8). Fluvastatin is mostly metabolized by CYP2C9. Even though pravastatin is metabolized by CYP3A4, pravastatin and rosuvastatin are mainly eliminated in the bile and *via* renal secretion.[38, 47, 48]

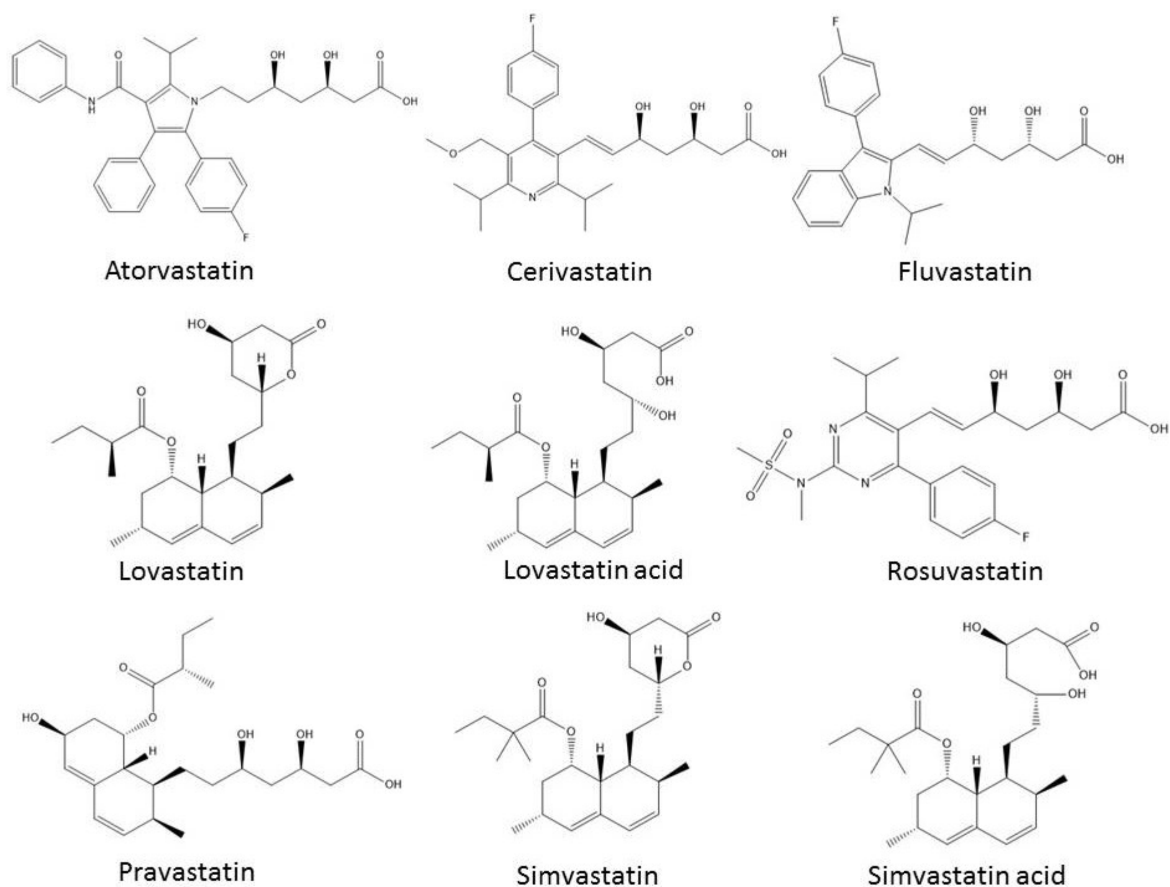


Figure 4. The structures of the statins that have been evaluated

Statins are generally well tolerated by patients, but their chronic use can sometimes be associated with two major side effects: asymptomatic elevation of hepatic enzymes or musculoskeletal disorders.[4, 51] Statin-associated myopathies present a large clinical spectrum of disorders. The symptoms are considered self-limiting, since they generally resolve upon cessation of the drug.[52] The mechanisms underlying statin-induced muscle disorders are still not well understood. Various hypotheses have been proposed to explain these adverse drug events including isoprenoid depletion, ubiquinone synthesis inhibition, sarcolemmal cholesterol alteration, calcium metabolism modification, apoptosis activation and immune reaction.[53, 54]

Statin-induced muscle symptoms can be classified based on CK levels and the presence of symptomatic myopathy. Myopathies are defined as muscle pain and weakness, which can be further classified as the following: 1) asymptomatic elevation of CK, 2) mild myalgia with or without CK elevation, 3) myalgia with CK mild elevation < 5 times upper limit of normal (ULN), 4) myositis with moderate CK elevation of between 5 and 10 times ULN, and 5) rhabdomyolysis with severe CK increase > 10 times ULN.[4, 55] The occurrence of severe myopathy (including myositis) and rhabdomyolysis induced by statins is rare (0.1-0.5% and 0.04-0.2%, respectively).[56] However, statins are more frequently (10-15%) associated with mild muscle symptoms including myalgia with or without creatine kinase elevation, cramps and muscle weakness.[49] Apart from high statin doses, the presence of drug-drug interactions or genetic polymorphisms increasing plasma levels seem to correlate with a higher frequency of muscular disorders.

Considering the relatively high frequency of clinically observed statin-induced myopathies, their occurrence in randomized controlled trials (RCTs) is relatively low (as low

as <0.1%, <1% and 1.5-3%), and comparable to placebo.[1, 23, 55, 57] The observed discrepancy can partly be explained by the fact that subjects chosen in RCTs are generally not at high risk for these ADR, while in clinic, patients can be subjected to polypharmacy and other predisposing factors (*e.g.* age, exercice level, pathology, sex).[23] This discrepancy can also be attributed to the definition of muscular disorder in RCTs *vs* clinical practice. Moreover, many patients are advised by their medical practitioner to be vigilant about any muscle pain that can possibly emerge during statin treament.[55] This can lead to a nocebo effect since patients may expect negative effects from their therapy, making them believe that statins can be toxic and therefore associating any myalgia to the statin.[55] Sometimes, this effect can even persist beyond the original regimen, affecting a statin rechallenge with a different statin and even with non-statin hypolipidemiants.[55]

Overall, statins play an important role in cholesterol lowering, which can lead to a better prevention and management of CVD.[4] Although their long-term use can cause a spectrum of muscular ADR, statin therapy discontinuation may result in an increased risk of cardiovascular events.[4] Since the mechanism of statin-induced myopathy is still not well understood, it is imperative for scientists to attempt to determine how these myotoxicities are caused so that patients can have the optimal treatment.

1.2.3 Other drugs that can induce myopathy

Other than statins, drugs that could be used concomitantly during lipid-lowering treatment, such as antihypertensives and angiotensin-converting enzyme (ACE) inhibitors, were analyzed to determine if they can exert an additional or synergistic effect. Analgesics

that could be administered to relieve muscle pain after the onset of drug-induced myopathies, specifically non-steroidal anti-inflammatory drugs (NSAID), were assessed to know if their use could exacerbate the pre-existing condition. Other acidic drugs known to cause myopathies were also studied, to determine if they cause muscle pain through the same mechanism as some statins. The characterized possible myopathy-inducing drugs relevant for this study are listed in Table III.

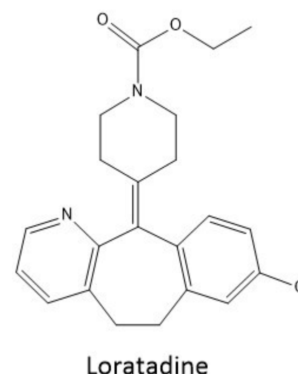
Table III. Potential myopathy-inducing drugs

Drug classes	Compounds
Statins	Atorvastatin Cerivastatin Fluvastatin Lovastatin hydroxy acid Lovastatin lactone Pravastatin Rosuvastatin Simvastatin hydroxy acid Simvastatin lactone
Fibrates	Gemfibrozil Clofibrate
Antihypertensives (Angiotensin-II receptor antagonists)	Irbesartan Losartan Valsartan
Non-steroidal anti-inflammatory drugs (NSAID)	Flurbiprofen Ibuprofen Naproxen Salicylic acid
Angiotensin-converting enzyme inhibitors (ACEi)	Captopril Enalapril
Acidic drugs	Colchicine Duloxetine Everolimus Loratadine Niacin Raltegravir Ropinirole Valproic acid

1.2.3.1 Loratadine

Loratadine is a tricyclic H_1-antihistamine commonly used to relieve allergy symptoms, such as allergic rhinitis and chronic urticaria.[58, 59] Loratadine is part of the second generation (non-sedating) antihistamine class characterized by their selective inverse agonist action on the peripheral histamine H_1-receptors.[58] Similar to other second-generation antihistamines, such as cetirizine, ebastine and terfenadine, loratadine competitively blocks the histamine receptor site instead of preventing histamine release.[58] Figure 5 represents the structure of loratadine.

Figure 5. Loratadine structure



Unlike their predecessors, the second-generation antihistamines do not present the major ADR of sedation. The first-generation antihistamines can cause these side effects due to their lack of selectivity for H_1-receptors (action on acetylcholine receptors, α-adrenergic receptors and 5-HT receptors) and their capacity to cross the brood-brain barrier.[58, 60] These second-generation antihistamines have a higher selectivity for the peripheral H_1-receptors than the ones in the central nervous system.[61] This selectivity is largely attributed to their polarity, with most of them being zwitterions (molecules that are both positively and negatively charged).[62] They are therefore unable to cross the blood-brain barrier, making them less susceptible to antihistamine-related sedation.[58, 61] Third-generation antihistamines are mainly active metabolites and enantiomers of second-generation antihistamines.[61] They do not present with any notable advantage, with the exception of fexofenadine, the active metabolite of

terfenadine, which has a lower risk for cardiac arrhythmia than the parent molecule.[61] A partial list of H_1-antihistamines is presented in Table IV.

Table IV. Examples of H_1-antihistamines from different generations (Brand names)[61, 63]

First generation	Second generation	Third generation
Brompheniramine (Dimetane®)	Ebastine (Ebast®)	
Chlorpheniramine (Chlor-Timeton®)	Loratadine (Claritin®) ⇨	Desloratadine (Clarinex®)
Dimenhydrinate (Gravol®)	Terfenadine (Seldane®) ⇨	Fexofenadine (Allegra®)
Diphenhydramine (Benadryl®)	Cetirizine (Zyrtec®) ⇨	Levocetirizine (Xyzal®)
Doxylamine (Unisom®)		

Loratadine allergy treatment can also be associated with muscle pain and other adverse effects, such as headache, dry mouth, fatigue, and gastrointestinal problems.[64-66] During clinical trials involving loratadine and its metabolite desloratadine, the frequency of muscular adverse events was low (~2%) and not significantly different from placebo.[67, 68] However, the occurrence of these events is not representative of clinical reality where patients are not in such controlled environments.[69] Loratadine was also found to be more likely to synergistically increase the risk of myopathy when paired with simvastatin (relative risk (RR) = 1.69), alprazolam (RR = 1.86), duloxetine (RR = 1.94), and ropinirole (RR = 3.21).[22] The mechanism by which loratadine causes these muscle side effects is also still unknown. Moreover, since loratadine is a widely used over-the-counter antihistamine, the frequency of the adverse events related to its use is not well reported and can lead to an underestimation of myopathies reported for this drug.

1.3 Mechanisms of drug-induced myotoxicities

Drug-induced myotoxicities, as mentioned previously, are associated with many widely-used drugs. Over the years, many mechanisms have been attributed to these adverse effects, such as alteration in cellular membrane cholesterol, mitochondrial impairments, increase of lysosomal activity, injuries to electrolyte homeostasis, alteration in protein synthesis and degradation, inhibition of myogenesis, oxidative stress, cell apoptosis and immune reactions.[6, 70, 71]

Corticosteroids constitute the most common class of drugs to cause muscle toxicity by inhibiting protein synthesis.[24] This results from the lowered expression of insulin-like growth factor I (IGF-I), which has antiapoptotic effects.[24] It can also be exacerbated by increased cytoplasmic protease activity (proteolysis) which leads to myofibrillar destruction.[24] In addition, steroids can lower glutamine synthase and glycogen phosphorylase activities, leading to muscular atrophy.[24]

Mitochondrial dysfunction is also associated with myalgia. The antiviral zidovudine (nucleoside reverse transcriptase inhibitor of γ-DNA polymerase) can interfere with the replication of mitochondrial DNA, whereas the immunosuppressant cyclosporin A blocks the mitochondrial permeability transition pore, leading to lowered cellular energy production.[2, 24]

Lysosomal activity can also play a pathogenic role in the antimalarial chloroquine-induced myopathy. An accumulation of inflammatory cells in the lysosomal system can be generated by chloroquine and hydroxychloroquine, leading to the alkalinisation of lysosomes as well as the alteration of protein glycosylation and membrane lipid metabolism.[2]

Other drugs, such as colchicine, can produce myotoxicities *via* lesions of the microtubular system by binding firmly to tubulin molecules.[2] D-penicillamine can cause

inflammatory myopathy with genesis of polymyositis and dermatomyositis.[2] NSAID analgesics can incite muscle necrosis and rhabdomyolysis, but they are rare adverse events.[2]

Despite the various mechanisms identified in drug-induced myopathy, the specific mechanisms related to statin-induced muscle pain are still unknown.[50] Different hypotheses have been proposed and will be discussed below.

1.3.1 Depletion of cholesterol, isoprenoids and coenzyme Q10

One of the first hypotheses proposed for statin-induced myopathy is that the reduction of cholesterol content in skeletal muscle cells can make their membrane unstable.[5, 52] Cholesterol is an essential component of the membrane structure and function, so its decrease during statin treatment can influence the membrane fluidity, changing in turn the membrane excitability.[5, 72] However, this mechanism has been proven not plausible since the specific *in vitro* inhibition of squalene synthase (downstream steps in cholesterol synthesis) did not precipitate to the observed myotoxicity.[5, 52]

This finding, combined with the ones that have shown that the addition of mevalonate or mevalonic acid (intermediate products of cholesterol synthesis) during statin treatment in rat muscle cells or mice can revert myotoxicity, suggest that the mechanism behind the statin-induced myopathy seems to involve the depletion of either isoprenoids or coenzyme Q10 (ubiquinone).[5, 40] The most important isoprenoids in the cholesterol synthesis pathway are farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which activate the regulatory guanosine 5'-triphosphate-binding proteins, promoting cell maintenance and reducing apoptosis (cell death) by a process known as protein prenylation.[5, 52] Statins can inhibit this

process, which increases cytosolic calcium, resulting in apoptosis following caspase-3 activation.[52]

The reduction in CoQ10 is another possible mechanism for statin-induced myopathy. Statins can inhibit the production of CoQ10, since it is one of the end products of the cholesterol synthesis pathway.[52] CoQ10 participates in the mitochondrial electron transport during oxidative phosphorylation. Therefore, a diminution of CoQ10 can impair the mitochondrial function in the respiratory chain. However, the effects of CoQ10 depletion on statin-induced myotoxicity remain controversial, since a supplement of CoQ10 during statin therapy did not result in a consistent improvement of symptoms in each study.[5]

1.3.2 Disturbed calcium metabolism

The impaired calcium metabolism has also been considered to be a possible mechanism for the observed muscular symptoms during statin treatment. *In vitro* studies on rat tissue have shown that statins activate the release of calcium from the sarcoplasmic reticulum by enhancing the expression of calcium channels ryanodine receptors 3 (RYR3).[5, 40] It has also been demonstrated that statins can increase calcium accumulation by activating mitochondrial depolarization through the permeability transition pore (mPTP) and the sodium-calcium exchanger (NCE).[5, 40] This increase in intracellular calcium levels (disturbance in calcium homeostasis) can lead to muscle contraction and cramps.[52]

1.3.3 Autoimmune

In recent years, other than the previously described self-limited statin-induced myopathies, there have been reports regarding the development of autoimmune myopathies during statin treatment.[73] The symptoms of these statin-associated autoimmune myopathies

persist or worsen even after statin discontinuation.[73] These immune-mediated necrotizing myopathies are rare, and are frequently associated with the production of anti-HMGCR autoantibody.[74] Therefore, patients suffering from these conditions can be identified using anti-HMGCR screening and treated with immunosuppressants.[75]

1.3.4 Drug-drug interactions

Patients with cardiovascular diseases are generally on long-term statin treatment and most of them are elderly with concomitant morbidities (heart disease, hypertension and diabetes).[14, 76] In general, these patients need a therapeutic arsenal including many drugs. This clinical reality increases the risk of drug-drug interactions, which in turn increases the occurrence of undesirable events and drug toxicity. The myopathy incidence rate is low with statin monotherapy; however, there is a significant increase in polymedicated patients.[47, 56] There is some data suggesting that drug-drug interactions that can affect the pharmacokinetics of statins, inducing a significant increase in statin plasma levels (CYP2C8, CYP2C9 and CYP3A4 inhibitors), are associated with a higher risk of musculoskeletal disorders such as myopathies and rhabdomyolysis.[47] However, this relation can only explain the higher potential of drug-related morbidity associated with statins. For example, the reported incidence rate for lovastatin monotherapy is 0.15%, but this is increased to 2%, 5% and even 28% when patients receive it in concomitance with niacin, niacin plus cyclosporine, and cyclosporine plus gemfibrozil, respectively.[47] A drug interaction prediction study by Duke *et al.* found that the combination of loratadine and simvastatin increased the risk for myopathy (RR = 1.69).[22, 77] Loratadine has also been reported to cause muscle pain.[64] It is therefore possible that this antihistamine may cause muscular toxicity through similar mechanisms as statins.

1.3.5 Genetic polymorphisms

Genetic polymorphisms in statin transporters can affect drug disposition and increase drug plasma levels, which lead to the observed ADR.[49] An important example of this is that the membrane expression of the SLCO1B1 V174A (*SLCO1B1 521T>C*) variant is reduced compared to the wild-type SLCO1B1 in human liver samples.[78] SLCO1B1 is a drug transporter expressed exclusively on the sinusoidal membrane of hepatocytes and it is implicated in the uptake of drugs from the blood into the liver.[7] In clinic, subjects homozygous for *SLCO1B1* 521CC have an increased exposure to pravastatin 3.21-fold higher than subjects with the *SLCO1B1* 521TT genotype.[79] However, the higher plasma levels can only partly explain the potential muscular toxicity.[80] Moreover, a genome-wide association study (GWAS) revealed an association between the *SLCO1B1* 521T>C polymorphism and the incidence of myopathy with simvastatin administration, with an odds ratio (OR) of 4.5 per copy of the C allele.[81] Drug transporters and their effects on statin pharmacokinetics will be discussed further in this thesis.

1.3.6 Inhibition of lactic acid efflux by MCT and drug-induced myopathy

Other than systemic or local drug levels, statin-induced myotoxicities can be precipitated through their effects on other drug transporters.

In recent studies, it was found that some statins (mostly lipophilic) can be transported by the proton-linked lactic acid transporters, MCT.[7, 82-84] This suggests that statins could inhibit lactic acid transport in a competitive manner.[84] Some published data obtained in transfected cell lines suggests that the inhibition of lactic acid efflux can cause an intracellular

accumulation of lactic acid and lead to muscle cramps and cell apoptosis (*via* activation of caspases).[54] We will further investigate this hypothesis in this thesis.

1.3.6.1 Muscle and lactic acid

The muscle is the largest organ in the human body, representing 45% of body weight, and is particularly prone to adverse drug reactions since it is highly vascularized, hence increasing its exposition to circulating drugs.[85] Indeed, it receives a large fraction of the blood supply and it is highly metabolically active. Skeletal muscle is the major producer of lactic acid in the organism through glycolysis, but it can also use lactic acid as a source of energy.[85] Briefly, muscle is defined by two distinct muscle fibers: type I and type II fibers. Type I fibers are highly oxidative and are considered slow twitch, whereas type II are highly glycolytic and are referred to as fast twitch.[86] The stereoselective transport of lactic acid through the muscle membrane is catalysed by the proton-linked monocarboxylate transporters.[87] The rapid transport of lactic acid through the membrane is crucial to maintain the intracellular pH homeostasis.[88]

During the first 5 to 10 minutes of moderate physical activity, ATP is produced *via* phosphorylative oxidation using glycogen catabolism as a primary fuel source.[29] When the effort or length of the physical activity is too high, glycolysis can start playing a major role and the anaerobic pathway becomes predominant as oxygen supplies are depleted.[29] This leads to an increased production of lactic acid. Following physical activity, muscle supplies of creatine phosphate, glycogen and oxygen are depleted. Oxygen is needed to metabolize lactic acid and restore normal lactic acid concentrations.[29]

Muscular fatigue can be induced by three mechanisms: impaired calcium metabolism (increased expression of RYR3, mPTP and NCE), lowered glucose replenishing (decreased glucose transporter GLUT4 expression) and increased lactic acid levels (inhibition of MCT).[40] Consequently, a lowered pH and higher lactic acid concentrations following exercise can alter protein conformation and activity in the muscle, such as actin and myosin, as well as proteins regulating calcium relargage.[5, 40, 89] Lactic acid intracellular accumulation will be discussed more in detail.

During glycolysis, pyruvate is formed.[29] At the end of this pathway, pyruvate can be processed in two different ways, depending on the oxygen supply. If the metabolism is in aerobic conditions, pyruvate enters the Krebs cycle, but in anaerobic conditions, pyruvate is converted to lactate. Lactate is the ionized form of lactic acid. The production of lactate through the glycolysis pathway is detailed in the figure below (Figure 6). Glucose metabolism in mammalian cells results in lactic acid production. Indeed, one glucose molecule breaks down to two lactic acid molecules and protons. Lactic acid production leads to a decrease in intracellular pH. MCT can transport lactic acid out of the cells (efflux), which increases the intracellular pH.[90]

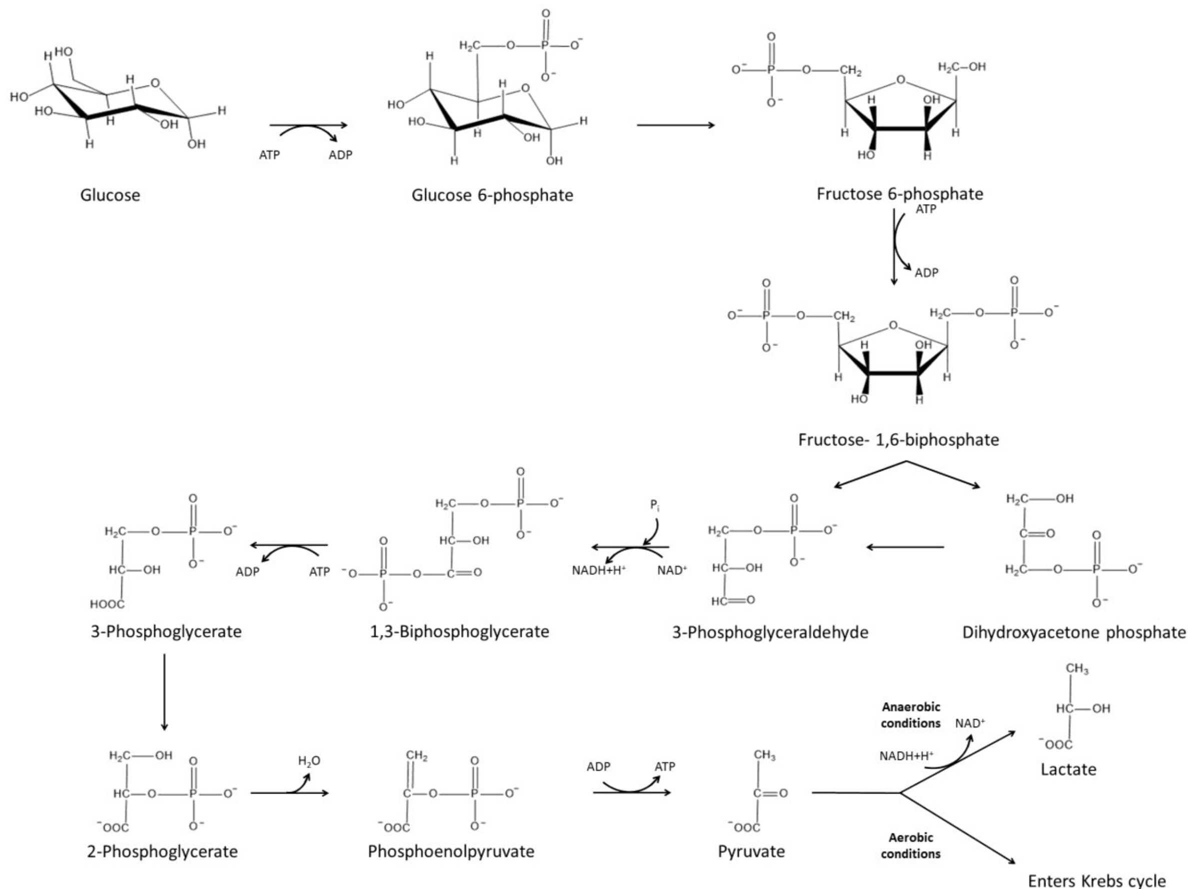


Figure 6. Glycolysis pathway
Figure adapted from Vander's Human Physiology- The Mechanisms of Body Function[29]

It has been reported that around 1,500 mM of lactic acid enters blood circulation every day from various tissues, such as muscle, skin, brain, red blood cells and intestine.[91] Lactic acid concentrations range from 0.5 to 2 mM at rest, but they can go as high as 10 to 15 mM during intensive exercise.[92] Lactic acid is generally considered as a byproduct of glycolysis. However, there is evidence suggesting that lactate can actually be used as an energy source in the neurons and cardiomyocytes.[93]

GPR81 is part of a GPCR subfamily recognized for binding hydroxy-carboxylic acids. This receptor binds lactate with an affinity (EC_{50}) of 1.5 to 3 mM and is expressed predominantly in adipocytes, although it is weakly expressed in skeletal muscle, liver, kidney

and brain.[91, 94] Several studies have supported the hypothesis that the signaling mechanism of GPR81 is mediated by its coupling to G_i- type G proteins, which results in an adenylyl cyclase inhibition. GPR81 activation by lactate is associated with lipolysis inhibition.[91, 92, 94] A study in cancer cells by Roland *et al.* showed that GPR81 activation increased the mRNA expression of the monocarboxylate transporters MCT1 and MCT4, critical for lactic acid transport.[95] Lactic acid transport by monocarboxylate transporters will be discussed further in this thesis.

During statin treatment, intense physical exercise can cause an increase of muscle intracellular lactic acid accumulation. It has been proposed that an inhibition of transport of major monocarboxylates related to exercise, such as L-lactate, can play a role in these drug-induced muscle disorders.[96-98]

1.4 Drug transporters

Transporters are transmembrane proteins located across physiological membranes. They mediate the uptake or efflux of numerous compounds, such as nutrients, endogenous substrates and xenobiotics at the cellular level.[7] Their role is to facilitate the transport of these compounds in and out of the cells, to either provide necessary elements or protect the organism against dietary and environmental toxins.[7] Transporters are mainly classified into two superfamilies, based on their transport mechanism: ATP-Binding Cassette (ABC) transporters and Solute Carrier (SLC) transporters.[7] ABC transporters are primary active transporters and mainly mediate efflux, whereas SLC transporters are mostly bidirectional, depending mainly on electrochemical gradient of the substrates, which classifies them as facilitated transporters.[99] However, some SLC can use an ion gradient, such as sodium or proton gradient across the membrane, to transport substrates against an electrochemical

potential difference; these are classified as secondary active transporters.[100] Efflux transporters pump the compounds out of the cells, while influx transporters bring them inside the cells. Membrane-bound transporters are found either on the apical or on the basolateral membrane, which determines their function.[7]

It was previously thought that drug pharmacokinetics and pharmacodynamics were mostly dependent on plasma concentrations.[7] However, it is now known that some transporters, which are identified as drug transporters, play an important role in drug absorption, distribution in specific organs and disposition.[7] These drug transporters are further classified into 13 different families depending on their function. The ABC superfamily comprises MDR, MRP and BCRP, whereas the SLC superfamily includes organic cation transporter (OCT), organic cation/carnitine transporter (OCTN), organic anion transporter (OAT), organic anion transporter polypeptide (OATP; SLCO), peptide transporter (PEPT), concentrative nucleoside transporter (CNT), equilibrative nucleoside transporter (ENT), bile acid transporter, sodium-coupled monocarboxylate transporter (SMCT) and MCT.

There are a multitude of drug transporters, but only the most relevant ones for this project will be discussed in detail.

1.4.1 ABC transporters

The ABC transporters superfamily, as their name suggests, require ATP hydrolysis to translocate their substrates across the membranes against their electrochemical gradient. ABC transporters share a common structure, generally including two nucleotide-binding domains and two transmembrane domains as the core unit. The nucleotide-binding domains are

required for ATP hydrolysis, whereas the transmembrane domains bind to the substrate and allow translocation across the membrane.[99]

The 49 ABC transporters genes are classified into 7 families (ABCA-ABCG), according to their sequence similarity.[99] Three of those families are better characterized: multidrug resistance protein (MDR; ABCB), multidrug resistance-associated protein (MRP; ABCC) and the breast cancer resistance protein (BCRP; ABCG).[7] The MDR family regroups 11 genes (*ABCB*), the MRP family contains 13 genes (*ABCC*), and there are only 5 members in the ABCG family, with BCRP (ABCG2) being the most studied.[99]

1.4.1.1 ABCB1

ABCB1 (P-gp; MDR1) is one of the most studied efflux transporters. It is expressed in multiple organs, such as the liver, the kidney and the gastrointestinal (GI) tract.[101] This transporter's localization and wide range of substrates make it determinant in the bioavailability of many drug classes: anticancer drugs, HIV protease inhibitors, analgesics, immunosuppressive agents, antibiotics, histamine H_2-receptor antagonists, etc.[7] In the liver, ABCB1 mediates the flux of drugs, such as statins, towards the bile and facilitates drug elimination.[101] There are around 30 characterized SNP for ABCB1. Two common ABCB1 polymorphisms (*2677G>T/A* and *3435C>T*) yield a lower ABCB1 expression and activity and may influence statin-associated myopathy.[7] However, regardless of ABCB1 genetic polymorphisms, only a weak association has been found between reduced ABCB1 transport activity and statin-induced myotoxicity. A stronger association was found between *ABCB1 3435C>T* carriers and drug-induced muscle symptoms when patients were treated with statins for more than 5 months.[40]

1.4.1.2 ABCC

The ABCC family mediates drug efflux and can be found in multiple tissues. ABCC1 and ABCC5 have a ubiquitous expression, with low ABCC1 levels in the liver, whereas ABCC4 is expressed in the muscle. ABCC1 can mediate the transport of a wide range of substrates, with a preference for organic anions, while ABCC4 and ABCC5 transport predominantly nucleotide analogues.[102] It was reported that these ABCC isoforms can mediate the transport of atorvastatin and rosuvastatin in heterologous expression systems.[80]

ABCC2 plays an important part in statin efflux from the liver towards the bile and ABCC2 inhibition may result in significantly higher drug exposure. In a study to determine whether ABCC2 polymorphisms could be associated with statin-induced myopathy, three common polymorphisms were characterized: *ABCC2 -24C>T*, *ABCC2 1249G>A* and *ABCC2 3972C>T*. They have found that these variants generate a higher drug exposure and could possibly contribute to statin-induced adverse events.[103]

1.4.1.3 ABCG2

ABCG2 is a main efflux transporter involved in statin elimination in the liver. Similar to P-gp, it mediates drug transport towards the bile canalicular duct. Therefore, a decrease in transport activity for ABCG2 may also lead to increased drug exposure. The *ABCG2 421C>A* polymorphism (frequency of 7.4-11.1% in Caucasians and 27-35% in Asians) results in decreased ABCG2 activity and higher drug exposure for fluvastatin, simvastatin, atorvastatin and rosuvastatin.[40]

ABCG2 is also expressed in the muscular cell membrane where it mediates the efflux of statins and other drugs. It has been proposed that decreased efflux transport *via* ABCG2

might result in statin accumulation in the muscle cell.[40] As previously mentioned, ABCG2 mediates the efflux of drugs at the hepatic level towards the bile canalicular duct and the intestine. Impaired ABCG2-mediated transport would therefore lead to higher drug plasma concentrations, and accumulation into the myocyte.[40] However, because several patients present with polymorphisms in ABCG2 and do not experience myopathy during statin treatment, it has been suggested that more than one SNP or predisposing muscle conditions are present in the same individual.[40]

1.4.2 SLC transporters

Most drugs transporters are part of the SLC transporters superfamily functioning either according to a concentration gradient of its substrate or by an electrochemical gradient created by other transporters.[100] The 395 SLC transporters are divided into 52 gene families (SLC1-SLC52). SLC transporters are classified in the same family if they share 20% of their sequence.[104] Transporters found in the SLC superfamily can have multiple transport mechanisms at the cell membrane, such as coupled transport, exchange and passive transport.[104]

1.4.2.1 SLCO transporters

SLCO transporters, coded by the SLC21 gene family, are also known as the organic anion-transporting polypeptides (OATP) family. Similar to the ABC transporters, the *SLCO* genes are classified by sequence similarity.[105] Indeed, SLCO transporters sharing over 40% of their sequence belong to the same family, whereas those sharing over 60% of their sequence are classified in the same subfamily.[105]

The SLCO transporters are characterized by 12 transmembrane domains and generally perform bidirectional transport, depending on the solute gradient.[106] A few members of the SLCO family are especially important in the liver, such as OATP1B1, OATP1B3 and OATP2B1 on the basolateral membrane of the hepatocytes.[107-109]

Various endogenous substrates have been found to be transported by the SLCO transporters, as well as drug classes including statins, angiotensin II receptor antagonists and ACE inhibitors.[110, 111]

1.4.2.1.1 SLCO1B1

SLCO1B1 is expressed in the liver and transports a wide variety of substrates, but the most relevant to this project are the statins cerivastatin, atorvastatin, rosuvastatin and pitavastatin.[7, 112-115] However, SLCO1B1 activity can be inhibited by structurally diverse compounds and commonly used drugs, such as ketoconazole, clarithromycin, erythromycin, verapamil and warfarin among others.[116]

Statin-induced myopathy has been associated with a decrease in SLCO1B1 function. Various genetic polymorphisms result in a reduced SLCO1B1-mediated transport activity, such as the missense variant 521T>C (*SLCO1B1*5*), found in 1% of Caucasians.[40, 117] The *SLCO1B1*1b* (388A>G) variant is observed at a frequency of 40% in Caucasians, 75% in African-American and 60% in Asians. The *SLCO1B1*1b* allele is in linkage disequilibrium (frequently associated with each other) with the *SLCO1B1*5* allele, resulting in the *SLCO1B1*15* variant. Both *SLCO1B1*5* and *SLCO1B1*15* result in decreased SLCO1B1-mediated transport activity.[7] This leads to a reduced influx of statins and other drugs (*i.e.* ezetimibe, fexofenadine, valsartan) into hepatocytes, lowers statin metabolism and increases

plasma concentrations.[7] It has been reported that *SLCO1B1 521CC* homozygotes have higher AUC for lovastatin acid (3.4-fold), pravastatin (1.9-fold), simvastatin (3.2-fold), pitavastatin (3.2-fold), atorvastatin (3.1-fold) and rosuvastatin (1.8-fold).[40, 118] The *SLCO1B1 521T>C* genotype has also been associated with statin intolerance defined by an increase of serum creatine kinase or a modification in statin regimen, with an odds ratio (OR) of 2.05.[40] Moreover, a GWAS correlated the prevalence of statin-induced myopathy during simvastatin treatment with the *SLCO1B1 521T>C* allele, with an OR of 4.5 per copy of the allele and an OR of 16.9 for *CC* homozygotes.[40] This mutation in SLCO1B1 could account for up to 60% of myopathies associated with simvastatin therapy.[1, 6] In summary, it has been reported that SLCO1B1 genetic polymorphisms can result in increased drug exposure, which could increase the risk of experiencing statin-induced myotoxicity.[119]

1.4.2.1.2 SLCO1B3

SLCO1B3 has been reported to be expressed in liver, small intestine and placenta.[7] SLCO1B3 is very similar to SLCO1B1 (80% sequence similarity) and has a comparable spectrum of substrates. As for SLCO1B1, decreased SLCO1B3-mediated transport could also affect statin concentrations. The two most common *SLCO1B3* variants are the *SLCO1B3 334T>G* and *SLCO1B3 699G>A*, which result in a lower SLCO1B3 activity, possibly increasing the risk of drug-induced myopathy *via* higher drug plasma concentrations.[120]

1.4.2.1.3 SLCO2B1

SLCO2B1 is expressed in a variety of organs, such as liver, kidney, intestine, brain and placenta.[7] This transporter facilitates the oral absorption of its substrates at the apical

membrane of enterocytes. It has also been reported in muscle, where its transport activity might influence local concentrations of drugs.[80]

SLCO2B1, like SLCO1B1 and SLCO1B3, also transports some statins (pravastatin, fluvastatin and rosuvastatin).[112, 121] Its activity is inhibited by multiple compounds found in the diet (orange and grapefruit juice, green tea), as well as some drugs like quercetin and salicylate.[122-124]

1.4.2.2 SLC22 transporters

The SLC22 family is also known as the organic anion transporter family or OAT.[7]

SLC22A6 is expressed in the kidney and the skeletal muscle. It has been shown to transport a wide range of xenobiotics, such as antihypertensives, statins, antibiotics, histamine H_2-receptor antagonists and NSAID.[125] SLC22A6 expression at the muscle cell membrane could also mediate the uptake of some statins (*i.e.* pitavastatin).[40]

SCL22A7 is expressed in the liver and transports a wide range of substrates (diuretics, antibiotics, antiviral agents, histamine H_2 receptor antagonists, NSAID, antineoplastic drugs, etc.).[125] SLC22A7 possibly mediates the influx transport of some statins.[40] However, no drug-drug interactions have been reported yet for this transporter.[125]

1.4.2.3 Monocarboxylate transporters

The proton-coupled monocarboxylate transporters, MCT, are part of the solute carrier 16 (*SLC16*) gene family. There are 14 sequence-related isoforms identified until now, but only 6 of them have been functionally characterized. Evidence suggests that MCT can play a role in the transport of some drugs that have monocarboxylate structures within the molecules, such

as statins.[84] The members of this family are characterized by 12 transmembrane helices, a large cytosolic loop between the transmembrane domains 6 and 7 and intracellular C- and N-termini.[7]

There are only four different MCT (MCT1, MCT2, MCT3 and MCT4) in human which can transport lactic acid, pyruvate, butyrate, and ketone bodies (β-hydroxybutyrate and acetoacetate). The skeletal muscle expresses MCT1 and MCT4, which act synergistically. The expression of MCT1 and MCT4 can increase in response to chronic endurance training and exercise in rat and in human.[7] Considering that lactic acid transport is crucial for many metabolic processes, it has been suggested that impairment in MCT-mediated activity might have significant effects and might be incompatible with life. This is consistent with the fact that very few significant polymorphisms in the MCT genes have been reported up to now.[126]

In order to understand the mechanism of MCT-mediated transport, site-directed mutagenesis has been performed on rat MCT1. These studies have identified a few amino acids essential for substrate recognition, such as arginine (R)306, aspartic acid (D)302 and glutamic acid (E)369.[127, 128] The binding of the monocarboxylate anion with the MCT transporter family may require R306, since it is highly conserved in all isoforms, whereas D302 and E369 are probably involved in proton binding. Substrate binding to R306 may lead to the proton moving from D302 to E369, causing a conformational change in the MCT that enables the transport of the monocarboxylate anion.[7, 127, 128] Briefly, lactate transport by MCT1 starts by proton binding, followed by lactate anion binding to the transporter in the open conformation. The transporter then changes to the closed conformation, releasing the lactate and the proton on the opposite side of the membrane. MCT1 then reverts to the open

conformation and is free to transport another lactate molecule.[129] MCT protein structure conformations are illustrated in Figure 7.

Similarly, R143, glycine (G)153 and phenylalanine (F)360 are critical to MCT activity, and mutations at those sites result in loss of transport activity. These mutations may also be associated with a decrease in MCT1 membrane expression, which could be caused by an inadequate association of MCT1 with CD147, a protein facilitating MCT translocation to the plasma membrane.[7, 128]

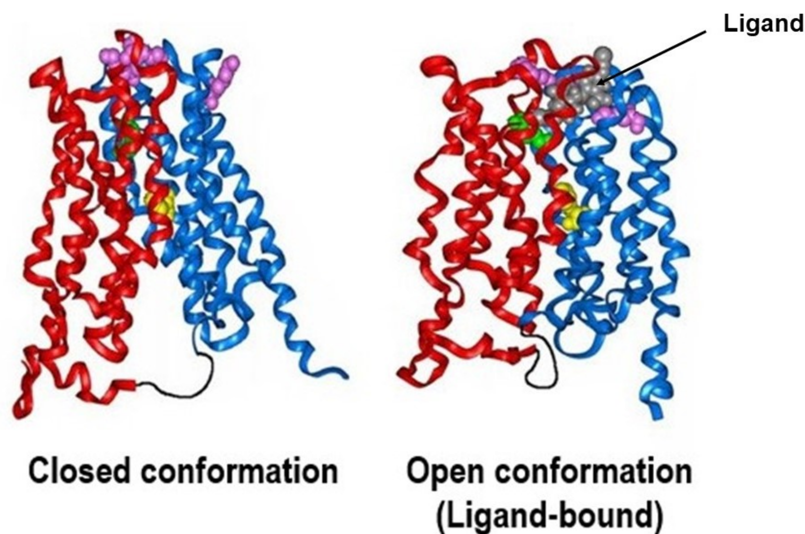


Figure 7. Protein structure of MCT1 under two conformational states
Figure adapted from School of Biochemistry from University of Bristol[130]

In the literature, other than the endogenous natural substrates, MCT can also transport some exogenous substrates that typically consist of anions of small, weak, monovalent organic acids, which are either hydrophobic or hydrophilic.[131] MCT also seem to facilitate the diffusion of weak organic acids such as acetate, propionate, benzoate, and nicotinate *in vitro* and *in vivo*.[7] Other drugs thought to be transported by MCT are nonsteroidal anti-

inflammatory drugs (NSAID) (*i.e.* salicylic acid and ibuprofen), several β-lactam antibiotics (*i.e.* phenethicillin, propicillin and carindacillin), statins (simvastatin and atorvastatin) and antiepileptic agent valproic acid.[82, 83, 132-135] A general list of MCT1/4 substrates and inhibitors is provided in Table V and kinetic parameters of human MCT are detailed in Table VI.

Table V. MCT1/4 substrates and inhibitors

Substrates	Inhibitors
L-lactate, D-lactate, pyruvate, β-hydroxybutyrate, γ-hydroxybutyrate, acetoacetate, α-ketobutyrate, α-ketoisocaproate, α-ketoisovalerate, salicylate, valproic acid, nateglinide, nicotinic acid	α-cyano-4-hydroxycinnamate (CHC), 4,4'-di-isothiocyanostilbene-2,2'-disulfonate (DIDS), 4,4'-dibenzamidostilbene2,2'-disulfonate (DBDS), phloretin, p-chloromercuribenzenesulfonate (pCMBS)

Table adapted from Halestrap (2013)[129]

Table VI. Kinetic parameters of human MCT

Transporter	Substrate	K_m (mM)
MCT1	L-Lactate	6.0
	Pyruvate	2.5
MCT2	L-Lactate	6.5
	Pyruvate	0.03
MCT3*	L-Lactate	5.8
MCT4	L-Lactate	28
	Pyruvate	150
	β-Hydroxybutyrate	130
	Acetoacetate	210

Table adapted from Drug Transporters- Molecular Characterization and Role in Drug Disposition (2007)[7]
* MCT3 kinetic parameters were determined in chicken

The expression of these monocarboxylate transporters in many barriers (*i.e.* gut, blood-brain barrier, placenta, etc.), combined with their ability to transport some drugs, makes them a good therapeutic target for the delivery of drugs that do not diffuse passively through these membrane barriers.[131]

1.4.2.3.1 MCT1

MCT1 (*SLC16A1*), is expressed in almost all tissues in humans and mediates the transport of lactate across the plasma membrane. In polarized cells (*i.e.* intestinal epithelium and choroid plexus), MCT1 is coexpressed with other MCT, depending on the tissue. Expression of MCT1 and other MCT isoforms on the apical or basolateral cell surface leads to

a differential flux of their substrates across the cellular membrane.[7] For example, MCT1 is expressed at the apical membrane of cells in the colon, whereas MCT4 is found at the basolateral membrane.[7] MCT1 is usually found at the apical membrane with its ancillary protein basigin.[129] Also, MCT1 expression was found in rat mitochondria of cardiac and skeletal muscle cells, which suggests that lactic acid could be transported from the cytosol into the mitochondria in order to be oxidized.[136]

A correlation has been established between MCT1 expression and the abundance of type I fibers in human muscle.[137] Indeed, type I fibers are highly oxidative and are able to use lactic acid as their energy source. In these fibers, MCT1 is predominant over MCT4 and mediates mainly lactic acid influx.[138] In some tissue, such as the liver and the kidney, MCT1 mediates lactic acid influx to be used in gluconeogenesis and lipogenesis. MCT1 expression in the skeletal muscle can also facilitate lactic acid use as respiratory fuel.[126]

It has been reported that transcriptional and translational mechanisms might regulate MCT1 expression.[7] Higher substrate concentrations could increase the expression of MCT1 by a still unknown mechanism.[139-141] Physical activity also impacts MCT expression in skeletal muscle.[10] During intense muscle activity, there is an increase in lactic acid production by glycolytic muscle cells. It has been noted that MCT1 in the plasma membrane increases by 76%, while MCT4 increased by 32% after 8 weeks of exercise training.[10] Several transcription factor-binding sites have been discovered within the MCT1 promoter region, such as upstream stimulatory factors (USF), nuclear factor (NFκB), stimulating protein 1 (SP1), activator protein 1 (AP1), and activator protein 2 (AP2). However, only USFI and USFII have been shown to regulate MCT1 expression *in vitro*.[7, 142, 143]

In the literature, some data suggest that MCT1 transcription can be regulated by hormones, such as the thyroid-stimulating hormone, upregulating MCT1 expression in a thyroid cell line *via* cAMP-dependent pathway.[144] The addition of leptin to the apical surface of CaCo-2 cells results in a modest increase in MCT1 protein, which is thought to be related to increased MCT1 mRNA production and enhanced translocation of the transporter protein to the apical plasma membrane.[145] Other studies have demonstrated that long-term exposure to phorbol ester resulted in a 5-fold increase in MCT1 expression and in protein kinase C down-regulation.[146]

Membrane transporters, ion channels and receptors are generally associated with ancillary proteins that facilitate their translocation to the plasma membrane. These proteins could also modulate transporter activity in some cases.[7, 147] CD147 (basigin) facilitates the translocation of various MCT isoforms to the plasma membrane.[138, 148] However, the interaction between CD147 and MCT still remains poorly understood.[7]

It has been shown that the activation of cAMP-dependent pathways reduced lactic acid transport *via* MCT1 from 40 to 60%.[149] This effect was thought to be linked with internalization of the transporter or inactivation by protein modification, leading to a loss of transporter function. These results suggest that phosphorylation of MCT1 or CD147 is probably involved in the cAMP response.[149]

There are mutations in the *SLC16A1* gene that can influence intracellular lactic acid concentrations. For example, the *SLC16A1 1470T>A* variant results in a MCT1 reduction or loss of function, hence suggesting lactic acid accumulation in the muscle.[40] Some studies have examined the effect of SLC16A1 polymorphisms on lactic acid concentrations and muscle

pain. However, since these mutations are rare, designing a study on their effects is problematic.[150, 151] Massidda *et al*. found that *SLC16A1 1470AA* homozygotes display a higher frequency of muscle injury in their participants.[150] Merezhinskaya *et al.* investigated the occurrence of subnormal red cell lactate transport and MCT1 genotype in 5 patients and identified 3 mutations in the *MCT1* gene within their samples. *MCT1 610A>G* resulted in a lactate transport rate 50% lower than the normal range, whereas *MCT1 1414G>A* gave a milder phenotype. In their study, they found that 3 of their patients carried the *MCT1 1470T>A*, but more than half their control group presented this mutation as well. Therefore, they supposed that this SNP had no effect on transport function.[151]

1.4.2.3.2 MCT4

MCT4, coded by the *SLC16A3* gene, is the major MCT isoform in white skeletal muscle (type II) and mediates L-lactic acid efflux.[84] MCT4 expression and activity is highly variable between individuals.[7] MCT4 has a lower affinity for MCT substrates and inhibitors (higher K_m) than MCT1.[152] In contrast to the ubiquitous MCT1 expression, predominant in red muscle fibers, MCT4 is highly expressed in tissues with high glycolytic activity, such as white muscle fibers.[152] Moreover, MCT4 has a high K_m for pyruvate (the substrate becoming lactate during glycolysis). This property ensures that pyruvate remains in the cell to allow glycolysis to continue.[126] MCT4 is also present in astrocytes, white blood cells, chondrocytes and placenta. The selective MCT4 expression suggests an important role in L-lactic acid efflux.[152]

Most studies related to MCT expression were conducted on MCT1. Therefore, data on MCT4 regulation is scarce and still incomplete. The available data shows that MCT4 can be regulated transcriptionally by the hypoxia-inducible factor 1α (HIF-1α).[153] Since cells with

high glycolytic activity need to export lactic acid, MCT4 is predominantly expressed in those cell types. This supports the fact that MCT4 would be upregulated in hypoxic conditions.[153] Moreover, MCT4 is predominantly expressed at the basolateral membrane in polarized cells.[154]

MCT1 and MCT4 constitute the major isoforms present in human skeletal muscle. Glycolytic white muscle fibers constitute the primary source of lactate formation during anaerobic glycolysis. Since MCT4 mostly mediates lactic acid efflux into the interstitial fluid, lactic acid can be transported in the red muscle fibers (expressing mostly MCT1).[138, 152]

Polymorphisms in these monocarboxylate transporters and drug-transporter interactions, which can alter the muscular lactic acid homeostasis, can lead to lactic acid intracellular accumulation resulting in muscle toxicity.

1.4.2.3.3 Other MCT

MCT1-4 are the most studied MCT, with a focus on the ubiquitous MCT1. It has been established that MCT require an ancillary protein to target them to the membrane. While MCT1, MCT3 and MCT4 bind to CD147, also known as basigin, MCT2 necessitates gp-70 (embigin) for its expression at the plasma membrane. It has also been suggested that these ancillary proteins might be important in MCT activity, as well as protein trafficking.[129] Since the focus of this thesis is the study of MCT1 and MCT4-mediated lactic acid transport, the other isoforms will only be treated briefly.

MCT2 has been reported in kidney, as well as in the brain. MCT2 exhibits a higher affinity than MCT1 for lactate, with a K_m of 0.7 mM, compared to 4-7 mM for MCT1.[126]

MCT3 is expressed at the basolateral membrane[154] and has been found in the retinal pigment epithelium and in the choroid plexus.[126]

MCT8 and MCT10 are the only other two characterized members of the MCT family, apart from the true monocarboxylate transporters MCT1-4. MCT8 transports the thyroid hormones T3 and T4, whereas MCT10 transports aromatic amino acids. MCT8 and MCT10 are ubiquitously expressed and their transport is sodium and proton-independent.[126, 129]

1.4.3 Statin transporters

Systemic and local statin concentrations can be regulated by the differential expression of several uptake and efflux transporters in various tissues. Whereas intestinal and hepatic drug transporters will modulate plasma drug concentrations, transporters expressed in the skeletal muscle cell will limit or facilitate statin accumulation in the cell.[80] The role of statin transporters will be discussed further in this section.

1.4.3.1 Intestinal and hepatic transporters

Statins must reach the liver to exert their hypolipidemiant effects by inhibiting the HMG-CoA reductase. In order to get to the hepatocytes, orally administered drugs must be absorbed in the enterocytes first. Statin absorption into the enterocytes is facilitated primarily by SLCO2B1 (OATP2B1). Then, its release into the mesenteric veins that lead to the portal vein and to the hepatocytes is mediated by various ABCC (MRP). Statin transport into the hepatocytes is mainly mediated by SLCO1B1 (OATP1B1) and SLCO1B3 (OATP1B3), where they are subsequently metabolized by hepatic drug-metabolizing enzymes. Statin efflux transport from the enterocytes and hepatocytes is mediated by ABCB1 (P-glycoprotein; P-gp; MDR1) and ABCG2 (BCRP), as well as ABCC2.[40, 155]

Statin plasma concentrations can be greatly influenced by intestinal and hepatic transport activity. A lower uptake into the enterocytes or reduced efflux towards the mesenteric veins due to transporter polymorphisms or drug-drug interactions will decrease statin plasma concentrations, as well as tissue concentrations. In contrast, lower influx into the hepatocytes or reduced efflux towards the bile will result in the increase of both statin plasma and tissue concentrations.[40]

Overall, the transporters discussed in this section can affect systemic drug concentrations. Nonetheless, increased drug plasma levels can only partly explain the observed drug-induced myopathies. Therefore, it is critical to characterize muscular membrane transporters, which can impact local drug exposure in myocytes.

1.4.3.2 Muscular transporters

Statin-induced muscle toxicity has been associated with the interplay between drug influx and efflux transporters on the muscle cell membrane, which are depicted in Figure 8. The uptake transporter SLCO2B1 and the efflux transporters ABCC1, ABCC4 and ABCC5 have been studied for their possible implication in drug-induced myopathy.[80]

SLCO mediate bidirectional transport according to the concentration gradient.[7] It has been reported that SLCO2B1 mediates atorvastatin and rosuvastatin influx in muscle cells.[80]

Other muscle membrane transporters, such as MCT, seem to also transport certain statins. This can inhibit MCT lactic acid transport competitively.[7, 82-84]

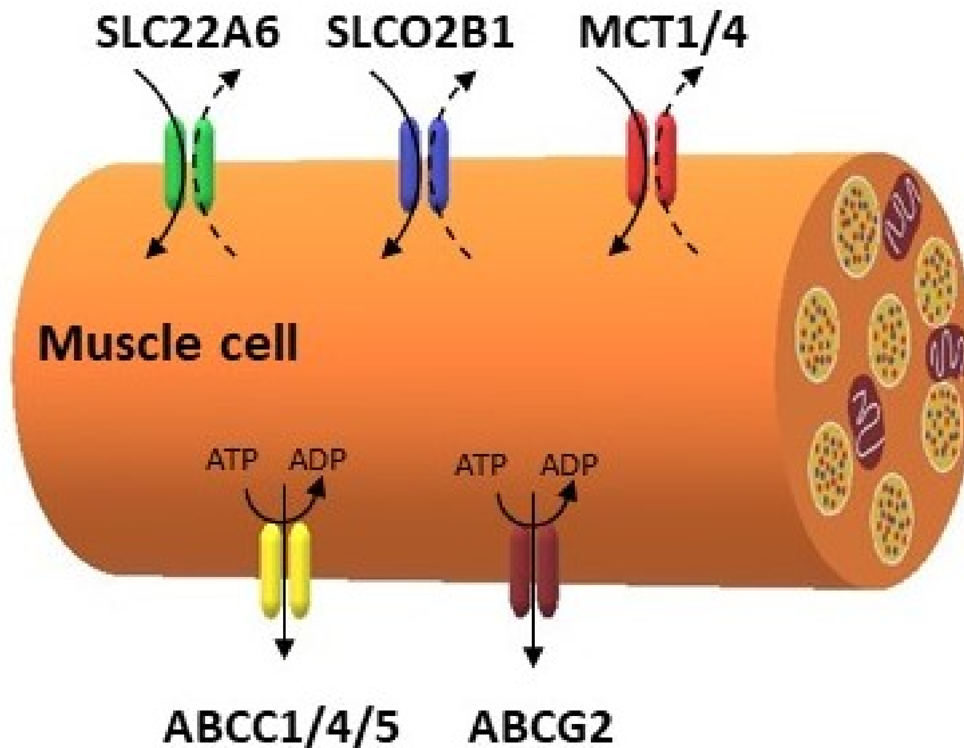


Figure 8. ABC and SLC statin transporters in muscle cells. MCT1 and MCT4 are possible contributors to the uptake and efflux of different statins.

Figure adapted from Du Souich *et al.* (2017)[40]

1.5 Rationale, hypothesis and objectives

Currently, statins are widely used as primary and secondary prevention for cardiovascular disease due to their efficiency in lowering LDL-cholesterol.[156] The risk/benefit ratio is more advantageous in secondary *vs* primary prevention since the patients have already experienced a cardiovascular event.[156, 157] It is well established for secondary prevention that the risk of myotoxicity in taking a statin treatment does not outweigh the benefit of lowered LDL, which can greatly diminish secondary cardiovascular events.[34, 35]

Regarding primary prevention, although the benefits are present for this population, they might not compensate for the possible adverse effects since only part of this population will be afflicted by a cardiovascular event during their lifetime.[35] Different statin treatments are known to be associated with muscular disorders, some asymptomatic, but others can affect patients' quality of life and their ability to perform daily functions.[4, 49, 55, 56] Therefore, in order to avoid or prevent these unwanted side effects, it is imperative to minimize the possible ADR with these statin therapies by trying to understand and elucidate the possible mechanism for statin-induced myopathy.

The hypothesis behind this doctorate project is that some statins and other acidic drugs can inhibit the transport, more specifically the efflux of lactic acid in the muscle and cause the clinically observed statin-induced myotoxicity. Indeed, some studies have shown that the expression of MCT does not seem to be affected by statins, but the statins might be able to affect their activity by diminishing directly the transport of lactic acid in muscle cells.[158] By diminishing the efflux of lactic acid *via* MCT1 and MCT4 in muscle cells, statins can cause an intracellular acidification. This can lead to the activation of certain caspases which in turn will activate apoptosis and cause the muscular disorder.[54, 159]

Therefore, the main objectives of this project were to determine whether the statins and other acidic drugs, including loratadine, can inhibit the efflux of L-lactic acid *via* MCT1 and MCT4, then more specifically in muscle cells in different physiological conditions.

In order to meet our objectives, the first study's aims were to describe the monocarboxylate transporters 1 and 4 for the transport of L-lactic acid. Two breast cancer cell lines expressing selectively MCT1 or MCT4 were identified and used as *in vitro* models.

L-lactic acid transport activity and the inhibitory potential of different statins and other acidic drugs were measured in these cells.

In our second study, we wanted to corroborate our first findings in a cellular model that is more physiologically representative. Therefore, cultured and differentiated human primary skeletal muscle cells (SkMC) were used in these studies. The L-lactic acid transport activity and the inhibitory potential of different statins and other acidic drugs were determined in these SkMC and at two physiological pH (pH7.0 and pH7.4), representing the level of physical activity. Pretreatments with statins and loratadine were also performed to determine if extended periods of these treatments could affect the transport of L-lactic acid or the inhibitory potential of the different compounds.

Chapter 2- Articles

2.1 Article 1: Effects of a series of acidic drugs on L-lactic acid transport by the monocarboxylate transporters MCT1 and MCT4

2.1.1 Introduction

Drug-induced myopathy has been associated with many currently administered medications. Some of these adverse drug-related events have established mechanisms, but they are unclear for most compounds. For example, the mechanism by which statins cause myotoxicities is still controversial with studies reporting contradictory results.

In this study, we are investigating the possible role of drug transporters in the development of drug-induced adverse events, more specifically the inhibition of lactic acid transport *via* MCT. The inhibition of lactic acid efflux from the muscle can cause intracellular acidification, which can lead to muscle cramps and activation of caspase 3/7, and induce apoptosis.

In vitro models can be a great tool to screen rapidly through various compounds in order to identify the ones that can affect certain physiological functions. To study the lactic acid transport activity of MCT, different models have been used, such as *Xenopus laevis* oocytes microinjected with human or rat MCT1 and MCT4 complementary RNA. Overall, this is a good model to study transport activity due to their large size (single cell model) and low amount of endogenous membrane transporters expressed at the cell surface. However, these oocytes have a short lifespan, low-throughput and transient protein expression, limiting this model.

In this article, we will identify and characterize different breast cancer cell lines, which can be used for reproducible lactic acid transport inhibition studies. The advantage of having established *in vitro* models for MCT1 and MCT4 is that we can study these MCT individually. This allows a better understanding of MCT-mediated transport and helps identify which transporters are inhibited by different compounds, leading to lactic acid accumulation in muscle.

2.1.2 Objectives

The main objective of this first article was to select *in vitro* models that can be used to study the transport of L-lactic acid *via* MCT1 and MCT4. Then, we aimed to characterize these models for their L-lactic acid transport activity before evaluating the inhibitory potential of a series of acidic drugs. After establishing *in vitro* models appropriate for this study, L-lactic acid inhibition studies were performed. L-lactic acid transport inhibition was evaluated on both the influx and efflux transport since these MCT are bidirectional.

2.1.3 Article

Current Pharmaceutical Biotechnology

RESEARCH ARTICLE

Effects of a Series of Acidic Drugs on L-Lactic Acid Transport by the Monocarboxylate Transporters MCT1 and MCT4

Yat Hei Leung[1,2], François Bélanger[2], Jennifer Lu[1,2], Jacques Turgeon[1] and Veronique Michaud[1,2,*]

[1]Faculty of Pharmacy, Université de Montréal, Montreal, Canada; [2]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, Canada

Received: November 09, 2017, Accepted: March 02, 2018, Published: March 07, 2018

Impact factor: 2.459

© 2017 Bentham Science Publishers

Effects of a series of acidic drugs on L-lactic acid transport by the monocarboxylate transporters MCT1 and MCT4

Yat Hei Leung,[a,b] Jennifer Lu,[a,b] François Bélanger,[b] Jacques Turgeon,[a] Véronique Michaud[a,b*]

[a]Faculty of Pharmacy, Université de Montréal, Montreal, Canada ;

[b]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, Canada

*Corresponding author:

Véronique Michaud, B.Pharm., Ph.D.

Centre de recherche du CHUM (CRCHUM),

900, rue St-Denis, Tour Viger, R08-480

Montreal, Quebec, Canada, H2X 0A9.

Tel: 514-890-8000 ext.15812

E-mail: v.michaud@umontreal.ca

© 2017 Bentham Science Publishers

RUNNING TITLE: Inhibition of L-lactic acid transport via MCTs

Number of words (abstract); 200

Number of references; 35

Number of tables; 2

Number of figures; 2

Number of supplemental figures; 3

Keywords: Adverse drug reactions, drug-transporters, MCT, statins, loratadine, lactic acid

ABSTRACT

Drug-induced myopathy is a serious side effect that often requires removal of a medication from a drug regimen. For most drugs, the underlying mechanism of drug-induced myopathy remains unclear. Monocarboxylate transporters (MCTs) mediate L-lactic acid transport, and inhibition of MCTs may potentially lead to perturbation of L-lactic acid homeostasis and muscular disorders. Therefore, we hypothesized that L-lactic acid transport may be involved in the development of drug-induced myopathy. The aim of this study was to assess the inhibitory potential of 24 acidic drugs on L-lactic acid transport using breast cancer cell lines Hs578T and MDA-MB-231, which selectively express MCT1 and MCT4, respectively. The influx transport of L-lactic acid was minimally inhibited by all drugs tested. The efflux transport was next examined: loratadine (IC_{50}: 10 and 61 μM) and atorvastatin (IC_{50}: 78 and 41 μM) demonstrated the greatest potency for inhibition of L-lactic acid efflux by MCT1 and MCT4, respectively. Acidic drugs including fluvastatin, cerivastatin, simvastatin acid, lovastatin acid, irbesartan and losartan exhibited weak inhibitory potency on L-lactic acid efflux. Our results suggest that some acidic drugs, such as loratadine and atorvastatin, can inhibit the efflux transport of L-lactic acid. This inhibition may cause an accumulation of intracellular L-lactic acid leading to acidification and muscular disorders.

1. INTRODUCTION

Several drugs can cause unforeseen toxicity in muscle tissues varying from asymptomatic biological abnormalities, mild nonspecific myalgia and myositis with weakness, up to severe life-threatening conditions. Muscles, which represent about 45% of human body weight are a well-perfused organ, are highly exposed to circulating drugs and are metabolically active, making them particularly susceptible to drug-related injury [1]. Although the incidence is unclear, drug-induced musculoskeletal disorders remains among the most common cause of muscle diseases.

Numerous drugs, including lipid lowering drugs (statins), antifungal agents, antimalarials, antivirals, cardiovascular agents and immunosuppressants, can cause adverse effects on muscles [2]. The underlying mechanisms by which drugs induce muscle disorders are not well understood and various hypotheses have been proposed including alteration in cellular membrane cholesterol, mitochondrial impairments, increased lysosomal activity, injuries to electrolyte homeostasis, alterations in protein synthesis and degradation, inhibition of myogenesis, oxidative stress, cell apoptosis and immune reactions [3-5].

Lipophilicity (greater penetration into muscle tissue), dosage, genetic polymorphisms and factors increasing plasma concentrations (increased bioavailability, decreased drug metabolism, or modulation of drug-transport) may augment the risk and partially explain intersubject variability in susceptibility to muscular adverse drug reactions [6-8]. For instance, drug-induced muscle disorders are often not related to a single agent but are more commonly observed in the context of drug–drug interactions [9]. In addition, exercise has been associated with an increase in muscular disorder induced by statins and transport of major

monocarboxylates related to exercise such as L-lactate has been proposed to play a role in drug-induced muscle disorder [10-15].

The monocarboxylate transporters (MCTs) are responsible for the influx/efflux of L-lactic acid from skeletal muscle cells and are essential for muscle homeostasis. The two proton-linked MCT transporters present in skeletal muscle cells are MCT1 and MCT4. MCT1, encoded by the *SLC16A1* gene, is recognized mostly as an uptake transporter and is ubiquitously expressed [16]. MCT4, encoded by the *SLC16A3* gene, is mostly responsible for the efflux of L-lactic acid in highly glycolytic tissues [17]. As skeletal muscles are the major producer of L-lactic acid, the transport of L-lactic acid is crucial for the maintenance of intracellular pH homeostasis. An accumulation of L-lactic acid would result in intracellular acidification causing apoptosis and muscular toxicity [18].

Studies suggest that some acidic drugs, such as statins, salicylic acid, bumetanide, and γ-hydroxybutyrate can be transported by the MCTs with the potential to interfere with MCT-mediated L-lactic acid transport [19]. RNA expression levels of MCTs in human muscle cells are not affected by statins, suggesting that a decrease in MCT activity could rather be due to inhibition of protein trafficking or competitive/non-competitive inhibition at the protein level [20, 21]. The main objective of our study was to characterize the effects of a series of acidic drugs, such as statins which are known to be associated with muscle adverse events, on the transport of L-lactic acid using cell lines that selectively express MCT1 or MCT4. Drugs that could be used concomitantly during statin treatment, such as antihypertensive agents and angiotensin-converting enzyme inhibitors (ACEi), were also evaluated to determine if they can exert any additional effects on L-lactic transport. The inhibitory effects of drugs that can be

administered to relieve muscle pain, specifically non-steroidal anti-inflammatory drugs (NSAID), were assessed to know if they could exacerbate the pre-existing condition.

2. MATERIALS AND METHOD

2.1 Materials

[^{14}C] L-lactic acid sodium salt was purchased from PerkinElmer (Walthman, MA, USA). Lactic acid sodium salt, captopril, colchicine, flurbiprofen, ibuprofen, naproxen, niacin, salicylic acid, valproic acid, and poly-L-lysine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Atorvastatin, cerivastatin, duloxetine, enalapril maleate salt, everolimus, fluvastatin, irbesartan, loratadine, losartan, lovastatin, lovastatin hydroxyl acid sodium salt, phloretin, pravastatin sodium salt, rosuvastatin, simvastatin, simvastatin hydroxyl acid ammonium salt and raltegravir were purchased from Toronto Research Center (Toronto, ON, Canada). DMEM, RPMI media and Trypsin/EDTA were obtained from Wisent Inc. (St. Bruno, QC, Canada). Fetal Bovine Serum was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The human breast cancer cell lines: Hs578T, MCF-7, MCF-10A, MDA-MB-231, MDA-MB-468, SKBR3, T47D, and ZR-75-1 were purchased from the American Type Culture Collection (Manassas, VA, USA). RNA extractions were performed using the QIAGEN RNA extraction Kit from Qiagen Sciences (Germantown, MD, USA). SuperScript II Reverse Transcriptase, random primers, RNaseOUT Recombinant Ribonuclease Inhibitor and Platinum SYBR Green qPCR SuperMix UDG were purchased from Invitrogen (Carlsbad, CA, USA). The 100mM dNTP mix was obtained from Wisent Inc. (St. Bruno, QC, Canada).

2.2 Cell culture

Hs578T, MCF-7, MDA-MB-231, MDA-MB-468 and SKBR3 were grown in DMEM media supplemented with 10% fetal bovine serum (FBS). T47D and ZR-75-1 were grown in RPMI also supplemented with 10% FBS. MCF-10A was grown in MEGM media (Lonza, Allendale, NJ, USA). These different human breast cancer cell lines were routinely cultured in plastic culture flasks (Sarstedt, Newton, NC, USA). Cells were used within 30 passages or less after thawing from liquid nitrogen, and at a maximum of 60 passages after receipt. Cells were cultured at 37°C with 5% CO_2. When they reached 80-95% confluence, they were harvested with 0.25% Trypsin/2.21 mM EDTA (Wisent Inc., St-Bruno, QC, Canada), resuspended and seeded into new flasks.

2.3 Total RNA extraction and cDNA synthesis

Seven different human breast cancer cell lines were grown to 70% confluence and total RNA was extracted using the RNAeasy kit according to manufacturer's instructions. The RNA concentration was measured by a spectrophotometer (UV absorption at 260 nm and 280 nm). RNA was stored at -80°C until used. The first-strand cDNA was prepared from 1 μg of isolated RNA using the SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random hexamer primers in a final volume of 20 μL according to the manufacturer's suggested protocol. cDNA, concentration fixed at 50 ng/μL, was aliquoted and stored at -80°C.

2.4 Quantitative real-time PCR

Gene expressions of *MCT1*, *MCT4* and *GAPDH* were evaluated by qPCR on a RotorGene RG6000 instrument (Corbett Research, San Francisco, CA, USA). All quantitative

PCR reactions were prepared using SYBR®Green PCR MasterMix with the synthesized first-strand cDNA (10 ng) and specific MCT1-, MCT4-, GAPDH-primer pairs (Invitrogen, Carlsbad, CA, USA). The sequences of the specific primers were as follows: for human MCT1, the sense sequence was 5'-TAA CAC CGT ACA GCA ACT ATA C-3', and the antisense sequence was 5'-AGC TTC CTC TCC ATC CAA AGA-3'; for human MCT4, the sense sequence was 5'-TGG CCT GGT GCT GCT GAT GGA-3', and the antisense sequence was 5'-CCA CCT CAGGCT GTG GCT CTT-3'; for human GAPDH, the sense sequence was 5'-GTC GGA GTC AAC GGA TTT GGT-3', and the antisense was 5'-GAT GAC AAG CTT CCC GTT CTC-3'. The thermal cycling conditions were 3 minutes at 95°C, followed by 40 amplification cycles of 95°C for 30 seconds, 60°C for 15 seconds and 72°C for 20 seconds. Reactions were performed in triplicate. Relative quantification of MCT gene expressions was performed using the $2^{-\Delta Ct}$ approach to calculate the fold change normalized to housekeeping gene (GAPDH); and $2^{-\Delta\Delta Ct}$ to compare the relative fold difference of individual gene expression within the cancer cell lines using SKBR3 and MDA-MB-231 as the references for the expression of MCT1 and MCT4, respectively.

2.5 Western blot analysis

Total protein content was extracted from Hs578T and MDA-MB-231 cells. Cells were suspended in a lysis buffer containing 1% SDS/0.2N NaOH. Protein concentration of the protein lysate was determined as described by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as a standard. For the Western blot analysis, samples were denatured at 100°C for 5 minutes in a loading buffer containing 50 mM Tris-HCL, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol and separated in 5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred by

electrophoresis onto a pure nitrocellulose membrane (BioTrace, Onenhuga, Auckland, New Zealand). Membranes were blocked with TBS containing 0.05% Tween 20 (TBS/T) and 5% dry milk. Membranes were washed with TBS/T and incubated with primary antibody mouse anti-GAPDH (Sigma-Aldrich, St. Louis, MO, USA) (diluted 1:10000), mouse anti-MCT1 (diluted 1:500), or rabbit anti-MCT4 (Santa Cruz Biotechnology, Dallas, Texas, USA) (diluted 1:500). Then, membranes were washed and incubated with secondary antibody conjugated with horseradish peroxidase goat-anti mouse (diluted 1:5000) or goat-anti rabbit (Santa Cruz Biotechnology, Dallas, Texas, USA) (diluted 1:5000). Bands were visualized on Hyblot CL autoradiography film (Denville Scientific, Holliston, MA, USA) with a standard enhanced chemiluminescence developing solution (GE Healthcare, Mississauga, ON, Canada).

2.6 Transport studies

Hs578T and MDA-MB-231 cells were seeded on 35 x 10 mm tissue culture plates (Sarstedt, Newton, NC, USA) at a density of 7.5 x 10^5 cells/mL and 1 x 10^6 cells/mL, respectively and cultured for 1 day to 90-100% confluence before use in transport assays. Before starting transport experiments, cells were washed and the medium replaced with HEPES (pH 7.4; 1 mL) buffer. Transport assays, including the pre-incubation period in HEPES, were performed at 37°C.

2.6.1 Assessment of influx transport. At the beginning of the experiment (*t*= 0), HEPES medium was replaced by MES (pH 6.0; 1 mL) buffer containing 0.0033-30 mM [^{14}C] L-lactic acid (0.2 µCi/mL). After incubation for 2.5 minutes, the radioactive media was removed from the milieu. Transport assays were stopped by placing culture plates on ice, rapidly aspirating the media and cells were washed 3-times with ice-cold HEPES buffer. Cells were then

solubilized using a solution of 0.2N NaOH and 1% SDS (500 µL). The suspension was passed through 27½ G needle 3-times. Aliquot of the cell lysate (400 µL) was transferred in a scintillation tube containing 5 mL of biodegradable scintillation counting cocktail buffer (Bio-Safe II, Research Products International Corp., Mt. Prospect, IL, USA). Radioactivity levels were quantified with a Tri-Carb liquid scintillation counter (LSC 1600TR, Packard Instrument Co., Meriden, CT, USA) to determine the intracellular [^{14}C] L-lactic acid concentrations. Protein concentrations was measured using Pierce BCA protein assay kit (Thermo Fisher Scientific, Walthman, MA, USA).

2.6.2 Assessment of efflux transport. A similar approach was used to measure efflux transport of L-lactic acid. For these experiments, distribution equilibrium of L-lactic acid was reached by adding MES buffer (pH 6.0; 1 mL) containing 6 mM [^{14}C] L-lactic acid (0.2µCi/mL). After 2.5 minutes, MES buffer containing [^{14}C] L-lactic acid was replaced by a L-lactic acid-free MES buffer for 2.5 minutes. Transport was stopped by placing culture plates on ice, rapidly aspirating the MES-media and cells were washed 3-times with ice-cold HEPES buffer. Cellular radioactivity levels were determined as described previously.

2.7 Inhibition studies

In the inhibition experiments, transport of L-lactic acid was assessed in the absence and presence of increasing concentrations of acidic drugs. In total, 24 compounds were investigated: atorvastatin, captopril, cerivastatin, colchicine, duloxetine, enalapril, everolimus, flurbiprofen, fluvastatin, ibuprofen, irbesartan, losartan, lovastatin hydroxy acid, lovastatin lactone, loratadine, naproxen, niacin, pravastatin, raltegravir, rosuvastatin, simvastatin hydroxy acid, simvastatin lactone, salicylic acid and valproic acid. Effects of acidic

compounds on L-lactic acid transport were tested at concentrations varying from 5-200 μM, except loratadine, which was tested from 0.05-250 μM.

To study the inhibition of L-lactic acid influx transport, the tested inhibitor was added at the beginning of the experiments, when the [^{14}C] L-lactic acid was loaded. Briefly, following pre-incubation of cells in MES buffer pH 6.0, [^{14}C] L-lactic acid (6 mM, 0.2 μCi/mL) and the potential inhibitor were added and incubated for 2.5 minutes. Culture plates were placed on ice to stop the reaction. Inhibition of the uptake transport of L-lactic acid was determined by measuring cellular [^{14}C] L-lactic acid concentration.

To study the inhibition of L-lactic acid efflux transport, tested inhibitor was added following the equilibrium period with [^{14}C] L-lactic acid. Tested inhibitors or vehicles were added to the L-lactic acid free MES buffer.

2.8 Data analysis

For kinetic studies, the K_m (Michaelis-Menten constant) and V_{max} (maximum uptake rate) of L-lactic acid transport were estimated by non-linear least-squares regression analysis program, GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA, USA) using the following equation: $v = V_{max} \cdot [S]/(K_m+[S])$,

where v and [S] are uptake rate of L-lactic acid at 2.5 minutes and concentration of L-lactic acid, respectively. The IC_{50} and K_i were also estimated using the GraphPad program.

3. RESULTS

3.1 Selection of cell lines for MCT1 and MCT4 transport assays

Numerous cell lines including Hs578T, MCF-7, MCF-10A, MDA-MB-231, MDA-MB-468, SKBR3, T47D and ZR-75-1 were tested in order to determine their respective specificity for MCT expression profile. Figure 1A compares the relative mRNA expression levels between MCT1 and MCT4 for each cell line. SKBR3, Hs578T and ZR-75-1 cell lines showed the highest selectivity for MCT1 mRNA expression compared to MCT4. MDA-MB-231 was the only cell line that displayed a specific mRNA expression for MCT4. MCF-10A and T47D cell lines expressed similar mRNA levels for MCT1 and MCT4. MCF-7 and MDA-MB-468 cells exhibited greater mRNA levels of MCT4, but a significant level of MCT1 mRNA was also observed. The relative mRNA expression levels for each MCT transporter were also compared across the various cell lines (Figure 1B and Figure 1C, respectively). Even though MCT1 was selectively expressed in ZR-75-1 cell line (Figure 1A, B and C), these cells expressed the lowest levels of MCT1 mRNAs compared to all tested cell lines. Based on qPCR analysis and technical features (adherence, ease to grow), Hs578T was selected over SKBR3. Hence, Hs578T and MDA-MB-231 were pre-selected as cell lines for *in vitro* MCT1 and MCT4 transport assays, respectively. In addition to the mRNA analysis, the selective expression of MCT1 (Hs578T) or MCT4 (MDA-MB-231) was confirmed by Western blot analyses. As shown in Figure 1D and Figure 1E, MCT1 protein was highly selectively expressed in Hs578T cells while only the MCT4 protein was detected in MDA-MB-231 cells.

3.2 Kinetic parameters of L-lactic acid transport in Hs578T and MDA-MB-231 cells

The concentration-dependent uptake of L-lactic acid into Hs578T and MDA-MB-231 cell lines were determined (Figure 2). The K_m values for transport of L-lactic acid by MCT1 and MCT4 were 2.3 ± 0.2 mM and 9.6 ± 0.9 mM in Hs578T and MDA-MB-231 cells, respectively (Figure 2A and Figure 2B). The estimated CL_{int} value for the transport of L-lactic acid for MCT1 was higher than MCT4; CL_{int} values were 24 µL/min/mg protein (V_{max}=54 nmol/min/mg protein) *vs* 15 µL/min/mg protein (V_{max}=149 nmol/min/mg protein), respectively. The intrinsic clearance could only be determined for the influx transport.

3.3 IC_{50} determination for inhibition of L-lactic acid uptake by different drugs

The inhibitory potential (IC_{50}) of different acidic drugs on the uptake of L-lactic acid by MCTs was evaluated in Hs578T and MDA-MB-231 cells in order to distinguish specificity of these compounds on MCT1 and MCT4 transporters (Table 1). Overall, L-lactic acid uptake by MCT1 and MCT4 was only slightly decreased by the series of acidic drugs tested. Atorvastatin, cerivastatin, fluvastatin, irbesartan and simvastatin hydroxy acid inhibited the influx transport through MCT1 and MCT4 with relatively high IC_{50} values ranging between 100-400 µM. No significant inhibition of L-lactic acid influx by neither MCT1 nor MCT4 was observed with other acidic compounds (such as lovastatin lactone, pravastatin, rosuvastatin, simvastatin lactone, captopril, enalapril, flurbiprofen, ibuprofen, naproxen and salicylic acid). Kinetic profiles for inhibition on L-lactic acid influx by statins and other compounds with measurable inhibition of MCTs are shown in Supplemental Figure 1.

3.4 IC_{50} determination for inhibition of L-lactic acid efflux transport by different drugs

The inhibition of MCT-mediated L-lactic acid efflux by acidic drugs was also evaluated in both Hs578T and MDA-MB-231 cells in order to distinguish specificity of these compounds for MCT1 *vs* MCT4. The IC_{50} values for the tested acidic drugs on the L-lactic acid efflux transport through MCT1 and MCT4 are shown in Table 2. Our results demonstrated that loratadine, a second-generation H1 histamine antagonist, was the most potent inhibitor with IC_{50} values of 10 μM (7-16) for MCT1 and 61 μM (39-94) for MCT4. For statins, atorvastatin exhibited the strongest inhibition of L-lactic acid efflux with IC_{50} of 78 μM (56-109) and 41 μM (29-58) in cells selectively expressing MCT1 and MCT4, respectively. Cerivastatin, fluvastatin, lovastatin hydroxy acid and simvastatin hydroxy acid showed weak inhibitory potency with estimated IC_{50} ranging between 100-500 μM. The angiotensin II receptor antagonists (ARBs) tested namely, irbesartan and losartan, also exhibited a weak inhibition (IC_{50} of 100-300 μM). No significant inhibition of L-lactic acid efflux by neither MCT1 nor MCT4 was observed with pravastatin, rosuvastatin, lovastatin lactone, simvastatin lactone, ACEi, NSAIDs and other tested acidic compounds. Kinetic profiles for inhibition on L-lactic acid efflux by statins and other compounds with measurable inhibition of MCTs are shown in Supplemental Figure 2.

Mechanisms involved in the inhibition of lactic acid transport through MCTs by atorvastatin have been further examined. Phloretin, a well-recognized MCT inhibitor, was used as a positive control of lactic acid transport inhibition in comparison to atorvastatin. Atorvastatin was selected based on our IC_{50} results and the frequency of muscle event complaints. K_i experiments for L-lactic acid uptake inhibition by atorvastatin *vs* phloretin were performed (Supplemental Method and Supplemental Figure 3).

4. DISCUSSION

In our study, Hs578T and MDA-MB-231 cell lines selectively expressing MCT1 and MCT4 were identified and used as experimental cell models to distinguish inhibition potency of acidic drugs towards these transporters. Our study showed that inhibitory potency of acidic drugs on L-lactic acid uptake was generally weak. In contrast, significant inhibition of L-lactic acid efflux by both MCTs was observed, especially for loratadine and atorvastatin.

MDA-MB-231 cells are known to selectively express MCT4 [22]. We built our study design on this observation and searched for cell lines selectively expressing MCT1. Hence, we demonstrated that SKBR3 and Hs578T cells selectively express MCT1. Although SKBR3 cells showed the highest mRNA expression for MCT1, we selected Hs578T cells as they showed higher adherence to culture plates and superior ease to culture.

MCT1 and MCT4 can mediate bidirectional transport of monocarboxylates: the directional flow depends on proton gradients present at a particular time. In addition to recognized endogenous monocarboxylates, such as L-lactate and pyruvate, there are evidences suggesting that drugs such as statins, salicylic acid, bumetanide, and γ-hydroxybutyrate can be transported by and/or inhibit transport by MCTs [19]. Consequently, administration of these pharmaceutical agents, substrates or inhibitors of MCTs, could interfere with the transport of L-lactic acid in skeletal muscle cells causing loss of cell homeostasis and ultimately, muscle pain. Based on these notions, various acidic drugs were tested as potential inhibitors of MCT transport of L-lactic acid.

The use of cell lines is however associated with some limitations. First, cancer cell lines selected exhibited a selective but high level of expression of MCT transporters. The use of supra-physiological L-lactic acid concentrations was required to saturate these transporters. Consequently, IC_{50} determined for inhibitors were also overestimated compared to the expected K_i determined with physiological concentrations of L-lactic acid. Second, efflux of L-lactic acid from the intracellular milieu is extremely rapid and almost completed after 2.5 minutes. Adding inhibitors in the extracellular L-lactic acid free medium allowed short period of time for diffusion of the drugs (inhibitors) and for inhibition of MCT efflux transporters. Such time-limited diffusion would have minimal impact if inhibitors bind to an extracellular domain but may significantly underestimate the inhibitor potency if inhibition occurs from an intracellular binding site. Nevertheless, we were able to demonstrate, for all potential inhibitors, greater inhibition of L-lactic acid efflux compared to inhibition of L-lactic acid influx by MCTs. Therefore, under true physiological situations, allowing sufficient time for drugs to reach the intracellular site (through either passive diffusion or active transported) could favor MCT inhibition.

Loratadine, an acidic antihistamine drug used to treat allergies, was found to be the most potent MCT1 and MCT4 L-lactic acid efflux inhibitor with average IC_{50} values of 10 µM and 61 µM, respectively. It has been reported that loratadine treatment can be associated with muscle pain, especially when it is used in concomitance with other medications [23]. Indeed, a study using a translational biomedical informatics approach recently identified drug-drug interactions with loratadine as a variable increasing the risk of drug-induced myopathy [9]. Their results suggested that these drug-drug interactions were unlikely to be associated with inhibition of CYP450 drug-metabolism or inhibition of hepatic uptake *via* OATP1B1/3

transporters [23]. These studies suggest that mechanisms related to drug-induced muscle disorders occur at the muscular cell level. Our results support this hypothesis and point towards the role of drug-induced MCT inhibition and L-lactic acid accumulation in drug-induced myopathy [24, 25].

Inhibition of L-lactic acid was not observed with all acidic drugs tested. Among various statins tested, atorvastatin was the most potent inhibitor of MCT1 and MCT4 L-lactic acid efflux (IC_{50} of 78 µM and 41 µM, respectively). Certain statins are associated with higher incidence of myopathy [26]. The higher frequency of muscle symptoms with statins may be partly explained by their pharmacologic characteristics including their drug metabolism pathways [27], lipophilicity [28], drug transporter specificity [29, 30], and drug-drug interactions [31, 32]. The low bioavailability of simvastatin (*i.e.* less than 5%) or the longer elimination half-life of atorvastatin compared to other statins may contribute to increased drug exposure [33-35].

Kobayashi and his group have conducted several studies in support of our hypothesis suggesting that statin-induced muscle disorder is associated with accumulation of L-lactic acid and intracellular pH alteration [18, 36, 37]. For instance, they demonstrated that cerivastatin was associated with intracellular acidification in a concentration-dependent manner [37]. In another series of experiments, they reported IC_{50} values for uptake inhibition of L-lactic acid *via* MCT4 of 32.4 ±3.2 µM, 32.6 ±2.1 µM, 44.2 ±9.7 µM, 79.4 ±2.5 µM, 96.0 ±5.5 µM, >100 µM, and >1000 µM for fluvastatin, atorvastatin, lovastatin acid, simvastatin acid, cerivastatin, rosuvastatin and pravastatin, respectively [21]. Their experimental model (CD147/MCT4 FLAG transfected LLC-PK1, porcine kidney epithelial cells) was based on inhibition of L-

lactic acid influx and did not allow for measurement of L-lactic acid efflux inhibition, a condition which could have been more relevant.

IC_{50} values of statin-mediated inhibition of L-lactic acid efflux by MCT1 or MCT4 in our experiments were higher than previously reported IC_{50} values (inhibition of L-lactic acid influx) [21]. Discrepancy between our results and their results could be explained by a much higher concentration of L-lactic acid used in our studies (6 mM) compared to those used by Kobayashi *et al.* (3 μM). Concentrations of L-lactic acid selected in our studies are close to their actual K_m for transport by MCTs (determined in cancer cell lines) and to the range of plasma concentrations in humans (0.5 to 2.2 mM). Obviously, inhibition study performed with higher concentrations (*i.e.* 6 mM *vs* 3 μM) will lead to higher IC_{50} values. In order to put in perspective the strength of inhibition, inhibition constant values were determined for atorvastatin and compared to phloretin a well-known MCT inhibitor. In Hs578T cells mediating lactic acid transport through MCT-1, K_i values of 36 μM and 115 μM were obtained for atorvastatin and phloretin, respectively (Supplemental Figure 3A and 3B). Moreover, atorvastatin and phloretin showed similar K_i values of 70 μM and 59 μM, respectively in MCT4-mediated transport of lactic acid (Supplemental Figure 3C and 3D). When compared to phloretin, these results put in perspective the *in vitro* potency of atorvastatin towards lactic acid transport through MCT1-4. Under physiological situations, *i.e.* in muscular skeletal cells instead of cancer cells (which overexpressed MCT transporters) and at normal lactic acid levels, inhibition of MCT transporters in muscle may remain relevant [24, 25].

An intermediate to weak inhibition of L-lactic acid efflux was observed with the angiotensin II receptor antagonists, irbesartan and losartan. ARBs have low incidence of

adverse effects including muscle cramps, fatigue and back pain. ARBs are commonly prescribed with concomitant medication, such as statins which could potentially work synergistically to induce muscle pain. This study demonstrated that neither NSAIDs nor antihypertensive agents contribute to a possible exacerbation of statin-induced myopathy through this mechanism. In addition, we established that the other acidic drugs known to cause muscle pain do not cause muscle toxicity through this mechanism.

In conclusion, we have characterized two breast cancer cell lines, Hs578T and MDA-MB-231, which can be used as selective *in vitro* models for the study of MCT1 and MCT4, respectively. Our experiments represent the first step for the determination of the inhibitory potential of different acidic drugs on the transport of L-lactic acid by MCTs. Our results demonstrated that loratadine and atorvastatin blocked L-lactic acid efflux transport to a significant extent in cell lines selectively expressing MCTs. Further studies are required to relate intracellular accumulation of L-lactic acid in skeletal muscle cells and the clinical observation of drug-induced muscle pain.

CONFLICT OF INTEREST

The authors report no declarations of interest. This work was supported by internal fundings from the CRCHUM, Fondation CHUM and Faculté de Pharmacie, Université de Montreal.

REFERENCES

1. Lee, A., Adverse drug reactions. Second ed. 2006: Pharmaceutical Press.
2. Valiyil, R. and L. Christopher-Stine, Drug-related myopathies of which the clinician should be aware. Curr Rheumatol Rep, 2010. 12(3): p. 213-20.
3. Dirks, A.J. and K.M. Jones, Statin-induced apoptosis and skeletal myopathy. Am J Physiol Cell Physiol, 2006. 291(6): p. C1208-12.
4. Dalakas, M.C., Toxic and drug-induced myopathies. J Neurol Neurosurg Psychiatry, 2009. 80(8): p. 832-8.
5. Mastaglia, F.L. and M. Needham, Update on toxic myopathies. Curr Neurol Neurosci Rep, 2012. 12(1): p. 54-61.
6. Smithson, J., Drug induced muscle disorders. 2009. 28(12): p. 1056-1062.
7. Guis, S., J.P. Mattei, and F. Liote, Drug-induced and toxic myopathies. Best Pract Res Clin Rheumatol, 2003. 17(6): p. 877-907.
8. Needham, M. and F.L. Mastaglia, Statin myotoxicity: a review of genetic susceptibility factors. Neuromuscul Disord, 2014. 24(1): p. 4-15.
9. Duke, J.D., et al., Literature based drug interaction prediction with clinical assessment using electronic medical records: novel myopathy associated drug interactions. PLoS Comput Biol, 2012. 8(8): p. e1002614.
10. Mikus, C.R., et al., Simvastatin impairs exercise training adaptations. J Am Coll Cardiol, 2013. 62(8): p. 709-14.
11. Parker, B.A., et al., Effect of statins on creatine kinase levels before and after a marathon run. Am J Cardiol, 2012. 109(2): p. 282-7.
12. Meador, B.M. and K.A. Huey, Statin-associated changes in skeletal muscle function and stress response after novel or accustomed exercise. Muscle Nerve, 2011. 44(6): p. 882-9.
13. Kearns, A.K., et al., The creatine kinase response to eccentric exercise with atorvastatin 10 mg or 80 mg. Atherosclerosis, 2008. 200(1): p. 121-5.
14. Thompson, P.D., et al., Lovastatin increases exercise-induced skeletal muscle injury. Metabolism, 1997. 46(10): p. 1206-10.
15. Reust, C.S., S.C. Curry, and J.R. Guidry, Lovastatin use and muscle damage in healthy volunteers undergoing eccentric muscle exercise. West J Med, 1991. 154(2): p. 198-200.
16. Bröer, S., et al., Characterization of the monocarboxylate transporter 1 expressed in Xenopus laevis oocytes by changes in cytosolic pH. Biochemical Journal, 1998. 333(1): p. 167-174.
17. Fox, J.E.M., D. Meredith, and A.P. Halestrap, Characterisation of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle. The Journal of Physiology, 2000. 529(2): p. 285-293.
18. Kobayashi, M., et al., Association between risk of myopathy and cholesterol-lowering effect: a comparison of all statins. Life Sci, 2008. 82(17-18): p. 969-75.
19. Morris, M.E. and M.A. Felmlee, Overview of the proton-coupled MCT (SLC16A) family of transporters: characterization, function and role in the transport of the drug of abuse gamma-hydroxybutyric acid. AAPS J, 2008. 10(2): p. 311-21.
20. Morikawa, S., et al., Analysis of the global RNA expression profiles of skeletal muscle cells treated with Statins. Journal of Atherosclerosis and Thrombosis, 2005. 12(3): p. 121-131.
21. Kobayashi, M., et al., Inhibitory effects of statins on human monocarboxylate transporter 4. Int J Pharm, 2006. 317(1): p. 19-25.

22. Gallagher, S.M., et al., Monocarboxylate transporter 4 regulates maturation and trafficking of CD147 to the plasma membrane in the metastatic breast cancer cell line MDA-MB-231. Cancer Res, 2007. 67(9): p. 4182-9.
23. Han, X., et al., Identification and Mechanistic Investigation of Drug-Drug Interactions Associated With Myopathy: A Translational Approach. Clin Pharmacol Ther, 2015. 98(3): p. 321-7.
24. Leung, Y.H., J. Turgeon, and V. Michaud, Study of Statin- and Loratadine-Induced Muscle Pain Mechanisms Using Human Skeletal Muscle Cells. Pharmaceutics, 2017. 9(42): p. 1-13.
25. Leung, Y.H., J. Turgeon, and V. Michaud, Supplementary Materials: Study of Statin- and Loratadine-Induced Muscle Pain Mechanisms Using Human Skeletal Muscle Cells. Pharmaceutics, 2017. 9(42): p. S1-S2.
26. Bruckert, E., et al., Mild to moderate muscular symptoms with high-dosage statin therapy in hyperlipidemic patients--the PRIMO study. Cardiovasc Drugs Ther, 2005. 19(6): p. 403-14.
27. Lennernas, H. and G. Fager, Pharmacodynamics and pharmacokinetics of the HMG-CoA reductase inhibitors. Similarities and differences. Clin Pharmacokinet, 1997. 32(5): p. 403-25.
28. Hamelin, B.A. and J. Turgeon, Hydrophilicity/lipophilicity: relevance for the pharmacology and clinical effects of HMG-CoA reductase inhibitors. Trends Pharmacol Sci, 1998. 19(1): p. 26-37.
29. Rodrigues, A.C., Efflux and uptake transporters as determinants of statin response. Expert Opin Drug Metab Toxicol, 2010. 6(5): p. 621-32.
30. Lee, H.H. and R.H. Ho, Interindividual and interethnic variability in drug disposition: polymorphisms in organic anion transporting polypeptide 1B1 (OATP1B1; SLCO1B1). Br J Clin Pharmacol, 2017. 83(6): p. 1176-1184.
31. Hirota, T. and I. Ieiri, Drug-drug interactions that interfere with statin metabolism. Expert Opin Drug Metab Toxicol, 2015. 11(9): p. 1435-47.
32. Bellosta, S. and A. Corsini, Statin drug interactions and related adverse reactions. Expert Opin Drug Saf, 2012. 11(6): p. 933-46.
33. Corsini, A., et al., New insights into the pharmacodynamic and pharmacokinetic properties of statins. Pharmacology & Therapeutics, 1999. 84(3): p. 413–428.
34. McKenney, J.M., Pharmacologic characteristics of statins. Clinical Cardiology, 2003. 26(S3): p. 32-38.
35. Kuncl, R.W., Agents and mechanisms of toxic myopathy. Curr Opin Neurol, 2009. 22(5): p. 506-15.
36. Kobayashi, M., et al., Effect of medium pH on the cytotoxicity of hydrophilic statins. J Pharm Pharm Sci, 2007. 10(3): p. 332-9.
37. Kobayashi, M., et al., Preventive effects of bicarbonate on cerivastatin-induced apoptosis. Int J Pharm, 2007. 341(1-2): p. 181-8.

TABLE 1: IC_{50} values of various drugs on the uptake of L-lactic acid (6 mM) by MCT1 in Hs578T cells and by MCT4 in MDA-MB-231 cells.

	Drugs	MCT1- IC_{50} (μM)	MCT4 - IC_{50} (μM)
Statins	Atorvastatin	>210	~138
	Cerivastatin	~150	>200
	Fluvastatin	~180	~187
	Lovastatin hydroxy acid	NI	>250
	Lovastatin lactone	NI	NI
	Pravastatin	NI	NI
	Rosuvastatin	NI	NI
	Simvastatin hydroxy acid	~180	~180
	Simvastatin lactone	NI	NI
ARB	Irbesartan	>409	>302
	Losartan	>400	NI
NSAID	Flurbiprofen	NI	NI
	Ibuprofen	NI	NI
	Naproxen	NI	NI
	Salicylic acid	NI	NI
ACEi	Captopril	NI	NI
	Enalapril	NI	NI
Others	Colchicine	NI	NI
	Duloxetine	NI	NI
	Everolimus	NI	NI
	Loratadine	~100	>300
	Niacin	NI	NI
	Raltegravir	NI	NI
	Valproic acid	NI	NI

NI: no inhibition observed (IC_{50} >1000 μM).
ARB: angiotensin II receptor blocker
NSAID: nonsteroidal anti-inflammatory drug
ACEi: angiotensin-converting-enzyme inhibitor

TABLE 2: IC_{50} values of various drugs on the efflux of L-lactic acid (6 mM) by MCT1 using Hs578T cells and MCT4 using MDA-MB-231 cells. (95% confidence interval)

	Drugs	MCT1- IC_{50} (μM)	MCT4 - IC_{50} (μM)
Statins	Atorvastatin	78 (56-109)	41 (29-58)
	Cerivastatin	>300	>400
	Fluvastatin	128	>210
	Lovastatin hydroxy acid	NI	>200
	Lovastatin lactone	NI	NI
	Pravastatin	NI	NI
	Rosuvastatin	NI	NI
	Simvastatin hydroxy acid	>180	>180
	Simvastatin lactone	NI	NI
ARB	Irbesartan	>229	~132
	Losartan	>300	>200
NSAID	Flurbiprofen	NI	NI
	Ibuprofen	NI	NI
	Naproxen	NI	NI
	Salicylic acid	NI	NI
ACEi	Captopril	NI	NI
	Enalapril	NI	NI
Others	Colchicine	NI	NI
	Duloxetine	NI	NI
	Everolimus	NI	NI
	Loratadine	10 (7-16)	61 (39-94)
	Niacin	NI	NI
	Raltegravir	NI	NI
	Valproic acid	NI	NI

NI: no inhibition was observed (IC_{50} >1000 μM).
ARB: angiotensin II receptor blocker
NSAID: nonsteroidal anti-inflammatory drug
ACEi: angiotensin-converting-enzyme inhibitor

FIGURE LEGENDS

Figure 1. Relative mRNA expression levels between MCT1 vs MCT4 in the 7 different human breast cancer cell lines determined by RT-PCR relative to GAPDH as the housekeeping gene and the $2^{-\Delta Ct}$ method (**A**). Panels **B** and **C** illustrate the relative mRNA expression of MCT1 and MCT4 calculated using the $2^{-\Delta\Delta Ct}$ approach where SKBR3 and MDA-MB-231 were used as reference for MCT1 and MCT4, respectively. Panels **D** and **E** illustrate Western blot analysis of MCT1 and MCT4 expression in Hs578T and MDA-MB-231, respectively. Proteins were separated by SDS-PAGE and Western blotted with antibodies against MCT1, MCT4 and GAPDH as indicated.

Figure 2. The uptake of L-lactic acid by MCT1 in Hs578T (**A**) and MCT4 in MDA-MB-231 (**B**). The intracellular [^{14}C] L-lactic acid was measured after 2.5 minutes of uptake at pH 6.0. Each point represents the mean ± S.D. of triplicate.

FIGURE 1

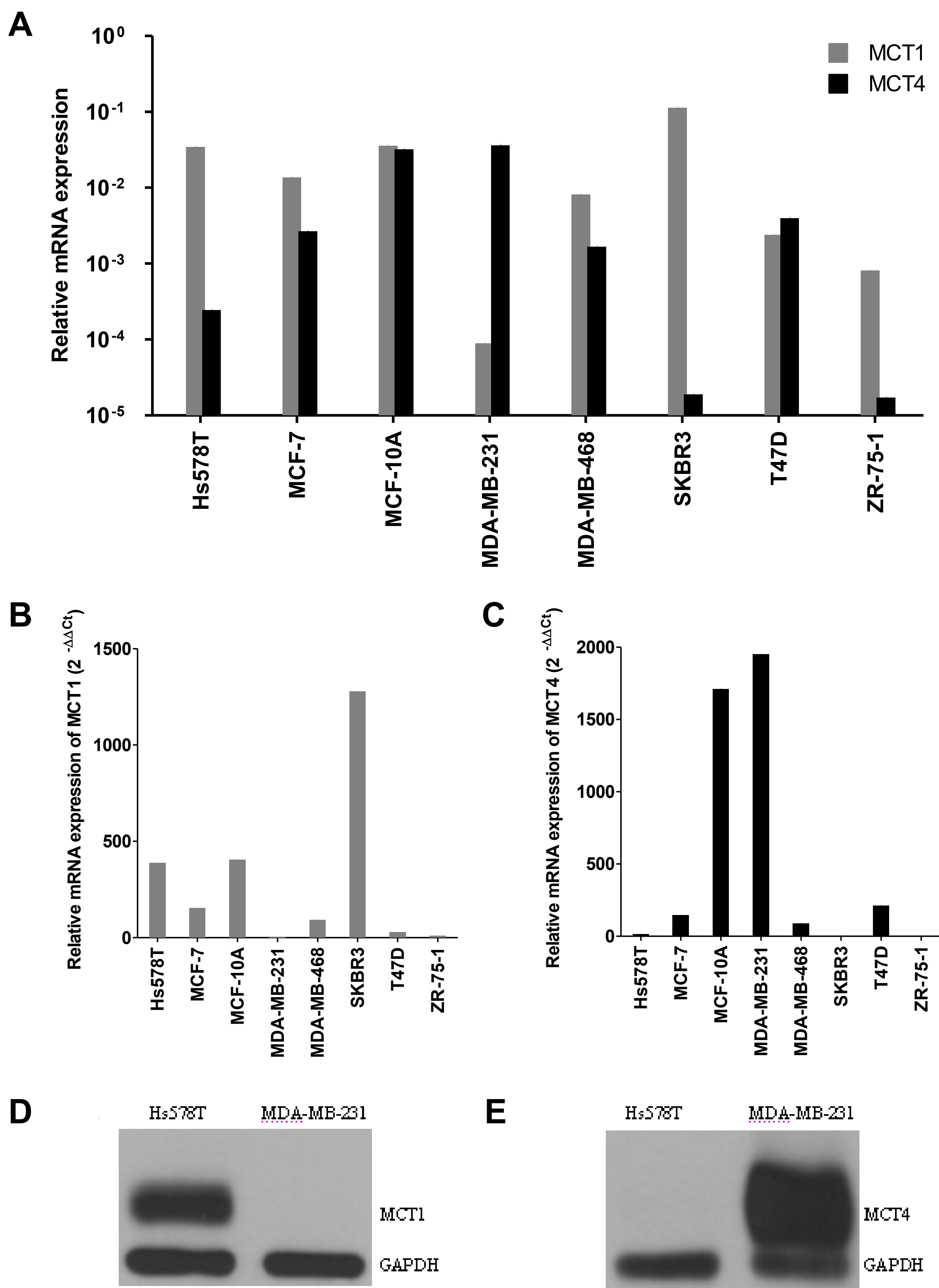
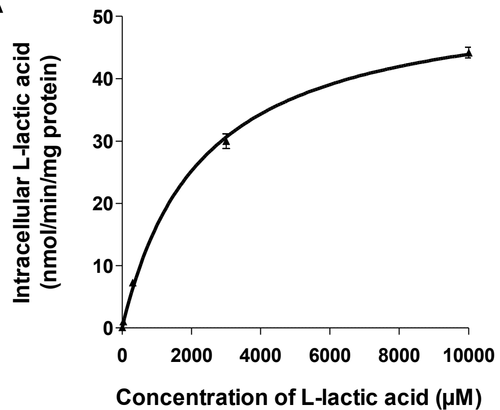
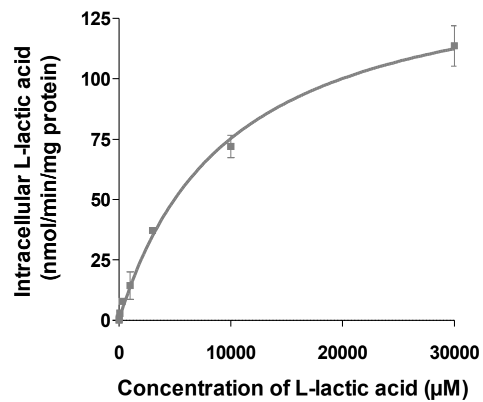


FIGURE 2





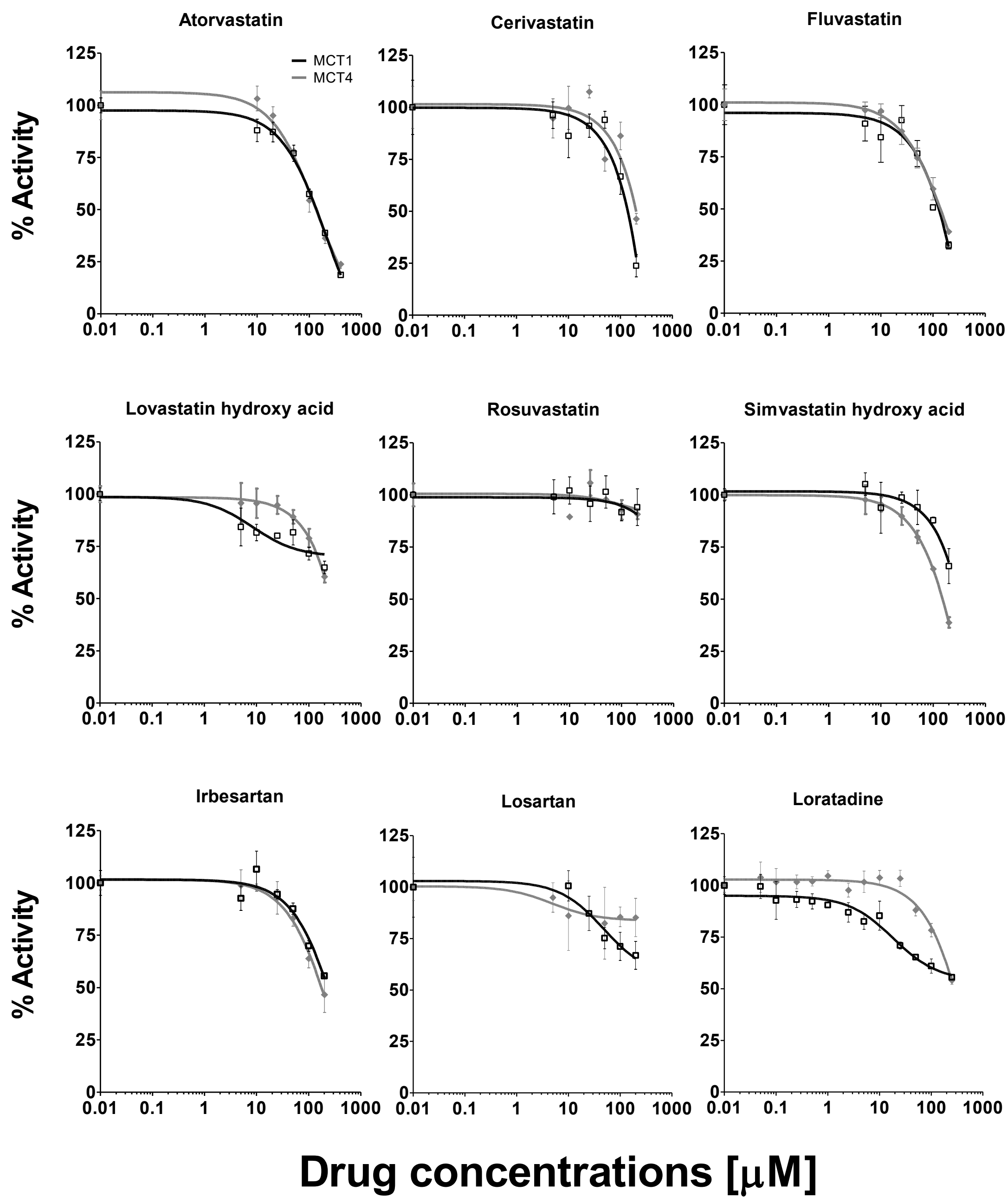
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Inhibitory effects of different acidic drugs on L-lactic acid (6 mM) uptake with Hs578T (MCT1) and MDA-MB-231 (MCT4) cell models. The intracellular [^{14}C] L-lactic acid was measured after 2.5 minutes of uptake at pH 6.0. Each point represents the mean ± S.D. of triplicate.

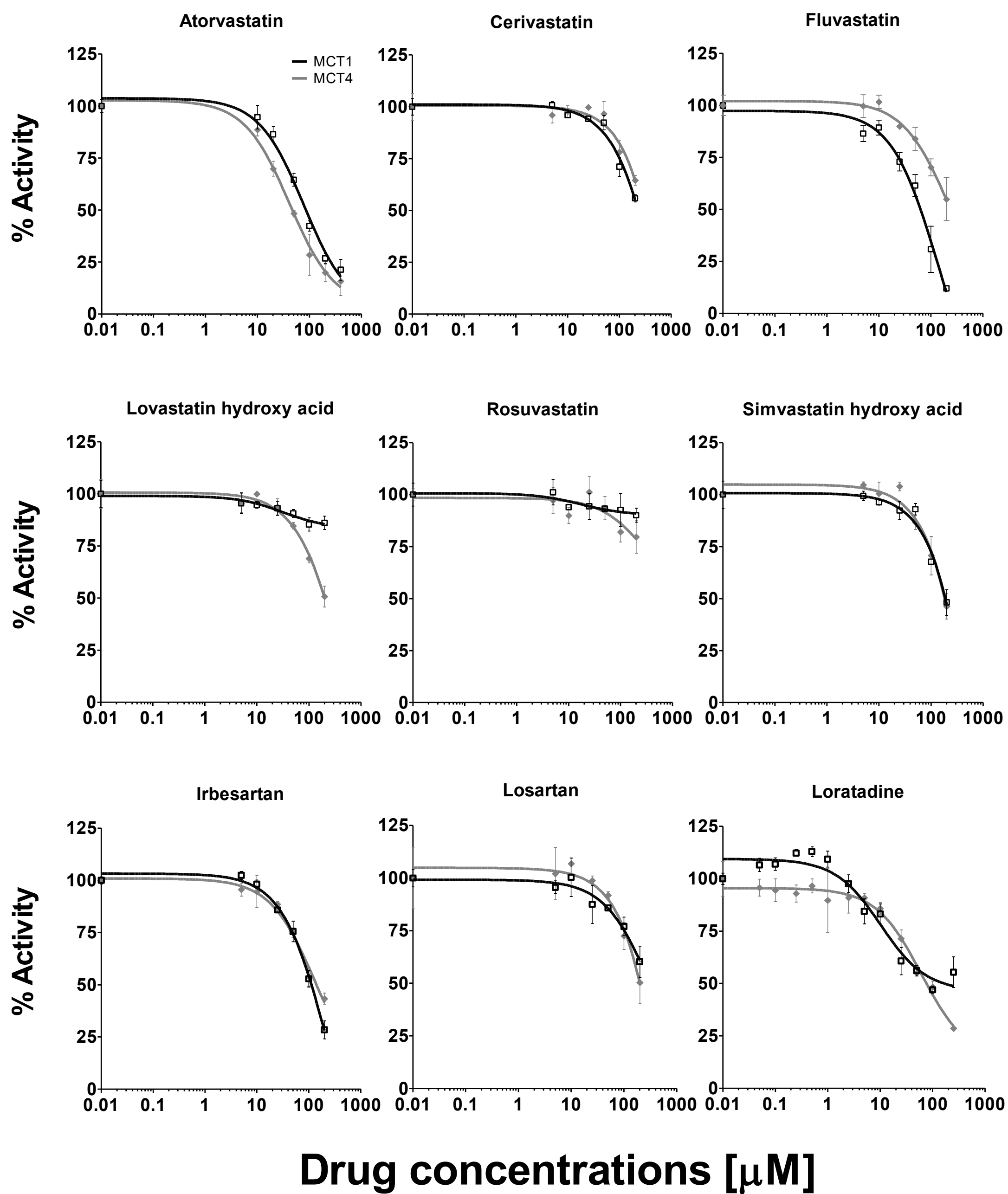
Supplemental Figure 2. Inhibitory effects of different acidic drugs on L-lactic acid (6 mM) efflux with Hs578T (MCT1) and MDA-MB-231 (MCT4) cell models. The intracellular [^{14}C] L-lactic acid was measured after 2.5 minutes of efflux at pH 6.0. Each point represents the mean ± S.D. of triplicate.

Supplemental Figure 3. Dixon plots of L-lactic acid uptake into Hs578T (MCT1) in presence of phloretin (**A**) and of atorvastatin (**B**). Panels **C** and **D** illustrate Dixon plots of L-lactic acid uptake into MDA-MB-231 (MCT4) in presence of phloretin and atorvastatin, respectively. The intracellular [^{14}C] L-lactic acid was measured after 2.5 min of uptake at pH 6.0. Each point represents the mean ± S.D. of three determinations.

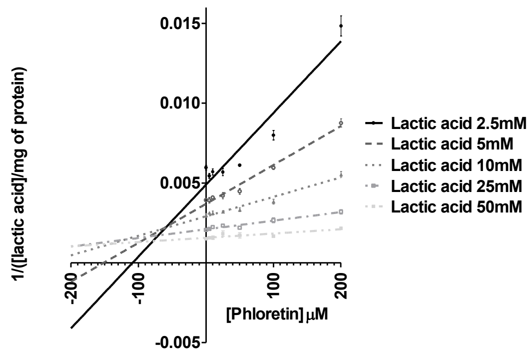
SUPPLEMENTAL FIGURE 1

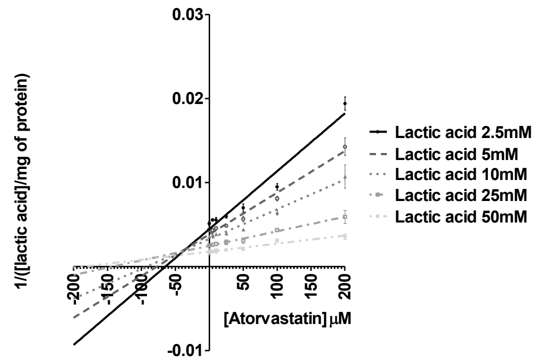


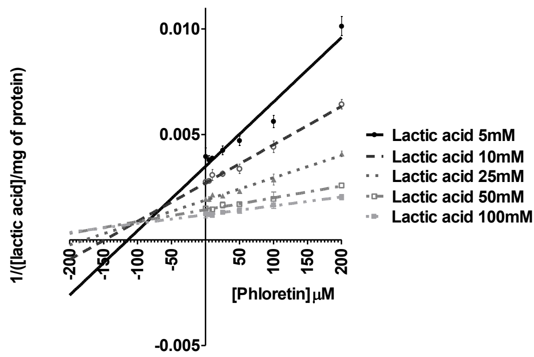
SUPPLEMENTAL FIGURE 2

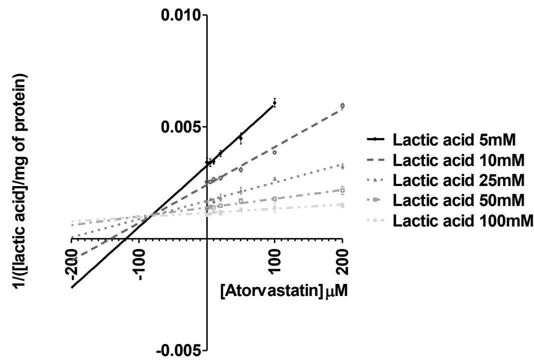


SUPPLEMENTAL FIGURE 3









SUPPLEMENTAL METHOD

K_i determination

To determine the inhibitor constant (K_i), L-lactic acid intracellular accumulation was determined in the absence and presence of varying concentrations of atorvastatin and phloretin, used as positive control (5-200 μM). The K_i values were only determined for the uptake experiments. The same incubation procedures were followed as described in the uptake inhibition section of *Inhibition studies*, except the concentrations of [^{14}C] L-lactic acid (0.2 μCi/mL) varied from 2.5-100 mM.

2.1.4 Discussion

In this study, we have identified two breast cancer cell lines, Hs578T and MDA-MB-231, which overexpress selectively MCT1 and MCT4, respectively. After characterization of the two transporters, lactic acid uptake and influx inhibition experiments have been performed for a series of acidic drugs. Loratadine and atorvastatin were found to have the greatest inhibitor potency for L-lactic efflux *via* both MCT1 and MCT4. Other compounds found with intermediate inhibitory potentials were fluvastatin, cerivastatin, simvastatin acid, lovastatin acid, irbesartan and losartan.

These findings, more specifically the IC_{50} for L-lactic acid uptake inhibition by statins, corroborate the ones determined by Kobayashi *et al.*[84] This suggests that statins and loratadine can possibly induce the clinically observed myopathy through drug transporter interaction, which can impede cell homeostasis. In perspective, a better model to represent the muscle can be used in order to have a more accurate assessment of the effect of L-lactic acid inhibition in physiologically relevant settings.

The clinically relevant plasma concentrations are 0.1 µM for atorvastatin and 0.07 µM for loratadine.[66, 160] Even though the determined IC_{50} in our study are higher than these concentrations, long term exposure to these drugs could lead to intracellular drug accumulation resulting in higher local concentrations predisposing to MCT inhibition.

2.1.5 Author contributions

Yat Hei Leung developed the methods and performed 95% of the experiments, as well as data analysis. The author also interpreted the data and wrote the paper.

François Bélanger provided technical assistance throughout the project.

Jennifer Lu assisted in preparing and performing 5% of the experiments.

Jacques Turgeon and Véronique Michaud conceived and designed the study. They also participated in data interpretation and writing the paper.

2.2 Article 2: Study of Statin- and Loratadine-Induced Muscle Pain Mechanisms Using Human Skeletal Muscle Cells

2.2.1 Introduction

As presented in the **1. Introduction** section of this thesis, drug-induced myopathy can be caused by the administration of various drugs. Different mechanisms have been associated with different medications. However, for some drugs, it remains unclear how myotoxicities are caused.

In the previous article, we have confirmed that lactic acid transport *via* MCT can be inhibited by statins and loratadine. This inhibition can be the cause of the drug-induced muscular symptoms associated with the use of those medications. To obtain a better assessment of this phenomenon in muscle, we will investigate statin- and loratadine-related inhibition of L-lactic acid transport in primary human skeletal muscle cells.

These cells are the only known *in vitro* model that can closely represent the muscle. The advantage of using this model is that these cells are clinically more relevant and allow the study of L-lactic acid inhibition under different conditions without resorting to the use of animals or clinical trials. However, the use of these cells comes with some inconvenience, such as fixed population doubling and passage and extensive cell culture periods, which can lead to high experimental costs.

In addition, it has been reported that higher physical activity levels, resulting in lowered pH, seem to exacerbate muscle pain in patients taking statins. Therefore,

accumulation of lactic acid during inhibition studies with the various compounds will be determined at different pH to account for those conditions. It also takes multiple doses before the occurrence of drug-induced myopathy. Therefore, SkMC under an extended period of treatment will also be evaluated for lactic acid transport activity and inhibitor potency.

2.2.2 Objectives

The main objective of this second article is to validate the results obtained with the cell line models overexpressing MCT in a physiological pertinent setting. Two different pH will be used for the inhibition studies as described previously to assess the effect of physical activity. The drugs that were identified to have strong inhibitory potentials will be used in pretreatment studies. After pre-exposure, MCT-mediated lactic acid transport activity will be evaluated, as well as the inhibitory potential of the medications.

2.2.3 Article

pharmaceutics

Article

Study of Statin- and Loratadine-Induced Muscle Pain Mechanisms Using Human Skeletal Muscle Cells

Yat Hei Leung [1,2], Jacques Turgeon [1] and Veronique Michaud [1,2,*]

1 Faculty of Pharmacy, Université de Montréal, Montreal, QC H2X 0A9, Canada; yat.hei.leung@umontreal.ca (Y.H.L.); turgeoja@gmail.com (J.T.)

2 Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, QC H2X 0A9, Canada

* Correspondence: v.michaud@umontreal.ca; Tel.: +1-514-890-8000 (ext. 15812)

Received: 31 August 2017; Accepted: 1 October 2017; Published: 10 October 2017

Impact factor: 2015 RG Journal Impact 3.1

Article

Study of Statin- and Loratadine-Induced Muscle Pain Mechanisms Using Human Skeletal Muscle Cells

Yat Hei Leung [1,2], Jacques Turgeon [1] and Veronique Michaud [1,2,*]

[1] Faculty of Pharmacy, Université de Montréal, Montreal, QC H2X 0A9, Canada

[2] Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, QC H2X 0A9, Canada

[*] Correspondence: Tel.: +1-514-890-8000 (ext. 15812)

Received: 31 August 2017 / Accepted: 1 October 2017 / Published: 10 October 2017

Abstract:

Many drugs can cause unexpected muscle disorders, often necessitating the cessation of an effective medication. Inhibition of monocarboxylate transporters (MCTs) may potentially lead to perturbation of L-lactic acid homeostasis and muscular toxicity. Previous studies have shown that statins and loratadine have the potential to inhibit L-lactic acid efflux by MCTs (MCT1 and 4). The main objective of this study was to confirm the inhibitory potentials of atorvastatin, simvastatin (acid and lactone forms), rosuvastatin, and loratadine on L-lactic acid transport using primary human skeletal muscle cells (SkMC). Loratadine (IC_{50} 31 and 15 μM) and atorvastatin (IC_{50} ~130 and 210 μM) demonstrated the greatest potency for inhibition of L-lactic acid efflux at pH 7.0 and 7.4, respectively (~2.5-fold L-lactic acid intracellular accumulation). Simvastatin acid exhibited weak inhibitory potency on L-lactic acid efflux with an intracellular lactic acid increase of 25–35%. No L-lactic acid efflux inhibition was observed for simvastatin lactone or rosuvastatin. Pretreatment studies showed no change in inhibitory potential and did not affect lactic acid transport for all tested drugs. In conclusion, we have demonstrated that loratadine and atorvastatin can inhibit the efflux transport of L-lactic acid in SkMC. Inhibition of L-lactic acid efflux may cause an accumulation of intracellular L-lactic acid leading to the reported drug-induced myotoxicity.

Keywords:

statins; loratadine; drug-transporters; MCT; monocarboxylate transporters; lactic acid; skeletal muscle cell; drug-induced muscle disorders

1. Introduction

Adverse drug reactions (ADRs) are an important public health problem. Death caused by ADRs has increased over the years and, since 2011, has actually surpassed motor vehicle traffic-related injuries [1]. There are many factors that can contribute to this situation, such as polypharmacy in the aging population, drug-drug interactions, and interindividual genetic variability modulating the pharmacodynamics and pharmacokinetics of drugs inside the organism [2,3,4]. Many common medications can induce musculoskeletal disorders, while their incidence is still unclear due to the lack of clear definitions (e.g., under drug-drug interaction conditions). However, drug-related musculoskeletal disorders have been reported more frequently since the introduction into the market of widely prescribed lipid lowering drugs, such as fibrates and statins [5]. Drug-induced myopathies can range from mild myalgias to myopathies with weakness and severe life-threatening rhabdomyolysis. While mild myalgias are more or less tolerable, chronic myopathies can affect quality of life. Therefore, an early recognition of these ADRs is really important for the patients, since most of them are partially or completely reversible when the offending drug is substituted or the dose is adjusted [6,7].

Statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, form the number one class of drugs prescribed in the United States for the prevention of cardiovascular disease [8]. However, muscle pain is a known side effect associated with statin treatment. Statin therapy is usually well tolerated, but muscle symptoms can limit treatment adhesion or even lead to its discontinuation [9]. The definitive mechanism of statin-induced muscle disorders is still not known, although different hypotheses have been proposed, including alteration in cellular membrane cholesterol, alterations in protein synthesis and degradation,

cell apoptosis, immune reactions, increased lysosomal activity, injuries to electrolytes homeostasis, inhibition of myogenesis, mitochondrial impairments, and oxidative stress [10,11,12].

Muscle is one of the largest human organs, and it is well-perfused, which means that it is also highly exposed to circulating drugs, making it quite susceptible to ADRs [13]. Since skeletal muscle is the major producer of L-lactic acid, the transport of L-lactic acid is critical for the maintenance of intracellular pH and homeostasis. We have previously hypothesized that drug-induced myotoxicities can be caused by an excess intracellular level of L-lactic acid. Indeed, we demonstrated that some statins are able to inhibit the efflux of L-lactic acid *via* MCT1 and MCT4 in breast cancer cell lines (Hs578T selectively expressing MCT1 and MDA-MB-231 selectively expressing MCT4). In those studies, atorvastatin and loratadine were associated with the greatest inhibitory potential on the efflux of L-lactic acid, leading to intracellular accumulation of lactic acid using cancer cells [14].

The main objective of our study was to corroborate our previous findings in physiologically relevant settings. Therefore, we proposed to characterize the effects of atorvastatin, loratadine, simvastatin lactone, simvastatin hydroxy acid, and rosuvastatin, on the transport of L-lactic acid using human skeletal muscle cells (SkMCs) at resting pH of 7.4 and at pH 7.0. A more acidic pH value was tested, since evidence suggests that drug-related muscle disorders can be exacerbated by exercise.

2. Materials and Methods

2.1. Materials

[^{14}C] L-lactic acid sodium salt was purchased from PerkinElmer (Walthman, MA, USA). L-lactic acid sodium salt was obtained from Sigma-Aldrich (St. Louis, MO, USA). Atorvastatin, loratadine, phloretin, rosuvastatin, simvastatin, and simvastatin hydroxyl acid ammonium salt were purchased from Toronto Research Center (Toronto, ON, Canada). Cryopreserved human primary skeletal muscle cells (from adult), Human Skeletal Muscle Cells Growth medium, Human Skeletal Muscle Cells Differentiation medium, and Subculture Reagent Kit were purchased from Cell Applications Inc. (San Diego, CA, USA).

2.2. Cell Culture

SkMCs were grown in all-in-one-ready-to-use Human Skeletal Muscle Cells Growth medium and were used within 5 passages or 15 population doublings after thawing upon arrival or from storage in liquid nitrogen. Cells were first cultured in plastic culture flasks (Sarstedt, Newton, NC, USA) at 37 °C with 5% CO_2. When they reached 60–80% confluence, they were harvested with Subculture Reagent Kit which includes HBSS, Trypsin/EDTA and Trypsin Neutralizing Solution, resuspended and seeded into new flasks. When the adequate amount of cells for the experiments was attained, they were again harvested, seeded on 35 × 10 mm tissue culture plates, and grown to reach 80–90% confluence before differentiation. After that, differentiation was initiated by changing the media from the Human Skeletal Muscle Cells Growth medium into the Human Skeletal Muscle Cells Differentiation medium for 6 days until the cells formed multinucleated syncytia, as seen in Figure 1A–D. The differentiation

was confirmed by immunomicroscopy for expression of myosin (Skeletal, Slow) described in the next section.

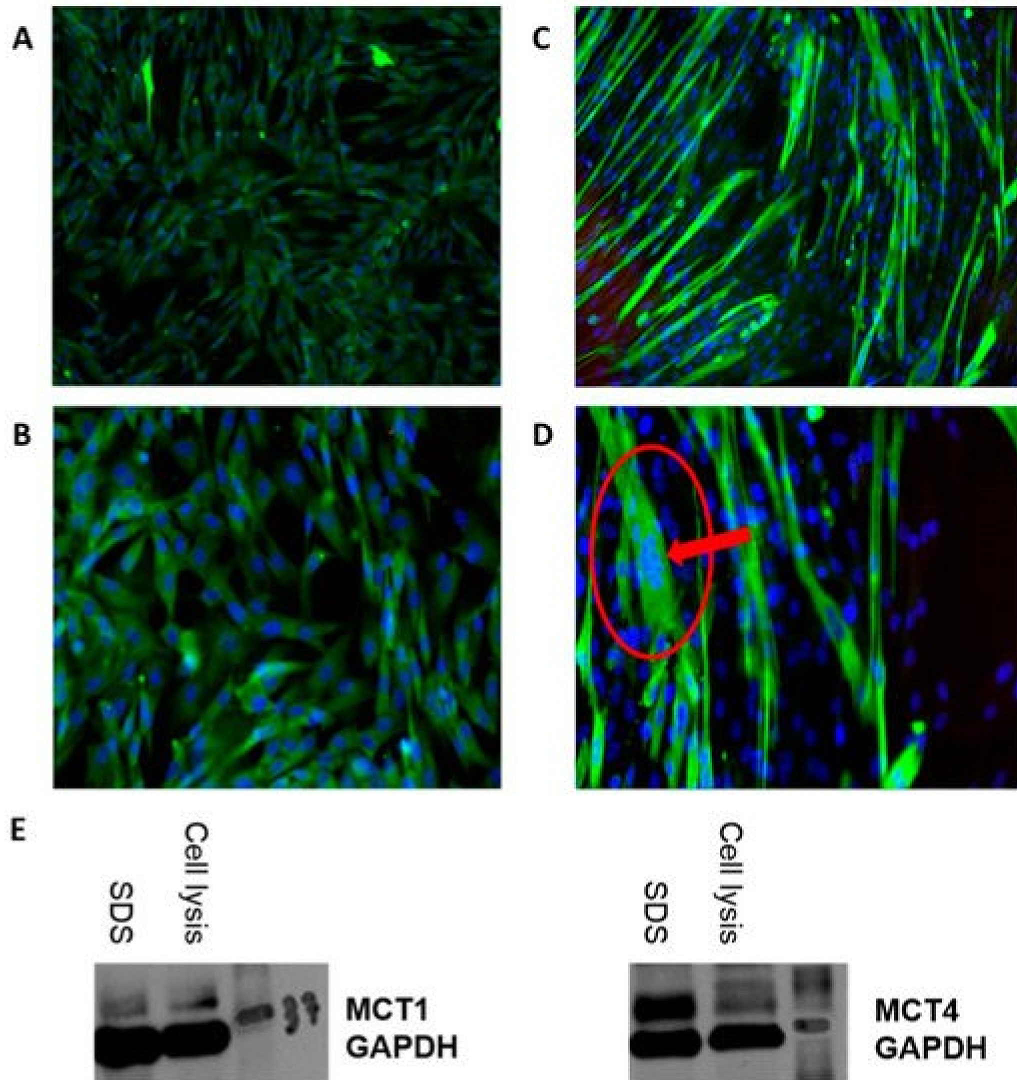


Figure 1. *SkMC*. Primary human myoblasts from Cell Applications Inc. were proliferated in 35 × 10 mm culture plates with SkMC growth medium for 25–35 days until 80–90% confluency and photographed at (A) 10× and (B) 20×. *SkMC in differentiation*. Differentiated SkMCs with multinucleated syncytia after exposition for 6 days with the SkMC differentiation medium are photographed at (C) 10× and (D) 20×. (E) Expression of MCT1 (left) and MCT4

(right) proteins in SkMCs was revealed by Western blotting using antibodies against MCT1, MCT4 and GAPDH (the 2 wells illustrated—SDS vs. cell lysis—represent 2 different methods tested for protein extraction).

2.3. Immunomicroscopy

The immunomiscroscopy images were obtained by having the SkMCs grown and differentiated on a glass slide cover. After removing the differentiation medium (or growth medim, if observations were made at an earlier stage), SkMCs were fixed with 3.7% formaldehyde for 15 min at room temperature and were washed twice with PBS 1× between every subsequent step. Samples were then quenched with glycine for 5 min, permeabilized with 0.2% Triton X-100 for 20 min and incubated for 3 h with BSA 3%. Cells were then incubated overnight at 4 °C with Monoclonal Anti-Myosin (Skeletal, Slow) antibody produced in mouse (Sigma-Aldrich, St. Louis, MO, USA). Cells were incubated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) in BSA 3% for an hour at room temperature, followed by incubation with Hoechst for 15 min. After a final wash, images were acquired using an EE (×10 and ×20) and Zen Imaging software. The expression of MCT1 and MCT4 proteins has been assessed by Western blot during the cell culture optimization step (Figure 1E) (details of Western blotting are described in Supplementary Materials).

2.4. Transport Studies

After differentiation, and before the beginning of the transport experiments, cells were washed, and the medium replaced with HEPES (pH 7.4; 1 mL) buffer. Transport assays, including the pre-incubation period in HEPES, were performed at 37 °C. Assay conditions were previously optimized by standard incubations with L-lactic acid (e.g., incubation times).

For the time-course experiments, different time points ranging from 5 s to 30 min were tested using one concentration (6 mM) of L-lactic acid.

Assessment of influx transport. At the beginning of the experiment ($t = 0$), HEPES medium was replaced by MES or HEPES (pH 7.0 or 7.4; 1 mL) buffer containing 0.03 to 30 mM [^{14}C] L-lactic acid (0.2 μCi/mL). After incubation for 2.5 min, the radioactive media was removed from the milieu. Transport assays were stopped by placing culture plates on ice, rapidly aspirating the media and cells were washed 3-times with ice-cold HEPES buffer. Cells were then solubilized using a solution of 0.2 N NaOH and 1% SDS (500 μL). The suspension was passed through 27½ G needle 3-times. Aliquot of the cell lysate (400 μL) was transferred in a scintillation tube containing 5 mL of biodegradable scintillation counting cocktail buffer (Bio-Safe II, Research Products International Corp., Mt. Prospect, IL, USA). Radioactivity levels were quantified with a Tri-Carb liquid scintillation counter (LSC 1600TR, Packard Instrument Co., Meriden, CT, USA) to determine the intracellular [^{14}C] L-lactic acid concentrations. Protein concentrations were measured using Pierce BCA protein assay kit (Thermo Fisher Scientific, Walthman, MA, USA).

Assessment of efflux transport. A similar approach was used to measure efflux transport of L-lactic acid. For these experiments, distribution equilibrium of L-lactic acid was reached by adding MES or HEPES (pH 7.0 or 7.4; 1 mL) containing 6 mM [^{14}C] L-lactic acid (0.2 μCi/mL). After 10 min (to allow L-lactic acid to reach equilibrium), MES or HEPES buffer containing [^{14}C] L-lactic acid was replaced by L-lactic acid-free buffer for 2.5 min. Transport was stopped by placing culture plates on ice, rapidly aspirating the media and cells were washed 3-times with ice-cold HEPES buffer. Cellular radioactivity levels were determined as previously described in the Method section (Assessment of influx transport).

2.5. Inhibition Studies

In the inhibition experiments, efflux of L-lactic acid was assessed in the presence and absence of increasing concentrations of statins and loratadine. In total, 5 compounds were investigated: atorvastatin, loratadine, rosuvastatin, simvastatin hydroxy acid, and simvastatin lactone. Effects of acidic drugs on L-lactic acid transport were tested at concentrations varying from 0.25 to 300 μM. Phloretin (1 to 300 μM) was also evaluated as a known potent MCT inhibitor.

At the beginning of the experiment ($t = 0$), medium was replaced by HEPES (pH 7.4; 1 mL) buffer to wash cells. After removing the wash buffer, cells were loaded with [^{14}C] L-lactic acid (6 mM, 0.2 μCi/mL) in MES or HEPES buffer (pH 7.0 or pH 7.4). Briefly, following pre-incubation of cells in buffer containing [^{14}C] L-lactic acid for 10 min (to allow L-lactic acid to reach equilibrium), the buffer was replaced by a buffer containing [^{14}C] L-lactic acid and the potential inhibitor. This mixture was incubated for an additional 3 min in order to allow diffusion of the inhibitor in the cells without perturbing L-lactic acid equilibrium. Then, cells were washed once rapidly with buffer containing the tested inhibitors or vehicle, but without L-lactic acid. In the final step, the tested inhibitors or vehicle were added to the L-lactic acid-free buffer and incubated for 2.5 min as described previously for the assessment of the efflux transport. Culture plates were placed on ice to stop the reaction. Inhibition of L-lactic acid efflux was determined by measuring intracellular [^{14}C] L-lactic acid concentration.

2.6. Effect of Pretreatment on Lactic Acid Transport

After differentiation, the cells were put in growth media for 24 h. After stabilization, cells were exposed to atorvastatin, simvastatin acid or loratadine (added to the media in DMSO) at clinically relevant concentrations (0.033 and 0.1 μM for atorvastatin, 0.033 and 0.1 μM for

simvastatin acid, and 0.023 and 0.07 μM for loratadine) for six days before conducting transport experiments. Separate experiments were thereafter carried out as described previously in the *Transport Studies*and *Inhibition Studies* sections.

2.7. Quantification of Intracellular Concentrations of Statins and Loratadine

HPLC-UV methods were used to quantify atorvastatin, loratadine, rosuvastatin, simvastatin hydroxyl acid, and simvastatin lactone in the intracellular compartment of the cells. Instruments used consisted of a SpectraSystem P4000 pump, a SpectraSystem AS3000 autosampler, a Finnigan SpectraSystem UV6000 ultraviolet detector and a SpectraSystem SN4000 System Controller from Thermo Electron Corporation (San Jose, CA, USA). An Agilent Zorbax Column, Eclipse XDB-C8, 4.6 mm × 150 mm (Agilent, Santa Clara, CA, USA) was used at a temperature of 40 °C. An isocratic mobile phase contained 10 mM ammonium formate pH 3 and acetonitrile with varying proportions, at a flow rate of 1.0 mL/min. Details for mobile phase proportions, internal standards and monitored UV wavelengths are listed in Supplementary Table S1.

The same protocol as described in the *Inhibition Studies* section was used to measure intracellular concentrations of statins and loratadine at the end of the experiments, but without radioactive product (cold L-lactic acid). After the final incubation, cells were washed twice with PBS 10% methanol and once with PBS alone. The cells were lysed with methanol containing appropriate internal standards for the compounds of interest, then transferred and centrifuged for 10 min at room temperature. The supernatant was transferred to a culture borosilicate glass tube, evaporated and reconstituted in 100 μL of 10 mM ammonium formate

pH 3 and acetonitrile (50:50 *v*/*v*). A volume of 20 µL per sample was injected. The ChromQuest Version 4.2.34 software was used for data acquisition.

2.8. Data Analysis

For kinetic studies, the K_m (Michaelis-Menten constant) and V_{max} (maximum uptake rate) of L-lactic acid transport were estimated by non-linear least-squares regression analysis program, GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA, USA) using the following equation:

$$v = V_{max} \cdot [S]/(K_m + [S]) \quad (1)$$

where v and [S] are uptake rate of L-lactic acid at 2.5 min and concentration of L-lactic acid, respectively. CL_{int} and IC_{50}were also estimated using the GraphPad program (Version 5.01). For the IC_{50} determination, the intracellular level of L-lactic acid measured at the end of the 10-min pre-incubation period (when equilibrium was reached, and before adding the inhibitor) was considered as the reference of the maximal intracellular concentration attained beforehand to inhibit the efflux transport.

3. Results

3.1. Kinetic Parameters of L-Lactic acid Transport in SkMC

The time-course for the uptake and efflux of L-lactic acid (6 mM) into SkMC was determined (Figure 2). The uptake and efflux of L-lactic acid were linear for the first minute while displaying a plateau thereafter. Those processes were rapid and most of the transport was completed within five minutes.

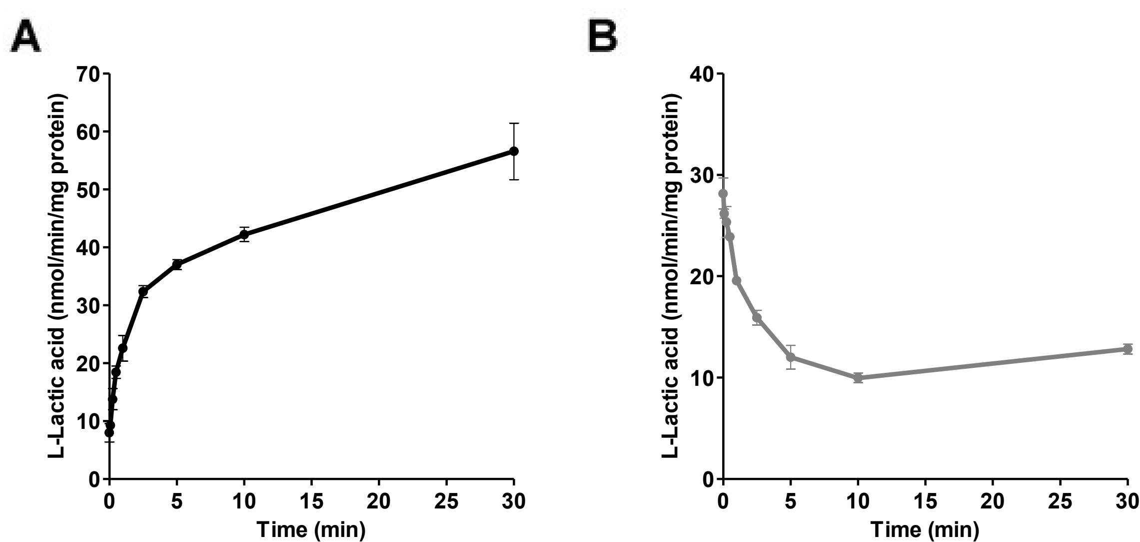


Figure 2. Intracellular concentrations of [^{14}C] L-lactic acid over time: (A) the uptake of L-lactic acid (6 mM), and (B) the efflux of L-lactic acid in SKMC at an extracellular pH of 7.4. Each point represents the mean ± S.D. of experiments performed in triplicate.

The kinetic parameters of the L-lactic acid influx transport in human skeletal muscle cells are illustrated in the Figure 3. The estimated CL_{int} value for the transport of L-lactic acid in SkMCs was higher at pH 7.0 than at pH 7.4; CL_{int} values were 5.2 µL/min/mg protein (V_{max} 90 nmol/min/mg protein; K_m 17 mM) vs. 3.6 µL/min/mg protein (V_{max} 82 nmol/min/mg

protein; K_m 23 mM), respectively (Figure 3). The intrinsic clearance could be determined only for the influx transport.

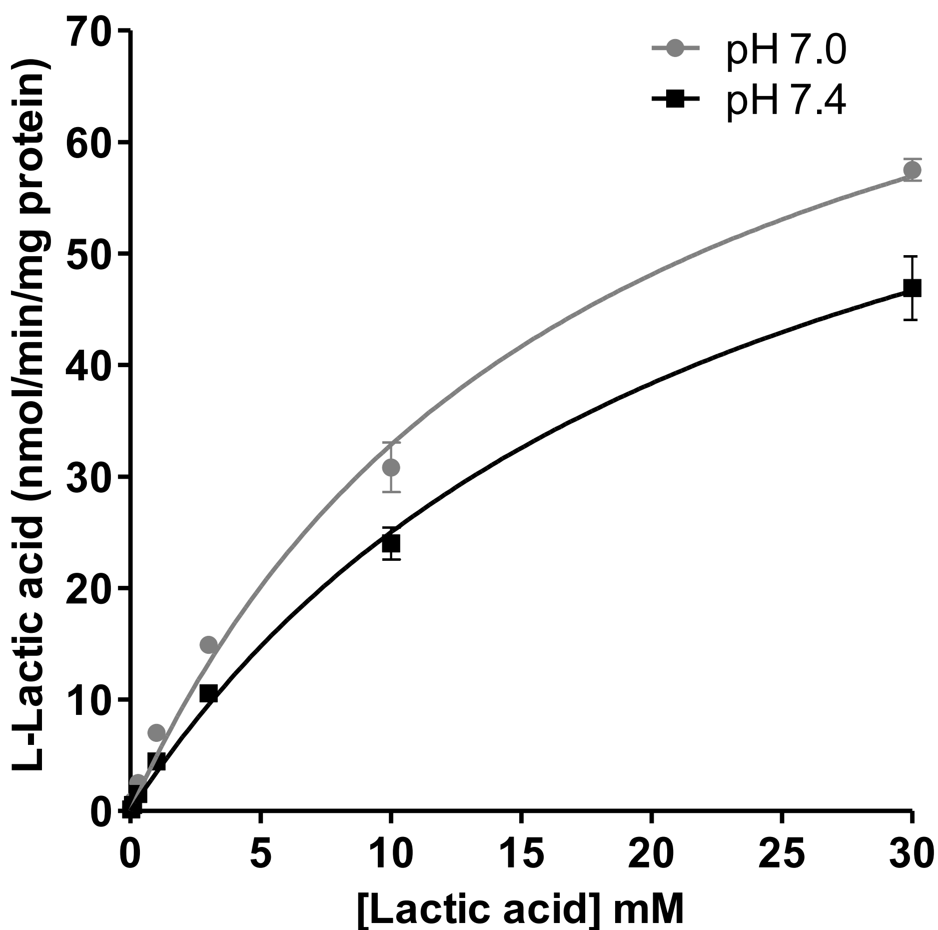


Figure 3. Kinetic parameters of L-lactic acid (0.03 to 30 mM) in SKMC determined at pH 7.0 and pH 7.4. Each point represents the mean ± S.D. of experiments performed in triplicate.

3.2. L-Lactic Acid Efflux Inhibition by Different Drugs

The inhibition of L-lactic acid efflux by statins and loratadine was tested at pH 7.0 and pH 7.4. In addition to pH 7.4, a more acidic pH value was assessed in order to determine whether an intense physical effort resulting in a lowered pH may modulate the inhibitory potential of the

different compounds compared to a physiological pH. Figure 4A and Figure 5A present the intracellular accumulation of L-lactic acid in presence of increasing concentrations of potential inhibitors, i.e., loratadine and statins, respectively. Among the drugs tested, loratadine and atorvastatin had the highest inhibitory potential on the efflux of L-lactic acid. The intracellular L-lactic acid increased 2.5-fold in the presence of 250 μM loratadine at pH 7.4 compared to the control (Figure 4A). Similarly, at the highest tested concentration of 300 μM of atorvastatin, intracellular L-lactic acid was increased by 2.5-fold (Figure 5A). For simvastatin acid (300 μM), the maximal increase of intracellular L-lactic acid was only 35%. No significant inhibitory effect on the efflux transport of L-lactic acid was observed with simvastatin lactone and rosuvastatin (Figure 5A). The IC_{50} values were estimated for the most potent inhibitors of L-lactic acid efflux observed in our study (i.e., atorvastain and loratadine) (Figure 6A,B). Our results showed that, at pH 7.4, loratadine was a more potent MCT inhibitor than atorvastatin on the L-lactic acid efflux, with IC_{50} values of 15 μM and 210 μM, respectively).

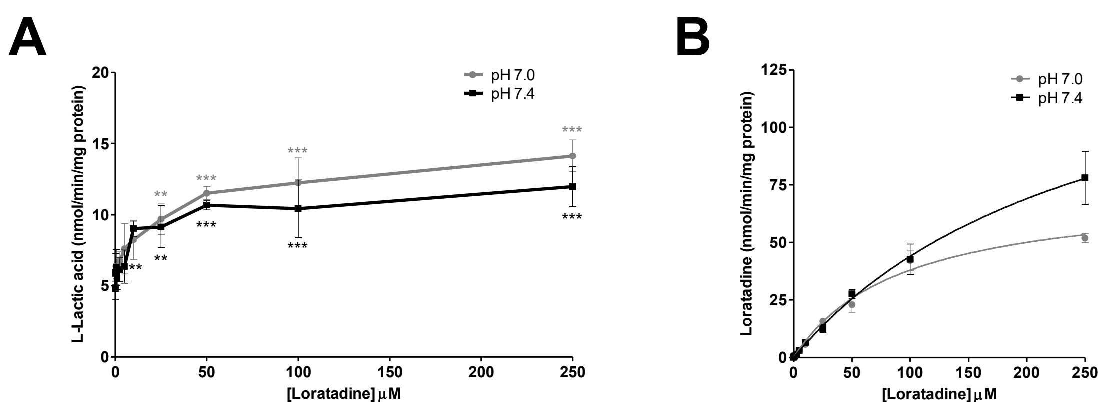


Figure 4. Drug inhibition studies with loratadine in SkMC. (A) Inhibitory effects of loratadine on L-lactic acid (6 mM) efflux in SkMC. The residual intracellular [^{14}C] L-lactic acid was

measured after 2.5 min of efflux at pH 7.0 and pH 7.4. (B) Intracellular concentrations of loratadine at the end of inhibition assays in SkMC at pH 7.0 and pH 7.4. Each point represents the mean ± S.D. of experiments performed in triplicate (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

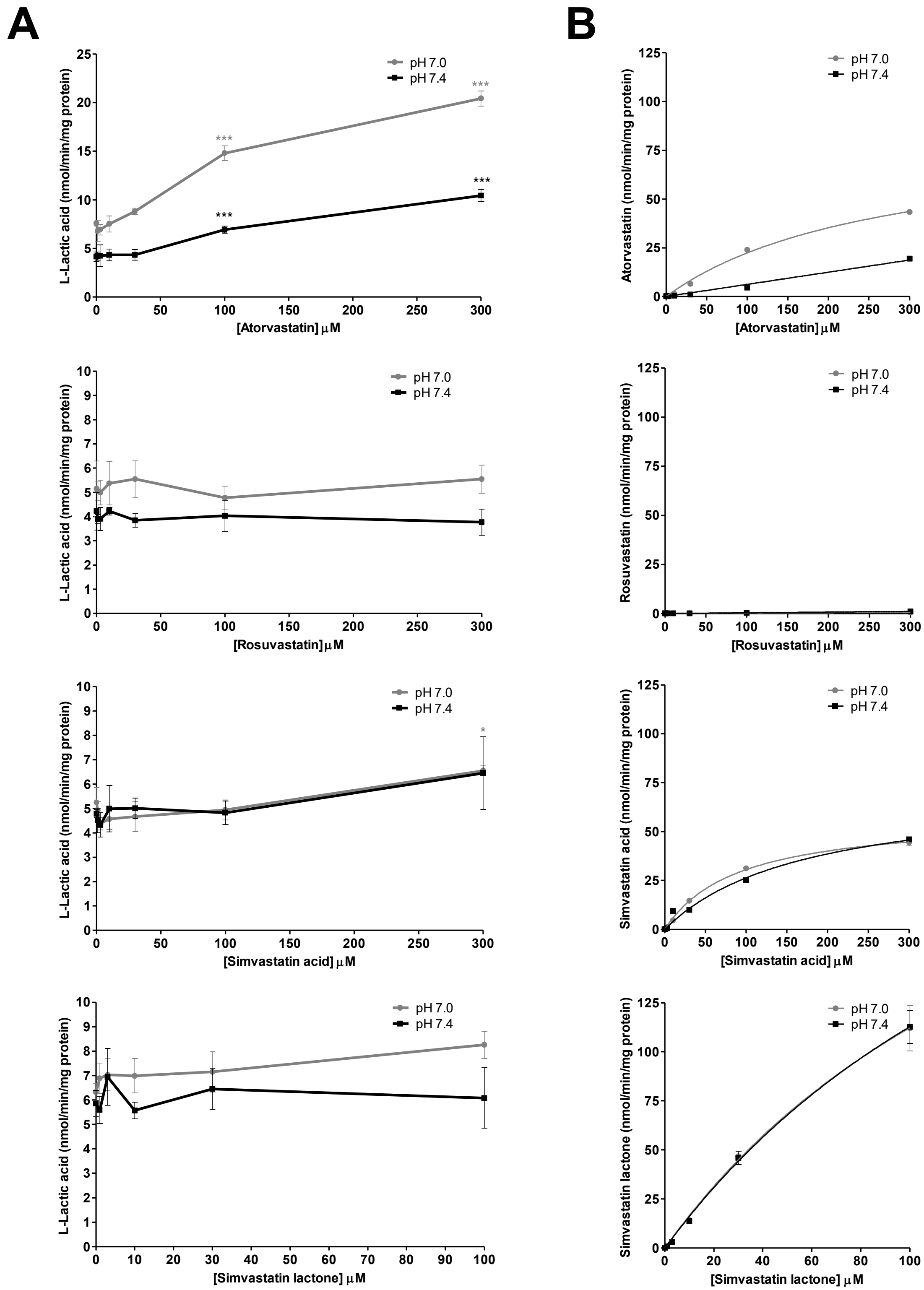


Figure 5. Drug inhibition studies with statins in SkMC. (A) Inhibitory effects of different

statins (atorvastatin, rosuvastatin, simvastatin hydroxy acid and simvastatin lactone) on L-lactic acid (6 mM) efflux in SkMC. The residual intracellular [^{14}C] L-lactic acid was measured after 2.5 min of efflux at pH 7.0 and pH 7.4. (B) Intracellular concentrations of statins (atorvastatin, rosuvastatin, simvastatin hydroxy acid and simvastatin lactone) at the end of inhibition assays in SkMC at pH 7.0 and pH 7.4. Each point represents the mean ± S.D. of experiments performed in triplicate (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

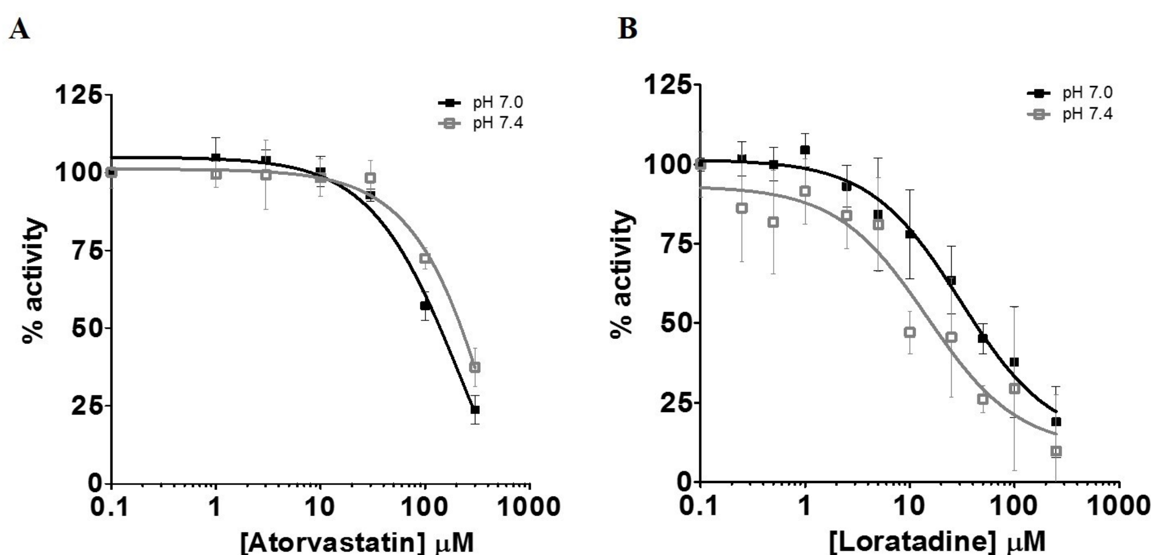


Figure 6. Inhibition of MCT-mediated efflux transport of L-lactic acid measured by the intracellular accumulation of L-lactic acid in the presence of atorvastatin (A) or loratadine (B). IC_{50} were determined at pH 7.0 and 7.4. The percentage of remaining activity was derived by substracting the maximal level of L-lactic acid after equilibrium to the residual intracellular L-lactic acid concentrations at the end of the experiment.

The pH value had an effect on the basal activity of lactic acid transport. The accumulation of L-lactic acid in the SkMC was higher at pH 7.0 compared to pH 7.4. However, a similar magnitude of inhibition with statin on L-lactic acid transport was observed at pH 7.0 and pH

7.4. Our results showed a 2.7-fold increase in the intracellular concentration of L-lactic acid by atorvastatin 300 μM at pH 7.0 (vs. 2.5-fold at pH 7.4). Similar observations were made with loratadine, which caused similar efflux transport inhibitions of L-lactic acid at pH 7.0 vs. 7.4 (L-lactic acid intracellular concentrations increased by 2.3- vs. 2.5-fold, respectively). Again, under these conditions, simvastatin, lactone and rosuvastatin had no significant inhibitory effect on the transport of L-lactic acid.

3.3. Uptake of Different Drugs during Lactic Acid Efflux Inhibition

Figure 4B and Figure 5B illustrate the intracellular concentrations of the tested potential inhibitors, namely, loratadine and statins, respectively. Our results showed a higher accumulation of atorvastatin in SkMC at pH 7.0 compared to pH 7.4. Overall, pH values did not affect the intracellular penetration of simvastatin and loratadine (except at supratherapeutic concentration). Furthermore, rosuvastatin did not have a significant uptake in SkMC.

3.4. Validation of L-Lactic Acid Efflux Inhibition Using a Known Potent MCT Inhibitor

In order to compare the relative potency of the inhibition on L-lactic acid efflux *via* MCTs obtained with loratadine and statins, inhibition assays were also conducted with phloretin, a potent known MCT inhibitor. As shown in Figure 7, phloretin produced a maximal intracellular L-lactic acid augmentation of 2.1- and 2.2-fold at pH 7.0 and 7.4, respectively, which was similar to the observed inhibition with loratadine or atorvastatin. These results also indicated that the extent of inhibition of phloretin on MCTs was not affected by the pH tested.

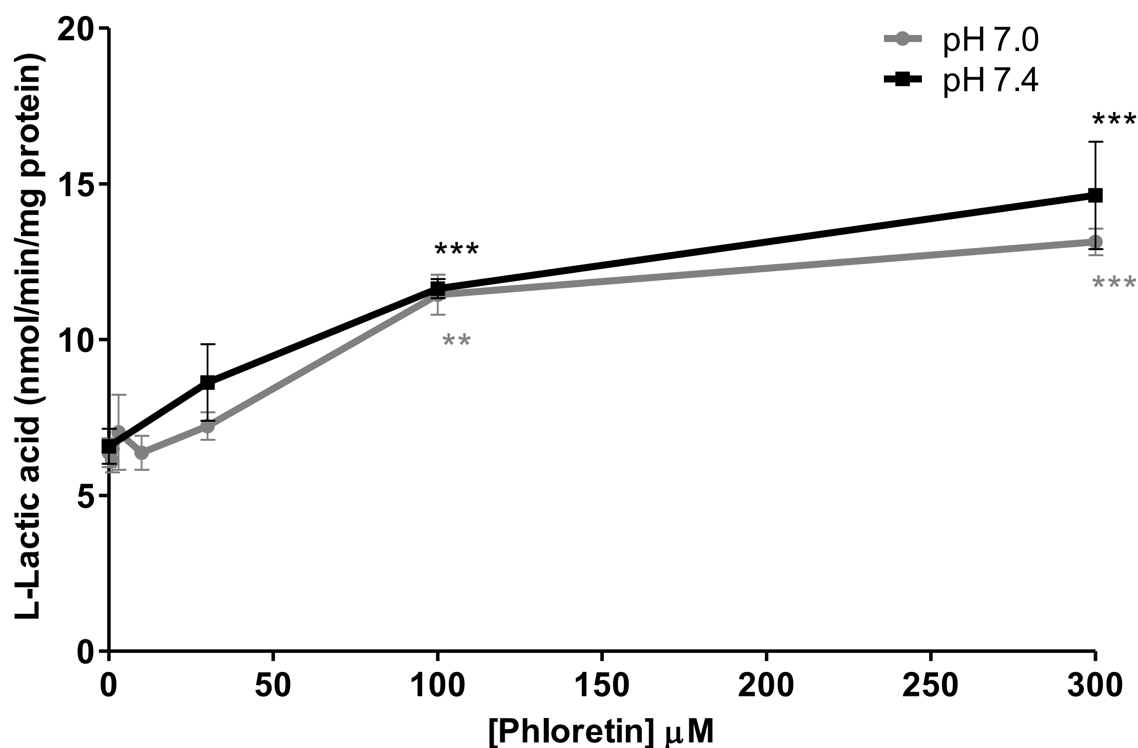


Figure 7. Inhibitory effects of phloretin, a known MCT inhibitor, on L-lactic acid efflux in SkMC. The intracellular [^{14}C] L-lactic acid was measured after 2.5 min of efflux at pH 7.0 and pH 7.4 (** $p < 0.01$ and *** $p < 0.001$).

3.5. Study of Pretreatment with Potential Inhibitors on L-Lactic Acid Transport in SkMCs

The three drugs with the highest potential inhibition (i.e., loratadine, atorvastatin and simvastatin hydroxy acid, based on prior data) of L-lactic acid efflux were selected for this study. Pretreatments with 0.033 μM and 0.1 μM of atorvastatin, 0.033 μM and 0.1 μM simvastatin acid, and 0.023 μM and 0.07 μM of loratadine were done to assess the transport capacity of L-lactic acid in SkMCs; these concentrations were selected based on clinically relevant concentrations, the highest tested concentrations were based on the maximal plasma concentrations for each substrate. Table 1 and Table 2 present the effects of various

pretreatments with loratadine, atorvastatin or simvastatin hydroxy acid on the basal L-lactic acid transport, both influx and efflux transport were evaluated. Our first observation was that the basal influx activity of L-lactic acid transporters did not change following a pretreatment with either of these drugs (CL_{int} at pH 7.0 vs. 7.4) as seen in Table 1. Our second observation was that pretreatment has no significant effect on the inhibition by statins and loratadine on L-lactic acid efflux transport, as shown in Table 2.

Table 1. Kinetic parameters of L-lactic acid influx following a six-day pretreatment with atorvastatin, simvastatin hydroxy acid and loratadine in SkMC at pH 7.0 and pH 7.4.

Compound	Concentration	CL_{int} (nL/min/mg Protein)	
		pH 7.0	pH 7.4
Atorvastatin	Control	5.2	3.6
	0.033 µM	4.1	3.7
	0.1 µM	4.4	2.7
Simvastatin hydroxy acid	Control	4.5	2.3
	0.033 µM	3.6	2.5
	0.1 µM	2.4	1.5
Loratadine	Control	4.5	2.3
	0.023 µM	3.4	2.4
	0.07 µM	5.3	2.4

Table 2. Intracellular L-lactic acid increase (%) during L-lactic acid efflux inhibition studies following a six-day pretreatment with atorvastatin, simvastatin hydroxy acid and loratadine in SkMC at pH 7.0 and pH 7.4.

Compound [1]	Concentrations Used for the Pretreatment	Intracellular L-Lactic Acid Increase (%)	
		pH 7.0	pH 7.4
Atorvastatin	Control	324	201
	0.033 µM	178 *	306 *
	0.1 µM	258	248
Simvastatin hydroxy acid	Control	91	65
	0.033 µM	103	51
	0.1 µM	31	35
Loratadine	Control	260	215
	0.023 µM	289	168
	0.07 µM	257	226

[1] The higest concentration of inhibitor tested during efflux experiments was used to determine the percentage of intracellular L-lactic acid increase i.e., atorvastatin 300 µM, simvastatin acid 300 µM and loratadine 250 µM and effects of pretreatment on inhibition potential were compared (* $p < 0.02$).

4. Discussion

Our previous studies demonstrated, using cell lines expressing selectively high levels of MCT1 (Hs578T) or MCT4 (MDA-MB-231), that certain acidic drugs inhibit the efflux of L-lactic acid *via* monocarboxylate transporters [14]. These breast cancer cell line models are great tools for rapidly screening drugs that can potentially cause an intracellular accumulation of L-lactic acid and lead to the observed muscular symptoms. However, the use of these cell lines has some limitations. First, they have a higher MCT expression due to their higher need of energy and metabolism to support their great capacity to proliferate. Second, they are not the most physiologically representative type of cells for studying muscles. To corroborate our previous findings in a more physiologically representative model, we proposed the use of primary SkMCs to confirm the effects of statins and loratadine on L-lactic acid transport.

Skeletal muscles are the major producers of L-lactic acid in the body. Therefore, it is essential that L-lactic acid transporters maintain pH homeostasis, especially during physical effort, where more L-lactic acid is formed. It was reported that physically active patients were more susceptible to experiencing drug-induced muscle disorders [15,16,17,18]. The reason and mechanisms underlying this association are not well known; although it has been postulated that coenzyme Q10 deficiency due to statin administration could lead to impaired mitochondrial energy metabolism in muscle cells, the results are still controversial [15,16]. Other hypotheses indicate that the ubiquitin proteasome pathway (UPP), involved in cell degradation and repair, or sarcoplasmic reticulum calcium cycling could be altered by statin therapy [16]. However, another proposed mechanism for drug-induced myopathies involves L-lactic acid transport. Our hypothesis and results are also supported by previous observations indicating that statins could inhibit L-lactic acid transport, causing its intracellular

accumulation [19]. It could also be speculated that this effect is mediated by co-transport of statins and L-lactic acid by MCTs, leading to competitive inhibition of the transporters.

Primary SkMCs were used in our experiments as an *in vitro* model of the actual muscle in order to study drug-induced myopathies. Among the statins tested, only atorvastatin (IC_{50} of 130–210 μM) and simvastatin hydroxy acid (35% increases in lactic acid intracellular levels at 300 μM) were found to be significant L-lactic acid efflux inhibitors. It is important to note that the inhibitory potency of L-lactic acid transport observed for atorvastatin was similar to that of the well characterized MCT inhibitor, phloretin.

As indicated previously, our results corroborate our previous findings, as well as other studies by Kobayashi et al., which showed that some statins—mainly the lipophilic ones, such as atorvastatin and simvastatin acid—can inhibit L-lactic acid transport *via* MCT4 [14,19]. In their model, they reported greater inhibitory potential for L-lactic acid uptake than the one we observed for its efflux. The differences between the results can be explained by the fact that we measured efflux inhibition, whereas they measured uptake inhibition. Furthermore, they used a much lower L-lactic acid concentration (3.3 μM) than the one used for our experiments (6 mM), as well as different cell models [19,20,21,22].

Our results are also in agreement with clinical data (Primo study), in which patients experienced muscular discomfort at a higher rate for statins with greater lipophilicity, such as atorvastatin (14.9%) and simvastatin (18.2%) [23]. Clinically, the higher frequency observed with simvastatin could be due to a greater propensity for drug-drug interactions, since simvastatin has a very low oral bioavailability (<5%) and greater potential for important increases in its exposure [24,25].

Loratadine, an H_1 histamine antagonist, has also been reported to cause muscle pain. It is therefore possible that this antihistaminic may cause muscular toxicity through similar mechanisms as statins. Loratadine was determined to be the most potent L-lactic acid efflux inhibitor in this study, with an average IC_{50} of 15 μM at pH 7.4. Since this drug can be obtained without prescription, it is more difficult to estimate the true frequency of loratadine-induced ADR. In the literature, it was reported that loratadine was associated with an increased risk of myopathy in some drug combinations. However, data suggests that these drug interactions do not involve inhibition of its metabolism [26]. It is hypothesized that the interaction might occur at the muscular cellular level. It has been reported that the combination of loratadine and simvastatin is associated with an increased risk for myopathy (RR = 1.69) [27].

We also investigated the effect of pH on the inhibitory potential of some drugs on L-lactic acid transporters. A pH 7.4 milieu was used as the physiological pH level and pH 7.0 was selected as a representative post-exercise physiological condition, since it is known that patients who are more physically active are generally more susceptible to these drug-induced muscular disorders. Overall, we could not demonstrate an increased potency in the blocking of L-lactic acid efflux with a more acidic pH. It could be suggested that a decrease in pH should favor intracellular accumulation of statins or loratadine due to their biophysical properties and passive diffusion. Indeed, we observed an increase in the intracellular concentrations of atorvastatin under a more acidic pH. A trend was observed between the IC_{50} values of loratadine and atorvastatin and their respective intracellular concentrations. A lower IC_{50} was estimated for loratadine at pH 7.4, where its concentrations tended to be higher. For

atorvastatin, the higher intracellular concentrations were observed at pH 7.0 and associated with lower IC_{50} for the efflux of L-lactic acid.

The use of primary human skeletal muscle cells also has some limitations. First, SkMCs take an extended period of time to grow and to produce the number of cells needed for the experiment. Second, it takes about one month for each batch of cells to reach maturity, which can impose an inter-batch variability. Moreover, the proportion of differentiated cells may vary for different batches, which could lead to differences in observed basal lactic acid transport activity.

In order to study the effects of statins or loratadine on skeletal muscle during a prolonged period of time, we pretreated the SkMCs with clinical concentrations of atorvastatin, simvastatin and loratadine for 6 days. The results showed that pretreatment with these drugs did not affect L-lactic acid transport activity. Pretreatment with these drugs did not affect their inhibitory potential either. Pre-exposure periods beyond 6 days were not recommended, because of the limited amount of time for which the cells could be kept in culture after differentiation. Additionally, we performed pretreatment assays at higher concentrations to assess the effect of short-term statin treatment on mRNA transporter expression in SkMCs (Supplementary Figure S1). No difference was observed in MCT1 and MCT4 expression between the control and the pretreated batches, which could explain the absence of change in L-lactic acid transport activity levels following pre-exposition to the drug.

In conclusion, we have developed a cell model that can be used to screen for different drugs that may contribute to drug-induced myopathy by inhibiting L-lactic acid efflux. Our experiments determined the inhibitory potential of different statins and loratadine on the

transport of L-lactic acid by MCTs in human skeletal cells. Our results demonstrated that loratadine and atorvastatin blocked L-lactic acid efflux transport to a significant extent, and that the magnitude of this effect was not affected by pH variation during physical activity. However, there was a higher basal accumulation of L-lactic acid at pH 7.0 vs pH 7.4. Further studies are required to relate intracellular accumulation of L-lactic acid in skeletal muscle cells and the clinical observation of drug-induced muscle pain.

Supplementary Materials

The following are available online at www.mdpi.com/1999-4923/9/4/42/s1, Figure S1: Relative mRNA expression levels of drug-transporters in SkMC following pretreatment with statins at different concentrations (0.2 and 2 µM of atorvastatin, 0.2 and 2 µM of simvastatin acid, 0.2 and 2 µM of rosuvastatin). Gene expression levels were normalized using GAPDH as an housekeeping gene and vehicle-treated SkMC were used as reference. OATP1B1 was also investigated and no expression of OATP1B1 was detected in any SkMC samples, Table S1: Summary of HPLC analytical method conditions for the quantification of statins and loratadine (flow rate of 1.0 mL/min). Details pertaining to Western blotting method are also available.

Acknowledgments

This work was supported by internal fundings from the CRCHUM, Fondation CHUM and Faculté de Pharmacie, Université de Montreal. Yat Hei Leung was a recipient of a studentship from the Fonds de la Recherche du Québec en Santé (FRQS). Veronique Michaud was the recipient of a research scholarship from FRQS in partnership with the Institut National d'Excellence en Santé et en Services Sociaux (INESSS). We are also grateful to Francois Belanger for his excellent technical assistance. We would like to thank Hasna Maachi for generating the cellular microscopy pictures.

Author Contributions

Veronique Michaud and Jacques Turgeon conceived and designed the study; Yat Hei Leung performed the experiments and the analysis; Yat Hei Leung, Veronique Michaud and Jacques

Turgeon interpreted the data; Yat Hei Leung, Veronique Michaud and Jacques Turgeon wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

References

Kochanek, K.D.; Murphy, S.L.; Xu, J.; Tejada-Vera, B. Deaths: Final data for 2014. *Natl. Vital Stat. Rep.* 2016, *65*, 1 – 122. [Google Scholar] [PubMed]

Brahma, D.K.; Wahlang, J.B.; Marak, M.D.; Sangma, M.C. Adverse drug reactions in elderly. *J. Pharmacol. Pharmacother.* 2013, *4*, 91 – 94. [Google Scholar] [CrossRef] [PubMed]

Roden, D.M.; George, A.L., Jr. The genetic basis of variability in drug responses. *Nat. Rev. Drug Discov.* 2002, *1*, 37 – 44. [Google Scholar] [CrossRef] [PubMed]

Liu, R.; AbdulHameed, M.D.M.; Kumar, K.; Yu, X.; Wallqvist, A.; Reifman, J. Data-driven prediction of adverse drug reactions induced by drug-drug interactions. *BMC Pharmacol Toxicol.* 2017, *18*, 44. [Google Scholar] [CrossRef] [PubMed]

Ghosh, B.; Sengupta, S.; Bhattacharjee, B.; Majumder, A.; Sarkar, S.B. Fenofibrate-induced myopathy. *Neurol. India*2004, *52*, 268 – 269. [Google Scholar] [PubMed]

Valiyil, R.; Christopher-Stine, L. Drug-related myopathies of which the clinician should be aware. *Curr. Rheumatol. Rep.* 2010, *12*, 213 – 220. [Google Scholar] [CrossRef] [PubMed]

Mor, A.; Mitnick, H.J.; Pillinger, M.H.; Wortmann, R.L. Drug-induced myopathies. *Bull. NYU Hosp. Jt. Dis.* 2009, *67*, 358 – 369. [Google Scholar] [PubMed]

Sathasivam, S. Statin induced myotoxicity. *Eur. J. Int. Med.* 2012, *23*, 317 – 324. [Google Scholar] [CrossRef] [PubMed]

Rizos, C.V.; Elisaf, M.S. Statin myopathy: Navigating the maze. *Curr. Med. Res. Opin.* 2017, *33*, 327 – 329. [Google Scholar] [CrossRef] [PubMed]

Dirks, A.J.; Jones, K.M. Statin-induced apoptosis and skeletal myopathy. *Am. J. Physiol. Cell Physiol.* 2006, *291*, C1208 – C1212. [Google Scholar] [CrossRef] [PubMed]

Dalakas, M.C. Toxic and drug-induced myopathies. *J. Neurol. Neurosurg. Psychiatry* 2009, *80*, 832 – 838. [Google Scholar] [CrossRef] [PubMed]

Mastaglia, F.L.; Needham, M. Update on toxic myopathies. *Curr. Neurol. Neurosci. Rep.* 2012, *12*, 54 – 61. [Google Scholar] [CrossRef] [PubMed]

Lee, A. *Adverse Drug Reactions*, 2nd ed.; Pharmaceutical Press: London, UK, 2006; p. 474. [Google Scholar]

Leung, Y.H.; Lu, J.; Papillon, M.-E.; Bélanger, F.; Turgeon, J.; Michaud, V. The role of MCT1 and MCT4 in drug-induced muscle disorders. Abstract. ASPET 2013. *FASEB J.* 2013, *27* (Suppl. 1), 674. [Google Scholar]

Bosomworth, N.J. Statin Therapy as Primary Prevention in Exercising Adults: Best Evidence for Avoiding Myalgia. *J. Am. Board Fam. Med.* 2016, *29*, 727 – 740. [Google Scholar] [CrossRef] [PubMed]

Parker, B.A.; Thompson, P.D. Effect of statins on skeletal muscle: Exercise, myopathy, and muscle outcomes. *Exerc. Sport Sci. Rev.* 2012, *40*, 188 – 194. [Google Scholar] [CrossRef] [PubMed]

Meador, B.M.; Huey, K.A. Statin-associated myopathy and its exacerbation with exercise. *Muscle Nerve* 2010, *42*, 469 – 479. [Google Scholar] [CrossRef] [PubMed]

Krishnan, G.M.; Thompson, P.D. The effects of statins on skeletal muscle strength and exercise performance. *Curr. Opin. Lipidol.* 2010, *21*, 324 – 328. [Google Scholar] [CrossRef] [PubMed]

Kobayashi, M.; Otsuka, Y.; Itagaki, S.; Hirano, T.; Iseki, K. Inhibitory effects of statins on human monocarboxylate transporter 4. *Int. J. Pharm.* 2006, *317*, 19 – 25. [Google Scholar] [CrossRef] [PubMed]

Kobayashi, M.; Kaido, F.; Kagawa, T.; Itagaki, S.; Hirano, T.; Iseki, K. Preventive effects of bicarbonate on cerivastatin-induced apoptosis. *Int. J. Pharm.* 2007, *341*, 181 – 188. [Google Scholar] [CrossRef] [PubMed]

Kobayashi, M.; Fujita, I.; Itagaki, S.; Hirano, T.; Iseki, K. Transport Mechanism for L-Lactic Acid in Human Myocytes Using Human Prototypic Embryonal Rhabdomyosarcoma Cell Line (RD Cells). *Biol. Pharm. Bull.* 2005, *28*, 1197 – 1201. [Google Scholar] [CrossRef] [PubMed]

Kobayashi, M.; Chisaki, I.; Narumi, K.; Hidaka, K.; Kagawa, T.; Itagaki, S.; Hirano, T.; Iseki, K. Association between risk of myopathy and cholesterol-lowering effect: A comparison of all statins. *Life Sci.* 2008, *82*, 969 – 975. [Google Scholar] [CrossRef] [PubMed]

Bruckert, E.; Hayem, G.; Dejager, S.; Yau, C.; Begaud, B. Mild to moderate muscular symptoms with high-dosage statin therapy in hyperlipidemic patients — The PRIMO study. *Cardiovasc. Drugs Ther.* 2005, *19*, 403 – 414. [Google Scholar] [CrossRef] [PubMed]

Merck & Co., Inc. *Zocor (Simvastatin) Tablets Prescribing Information*; West Point: New York, NY, USA, 2015. [Google Scholar]

McKenney, J.M. Pharmacologic characteristics of statins. *Clin. Cardiol.* 2003, *26* (Suppl. 3), III32 – III38. [Google Scholar] [CrossRef] [PubMed]

Han, X.; Quinney, S.K.; Wang, Z.; Zhang, P.; Duke, J.; Desta, Z.; Elmendorf, J.S.; Flockhart, D.A.; Li, L. Identification and Mechanistic Investigation of Drug-Drug Interactions Associated With Myopathy: A Translational Approach. *Clin. Pharmacol. Ther.* 2015, *98*, 321 – 327. [Google Scholar] [CrossRef] [PubMed]

Duke, J.D.; Han, X.; Wang, Z.; Subhadarshini, A.; Karnik, S.D.; Li, X.; Hall, S.D.; Jin, Y.; Callaghan, J.T.; Overhage, M.J.; et al. Literature based drug interaction prediction with clinical assessment using electronic medical records: Novel myopathy associated drug interactions. *PLoS Comput. Biol.* 2012, *8*, e1002614. [Google Scholar] [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Supplementary Materials: Study of Statin- and Loratadine-Induced Muscle Pain Mechanisms Using Human Skeletal Muscle Cells

Yat Hei Leung, Jacques Turgeon and Veronique Michaud

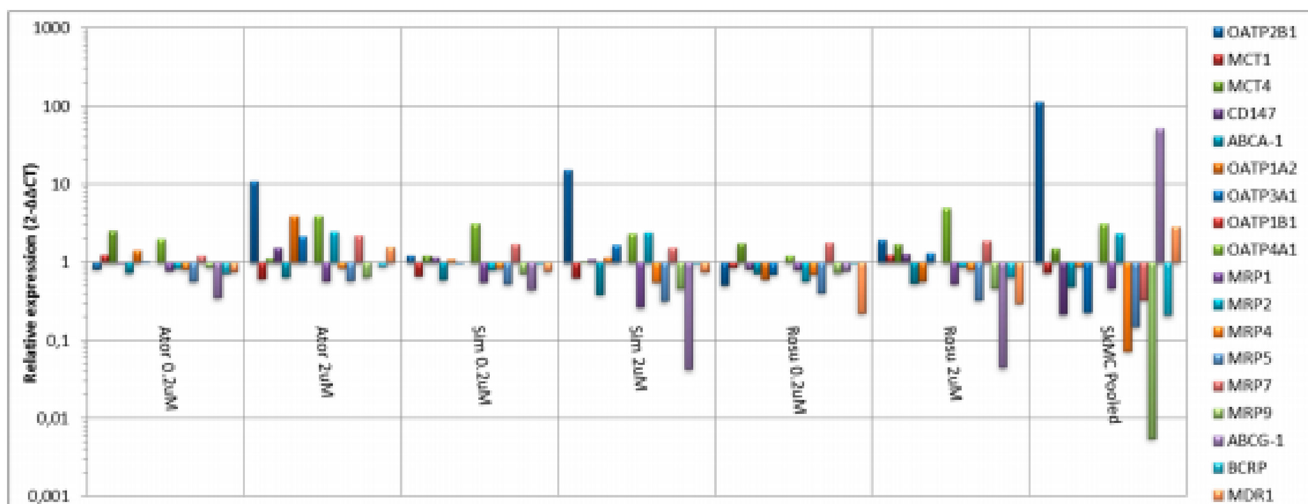


Figure S1. Relative mRNA expression levels of drug-transporters in SkMC following pretreatment with statins at different concentrations (0.2 and 2 µM of atorvastatin, 0.2 and 2 µM of simvastatin acid, 0.2 and 2 µM of rosuvastatin). Gene expression levels were normalized using GAPDH as an housekeeping gene and vehicle-treated SkMC were used as reference. OATP1B1 was also investigated and no expression of OATP1B1 was detected in any SkMC samples.

Table S1. Summary of HPLC analytical method conditions for the quantification of statins and loratadine (flow rate of 1.0 mL/min).

Compound	Proportion of Mobile Phase: 10 mM Ammonium Formate pH 3: Acetonitrile (v:v)	Internal Standard	Monitored UV Wavelength (nm)
Atorvastatin	53:47	Naproxen	250
Loratadine	60:40	Lovastatin	248
Rosuvastatin	53:47	Naproxen	243
Simvastatin hydroxyl acid	53:47	Loratadine	248
Simvastatin lactone	60:40	Loratadine	246

Western blot analysis

Total protein content was extracted from SkMC. Cells were lysed in a sample buffer containing 1% SDS/0.2N NaOH or commercially available cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA). Protein concentration of the protein lysate was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Walthman, MA, USA) with bovine serum albumin as a standard, following the manufacturer's recommendations. For the Western blot analysis, samples were denatured at 100°C for 5 minutes in a loading buffer containing 50 mM Tris-HCL, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol and separated in 5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred by electrophoresis onto a pure nitrocellulose membrane (BioTrace, Onenhuga, Auckland, New Zealand). Membranes were blocked with TBS containing 0.05% Tween 20 (TBS/T) and 5% dry milk. Membranes were washed with TBS/T and incubated with primary antibody mouse anti-GAPDH (Sigma-Aldrich, St. Louis, MO, USA) (diluted 1:10000), mouse anti-MCT1 (diluted 1:500), or rabbit anti-MCT4 (Santa Cruz Biotechnology, Dallas, TX, USA) (diluted 1:500). Then, membranes were washed and incubated with secondary antibody Pharmaceutics 2017, 9, 42 S2/S2 conjugated with horseradish peroxidase goat-anti mouse (diluted 1:5000) or goat-anti rabbit (Santa Cruz Biotechnology, Dallas, TX, USA) (diluted 1:5000). Bands were visualized on Hyblot CL autoradiography film (Denville Scientific, Holliston, MA, USA) with a standard enhanced chemiluminescence developing solution (GE Healthcare, Mississauga, ON, Canada).

2.2.4 Discussion

In this study, we have selected the primary human SkMC from Cell Applications Inc. to be used as the *in vitro* models for the experiments. These cells had to be differentiated before use. They were then characterized for their L-lactic acid transport activity. Uptake and efflux of lactic acid were found to be a rapid process.

L-lactic acid efflux inhibition by statins and loratadine were evaluated for the first time in SkMC (physiologically representative model). The results obtained corroborate the ones we have reported in the previous study. Loratadine had the highest inhibitory potential for the efflux of L-lactic acid. Atorvastatin also showed great inhibitor potency, while simvastatin acid only showed an intermediate inhibitor potency for L-lactic acid efflux. As previously reported, rosuvastatin did not affect the transport of L-lactic acid. The extent of the drugs' ability to block L-lactic acid efflux was also compared to a known MCT inhibitor, phloretin. Atorvastatin and loratadine were actually found to be more potent inhibitors than phloretin.

To consider the effect of physical activity on the incidence rate of drug-induced myopathy, we have performed inhibition studies at physiological (pH 7.4) and post-exercise (pH 7.0) conditions. Overall, the different pH did not affect the inhibitory potential of the tested compounds. However, depending on the compounds, their intracellular concentration can increase or decrease with the pH. This appears to affect the accumulation of lactic acid. Prolonged statin and loratadine exposure did not show any effect on the L-lactic acid transport activity or the inhibitory potency of these compounds.

2.2.5 Author contributions

Yat Hei Leung developed the methods and performed all the experiments, as well as data analysis. The author also interpreted the data and wrote the paper.

Jacques Turgeon and Véronique Michaud conceived and designed the study. They also participated in data interpretation and writing the paper.

Chapter 3 - Discussion and Conclusion

3.1 General discussion

For a long time, it was believed that drugs' pharmacokinetics and pharmacodynamics depended mainly upon their systemic metabolism, leading to the observed plasma levels and therapeutic effects. However, it is now known that drug transporters also play an important role in the absorption, distribution, metabolism and elimination of most medication.[7]

To exert their therapeutic effects, drugs have to reach their site of action, and this sometimes requires the action of drug transporters. Many drug transporters have been characterized over the years on their ability to affect drug efficacy, disposition and toxicity. In this doctoral project, we have evaluated the possible role of drug transporters in drug-induced myopathies, more specifically the ones induced by statins, loratadine and other acidic drugs.

Different factors influencing drug transport activity, potentially modifying plasma and local concentration of drugs, and possibly causing the drug-induced myotoxicities, are discussed in the **1.4 Drug transporters** section. Indeed, gene polymorphisms and drug-drug interactions during concomitant medications can increase drug plasma levels by lowering metabolism or excretion (modification of hepatic and intestinal influx transporter or hepato-biliary efflux transporter activities). The most relevant polymorphism found to affect statin plasma concentrations is *SLCO1B1 521T>C*, and it has actually shown to be highly associated with statin (simvastatin)-induced muscle pain.[1, 40, 119]

However, systemic concentrations of medication can only partly explain the clinically observed drug-associated myotoxicity.[80] Therefore, drug transporters regulating the local exposure have also been studied as a possibly more relevant cause for muscular ADR. For statins, the uptake transporter SLCO2B1 and the efflux transporters ABCC1, ABCC4, and

ABCC5 have been evaluated by Knauer *et al.*[80] They have found that, in fact, SLCO2B1 is able to increase the local exposure of atorvastatin and rosuvastatin in their differentiated human skeletal muscle myoblast model (HSMM) overexpressing *SLCO2B1*. They have also overexpressed ABCC1 in their model and found that this efflux transporter can abolish statin accumulation, suggesting that inhibition or polymorphism of these transporters can promote statin myopathy.

There are many hypotheses proposed for these drug-induced myopathies, but they still remain not well known. Increased drug plasma and local concentrations due to modified drug transport activity seem to be the cause, but the underlying mechanism is uncertain.[40] In the **1.3 Mechanisms of drug-induced myotoxicities** section, we have discussed the different plausible mechanisms behind statin myotoxicities, such as depletion of essential compounds during cholesterol biosynthesis (cholesterol, isoprenoids and ubiquinone), impaired mitochondrial respiration, disturbed calcium metabolism, autoimmunity or increased plasma concentration caused by genetic polymorphisms and drug-drug interactions.[5, 14, 22, 40, 47, 52, 56, 72-77, 81] However, most of them are still controversial, with studies reporting conflicting results.

It has been shown in recent studies that some statins can be translocated into muscle cells not only by the more recognized drug transporters, such as the SLCO, but also by the monocarboxylate transporters, which mediate lactate transport and pH homeostasis.[82, 84] In addition to these findings, it is also known that physical exercise during statin therapy can exacerbate drug-induced muscular symptoms.[96-98] The main byproduct formed during high intensity exercise (energy supply *via* glycolysis pathway) and depleted oxygen (anaerobic) conditions is L-lactic acid. Consequently, we have proposed in this study that the local drug exposure can affect the physiological role of the L-lactate transporters situated in muscle,

leading to an increase of intracellular lactic acid concentrations, which can in turn cause the clinically observed drug-induced myopathy.

In order to prove our hypothesis, different *in vitro* models expressing MCT1 and/or MCT4 have been considered. First, we wanted to characterize the two proton-linked MCT transporters. In this study (Chapter 2), we have evaluated breast cancer cell lines expressing selectively MCT1 or MCT4, since it has been found by Gallagher *et al.* that breast cancer cells, MDA-MB-231, selectively express high levels of MCT4.[161] Based on this observation, we screened eight breast cancer cell lines to see if some also express selectively MCT1 or MCT4. Through RT-PCR analysis, we have found two cell lines that highly express MCT1 selectively (Hs578T and SKBR3) and confirmed that MDA-MB-231 indeed express selectively MCT4. The Hs578T was chosen as the MCT1 model due to its greater adherence to the cell culture plastic wares and its ease to culture. Kinetic parameters of L-lactic acid transport were determined, with K_m values of 2.3 and 9.6 mM for MCT1 and MCT4, respectively. These findings were similar to the ones reported by Manning Fox *et al*., Dimmer *et al*., Bröer *et al*., Lin *et al*., Wilson *et al*. and Carpenter *et al*., which were determined either in *Xenopus laevis* oocytes (expressing MCT1 or MCT4) or tumor cells.[90, 162-166] Their experiments involved either human or rat MCT1 and MCT4 cRNA microinjected into *Xenopus* oocytes or Ehrlich-Lettre ascites tumor cells isolated from white Balb/C mice after implantation.[90, 162-166] The L-lactic acid transport in these studies was assessed either by monitoring changes in intracellular pH with BCECF (fluorescent dye) or by directly recording the pH_i with double-barreled pH-sensitive microelectrodes to measure pH_i and membrane potential.[90, 162-166] pH was measured in those studies since MCT are monocarboxylate transporters coupled with proton translocation. In contrast, we monitored L-lactic acid

transport by measuring the intracellular levels of radiolabeled [^{14}C] L-lactic acid. Despite these differences in methods and models used, we have found comparable L-lactic acid transport kinetics. Comparative data are found in Table VII.

Table VII. MCT1 and MCT4 K_m values from different studies

Author (year)	Model	MCT1 (mM)	MCT4 (mM)
Leung *et al.* (2017)	Hs578T	2.3	-
Leung *et al.* (2017)	MDA-MB-231	-	9.6
Manning Fox *et al.* (2000)[162]	*Xenopus* oocytes	4.4*	28
Dimmer *et al.* (2000)[163]	*Xenopus* oocytes	6.0	-
Bröer *et al.* (1998)[90]	*Xenopus* oocytes	3.5*	-
Lin *et al.* (1998)[164]	*Xenopus* oocytes	-	34*
Wilson *et al.* (1998)[165]	Tumor (Ehrlich-Lettre)	6.4	-
Carpenter *et al.* (1994)[166]	Tumor (Ehrlich-Lettre)	4.5	-

* Rat MCT expression

After characterizing the individual MCT transporters, we used our cell models to study the inhibitory potential of a series of acidic drugs including statins and possible comedications, analgesics (used to relieve myopathy symptoms), and other known myopathy-inducing drugs. Several lipophilic statins were identified to inhibit both the influx and efflux transport of L-lactic acid: atorvastatin, cerivastatin, fluvastatin, lovastatin acid and simvastatin acid. In our studies, the inhibition of L-lactic acid efflux *via* MCT1 and/or MCT4 was considered more critical for the clinically observed drug-induced muscular events. However, we have also investigated the inhibition of L-lactic acid uptake in order to corroborate the findings by

Kobayashi *et al.*[84] They are the only other investigators to have examined the inhibition of L-lactic acid by statins as the cause of statin-induced myopathy. In their research, they have found that all tested lipophilic statins were strong MCT4-mediated L-lactic acid influx inhibitors but not the hydrophilic ones, which is similar to our findings. Comparative IC_{50} results for L-lactic acid MCT4 transport are shown in Table VIII.

Table VIII. IC_{50} values (μM) of statins for L-lactic acid MCT4 transport in different studies

Author (year)	Leung *et al.* (2017)	Leung *et al.* (2017)	Kobayashi *et al.* (2006)[84]
Model (transport direction)	MDA-MB-231 (efflux)	MDA-MB-231 (influx)	LLC-PK1/MCT4 (influx)
Atorvastatin	41 (29-58)	~138	32.6 ±2.1
Cerivastatin	>400	>200	96.0 ±5.5
Fluvastatin	>210	~187	32.4 ±3.2
Lovastatin Acid	>200	>250	44.2 ±9.7
Pravastatin	>1000	>1000	>1000
Rosuvastatin	>1000	>1000	>100
Simvastatin acid	>180	>180	79.4 ±2.5

In summary, the study by Kobayashi *et al.* and ours have found the same inhibitory potential pattern for the different statins. Atorvastatin has the highest effect, while pravastatin and rosuvastatin had no detectable transport inhibition. Fluvastatin, simvastatin acid, lovastatin acid and cerivastatin all displayed intermediate inhibitory potential. However, our determined IC_{50} values are overall higher than the ones reported by Kobayashi *et al.*[84] This can be due either to the different *in vitro* models used (breast cancer cell lines *vs* stable transfected cell line), incubation time (2.5 *vs* 30 min) and/or L-lactic acid loading concentration (6 mM *vs* 3.3 μM) for our study and theirs, respectively.[84] Our cell lines overexpress MCT transporters

while Kobayashi *et al*. co-transfected CD147/MCT4 into LLC-PK1 (porcine kidney epithelial cells), which present different lactic acid transport K_m values of 9.6 mM and 28.4 mM, respectively for our study and theirs.[84] Their transport assay was substantially longer, which may affect their transport activity measurement as it is a rapid process. We have also used a considerably greater amount of lactic acid during the incubation step for L-lactic acid uptake study. The L-lactic acid concentration selected in our studies is comparable to the physiological concentration, as well as to the K_m obtained in our model characterization experiments. We have used approximately 2000 times more lactic acid than their study, which can explain the higher obtained IC_{50} values since more inhibitor would be needed to compete with the higher substrate levels during competitive inhibition.[83]

Considering the transporter overexpression in our *in vitro* models, these cell lines can be very useful tools for the screening of medication or compounds that can possibly inhibit the transport of L-lactic acid *via* MCT1 or MCT4. Loratadine, an antihistamine known to cause myopathy, was identified to be a strong MCT inhibitor, while irbersartan and losartan showed intermediate inhibition. Other tested acidic drugs had no observed L-lactic acid transport inhibition. However, these models cannot account for the interaction between different drugs and membrane transporters within the muscle. Hence, a second *in vitro* model was developed during this doctoral project. We have tested different commercially available primary human skeletal muscle cells in order to find a batch of cells that was appropriate for our studies. There are some difficulties associated with the use of primary cells and especially differentiated ones. These SkMC have fixed population doubling and passage numbers, slow growth (prolonged experiment preparation phase), and frequent media change (increased experimental

cost). In addition, these cells need to be differentiated, which can add an inter-batch variability because they might not be differentiated to the same extent.

With this more physiologically relevant SkMC model to study drug-induced myopathy, we wanted to account for physical activity in patients under statin therapy and the prolonged periods of the treatments, which can exacerbate muscular symptoms. Intracellular concentrations of the tested compounds were measured since it is recognized that higher drug concentrations also induce or aggravate muscle pain. L-lactic acid efflux inhibition assays were done at pH7.0 and pH7.4 representing physical activities and resting pH, respectively. Even though there was no inhibitory potential difference discovered between the different pH for the tested compounds, higher levels of remaining intracellular L-lactic acid were found at pH7.0. This further supports the hypothesis that inhibition of L-lactic acid transport, which promotes its accumulation in the muscle cells, is associated with drug-induced myotoxicity. Decreased physiological pH during exercise can also increase the cellular uptake of acidic drugs due to their physicochemical properties, possibly increasing the inhibition of L-lactic acid efflux. This only applies if MCT-mediated efflux of L-lactic acid has to be blocked from within the muscle cells. Pretreatments with pertinent compounds at clinically relevant concentrations were also performed to study their effect in muscle cells for an extended period of time. MCT1 and MCT4 expression levels do not seem to have been modified, thus explaining the absence of variation in L-lactic acid transport activities. The pretreatment did not alter the inhibitor potency of the compounds either, indicating that it is most likely the lactic acid transport inhibition alone that is associated with myopathy and that the prolonged exposure probably facilitates intracellular drug accumulation.

Overall, we have identified atorvastatin and loratadine as the strongest L-lactic acid efflux inhibitors. Simvastatin acid had an intermediate inhibitory potential. These results are in agreement with clinical data (PRIMO study), in which higher myopathy occurrence rates are associated with lipophilic statins such as atorvastatin (14.9%) and simvastatin (18.2%).[167] The higher adverse event frequency associated with simvastatin treatment could be explained by its very low oral bioavailability (<5%), which can result in important increases in its exposure during drug-drug interactions.[167, 168]

An *in vitro* cell viability study by Kobayashi *et al.* in a rhabdomyosarcoma cell line showed that the most cytotoxic statins were as follows: cerivastatin, simvastatin acid, fluvastatin, atorvastatin, lovastatin acid, pitavastatin, rosuvastatin and pravastatin.[43] They have correlated statin myotoxicity with their partition coefficient, meaning that the most lipophilic statins were the most cytotoxic in their model. This might be partly explained by the fact that there is a greater accumulation of lipophilic statins in the cells since the cytotoxicity is dose-dependent. Their group also conducted experiments in a hepatocyte model (HepG2 cells) and found no correlation between the cholesterol-lowering effect of statins and cytotoxicity.[43]

According to the literature, the most myotoxic statin is cerivastatin, which is now withdrawn from the market, followed by simvastatin, lovastatin, pravastatin, atorvastatin and fluvastatin.[6] *In vivo* data matched for the lowest therapeutic dose showed that the reduction of LDL-C is the greatest for rosuvastatin and cerivastatin, followed by atorvastatin, simvastatin, pravastatin, fluvastatin and lovastatin (See Table I, p.12).[38, 44]

It is reported in the literature, that the combination of loratadine and simvastatin is associated with an increased risk for myopathy.[22] However, in our study, there was no

significant difference between the effects of loratadine or simvastatin used alone or in combination *(data not shown)*.

Loratadine is an over-the-counter allergy relief treatment, so not all ADR that are caused by this medication are reported, underestimating the frequency of myopathies related to its use and overestimating the occurrence of muscular ADR for other drugs taken concomitantly. The IC_{50} determined in this study were all greater than clinically relevant concentrations, but patients are under statin therapy for extended periods of time, possibly increasing drug accumulation in the muscle over time. To assess the extent of L-lactic acid transport inhibition, inhibitor potencies of these drugs were compared to a known strong MCT inhibitor, phloretin. Atorvastatin and loratadine had lower IC_{50} than phloretin, indicating that these drugs are more potent inhibitors in these models.

3.2 Conclusion

In conclusion, the mechanism for drug-induced myopathies, especially the ones caused by statins and loratadine, still remains not well understood. This project is the first to evaluate the role of L-lactic acid efflux inhibition *via* MCT in drug-associated myotoxicities. Our results show that certain acidic drugs can inhibit the MCT-mediated transport of L-lactic acid in different *in vitro* models. Indeed, atorvastatin and loratadine had the highest inhibitory potency for L-lactic acid efflux, which will lead to intracellular accumulation of this glycolysis byproduct. This over-accumulation can induce myotoxicity related to drug therapy, which may result in the activation of caspases 3/7 leading to cell apoptosis.

The major limitation associated with the use of these *in vitro* models is that they cannot connect the L-lactic acid accumulation to the reported drug-induced myopathies. An *in vivo* model in which the level of muscle discomfort can be measured would provide valuable insight into the possible mechanism of drug-related muscle pain. For example, a study could be conducted on rats treated with atorvastatin, simvastatin acid, rosuvastatin, loratadine, a combination of drugs or vehicle for 2 weeks. Exercise tolerance tests would be performed to assess the effect of drug administration on muscle function. After treatment, the hindlimb muscles would be removed and used for further analysis. Drug concentrations in muscle would be measured, as well as lactic acid, pyruvate and creatine kinase. The mRNA expression of various drug transporters in muscle would also be studied in order to better characterize their distribution.

Moreover, a clinical trial involving symptomatic and asymptomatic patients would allow the discovery of differences between the groups and potentially explain the occurrence

of these adverse events in some individuals. The objectives of these studies would be to determine, for the different groups, drug transporter expression by RT-PCR in muscle before and after treatment, as well as the lactate/pyruvate ratio in plasma and muscle. Moreover, it would be relevant to compare the plasma/muscle ratio of drugs before and after exercise. MCT-mediated transport activity could be assessed in muscle biopsy to correlate with our *in vitro* data.

Data obtained for this project combined with the suggested studies can help to identify the cause of certain drug-induced myopathies and possibly reduce their frequency. Statins, as well as lifestyle change, lead to better cholesterol management and are effective at lowering the risk of CVD. Even though statins are sometimes associated with myopathy, their use has been proven beneficial as a preventive measure for cardiovascular events. We have shown that some statins inhibit MCT-mediated lactic acid transport less than others (also reported to be less cytotoxic) and these should be the first options in statin therapy. Better knowledge of the extent of MCT inhibition as an underlying mechanism in drug-induced myopathies may facilitate the physician's choice between the different treatment options.

As for allergy symptom relief, we have found that loratadine is a potent MCT inhibitor, which might cause myopathy in clinical settings. Studies on other antihistamines might help understand the effects of other drugs of the same class on MCT-mediated transport and guide patients in their choice between these over-the-counter drugs.

Overall, further testing is necessary to confirm our *in vitro* data. Studies similar to ours and our proposed studies could be conducted for various drugs associated with myotoxicities to see if they cause myopathy through the same mechanism.

Bibliography

1. Valiyil R, Christopher-Stine L. Drug-related myopathies of which the clinician should be aware. Curr Rheumatol Rep. 2010;12(3):213-20.
2. Guis S, Mattei JP, Liote F. Drug-induced and toxic myopathies. Best Pract Res Clin Rheumatol. 2003;17(6):877-907.
3. Schachter M. Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update. Fundam Clin Pharmacol. 2005;19(1):117-25.
4. Rizos CV, Elisaf MS. Statin myopathy: navigating the maze. Curr Med Res Opin. 2017;33(2):327-9.
5. Bitzur R, Cohen H, Kamari Y, Harats D. Intolerance to statins: mechanisms and management. Diabetes Care. 2013;36 Suppl 2:S325-30.
6. Dalakas MC. Toxic and drug-induced myopathies. J Neurol Neurosurg Psychiatry. 2009;80(8):832-8.
7. You G, Morris ME. Drug Transporters- Molecular Characterization and Role in Drug Disposition. Hoboken, New Jersey: John Wiley & Sons; 2007. 889 p.
8. Parker BA, Thompson PD. Effect of statins on skeletal muscle: exercise, myopathy, and muscle outcomes. Exerc Sport Sci Rev. 2012;40(4):188-94.
9. Meador BM, Huey KA. Statin-associated myopathy and its exacerbation with exercise. Muscle Nerve. 2010;42(4):469-79.
10. Pilegaard H, Domino K, Noland T, Juel C, Hellsten Y, Halestrap AP, et al. Effect of high-intensity exercise training on lactate/H+ transport capacity in human skeletal muscle. Am J Physiol. 1999;276(2 Pt 1):E255-61.
11. Kochanek KD, Murphy SL, Xu J, Tejada-Vera B. Deaths: Final Data for 2014. National Vital Statistics Reports. 2016;65(4):1-122.
12. Lazarou J, Pomeranz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. JAMA. 1998;279(15):1200-5.
13. Pirmohamed M, James S, Meakin S, Green C, Scott AK, Walley TJ, et al. Adverse drug reactions as cause of admission to hospital: prospective analysis of 18820 patients. BMJ. 2004;329:15-9.
14. Brahma DK, Wahlang JB, Marak MD, Ch Sangma M. Adverse drug reactions in the elderly. J Pharmacol Pharmacother. 2013;4(2):91-4.
15. Liu R, AbdulHameed MDM, Kumar K, Yu X, Wallqvist A, Reifman J. Data-driven prediction of adverse drug reactions induced by drug-drug interactions. BMC Pharmacol Toxicol. 2017;18(1):44.
16. Rodine RJ, Tibbles AC, Kim PSY, Alikhan N. Statin induced myopathy presenting as mechanical musculoskeletal pain observed in two chiropractic patients. J Can Chiropr Assoc. 2010;54(1):43-51.
17. Ferdinandy P, Hausenloy DJ, Heusch G, Baxter GF, Schulz R. Interaction of risk factors, comorbidities, and comedications with ischemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. Pharmacological reviews. 2014;66(4):1142-74.
18. Backman JT, Filppula AM, Niemi M, Neuvonen PJ. Role of Cytochrome P450 2C8 in Drug Metabolism and Interactions. Pharmacological reviews. 2016;68(1):168-241.

19. Jordan KP, Kadam UT, Hayward R, Porcheret M, Young C, Croft P. Annual consultation prevalence of regional musculoskeletal problems in primary care: an observational study. BMC Musculoskelet Disord. 2010;11:144.
20. Lee A. Adverse drug reactions. 2nd edition ed: Pharmaceutical Press; 2006. 474 p.
21. Mor A, Mitnick HJ, Pillinger MH, Wortmann RL. Drug-induced myopathies. Bull NYU Hosp Jt Dis. 2009;67(4):358-69.
22. Duke JD, Han X, Wang Z, Subhadarshini A, Karnik SD, Li X, et al. Literature based drug interaction prediction with clinical assessment using electronic medical records: novel myopathy associated drug interactions. PLoS Comput Biol. 2012;8(8):e1002614.
23. Smithson J. Drug induced muscle disorders. Australian pharmacist. 2009;28(12):7.
24. Owczarek J, Jasinska M, Orszulak-Michalak D. Drug-induced myopathies. An overview of the possible mechanisms. Pharmacol Rep. 2005;57(1):23-34.
25. CDC. Heart disease fact sheet. 2016.
26. Gillespie C, Kuklina EV, Briss PA, Blair NA, Hong Y. Vital Signs: Prevalence, Treatment, and Control of Hypertension-United States, 1999-2002 and 2005-2008 (Reprinted from MMWR, vol 60, pg 103-108, 2011). Jama-J Am Med Assoc. 2011;305(15):1531-+.
27. WHO. Risk factors.
28. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. Circulation. 2011;123(4):e18-e209.
29. Widmaier EP, Raff H, Strang KT. Vander's Human Physiology- The Mechanisms of Body Function. Eleventh Edition ed. New York: McGraw-Hill; 2008. 775 p.
30. Charlton-Menys V, Durrington PN. Human cholesterol metabolism and therapeutic molecules. Exp Physiol. 2008;93(1):27-42.
31. Dadu RT, Ballantyne CM. Lipid lowering with PCSK9 inhibitors. Nat Rev Cardiol. 2014;11(10):563-75.
32. Collaborators CTTC. The effects of lowering LDL cholesterol with statin therapy in people at low risk of vascular disease: meta-analysis of individual data from 27 randomised trials. Lancet. 2012;380:581–90.
33. Force USPST, Bibbins-Domingo K, Grossman DC, Curry SJ, Davidson KW, Epling JW, Jr., et al. Statin Use for the Primary Prevention of Cardiovascular Disease in Adults: US Preventive Services Task Force Recommendation Statement. JAMA. 2016;316(19):1997-2007.
34. Brown F, Singer A, Katz A, Konrad G. Statin-prescribing trends for primary and secondary prevention of cardiovascular disease. Can Fam Physician. 2017;63(11):e495-e503.
35. Ellis JJ, Erickson SR, Stevenson JG, Bernstein SJ, Stiles RA, Fendrick AM. Suboptimal Statin Adherence and Discontinuation in Primary and Secondary Prevention Populations: Should We Target Patients with the Most to Gain? J Gen Intern Med. 2004;19:638-45.
36. IMS Health. National Prescription Audit. IMS Institute for Healthcare Informatics; Dec 2010.
37. IMS Brogan. 2012.
38. Vaughan CJ, Gotto AM, Jr. Update on statins: 2003. Circulation. 2004;110(7):886-92.
39. Liao JK, Laufs U. Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol. 2005;45:89-118.

40. du Souich P, Roederer G, Dufour R. Myotoxicity of statins: Mechanism of action. Pharmacol Ther. 2017;175:1-16.
41. Mabuchi H, Higashikata T, Kawashiri M, Katsuda S, Mizuno M, Nohara A, et al. Reduction of serum ubiquinol-10 and ubiquinone-10 levels by atorvastatin in hypercholesterolemic patients. J Atheroscler Thromb. 2005;12(2):111-9.
42. Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. Science. 2001;292(5519):1160-4.
43. Kobayashi M, Chisaki I, Narumi K, Hidaka K, Kagawa T, Itagaki S, et al. Association between risk of myopathy and cholesterol-lowering effect: a comparison of all statins. Life Sci. 2008;82(17-18):969-75.
44. Stein E, Isaacsohn J, Stoltz R, Mazzu A, Liu MC, Lane C, et al. Pharmacodynamics, safety, tolerability, and pharmacokinetics of the 0.8-mg dose of cerivastatin in patients with primary hypercholesterolemia. Am J Cardiol. 1999;83(10):1433-6.
45. Muck W. Clinical pharmacokinetics of cerivastatin. Clin Pharmacokinet. 2000;39(2):99-116.
46. Hansch C, Leo A, Hoekman D. Exploring QSAR: Hydrophobic, Electronic, and Steric Constants. Washington, DC American Chemical Society 1995.
47. Bellosta S, Paoletti R, Corsini A. Safety of statins: focus on clinical pharmacokinetics and drug interactions. Circulation. 2004;109(23 Suppl 1):III50-7.
48. White CM. A review of the pharmacologic and pharmacokinetic aspects of rosuvastatin. J Clin Pharmacol. 2002;42(9):963-70.
49. Abd TT, Jacobson TA. Statin-induced myopathy: a review and update. Expert Opin Drug Saf. 2011;10(3):373-87.
50. Rosenson RS. Current overview of statin-induced myopathy. Am J Med. 2004;116(6):408-16.
51. Josan K, Majumdar SR, McAlister FA. The efficacy and safety of intensive statin therapy: a meta-analysis of randomized trials. CMAJ. 2008;178(5):576-84.
52. Sathasivam S. Statin induced myotoxicity. Eur J Intern Med. 2012;23(4):317-24.
53. Baker SK. Molecular clues into the pathogenesis of statin-mediated muscle toxicity. Muscle Nerve. 2005;31(5):572-80.
54. Kobayashi M, Kagawa T, Narumi K, Itagaki S, Hirano T, Iseki K. Bicarbonate supplementation as a preventive way in statins-induced muscle damage. J Pharm Pharmaceut Sci. 2008;11(1):1-8.
55. Backes JM, Ruisinger JF, Gibson CA, Moriarty PM. Statin-associated muscle symptoms-Managing the highly intolerant. J Clin Lipidol. 2017;11(1):24-33.
56. Omar MA, Wilson JP. FDA adverse event reports on statin-associated rhabdomyolysis. Ann Pharmacother. 2002;36(2):288-95.
57. Group MBHPSC. Effects of simvastatin 40 mg daily on muscle and liver adverse effects in a 5-year randomized placebo-controlled trial in 20,536 high-risk people. BMC Clinical Pharmacology. 2009;9(6).
58. Devillier P, Roche N, Faisy C. Clinical pharmacokinetics and pharmacodynamics of desloratadine, fexofenadine and levocetirizine : a comparative review. Clin Pharmacokinet. 2008;47(4):217-30.
59. Information NCfB. PubChem Compound Database; CID=3957 2018 [Available from: https://pubchem.ncbi.nlm.nih.gov/compound/3957.

60. Kalpaklioglu F, Baccioglu A. Efficacy and safety of H1-antihistamines: an update. Antiinflamm Antiallergy Agents Med Chem. 2012;11(3):230-7.
61. Ellis AK, Day JH. Second- and third-generation antihistamines. Dermatologic Therapy. 2000;13:327-36.
62. Timmerman H. Why are non-sedating antihistamines non-sedating? Clin Exp Allergy. 1999;29 Suppl 3:13-8.
63. Simons FER, Simons KJ. The Pharmacology and Use of H1-Receptor-Antagonist Drugs. N Engl J Med. 1994;330(23):1663-70.
64. Carlsen KH, Kramer J, Fagertun HE, Larsen S. Loratadine and terfenadine in perennial allergic rhinitis. Treatment of nonresponders to the one drug with the other drug. Allergy. 1993;48(6):431-6.
65. Inc. B. Product monograph- Claritin. 2016.
66. Apotex. Loratadine- Prescribing information. 2001.
67. Co M. Clarinex- Prescribing information. 2018.
68. Portal D-DI. Claritin (Loratadine) - Side Effects and Adverse Reactions 2006 [Available from: http://www.druglib.com/druginfo/claritin/side-effects_adverse-reactions/.
69. Estey E. Do commonly used clinical trial designs reflect clinical reality? Haematologica. 2009;94(10):1435-9.
70. Mastaglia FL, Needham M. Update on toxic myopathies. Curr Neurol Neurosci Rep. 2012;12(1):54-61.
71. Dirks AJ, Jones KM. Statin-induced apoptosis and skeletal myopathy. Am J Physiol Cell Physiol. 2006;291(6):C1208-12.
72. Tomaszewski M, Stępień KM, Tomaszewska J, Czuczwar SJ. Statin-induced myopathies. Pharmacological Reports. 2011;63(4):859-66.
73. Albayda J, Mammen AL. Is statin-induced myositis part of the polymyositis disease spectrum? Curr Rheumatol Rep. 2014;16(8):433.
74. Mammen AL. Necrotizing myopathies: beyond statins. Curr Opin Rheumatol. 2014;26(6):679-83.
75. Mammen AL, Chung T, Christopher-Stine L, Rosen P, Rosen A, Doering KR, et al. Autoantibodies against 3-hydroxy-3-methylglutaryl-coenzyme A reductase in patients with statin-associated autoimmune myopathy. Arthritis Rheum. 2011;63(3):713-21.
76. Bhardwaj S, Selvarajah S, Schneider EB. Muscular effects of statins in the elderly female: a review. Clin Interv Aging. 2013;8:47-59.
77. Han X, Quinney SK, Wang Z, Zhang P, Duke J, Desta Z, et al. Identification and Mechanistic Investigation of Drug-Drug Interactions Associated With Myopathy: A Translational Approach. Clin Pharmacol Ther. 2015;98(3):321-7.
78. Tirona RG, Leake BF, Merino G, Kim RB. Polymorphisms in OATP-C: Identification of multiple allelic variants associated with altered transport activity among European- and African-Americans. J Biol Chem. 2001;276(38):35669–75.
79. Kellick KA, Bottorff M, Toth PP, The National Lipid Association's Safety Task F. A clinician's guide to statin drug-drug interactions. J Clin Lipidol. 2014;8(3 Suppl):S30-46.
80. Knauer MJ, Urquhart BL, Meyer zu Schwabedissen HE, Schwarz UI, Lemke CJ, Leake BF, et al. Human skeletal muscle drug transporters determine local exposure and toxicity of statins. Circulation research. 2010;106(2):297-306.
81. Link E, Parish S, Armitage J, Bowman L, Heath S, Matsuda F, et al. SLCO1B1 variants and statin-induced myopathy--a genomewide study. N Engl J Med. 2008;359(8).

82. Wu X, Whitfield LR, Stewart BH. Atorvastatin transport in the Caco-2 cell model: contributions of P-glycoprotein and the proton-monocarboxylic acid co-transporter. Pharm Res. 2000;17(2):209-15.
83. Tsuji A, Saheki A, Tamai I, Terasaki T. Transport Mechanism of 3-Hydroxy-3-Methylglutaryl Coenzyme a Reductase Inhibitors at the Blood-Brain-Barrier. J Pharmacol Exp Ther. 1993;267(3):1085-90.
84. Kobayashi M, Otsuka Y, Itagaki S, Hirano T, Iseki K. Inhibitory effects of statins on human monocarboxylate transporter 4. Int J Pharm. 2006;317(1):19-25.
85. Lee A. Adverse drug reactions. Second ed: Pharmaceutical Press; 2006.
86. Herbison GJ, Jaweed MM, Ditunno JF. Muscle fiber types. Arch Phys Med Rehabil. 1982;63(5):227-30.
87. Halestrap AP. The monocarboxylate transporter family--Structure and functional characterization. IUBMB Life. 2012;64(1):1-9.
88. Kikutani Y, Kobayashi M, Konishi T, Sasaki S, Narumi K, Furugen A, et al. Involvement of Monocarboxylate Transporter 4 Expression in Statin-Induced Cytotoxicity. J Pharm Sci. 2016;105(4):1544-9.
89. Wray S, Smith RD. Mechanisms of action of pH induced effects on vascular smooth muscle. Mol Cell Biochem. 2004;263:163-72.
90. Bröer S, Schneider HP, Bröer A, Rahman B, Hamprecht B, Deitmer JW. Characterisation of MCT1 expressed in Xenopus oocytes by changes in cytosolic pH. Biochem J. 1998;333:167-74.
91. Sun S, Li H, Chen J, Qian Q. Lactic Acid: No Longer an Inert and End-Product of Glycolysis. Physiology (Bethesda). 2017;32(6):453-63.
92. Ahmed K, Tunaru S, Tang C, Muller M, Gille A, Sassmann A, et al. An autocrine lactate loop mediates insulin-dependent inhibition of lipolysis through GPR81. Cell Metab. 2010;11(4):311-9.
93. Bergersen LH. Is lactate food for neurons? Comparison of monocarboxylate transporter subtypes in brain and muscle. Neuroscience. 2007;145(1):11-9.
94. Ahmed K, Tunaru S, Offermanns S. GPR109A, GPR109B and GPR81, a family of hydroxy-carboxylic acid receptors. Trends Pharmacol Sci. 2009;30(11):557-62.
95. Roland CL, Arumugam T, Deng D, Liu SH, Philip B, Gomez S, et al. Cell surface lactate receptor GPR81 is crucial for cancer cell survival. Cancer Res. 2014;74(18):5301-10.
96. Kearns AK, Bilbie CL, Clarkson PM, White CM, Sewright KA, Fallon KSO, et al. The creatine kinase response to eccentric exercise with atorvastatin 10 mg or 80 mg. Atherosclerosis. 2008;200(1):121-5.
97. Thompson PD, Zmuda JM, Domalik LJ, Zimet RJ, Staggers J, Guyton JR. Lovastatin increases exercise-induced skeletal muscle injury. Metabolism. 1997;46(10):1206-10.
98. Reust CS, Curry SC, Guidry JR. Lovastatin Use and Muscle Damage in Healthy-Volunteers Undergoing Eccentric Muscle Exercise. Western J Med. 1991;154(2):198-200.
99. Vasiliou V, Vasiliou K, Nebert DW. Human ATP-binding cassette (ABC) transporter family. Human Genomics. 2009;3(3):281-90.
100. Lin L, Yee SW, Kim RB, Giacomini KM. SLC transporters as therapeutic targets: emerging opportunities. Nat Rev Drug Discov. 2015;14(8):543-60.
101. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst. 2000;92(16):1295-302.

102. Choudhuri S, Klaassen CD. Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. Int J Toxicol. 2006;25(4):231-59.
103. Becker ML, Elens LLFS, Visser LE, Hofman A, Uitterlinden AG, van Schaik RHN, et al. Genetic variation in the ABCC2 gene is associated with dose decreases or switches to other cholesterol-lowering drugs during simvastatin and atorvastatin therapy. Pharmacogenomics Journal. 2013;13(3):251-6.
104. Hediger MA, Clemencon B, Burrier RE, Bruford EA. The ABCs of membrane transporters in health and disease (SLC series): introduction. Mol Aspects Med. 2013;34(2-3):95-107.
105. Hagenbuch B, Meier PJ. Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. Pflugers Arch. 2004;447(5):653-65.
106. Hagenbuch B, Meier PJ. The superfamily of organic anion transporting polypeptides. Biochimica et Biophysica Acta. 2003:1-18.
107. Konig J, Cui Y, Nies AT, Keppler D. A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. Am J Physiol Gastrointest Liver Physiol. 2000;278(1):G156-64.
108. Konig J, Cui Y, Nies AT, Keppler D. Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide. J Biol Chem. 2000;275(30):23161-8.
109. Kullak-Ublick GA, Ismair MG, Stieger B, Landmann L, Huber R, Pizzagalli F, et al. Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. Gastroenterology. 2001;120(2):525-33.
110. Yarim M, Moro S, Huber R, Meier PJ, Kaseda C, Kashima T, et al. Application of QSAR analysis to organic anion transporting polypeptide 1a5 (Oatp1a5) substrates. Bioorg Med Chem. 2005;13(2):463-71.
111. Chang C, Pang KS, Swaan PW, Ekins S. Comparative pharmacophore modeling of organic anion transporting polypeptides: a meta-analysis of rat Oatp1a1 and human OATP1B1. J Pharmacol Exp Ther. 2005;314(2):533-41.
112. Ho RH, Tirona RG, Leake BF, Glaeser H, Lee W, Lemke CJ, et al. Drug and bile acid transporters in rosuvastatin hepatic uptake: function, expression, and pharmacogenetics. Gastroenterology. 2006;130(6):1793-806.
113. Lau YY, Okochi H, Huang Y, Benet LZ. Multiple transporters affect the disposition of atorvastatin and its two active hydroxy metabolites: application of in vitro and ex situ systems. J Pharmacol Exp Ther. 2006;316(2):762-71.
114. Fujino H, Saito T, Ogawa S, Kojima J. Transporter-mediated influx and efflux mechanisms of pitavastatin, a new inhibitor of HMG-CoA reductase. J Pharm Pharmacol. 2005;57(10):1305-11.
115. Shitara Y, Hirano M, Sato H, Sugiyama Y. Gemfibrozil and its glucuronide inhibit the organic anion transporting polypeptide 2 (OATP2/OATP1B1:SLC21A6)-mediated hepatic uptake and CYP2C8-mediated metabolism of cerivastatin: analysis of the mechanism of the clinically relevant drug-drug interaction between cerivastatin and gemfibrozil. J Pharmacol Exp Ther. 2004;311(1):228-36.
116. Hirano M, Maeda K, Shitara Y, Sugiyama Y. Drug-drug interaction between pitavastatin and various drugs via OATP1B1. Drug Metab Dispos. 2006;34(7):1229-36.

117. Ramsey LB, Johnson SG, Caudle KE, Haidar CE, Voora D, Wilke RA, et al. The clinical pharmacogenetics implementation consortium guideline for SLCO1B1 and simvastatin-induced myopathy: 2014 update. Clin Pharmacol Ther. 2014;96(4):423-8.
118. Tornio A, Vakkilainen J, Neuvonen M, Backman JT, Neuvonen PJ, Niemi M. SLCO1B1 polymorphism markedly affects the pharmacokinetics of lovastatin acid. Pharmacogenet Genomics. 2015;25(8):382-7.
119. Needham M, Mastaglia FL. Statin myotoxicity: a review of genetic susceptibility factors. Neuromuscul Disord. 2014;24(1):4-15.
120. Schwarz UI, Meyer zu Schwabedissen HE, Tirona RG, Suzuki A, Leake BF, Mokrab Y, et al. Identification of novel functional organic anion-transporting polypeptide 1B3 polymorphisms and assessment of substrate specificity. Pharmacogenet Genomics. 2011;21(3):103-14.
121. Kobayashi D, Nozawa T, Imai K, Nezu J, Tsuji A, Tamai I. Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. J Pharmacol Exp Ther. 2003;306(2):703-8.
122. Sai Y, Kaneko Y, Ito S, Mitsuoka K, Kato Y, Tamai I, et al. Predominant contribution of organic anion transporting polypeptide OATP-B (OATP2B1) to apical uptake of estrone-3-sulfate by human intestinal Caco-2 cells. Drug Metab Dispos. 2006;34(8):1423-31.
123. Satoh H, Yamashita F, Tsujimoto M, Murakami H, Koyabu N, Ohtani H, et al. Citrus juices inhibit the function of human organic anion-transporting polypeptide OATP-B. Drug Metab Dispos. 2005;33(4):518-23.
124. Fuchikami H, Satoh H, Tsujimoto M, Ohdo S, Ohtani H, Sawada Y. Effects of herbal extracts on the function of human organic anion-transporting polypeptide OATP-B. Drug Metab Dispos. 2006;34(4):577-82.
125. Riedmaier AE, Nies AT, Schaeffeler E, Schwab M. Organic Anion Transporters and Their Implications in Pharmacotherapy. Pharmacological reviews. 2012;64(3):421-49.
126. Halestrap AP, Meredith D. The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pflugers Arch. 2004;447(5):619-28.
127. Rahman B, Schneider HP, Broer A, Deitmer JW, Broer S. Helix 8 and helix 10 are involved in substrate recognition in the rat monocarboxylate transporter MCT1. Biochemistry. 1999;38(35):11577-84.
128. Galic S, Schneider HP, Broer A, Deitmer JW, Broer S. The loop between helix 4 and helix 5 in the monocarboxylate transporter MCT1 is important for substrate selection and protein stability. Biochemical Journal. 2003;376:413-22.
129. Halestrap AP. The SLC16 gene family - structure, role and regulation in health and disease. Mol Aspects Med. 2013;34(2-3):337-49.
130. Biochemistry So. Structure and different isoforms Bristol: University of Bristol; 2017 [updated 24 October 2017. Available from: http://www.bristol.ac.uk/biochemistry/halestrap/structure.html.
131. Enerson BE, Drewes LR. Molecular features, regulation, and function of monocarboxylate transporters: implications for drug delivery. J Pharm Sci. 2003;92(8):1531-44.
132. Utoguchi N, Audus KL. Carrier-mediated transport of valproic acid in BeWo cells, a human trophoblast cell line. Int J Pharm. 2000;195(1-2):115-24.

133. Tamai I, Sai Y, Ono A, Kido Y, Yabuuchi H, Takanaga H, et al. Immunohistochemical and functional characterization of pH-dependent intestinal absorption of weak organic acids by the monocarboxylic acid transporter MCT1. J Pharm Pharmacol. 1999;51(10):1113-21.
134. Terasaki T, Takakuwa S, Moritani S, Tsuji A. Transport of Monocarboxylic Acids at the Blood-Brain-Barrier - Studies with Monolayers of Primary Cultured Bovine Brain Capillary Endothelial-Cells. J Pharmacol Exp Ther. 1991;258(3):932-7.
135. Itoh T, Tanno M, Li YH, Yamada H. Transport of phenethicillin into rat intestinal brush border membrane vesicles: role of the monocarboxylic acid transport system. International Journal of Pharmaceutics. 1998;172(1-2):103-12.
136. Brooks GA, Brown MA, Butz CE, Sicurello JP, Dubouchaud H. Cardiac and skeletal muscle mitochondria have a monocarboxylate transporter MCT1. American Physiological Society. 1999.
137. Bonen A. The expression of lactate transporters (MCT1 and MCT4) in heart and muscle. Eur J Appl Physiol. 2001;86(1):6-11.
138. Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN, Halestrap AP. CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. Embo J. 2000;19(15):3896-904.
139. Leino RL, Gerhart DZ, Drewes LR. Monocarboxylate transporter (MCT1) abundance in brains of suckling and adult rats: a quantitative electron microscopic immunogold study. Brain Res Dev Brain Res. 1999;113(1-2):47-54.
140. Leino RL, Gerhart DZ, Duelli R, Enerson BE, Drewes LR. Diet-induced ketosis increases monocarboxylate transporter (MCT1) levels in rat brain. Neurochem Int. 2001;38(6):519-27.
141. Cuff MA, Lambert DW, Shirazi-Beechey SP. Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1. J Physiol. 2002;539(Pt 2):361-71.
142. Hadjiagapiou C, Borthakur A, Dahdal RY, Gill RK, Malakooti J, Ramaswamy K, et al. Role of USF1 and USF2 as potential repressor proteins for human intestinal monocarboxylate transporter 1 promoter. Am J Physiol-Gastr L. 2005;288(6):G1118-G26.
143. Cuff MA, Shirazi-Beechey SP. The human monocarboxylate transporter, MCT1: genomic organization and promoter analysis. Biochem Biophys Res Commun. 2002;292(4):1048-56.
144. Fanelli A, Grollman EF, Wang D, Philp NJ. MCT1 and its accessory protein CD147 are differentially regulated by TSH in rat thyroid cells. Am J Physiol Endocrinol Metab. 2003;285(6):E1223-9.
145. Buyse M, Sitaraman SV, Liu X, Bado A, Merlin D. Luminal leptin enhances CD147/MCT-1-mediated uptake of butyrate in the human intestinal cell line Caco2-BBE. J Biol Chem. 2002;277(31):28182-90.
146. Alrefai WA, Tyagi S, Gill R, Saksena S, Hadjiagapiou C, Mansour F, et al. Regulation of butyrate uptake in Caco-2 cells by phorbol 12-myristate 13-acetate. Am J Physiol Gastrointest Liver Physiol. 2004;286(2):G197-203.
147. McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature. 1998;393(6683):333-9.
148. Philp NJ, Ochrietor JD, Rudoy C, Muramatsu T, Linser PJ. Loss of MCT1, MCT3, and MCT4 expression in the retinal pigment epithelium and neural retina of the 5A11/basigin-null mouse. Invest Ophthalmol Vis Sci. 2003;44(3):1305-11.

149. Smith JP, Drewes LR. Modulation of monocarboxylic acid transporter-1 kinetic function by the cAMP signaling pathway in rat brain endothelial cells. J Biol Chem. 2006;281(4):2053-60.
150. Massidda M, Eynon N, Bachis V, Corrias L, Culigioni C, Piras F, et al. Influence of the MCT1 rs1049434 on Indirect Muscle Disorders/Injuries in Elite Football Players. Sports Med Open. 2015;1(1):33.
151. Merezhinskaya N, Fishbein WN, Davis JI, Foellmer JW. Mutations in MCT1 cDNA in patients with symptomatic deficiency in lactate transport. Muscle Nerve. 2000;23(1):90-7.
152. Lean CB, Lee EJD. Genetic Variations of the MCT4 (SLC16A3) Gene in the Chinese and Indian Populations of Singapore. Drug Metabolism and Pharmacokinetics. 2012;27(4):456-64.
153. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem. 2006;281(14):9030-7.
154. Castorino JJ, Deborde S, Deora A, Schreiner R, Gallagher-Colombo SM, Rodriguez-Boulan E, et al. Basolateral sorting signals regulating tissue-specific polarity of heteromeric monocarboxylate transporters in epithelia. Traffic. 2011;12(4):483-98.
155. Shugarts S, Benet LZ. The role of transporters in the pharmacokinetics of orally administered drugs. Pharm Res. 2009;26(9):2039-54.
156. Tziomalos K, Athyros VG, Mikhailidis DP. Statin discontinuation: an underestimated risk? Curr Med Res Opin. 2008;24(11):3059-62.
157. Brugts JJ, Yetgin T, Hoeks SE, Gotto AM, Shepherd J, Westendorp RG, et al. The benefits of statins in people without established cardiovascular disease but with cardiovascular risk factors: meta-analysis of randomised controlled trials. BMJ. 2009;338:b2376.
158. Analysis of the global RNA expression profiles of Skeletal Muscle Cells treated with statins. 2005.
159. Kobayashi M, Kaido F, Kagawa T, Itagaki S, Hirano T, Iseki K. Preventive effects of bicarbonate on cerivastatin-induced apoptosis. Int J Pharm. 2007;341(1-2):181-8.
160. Lins RL, Matthys KE, Verpooten GA, Peeters PC, Dratwa M, Stolear JC, et al. Pharmacokinetics of atorvastatin and its metabolites after single and multiple dosing in hypercholesterolaemic haemodialysis patients. Nephrology Dialysis Transplantation. 2003;18(5):967-76.
161. Gallagher SM, Castorino JJ, Wang D, Philp NJ. Monocarboxylate transporter 4 regulates maturation and trafficking of CD147 to the plasma membrane in the metastatic breast cancer cell line MDA-MB-231. Cancer Res. 2007;67(9):4182-9.
162. Manning Fox JE, Meredith D, Halestrap AP. Characterisation of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle. Journal of Physiology. 2000;529(2):285-93.
163. Dimmer KS, Friedrich B, Lang F, Deitmer JW, Broer S. The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochemical Journal. 2000;350:219-27.
164. Lin RY, Vera JC, Chaganti RSK, Golde DW. Human monocarboxylate transporter 2 (MCT2) is a high affinity pyruvate transporter. Journal of Biological Chemistry. 1998;273(44):28959-65.

165. Wilson MC, Jackson VN, Heddle C, Price NT, Pilegaard H, Juel C, et al. Lactic acid efflux from white skeletal muscle is catalyzed by the monocarboxylate transporter isoform MCT3. J Biol Chem. 1998;273(26):15920-6.
166. Carpenter L, Halestrap AP. The kinetics, substrate and inhibitor specificity of the lactate transporter of Ehrlich-Lettre tumour cells studied with the intracellular pH indicator BCECF. Biochem J. 1994;304 (Pt 3):751-60.
167. Bruckert E, Hayem G, Dejager S, Yau C, Begaud B. Mild to moderate muscular symptoms with high-dosage statin therapy in hyperlipidemic patients--the PRIMO study. Cardiovasc Drugs Ther. 2005;19(6):403-14.
168. Merck & Co. I. Zocor (simvastatin) tablets prescribing information. West Point, PA2015.
169. McKenney JM. Pharmacologic characteristics of statins. Clin Cardiol. 2003;26(4 Suppl 3):III32-8.