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The Pharmacogenetics of OATP1B1 Polymorphisms and Drug-Drug Interactions in Cerivastatin Associated Rhabdomyolysis

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The Pharmacogenetics of OATP1B1 Polymorphisms and Drug-Drug Interactions in Cerivastatin Associated Rhabdomyolysis

by

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DISSERTATION

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Inspiration and will power represent only one part of the equation that inspires one to set out on the path of graduate work. The balance of the equation lies in the hands of the mentor. I believe an exceptional teacher is one that inspires students to create their own image and does not in any way force his way on students, rather he guides them to discover the threshold of their own minds. Dr. Pui-Yan Kwok is such a teacher. Pui's methods of inspiration and guidance include not only his exceptional command of genetic science and technology but also his humble, polite and kind demeanor. He has ALWAYS been available to listen and he has demonstrated a genuine interest in helping. His support, thoughtfulness and guidance at times when I was at the mercy of intractable steps in a project were more than words can describe. He is a master at creating an atmosphere of joy and an environment conducive to working and learning. It was clear after spending a month in his lab as a rotation student that I was in good company. My decision to join Pui's group is one that I am particularly proud of. I only hope that in future I can return a fraction of the kindness and support that I have received from Pui.

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ABSTRACT

The Pharmacogenetics of OATP1B1 Polymorphisms and Drug-Drug Interactions in Cerivastatin Associated Rhabdomyolysis

Bani Tamraz

In this dissertation, the two main mechanisms of adverse event occurrence namely drug-gene and drug-drug interaction were studied to identify the cause of CER induced rhabdomyolysis. Genetic variation in drug metabolizing enzymes and membrane transporters as well as other drugs can modulate the beneficial, as well as the deleterious, effects of drugs. In a study of 126 patients who developed rhabdomyolysis while taking the HMG-CoA reductase inhibitor, cerivastatin, we sought to identify genetic variants and drug-drug interactions that might explain the high incidence of rhabdomyolysis. We re-sequenced three transporter genes, *ABCC2* (coding for MRP2), *ABCG2* (coding for BCRP) and *SLCO1B1* (coding for OATP1B1), three metabolizing enzyme genes *CYP2C8*, *UGT1A1* and *UGT1A3* and *HMGCR* involved in transport, metabolism and target of cerivastatin, respectively. A total of 203 SNPs were identified in these samples and of these 52 were in the coding region.

In a previously published case-control analysis of polymorphisms identified in our *CYP2C8*, *SLCO1B1*, *UGT1A1* and *UGT1A3* genes, the V174A SNP was found to be significantly associated with CER induced rhabdomyolysis with an odds ratio of 1.89 (95% CI,1.40-2.56). We were able to only complete in *in vitro* functional analysis of variants in *SLCO1B1* gene on the uptake of cerivastatin in HEK293/frt cells stabely expressing *SLCO1B1* reference, polymorphisms and haplotypes. The V174A SNP, along with R57Q, P155T, FS and OATP1B1*15 and N1 haplotypes were shown in *in vitro* assays to be associated with significant reduction (P>0.001) in CER uptake (32%,

17.9%, 72%, 3.4%, 2.1% and 5.7% of reference, respectively) compared to reference. Furthermore, clopidogrel and rofecoxib, previously identified in our cases to be associated with cerivastatin induced rhabdomyolysis at odds ratio of 29.6 (95% CI, 6.1-143) and 4.9 (95% CI, 1.1-20.8), were shown *in vitro* to have a significant OATP1B1 mediated interaction, inhibiting cerivastatin uptake with IC50 values of 0.32 µM (95% CI, 0.06-1.71) and 0.73 µM (95% CI, 0.3-1.8), respectively. The calculated R value for clopidogrel and rofecoxib were greater than 2, supporting a further clinical evaluation of this drug interaction.

PHARMACOGENETICS – A BRIEF HISTORY

The science of studying the influence of heredity on individual variation in disposition and response to drugs is known as pharmacogenetics. The identification of the cause of the observed variation in response to xenobiotics and other exogenous substances has been the main challenge of this field to date. Yet first documented observations of interindividual variability dates back to 510 BC where Pythagoras observed variation in occurrence of hemolytic anemia in response to ingestion of fava beans.[1] Pythagoras's observation was one which molecular genetics was able to partially explain in a 1971 publication linking alleles of the acid phosphatase gene (ACP1) in male subjects deficient in glucose-6-phosphate dehydrogenase (G6PD) to having hemolytic clinical favism.[2]

Pharmacogenetics on the map of scientific fields is located at the crossroads between pharmacology, genetics and biochemistry. The building of the crossroads began with the growth of organic chemistry in the later part of the 19th century in a period closely related to the discovery of the laws of heredity in 1865 by Gregor Mendel and their rediscovery again at the turn of the 20th century. A pioneering individual that created the nexus between Mendel's gene and their affect on biochemical processes was Archibald Garrod[3]. Garrod from his work on studying alkaptonuria, a rare inherited disease where tyrosine byproduct called homogentisic acid (or alkapton) accumulates in the blood and is excreted in large quantities in the urine, concluded that

"alkaptonuria is not the manifestation of a disease but is rather of the nature of an alternative course of metabolism, harmless and usually congenital and lifelong. Witness is borne to its harmlessness by those who have manifested the peculiarity without any apparent detriment to health from infancy on into adult and even advanced life."[3]

and that alkaptonuria was more common in families with this history which he could explain through Mendel's genetics.[3]

While Garrod was still active there were others that were trying to explain similar observation as Garrod but this time in peculiarities associated with taste. Arthur Fox a scientist in Jackson laboratory at du Pont was searching for a new sugar substitute when he noticed that some people in the lab, including himself, could not detect the bitter taste "non-tasters" in crystals of phenyl thio carbamide.[4] Blakeslee[5] showed that the variation in taste perception associated with Fox's crystal among "tasters" and "non-tasters" followed a Mendelian pattern of inheritance. These early works on variation in response also elucidate an important principle in pharmacogenetics and that is phenotypes are observed upon exposure to a particular drug or chemical.

It was not until the advent of new technologies in the 1950s which enabled separation of closely related proteins as well as identification of unique pattern of metabolites of drugs that enabled scientists to connect the genetic influence on drug disposition. Interindividual variation in response to suxamethonium (succinylcholine)[6-8] and primaquine[9-11] and isoniazid[12,13] were among the first set of drugs that were studied from the perspective of pharmacogenetics and provided proof that the observed phenotypes were inherited.

Succinylcholine, a muscle relaxant used as an adjuvant to general anesthesia, is destroyed normally by serum cholinesterases to its inactive metabolites. However, malignant hyperthermia[6] observed in subset of patients and their respective families that underwent general anesthesia with succinylcholine was first explained by Werner Kalow[7] to be due to presence of at least two types of human serum cholinesterase. Kalow refers to these variations in enzyme as “inborn error of metabolism” a term that one comes across in the literature of that period. Prior to Kalow’s explanation of polymorphisms in the choniesterase enzyme, Lehmann et al[8] reported familial incidence of low pseudocholinesterase levels and concluded his paper by saying that it is important for people to know if they have a low pseudocholinesterase level. Furthermore, he goes on to say that people identified with low levels of this enzymes should be given a letter to be handed to the anesthesiologists should they ever require an operation.

Primaquine, an antimalarial drug, first licensed by FDA in 1944 was associated with high occurrence of hemolytic anemia in African American males. Although the cause of this was not initially known, Alf Alving and his colleagues were the first to show that the destructive effect of primaquine on red blood cells was due to a deficiency in glucose-6-phosphate dehydrogenase (G6PD).[14]

Isoniazid was first synthesized in 1912[12] but its therapeutic value was not discovered until 1952[15] . Subsequent to the wide use of Isoniazid, symptoms of peripheral neuropathy such as numbness, pain and tingling began to manifest in arms and legs of some patients on therapeutic dose of Isoniazid. The first large

study conducted was published in 1960 that showed a bimodal distribution of plasma Isoniazid concentration in 484 individuals in 53 Caucasian families concluding that the human metabolism of Isoniazid is controlled by two different alleles of a gene; “slow inactivator” and “rapid inactivator”.[12] Four years later it was identified by Evans and White[13] that the polymorphism observed with Isoniazid was due to differences in hepatic N-acetyltransferase (NAT) enzyme.

What marked the true beginning of the field of pharmacogenetics was a seminal paper by Motulsky[16] in 1957 where he clearly states that the idiosyncrasies observation in response to drugs, including failed efficacy at normal therapeutic doses, are due to genetic differences that give rise to enzymatic deficiencies that ultimately cause the observed variations in drug response. Following Motulsky, in 1959 Vogel[17] proposed officially the term pharmacogenetics to be applied to “the study of the role of genetics in drug response.”[18]

In the ensuing decades our knowledge in the field of pharmacogenetic has increased dramatically and we are beginning to see the integration of this field with the practice of medicine. Table 1 summarizes the drug classes that contained pharmacogenetic data as of 2004.[19] As of 2004, 22 drugs have references within their package insert for genetic testing to guide therapeutic decision.[19] Although there are various factors that are limiting the translation of pharmacogenetic testing to clinics[20] one thing remains certain that we are resolute in the goal of translating pharmacogenetics into clinical practice. I

believe the work that I am about to present in my dissertation will contribute to such foundation.

Table 1: Drug classes containing pharmacogenetic data in their package insert as of 2004.[19]

Drug Class	Breakdown of 76 identified package inserts with PG-containing information (%)
Anti-infective	30.7
Psychotropic	20
Gastrointestinal	10.7
Cardiovascular	9.3
Neurologic	9.3
Analgesis	4
Antineoplastic	4
Dermatological	2.7
Hormone/hormone modifier	2.7
Genitourinary	2.7
Hematological	1.3
Antihistamine	1.3
Respiratory	1.3

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TABLE OF CONTENTS

Chapter 2

Identification of genetic polymorphisms in 126 cases with cerivastatin associated Rhabdomyolysis

Chapter 3

OATP1B1 and Cerivastatin Uptake

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

LIST OF FIGURES

Chapter 4

CHAPTER 1

An introduction to HMG-CoA reductase inhibitors

1.1. Introduction

Modern medicine is powered by discovery of new medications that stem from advancement of knowledge in various areas of science. As valuable as these drugs are, the process of drug development has become an evolutionary struggle between the manufacturers' desire to maximize profit and the public need to ensure safety and efficacy. The regulatory process designed to balance these competing needs, though rational, has certain limitations.[1] At the time of regulatory approval of a new medication, a number of issues remain unknown, including the occurrence of rare but serious adverse drug reactions (ADR). Between 1975 and 1999, 548 new chemical entities were approved by the FDA.[2] Of these, 56 (10.2%) acquired new black-box warnings or were withdrawn subsequently.[2] Although the premarketing data analysis and the post marketing surveillance conducted by FDA is found to be adequate[3], premarketing drug trials are not powered to detect rare ADRs.[4] While a number of drug withdrawals are due to drug-drug interactions[3], for some medications withdrawn from the market the exact mechanism of the ADR is not fully understood[1]. In the advent of genomic era, attention is turned to the possibility that the presence of genetic variants in certain individuals may predispose them to higher risk of experiencing ADRs.

Over time and across populations, a large number of variants have

appeared in genes that are now known to code for drug receptors, drug metabolizing enzymes and drug response pathways. Under a variety of selection pressures, some genetic variant alleles involved in the mentioned systems became common long before the appearance of modern pharmacotherapies. As many people in the population are exposed to drugs on a daily basis (for example, older adults recruited to the Cardiovascular Health Study (CHS) in 1990 took an average of 2.28 prescription medications[5]), there is ample opportunity for common or powerful drug-gene interactions to occur. In 1994, 2.2 million hospitalized persons experienced serious ADRs (SADRs), and 106,000 had fatal SADRs.[6] When a plane or a ferry crashes, the National Transportation and Safety Board investigate the incident fully. When a new drug causes SADRs, the FDA encourages the manufacturer to withdraw the drug without conducting an official investigation. In the absence of additional information, preventive efforts are difficult to mount. The purpose of the project, a kind of drug-disaster autopsy, is to identify several causes of these extreme responses, help prevent future accidents, and perhaps improve the safety profile of medications already in common use.

1.2. The Statins

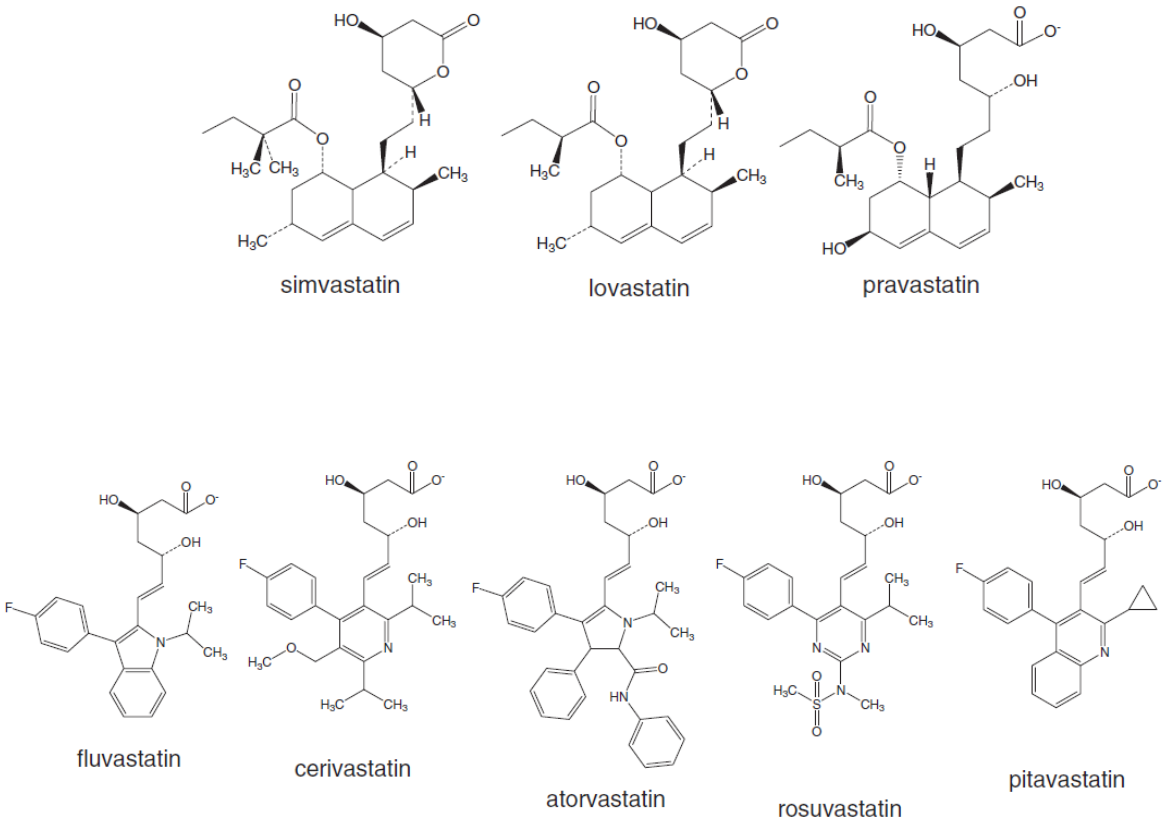
The Hydroxymethylglutaryl Coenzyme-A Reductase (HMG-CoA) Inhibitors (statins) are the most powerful and commonly used cholesterol lowering agents. Today there are seven (Figure 1.1, except for cerivastatin) statins available in the United States. The first to be approved was lovastatin (Mevacor®) (08/1987), followed by pravastatin (Pravachol®) (10/1991), simvastatin (Zocor®) (12/1991),

fluvastatin (Lescol®) (12/1993), atorvastatin (Lipitor®) (12/1996), rosuvastatin (Crestor®) (8/2003) and pitavastatin (Livalo®) (08/2009). Lovastatin, pravastatin, and simvastatin are manufactured by "natural" fermentation, while synthetic means are used to produce atorvastatin, fluvastatin, rosuvastatin and pitavastatin. According to Adult Treatment Panel (ATP) III recommendations[7], statins are the first-line cholesterol lowering drug therapy after failure of the following therapeutic lifestyle changes dietary changes, weight reduction and increased physical activity. Data derived to date from 44[8] large and well controlled randomized clinical trials (4S trial[9]; WOSCOPS trial[10]; CARE trial[11]; LIPID trial[12]; AFCAPS/TexCAPS[13];CARDS trial[14]) have established the benefit of statins in primary and secondary prevention of cardiovascular events.

1.2.1. Molecular Structure of Statins

Structurally all statins include the 7-carbon side chain, lactone or open β-hydroxy acid form (heptanoic or dihydroxy heptanoic acid, respectively), in their molecule that mimics the natural substrate of HMG-CoA reductase that is necessary for inhibitory activity (Figure 1.1). The differences lie in the nucleus and other residual parts of their chemical structures. The active form of statins is the acid form. Lovastatin and simvastatin are the only prodrugs that must be hydrolyzed in the liver to active forms, while all other statins are administered as active acid form.

Figure 1.1: Chemical structures of all 8 statins[15]



1.2.2. Clinical Pharmacology of Statins

The statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase, an enzyme that facilitates conversion of HMG-CoA to mevalonate, a rate-determining and early step in sterol production (including cholesterol). This inhibition reduces the concentrations of cholesterol in hepatocytes, thus stimulating the synthesis of low-density lipoprotein (LDL) cholesterol receptor and LDL particle uptake from the bloodstream into the hepatocyte. This reduces plasma total cholesterol concentrations, LDL cholesterol (LDL-C), apolipoprotein

B, and triglycerides, and increases high-density lipoprotein (HDL) cholesterol (HDL-C).[16] The enzyme inhibitory activity of statins may vary among statins as evidenced by differences in dose-response relationships. All available statins reduce LDL levels in a nonlinear dose-dependent manner and are effective when given as a once-daily dose.

Although statins exert their benefits on coronary heart disease mainly by improving the serum lipid profile, recent studies indicate that there are other potential mechanisms through which the statins exert their cardioprotective effects. The statins may improve endothelial function[17], stabilize plaques by preventing thrombosis[18] and reduce vascular inflammation[19]. However, these non-lipid mechanisms of action of statins are not yet well established.

1.2.3. Statin Pharmacokinetics

It is well known that drug disposition (absorption, metabolic transformation and subsequent elimination) is greatly influenced by drug metabolizing enzymes as well as by membrane transporters. The metabolic biotransformations in drug disposition are classified generally as either a Phase I or Phase II reactions. Phase I reactions, through oxidation, increase the polarity of a compound. This is typically accomplished by cytochrome P450 (CYP) enzymes. Phase II reactions come in play if phase I metabolites are not sufficiently polar. UGT's (UDP-Glucuronosyltransferases) are the major phase II drug metabolizing enzymes. These enzymes add functional groups such as glucuronic acid and convert xenobiotics to a highly polar conjugate that facilitate their transport to excretory organs and subsequent elimination via bile or urine. The liver is the

principal site of such metabolic biotransformation reactions. Table 1.1 tabulates some of the pharmacokinetic properties of statins.

Table 1.1: Pharmacokinetics of statins

Statins	Absolute Bioavailability (%)	T(max) after PO Dose (hrs)	Systemic Active Metabolites	Metabolism	OATP Transporters	Protein Binding (%)	Elimination Half-life (hr)	Renal Excretion (%)	Fecal Excretion (%)
Atorvastatin	14	1-2	Yes	CYP3A4	OATP1B1	>98	14-Jul	<2	>98
Fluvastatin	20-30	IR:<1; XL:3	No	CYP2C9,CYP2C8,CYP3A4,CYP2D6	OATP1B1	98	Cap<3, XL Cap 9	5	95
Lovastatin	5	IR:2-2.4; XR:11.1-24	Yes	CYP3A4	---	>95	1.1-1.7	10	83
Pravastatin	17	1-1.5	Yes	----	OATP1B1	43-55	2.6-3.2	20	71
Simvastatin	5	1.3-2.4	Yes	CYP3A4	OATP1B1	95	3.45	13	60
Rosuvastatin	20	3-5	Yes (1/6 to 1/2 as active)	*minimally metabolized (10%)* CYP2C9, CYP3A4	OATP1B1	88	19	10	90
Pitavastatin	51	1	Yes	CYP2C9, CYP2C8, *Primary* UGT1A3, UGT2B7	OATP1B1, OATP1B3	>99	11-12	15	79
Cerivastatin	60	2-3	yes	CYP3A4, CYP2C8, UGT1A1, UGT1A3	OATP1B1	>99	1.5-3	26-30	70

Sources: Cerivastatin[20], Pitavastatin[21,22], Rosuvastatin[23,24], Simvastatin[25,26], Pravastatin[27-29],Lovastatin [30,31],Fluvastatin[32-35], Atorvastatin[36,37]

All statins except rosuvastatin and pravastatin undergo extensive first-pass metabolism.

1.2.4. Pharmacology of Cerivastatin

Cerivastatin (Baycol®, MW=481.5 g/mol), a product of Bayer Pharmaceuticals, was the fifth statin that received regulatory approval in the United States. It is entirely synthetic and an enantiomerically pure inhibitor of HMG-CoA reductase.[38] Of all the statins, cerivastatin has the strongest affinity for HMG-CoA reductase, with a K_i of 1.3 nmol/L – approximately 100-fold more

potent than lovastatin[38] – and therefore the approved dosages were 0.1 to 0.8 mg. The absolute bioavailability of cerivastatin is 60% (90% CI 53-68%).[39] This number was determined based on a single dose, randomized crossover study comparing 0.1 mg intravenous bolus dose of cerivastatin with oral administration of two 0.1 mg tablets and a 0.2 mg solution to 12 healthy adult males.[39] The cerivastatin tablet and solution showed identical drug plasma concentration-time curves with the mean relative bioavailability of solution compared to the tablet of 100.7% (90% CI 89-114%).[39] The mean absorption half-life was estimated to be 1-2 hours. Following oral administration cerivastatin was rapidly and completely absorbed from the gastrointestinal tract (>98%).[40]

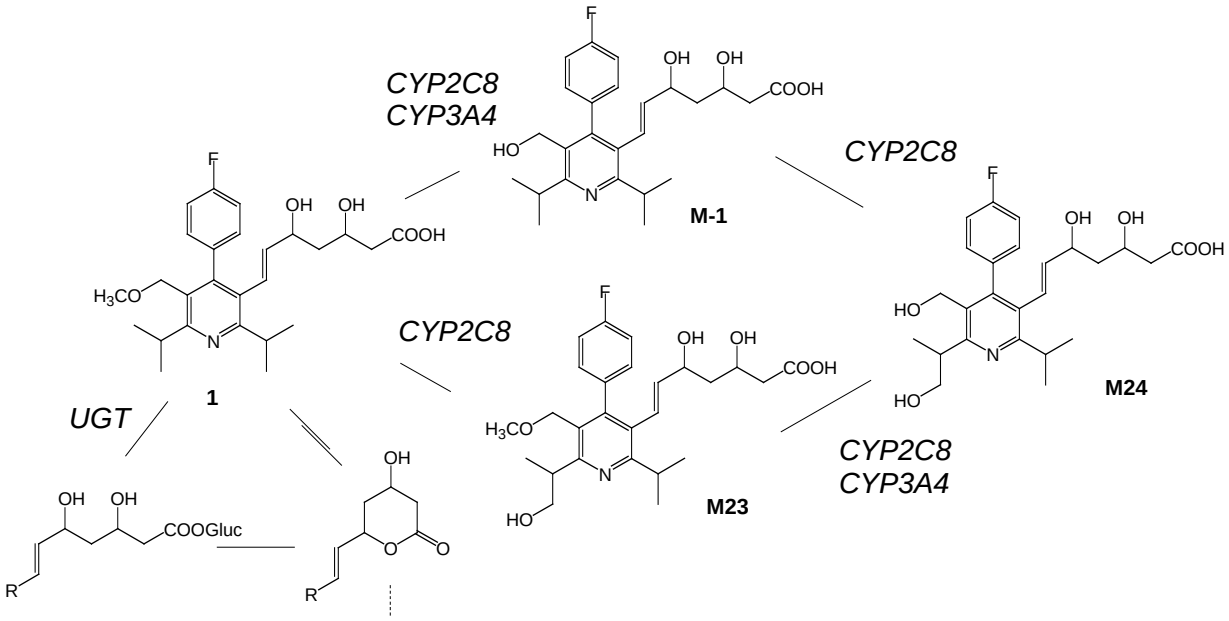
Based on these numbers cerivastatin was and is considered a highly soluble and highly permeable drug. Of course these findings were before the publication of BDDCS system of drug disposition classification by Wu and Benet in 2005.[41] Although cerivastatin does not appear in this seminal paper, cerivastatin is considered a class I drug (confirmed with Dr. Leslie Z. Benet) and as such it possess all the characteristics of this class of drugs. This means that efflux and uptake transporters are not important clinically in the intestine, even though *in vitro* cellular assay may show cerivastatin to be a substrate for such transporters. Furthermore, the primary route of elimination of class I drugs is through metabolism and cerivastatin holds true to that characteristic. Approximately 70% of the administered dose of cerivastatin is excreted as metabolite in the feces and the rest of the metabolites are excreted in the urine.[39]

No unchanged drug is found in urine and 2% of the dose is found as intact cerivastatin in the feces.[39] Moreover, as expected for class I drugs, high-fat meal have no effect on the extent of absorption (F_{extent}) for cerivastatin.[40,41]

1.2.5. Metabolism of Cerivastatin

Upon oral ingestion and gastrointestinal absorption, cerivastatin is transported to the liver for metabolism. At the liver, cerivastatin enters the hepatocyte by a combination of passive diffusion and uptake by transporter(s) (often named phase 0) such as Organic Anion Transporting Polypeptide 1B1 (OATP1B1).[42] Once absorbed into the hepatocyte, cerivastatin is metabolized mainly through 2 equally important pathways: demethylation of the benzylic methyl ether moiety catalyzed by CYP2C8 and CYP3A4 leading to the metabolite M-1 and stereoselective hydroxylation of one methyl group of the 6-isopropyl substituent leading to metabolite M-23 catalyzed only by CYP2C8 (Figure 1.2). The product of the combined biotransformation reactions is a secondary minor metabolite, M-24, which is not detectable in human plasma.[20,43] All 3 metabolites are active inhibitors of HMG-CoA reductase with a M-23 and M-24 having similar potency to the parent drug and M-1 exhibiting only 30-50% of parent compound activity.[44,45]

Figure 1.2: Cerivastatin metabolism[20,46]



However, the affinity of cerivastatin for CYP2C8 is much higher than for CYP3A4 [20], therefore inhibiting CYP3A4 has negligible effect on the area under the curve of cerivastatin.[20]

Prueksaritanont and colleagues also identified a novel mechanism of statin metabolism that further enhanced our understanding of cerivastatin metabolism.[46,47] This group provided the first evidence for UGT-mediated glucuronidation of cerivastatin producing an unstable compound that undergoes spontaneous cyclization to form an inactive lactone. The recombinant isoforms, UGT1A1 and UGT1A3, were shown to be responsible for the glucuronidation and subsequent lactonization of the tested statins.[46] Their explanation provided the

first mechanism describing the formation of cerivastatin lactone that has been detected *in vivo.*[44]

1.2.6. Cerivastatin Associated Rhabdomyolysis

Generally, statins are very well tolerated by the majority of patients and have acceptable safety profiles. Adverse events associated with statins are rare and include asymptomatic elevation of hepatic transaminases, extremely rare cases of hepatitis and skeletal muscle related complaints or myopathies. Myopathies, in very rare cases, progress to rhabdomyolysis, the most dangerous side effect of these drugs. Rhabdomyolysis, where the breakdown of skeletal muscle cells leads to muscle pain and weakness and in some rare cases to death secondary to hyperkalemia, cardiac arrhythmia, renal failure and disseminated intravascular coagulation, are a major side effect for statin therapy. Cerivastatin was first approved and marketed by Bayer Pharmaceuticals in the United States in 1998 [48] for treatment of dyslipidemia. Soon after its introduction, suspected adverse drug reaction reports cited rhabdomyolysis in cerivastatin patients. Rhabdomyolysis generally occurred within a few weeks after starting cerivastatin. By the time cerivastatin was removed from the market in August 2001, the Adverse Event Reporting System (AERS) – a post-marketing data base maintained by the US Food and Drug Administration (FDA) - had recorded 1,899 SADRs for rhabdomyolysis associated with cerivastatin compared to 1,440 for all other statins combined.[49]

In an analysis of 12 years of FDA post-marketing data of domestic cases meeting a strict definition of rhabdomyolysis (creatine kinase (CK) ≥10,000 IU/L,

signs and symptoms of myopathy and a clinical diagnosis of rhabdomyolysis), the relative reporting rate (RRR) for fatal rhabdomyolysis was 18 to 95 times higher among cerivastatin users than among users of other statins (Table 1.2).[50] Furthermore, the RRR of fatal rhabdomyolysis was 40 times higher among users of cerivastatin than all other statins combined (Table 1.2).[50] For all reported cases of rhabdomyolysis, fatal and non-fatal, the RRR for cerivastatin was 24 to 1634 times higher than those found in users of the other statins, and it was 54 times higher than for all the other statins combined (Table 1.2).[50] The reporting rate of rhabdomyolysis for all statins, excepting cerivastatin, was less than 1 case in 100,000 prescriptions (Rx's) while it was 4.29 cases per 100,000 prescriptions for cerivastatin.[50]

Table 1.2: All domestic reported cases of statin associated rhabdomyolysis (data source[50])

HMG-CoA Reductase Inhibitors	# of Rx	Reporting Years	All Cases	Reporting Rate*	RRR	Fatal Cases	Reporting Rate*	RRR
Cerivastatin	11172000	1998-2001	479	4.29	----	38	0.340	----
Atorvastatin	149706000	1997-2001	51	0.03	125.86	6	0.004	84.87
Fluvastatin	38119000	2000	1	0.003	1634.35	0	0.000	----
Lovastatin	99485000	1987-2001	180	0.18	23.70	19	0.019	17.81
Pravastatin	83673000	1993-2001	19	0.02	188.81	3	0.004	94.87
Simvastatin	120188000	1993-2001	136	0.11	37.89	14	0.012	29.20
All statins except Cerivastatin	491171000	-----	387	0.08	**54.42**	42	0.009	**39.78**

Reporting Rate* = rate per 100,000 Rx's
RRR= Relative reporting rate to cerivastatin

In the aforementioned FDA analysis, the RRR for rhabdomyolysis was 31 times higher among cerivastatin monotherapy users than among all other statin monotherapy users combined, and was 726 times higher among cerivastatin-gemfibrozil combination therapy users than among users of other statin-

gemfibrozil combinations combined (Table 1.3).[50]

Table 1.3: Reported cases of rhabdomyolysis associated with statin monotherapy and combination with gemfibrozil (data source[50])

	Monotherapy		Monotherapy			with Gemfibrozil			
HMG-CoA Reductase Inhibitors	# of Rx	Reporting Years	Cases	Reporting Rate*	RRR	# of Rx	Combination Cases	Reporting Rate*	RRR
Cerivastatin	11038000	1998-2001	200	1.81	----	22000	279	1268.182	----
Atorvastatin	147610000	1997-2001	45	0.03	59.44	1198000	6	0.501	2532.14
Fluvastatin	37791000	2000	1	0.003	684.74	316000	0	0.000	----
Lovastatin	97336000	1987-2001	120	0.12	14.70	2109000	60	2.845	445.77
Pravastatin	82000000	1993-2001	17	0.02	87.40	1422000	2	0.141	9016.77
Simvastatin	118986000	1993-2001	99	0.08	21.78	962000	37	3.846	329.73
All statins except Cerivastati	483723000	-----	282	0.06	**31.08**	6007000	105	1.748	**725.52**

Reporting Rate* = rate per 100,000 Rx's
RRR= Relative reporting rate to cerivastatin

The relatively high RRR with cerivastatin complements the relative risk of rhabdomyolysis reported yet in another cohort study. In this cohort study[51] of 252,460 patients on lipid lowering agent identified via claims established from 11 managed care health plans across the United States, 24 cases of hospitalized rhabdomyolysis occurred during treatment. Average incidence per 10,000 person-years for monotherapy with atorvastatin, pravastatin, or simvastatin was 0.44 (95% confidence interval [CI], 0.20-0.84); for cerivastatin, 5.34 (95% CI, 1.46-13.68); and for fibrate, 2.82 (95% CI, 0.58-8.24).[51] By comparison, the incidence during unexposed person-time was 0 (95% CI, 0-0.48; P = 0.06). The incidence increased to 5.98 (95% CI, 0.72-216.0) for combined therapy of

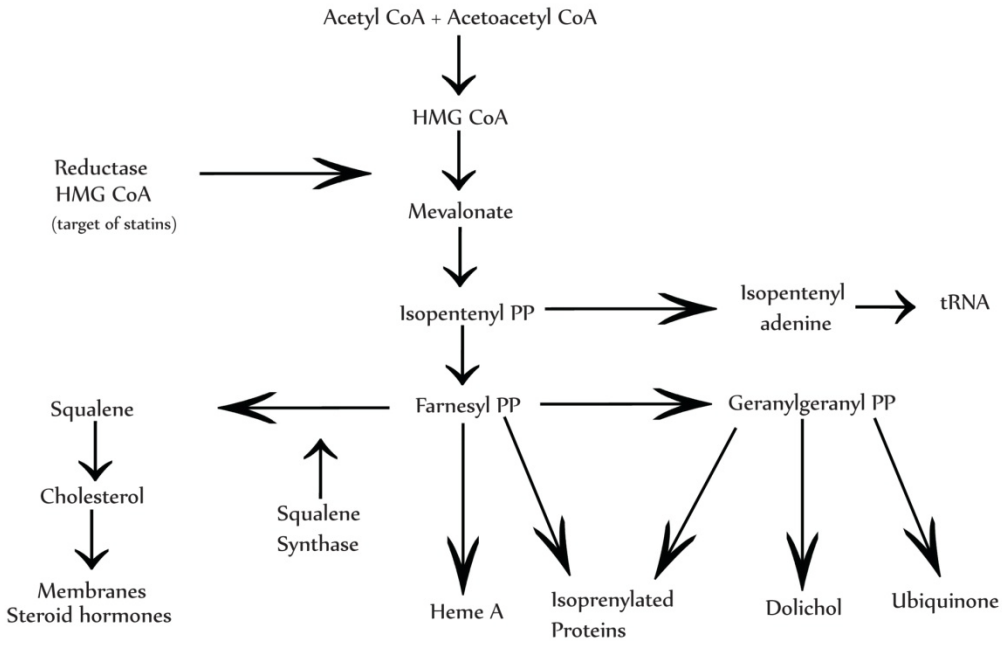
atorvastatin, pravastatin, or simvastatin with a fibrate (gemfibrozil or fenofibrate), and to 1,035 (95% CI, 389-2,117) for combined cerivastatin-fibrate use. [51]

It is important to note that detection of such rare adverse event is a limitation of premarketing phase of drug development and not an oversight on the part of the pharmaceutical companies and regulatory agencies. Such rare occurrences remain undetected due to small premarketing trial sizes, the relatively short duration of the study and the control nature of the trials. As an example to detect an event that happens in 1 out of 1,000 patients, 3,000 patients need to be receiving the drug in order to identify the side effect within a 95% confidence interval.[52]

1.2.7. Mechanism of Rhabdomyolysis

The mechanism of statin induced muscle injury is not known but there are several theories. The most promising theory is based on data showing that depletion of geranylgeranyl pyrophosphate (PP), an intermediary product in the synthesis of ubiquinone and isoprenylated proteins in the mevalonate pathway (Figure 1.3), not inhibition of cholesterol synthesis, is the primary cause of statin induced myotoxicity.[53] Important in protein prenylation, a post-translational modification step, are small GTPase binding regulatory proteins such as Ras, Rac and Rho that promote cell growth and inhibit apoptosis.[49,54] A decline in the amount of these proteins can leads to inappropriate activation of apoptosis that can have pathological consequences such as rhabdomyolysis.[49]

Figure 1.3: The mevalonate pathway[53]



As mentioned, the mechanism of rhabdomyolysis is not yet elucidated but what is known is that statins on their own are capable of causing this adverse event and the relative risk of occurrence of rhabdomyolysis increases even more when statins are administered concomitantly with drugs that inhibit their catabolism thereby increasing statin plasma concentration.[50,55,56] Gemfibrozil is such a drug. From data presented in Table 1.3 the RRR for rhabdomyolysis was 31 and 726 times higher among cerivastatin monotherapy and cerivastatin-gemfibrozil combination therapy, respectively. In a randomized double-blind pharmacokinetic crossover study, 10 subjects took 600 mg gemfibrozil or placebo twice daily for 3 days and on the third day they took a single dose of 0.3 mg cerivastatin. The cerivastatin AUC was increased in gemfibrozil recipients by an average of 559% (range 138% to 995%).[57]

1.3. Statement of Purpose

In the first chapter I introduced background information upon which I developed my dissertation project to elucidate the pharmacogenetics of cerivastatin associated rhabdomyolysis. Although the mechanism of rhabdomyolysis is not known, its risk of occurrence is exacerbated by disease states such as compromised renal and hepatic function or hypothyroidism, or by concomitant use of certain medications. We hypothesized that patients exhibiting rhabdomyolysis on cerivastatin had one or both of the following two risk factors: (1) they were taking medications that are known inhibitors of the cerivastatin relevant enzymes or membrane transporters; or (2) they possessed genetic variants in one or more of transporter or metabolizing enzymes such that the presence of these polymorphism was associated with increased plasma cerivastatin level.

Three specific aims were designed to test the proposed hypothesis and they are the topics of the next three chapters.

The first is a **pharmacogenetic aim** in which we sequenced 126 patients who developed rhabdomyolysis while taking cerivastatin to identify genetic polymorphisms, rare or common, in 6 genes involved in cerivastatin biotransformation and the HMG-CoA reductase gene, the target of all statins.

The second is a **pharmacologic aim** designed to evaluate the functional significance of identified genetic variants in *in vitro* assays.

The third is a **pharmacoepidemiologic aim** to identify the influence of other drugs in our patient population on the pharmacokinetics of cerivastatin in an *in vitro* cell based assay.

1.4. Summary of Subsequent Chapters

1.4.1. Chapter 2

This chapter encompasses all the work surrounding the pharmacogenetic aim of this project. We take a candidate gene approach targeting genes of enzymes and transporters known to be involved in cerivastatin disposition. We hope to identify genetic polymorphisms that help explain the occurrence of rhabdomyolysis in our cerivastatin users. This chapter will discusses the basis of selection of 7 genes, *UGT1A1, UGT1A3, CYP2C8, SLCO1B1, ABCC2, HMGCR* and *ABCG2*, their sequencing method and results.

1.4.2. Chapter 3

In this chapter, I describe the OATP1B1 *in vitro* work of studying the drug-gene interaction between cerivastatin and *SLCO1B1*. We created stable cell lines expressing the non-synonymous SNPs and their common (frequency >1%) haplotypes identified through sequencing rhabdomyolysis cases. I conducted *in vitro* uptake assays with cerivastatin and estrone-3-sulfate, our positive control substrate, in HEK293 cells transfected with variants of the *OATP1B1* gene to determine the functional effect of each variant on the OATP1B1 transporter. In this chapter I also discuss the CYP2C8 *in vitro* findings that were conducted and published by our collaborators in Seattle.[58]

1.4.3. Chapter 4

As mentioned in the brief history of pharmacogenetics section of my dissertation, thinking of drug related adverse events in the context of drug-gene interaction is relatively novel. A more conventional way of assessing an adverse drug reaction is to look for other drugs being taken by the patient at the same time. In this chapter, I present the medications that were taken by the patients in addition to cerivastatin at the time of rhabdomyolysis occurrence. We designed *in vitro* drug-drug interaction assays to assess the influence of each identified drug on cerivastatin and estrone-3-sulfate, our positive control drug, on their uptake by OATP1B1. The clinical significance of our *in vitro* findings is discussed. I also briefly discuss the influence of the same drugs on *CYP2C8* metabolism of cerivastatin, the *in vitro* work that was completed by our collaborator, Rüdiger Kaspera, in Seattle.

1.5. Final Thoughts

I will conclude by giving my assessment of the future of pharmacogenetics and the challenges that hinder the translation of pharmacogenetic, pharmacologic, and pharmcoepidemiologic data, such as that presented in this dissertation, to clinics.

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CHAPTER 2

Identification of genetic polymorphisms in 126 cases with cerivastatin associated rhabdomyolysis

2.1. Introduction

As described in Chapter 1, the relative reporting rate (RRR) of cerivastatin (CER) associated rhabdomyolysis was 31 times higher compared to other listed statin monotherapies combined and RRR increased to 726 times higher for CER relative to other statins when gemfibrozil was coadministered.[1] In pharmacokinetic studies we have evidence that gemfibrozil not only inhibits phase I and phase II metabolizing enzymes, CYP2C8, UGT1A1 and UGT1A3 but it also inhibits OATP1B1 transporter mediated uptake of CER into hepatocytes (Figure 2.1).[2-4] Indeed, there is clinical evidence in support of the association of elevated CER and its metabolite with rhabdomyolysis.

In a case study of a 74 year old woman that was started on 0.15 mg of CER daily, after 22 days she developed rhabdomyolysis. An analysis of CER and its metabolites in her serum showed that at 6, 22.4 and 48 hours after the last dose, CER levels were 8,062 ng/L, 4,931 ng/L and 1,993 ng/L respectively.[5] In comparison, the CER levels for doses of 0.1 mg and 0.2 mg in healthy male individuals ages 18-42 are 1,010 ng/L and 2,150 ng/L.[6] Table 2.1 summarizes the data on serum concentration of CER and its two metabolites in this patient and that of a normal subject taking 0.3 mg doses of CER for 7 days.

Table 2.1: Serum concentration of CER and its metabolites.[5] For The patient values are 6 hours after the last 0.15 mg CER dose. The values for control are based on maximum drug levels achieved on a 0.3 mg/day dose for 7 days.

	Cerivastatin (µg/L)	M1 (µg/L)	M23 (µg/L)	M23/M1
Patient	8.06	5.22	3.35	0.64
Control	3.36	0.26	0.57	2.19

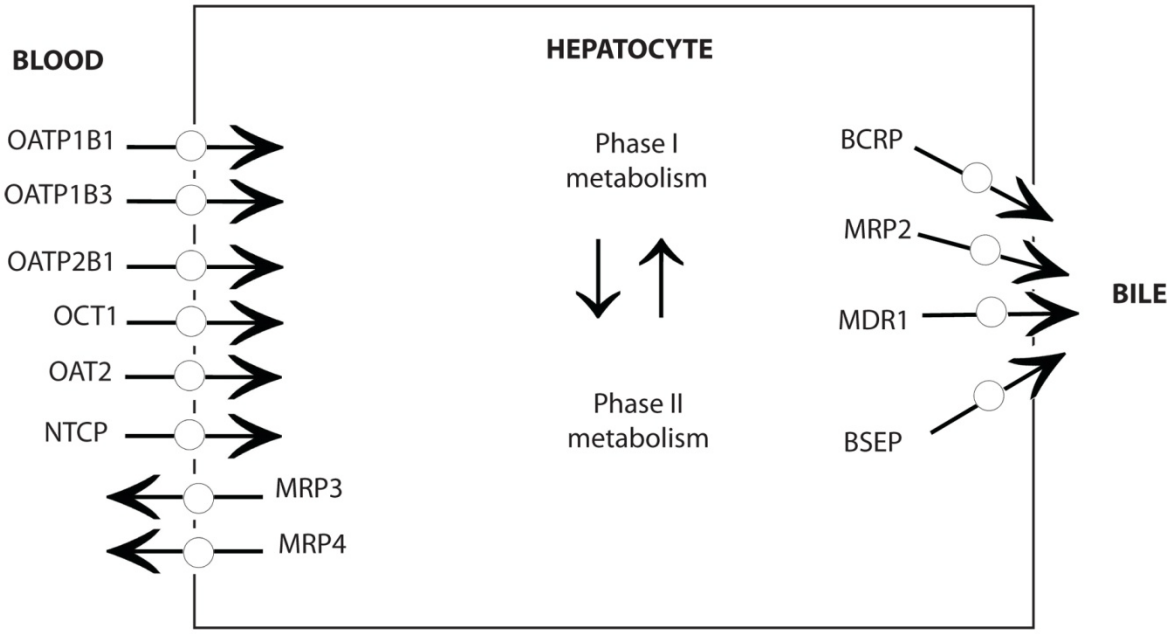
CER half life, $t_{1/2}$, for this patient was 22.4 hours[5] whereas expected values are 1.5-3 hours[6]. The predicted plasma concentration of CER for this patient was 2,600 ng/L, less than 1/3 of the observed value. Significant increases in CER's two major active metabolites are observed in this patient but, interestingly, it would seem that the production of the M23 metabolite (via CYP2C8) is less affected compared to M1 (produced via CYP2C8 and CYP3A4).

In an effort to isolate the cause of rhabdomyolysis in this patient, Ishikawa and colleagues sequenced *CYP3A4, CYP2C8* and *OATP1B1* genes in search of unique genetic polymorphisms for this patient[7]. They identified the patient to be a homozygous carrier of the 475delA genetic polymorphism in CYP2C8 enzyme that leads to a frame shift and premature termination causing a 64% loss of protein structure.[7] Although there is no experimental data verifying the functional influence of this polymorphism, they conclude that this patient lacks CYP2C8 enzyme activity. Indeed, the marked decrease in the M23/M1 ratio (Table 2.1) due to a smaller increase in the level of M23 relative to M1 supports the CYP2C8 deficiency hypothesis. This deficiency or lack of enzyme activity may in part

explain the occurrence of rhabdomyolysis in this patient. These two papers[5,7] are a great example of how a genetic variation causes a significant change in pharmacokinetics of cerivastatin leading to rhabdomyolysis.

Although the exact mechanism of rhabdomyolysis with statins is not known, more than half of the reported CER associated rhabdomyolysis were in subjects who were being treated with combination of CER and gemfibrozil (Chapter 1, Table 1.3).[1] Pharmacokinetic and clinical data demonstrate that in the presence of gemfibrozil patients are exposed to significantly higher levels of CER and its active metabolites, leading to a significant increase in rhabdomyolysis cases among these patients. The increase in exposure is in part due to gemfibrozil inhibition of both major metabolic pathways for CER – CYP450 mediated oxidation (phase I) and conjugation with acyl-glucuronides (phase II) by the uridine diphosphate glucuronysyl transferases (UGT) [6,8] and OATP1B1 mediated uptake of CER[3] (Figure 2.1). Additionally, membrane transporters such as BCRP, MRP2, and P-gp have been shown to be involved in shuttling CER across cellular membranes.[9,10] Given that changes in pharmacokinetics of drugs can be due to inhibition, induction or genetic polymorphisms in enzymes and transporters, we hypothesize that patients exhibiting rhabdomyolysis who were taking CER possess functional genetic variants in one or more enzymes and/or transporters that contribute to systemic accumulation of cerivastatin. We took a candidate gene approach to test this hypothesis.

Figure 2.1: Uptake and efflux transporters and phase I and phase II metabolizing enzymes and their interplay in hepatic elimination of drugs. [11]



2.2 Pharmacogenetic Aim

We scanned for genetic variants by sequencing the genes encoding metabolizing enzymes (CYP2C8, UGT1A1, UGT1A3), membrane transporters (OATP1B1, MRP2, BCRP) and HMG-CoA reductase (*HMGCR*) in patients who developed rhabdomyolysis while on CER.

2.3. A Brief Overview of Targeted Genes

In this section I will, in brief, introduce each gene that I sequenced. Each gene is the subject of countless papers and years of research. There are numerous review papers available on these genes that provide comprehensive tables of substrates, inhibitors, genetic variants and their functional impact.

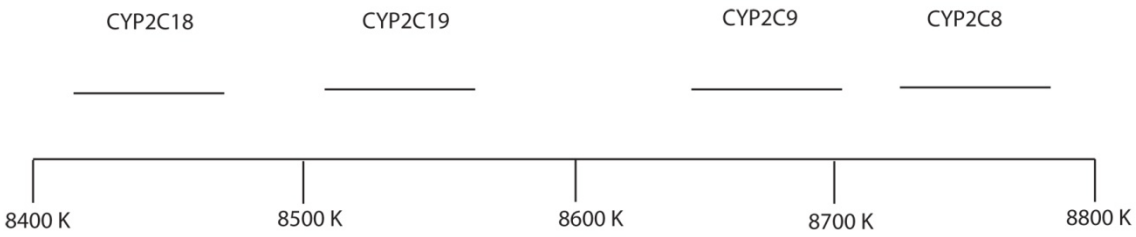
To avoid unproductive repetition of information, I will focus mostly on some generalizations and refer the reader to reviews that I have cited on each gene in preparation of this chapter.

2.3.1 CYP2C8 Review

CYP2C8 is a member of the four known human CYP2C metabolizing enzymes on chromosome 10q24 (Figure 2.2) that also includes CYP2C9, CYP2C18 and CYP2C19, which collectively are responsible for the metabolism of 20% of drugs used in clinics. [12,13] CYP2C8 represents about 6-7% of the total hepatic cytochrome P450 content.[14] The genetic structure of CYP2C8 was first determined in 1999 and it was found to contain 9 exons that spanned 37 kilobases.[15] To date at least 17 genetic variants of CYP2C8 with variable effects on drug metabolism have been identified (www.cypalleles.ki.se+cyp2c8.htm).

There are many known inhibitors and inducers of CYP2C8, making it an important enzyme in drug metabolism. Gemfibrozil[2,14], trimethoprim, troglitazone, terfenadine, triazolam, ketoconazole and clotrimazole are known inhibitors.[16,17] Inducers of CYP2C8 include rifampin, phenobarbital and cortisol. [15,17,18] Colchicine decreases both basal and rifampin and phenobarbital induced expression of CYP2C8.[19]

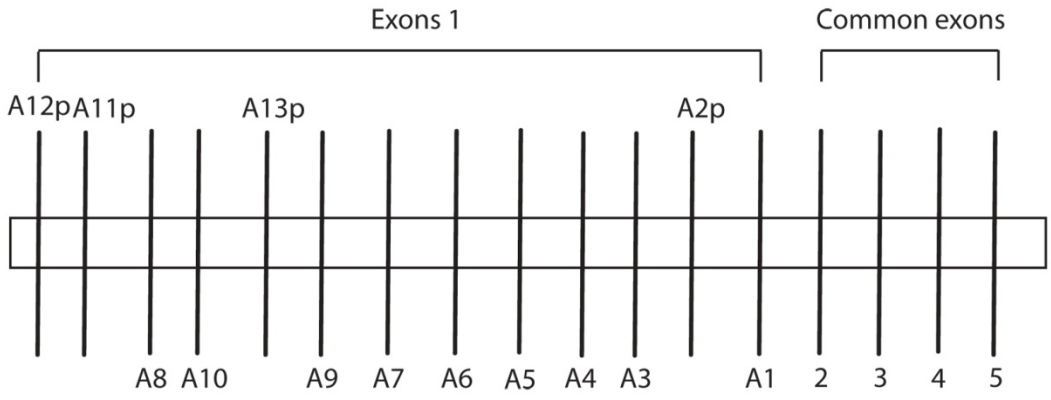
Figure 2.2: Schematic of human CYP2C gene cluster on chromosome 10q24[13]



2.3.2. UGT1A1 and UGT1A3 Review

Glucuronidation is a major phase II conjugation reaction that is involved in excretion of endobiotics and xenobiotics. The conjugation reaction is catalyzed by a superfamily of enzymes called uridine diphospho glucuronosyl transferases (UGTs). The superfamily is divided into two subfamilies designated UGT1A and UGT2B.[20] The UGT1A gene is located on human chromosome 2q37. In the UGT1A family system, 13 first exons, each with its own promoter and enhancer region, are spliced to exons 2-5, which spans 6 kb and encode the C-terminal portion of all 13 UGT1A enzymes (Figure 2.3). [20,21]

Figure 2.3: Schematic of UGT1A gene complex[20]



UGT's are involved in metabolism of numerous drugs.[22] UGT1A1 is the only isoform that contributes to the conjugation of bilirubin and mutations in this gene are best known for the familial unconjugated hyperbilirubinemias of the autosomal recessive disease called Crigler-Najjar syndromes Type I and II and Gilbert's syndrome.[23] UGT1A1 substrates include morphine and other structurally similar opioids [24], aspirin, acetaminophen and coumadin [25]. UGT1A3 is involved in glucuronidating various compounds such as coumadin, opioids (e.g. morphine, buprenorphine), nonsteroidal anti-inflammatory agents (e.g. naproxen, ibuprofen) and fibrates (gemfibrozil) [26]. Phenytoin, phenobarbital and carbamazepine are broad spectrum inducers of the UGT enzyme system.[27,28] Valproic acid is a broad spectrum inhibitor of the UGT enzyme system. Other inhibitors include ethynyl estradiol, naproxen, diclofenac, morphine, diazepam, chloramphenicol, probenecid, oxazepam, ranitidine and furosemide [28,29].

Genetic polymorphisms are known to exist in the UGT enzyme system and many studies have looked at the effect of polymorphism in this enzyme system in diseases and drug metabolism.[20] However, while genetic polymorphism of various UGT isoforms have been reported, in most of these the functional significance of the polymorphism is unclear.[30] Genetic polymorphism have been identified for only a subset of the UGT enzymes that does not include UGT1A3.[30] Due to physiological importance of UGT1A1 in Crigler-Najjar syndromes Type I and II and Gilbert's syndrome more than 60 UGT1A1 variants

have been discovered that contribute to these diseases.[20,22,31] The genetic variants responsible for Gilbert's syndrome include several non-synonymous mutations and a common TA insertion in the TATA box within the UGT1A1 promoter. While the wild-type has six TA repeats, $A(TA)_6TAA$, the low activity mutant, UGT1A1*28, has seven TA inserts, $A(TA)_7TAA$, which leads to low levels of expression; about 30% of normal.[30] There are ample studies demonstrating that this polymorphism leads to decreased glucuronidation of substrates such as L-thyroxine[32] and SN-38[32,33] giving rise to inter-individual differences in response to these drugs.

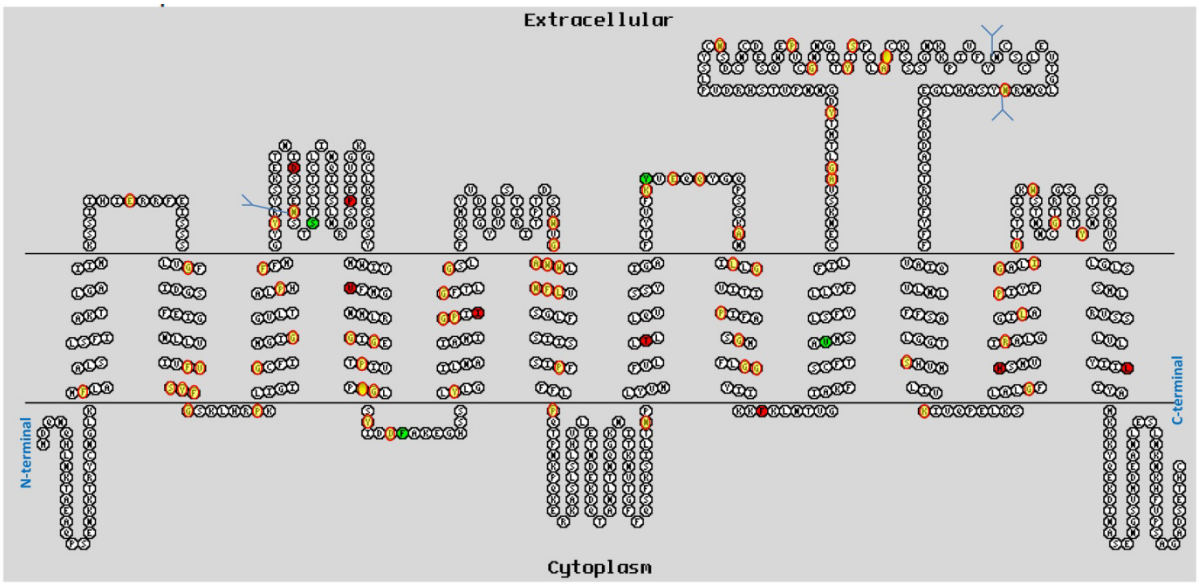
2.3.3. OATP1B1 Overview

Hepatic uptake is in large part mediated by three types of transporters: the sodium-taurocholate cotransporter (*SLC10*), organic anion or cation transporters (*SLC22A*) and organic anion transporting polypeptides (*SLCO*).[34] The human Organic Anion Transporter Polypeptide (OATP) family consists of 11 members including 10 OATPs and the prostaglandin transporter OATP2A1.[35] They are involved in the first step of hepatic elimination by facilitating the uptake of substrates incoming from the portal vein. OATP1B1 uptake transporter, was first discovered in efforts to find novel molecular mechanism that would describe the transport of pravastatin inside the liver.[36] It is a member of the OATP1 family, the largest and best characterized of all six OATP families.[37] This particular transporter is unique in this class. It is sequentially divergent, with only 42% sequence identity to the other members of the family. OATP1B1 and OATP1B3, which has 80% sequence similarity to OATP1B1, are found to be exclusively

expressed in the liver.[36,38]

All members of the OATP family share the following common features. Computationally they all have 12 transmembrane domains (TMDs), however the 12 TMD feature has not been verified experimentally.[37] They all have: a.) a large extracellular domain between TM 9 and 10 that contain many conserved cysteine residues; b.) three N-glycosylation sites in extracellular loops 2 (two sites) and 5 (one site); and c) the OATP super family signature D-X-RW-(I,V)-GAWW-X-G-(F,L)-L at the border between extracellular loop 3 and TM domain VI (Figure 2.4).[37,38] The human OATP1B1 has 691 amino acids with a molecular mass of 84 kDa.[39]

Figure 2.4: Topological model of human OATP1B1. Amino acids conserved in 77 out of 97 mammalian OATPs are indicated in yellow. Three N-glycosylation sites are indicated with (Y).[37]



The exact mechanism of transport for OATP transporters is not known, but what is known is that this protein and other members of its family transport substrates independent of Na^{+}, K^{+},Ca^{+2}, membrane potential, pH and ATP.[40] At least for OATP1B1 and OATP1B3, there is evidence that they are bidirectional facilitated diffusion transporters, that is the transport is facilitated by an electrochemical substrate gradient that is not coupled with a simultaneous movement of another substrate.[34] The transport takes effect under an electrically neutral condition where an anion entering the cell is followed by one leaving the cell; they are not exchangers or cotransporters of ions.[34] Meier-Abt et al.[41] has proposed that for all OATPs the substrate movement occurs through a central and positively charged pore in a rocker-switch type mechanism.

Numerous endogenous substrates, drugs and inhibitors for the OATP1B1 have been identified.[42,43] Some OATP1B1 substrates are cerivastatin[3], rosuvastatin [44], repaglinide[45], fluvastatin [46], atorvastatin[47], pravastatin[48], pitavastatin[49] and simvastatin[50]. Using cerivastatin as a substrate for the OATP1B1 transporter, gemfibrozil and cyclosporine were identified as two inhibitors of this transporter.[3,51] A more comprehensive review of the pharmacogenetics of OATP1B1 and statins is presented in Chapter 3.

2.3.4. ABC Transporters

The human ATP-binding cassette (ABC) transporter family includes at least 48 members and seven subfamilies designated A to G.[53,54] Members of

these transporters are ubiquitously and differentially expressed in most tissues, on various cell types and cell membranes. They facilitate unidirectional transport of chemically diverse substrates important to human physiology, toxicology, pharmacology and disease. Members of this family of transporters are involved in protection of tissues and organs against an array of chemicals and their metabolites. Unlike the OATP transporter, the ABC transporters require the energy of hydrolysis of ATP to power the transporter function. The exact mechanism of transport is not well known.

The typical characteristics of an ABC transporter is the presence of three motifs: Walker A, Walker B and the ABC signature sequence (ALSGGQ).[55,56]

2.3.5. MRP2 Overview

The first member of the ABCC subfamily (or MRP) to be cloned was ABCC1 (MRP1) in 1992.[57] The human ABCC subfamily has 12 members and it includes nine MRPs, the cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7), sulfonylurea receptors SUR1 (ABCC8) and SUR2A/B (ABCC9).[58] MRP2 was first cloned out from the canalicular membrane of rat hepatocyte in 1996.[59] In 1997 Paulusma et al.[60] identified MRP2, which they called canalicular membrane organic transporter or cMOAT, in human hepatocytes. In the same publication they showed that polymorphism in this transporter is associated with Dubin-Johnson syndrome, a disease of chronic conjugated hyperbilirubinemia secondary to lack of secretion of conjugated bilirubin into the bile.

The MRP transporter structure come in two general types: a 17 TMD that includes MRP 1,2,3 and 6 and a 12 TMD that includes MRP4, 5, 7 and 9.[54] However, different algorithms used to predicted the formation of TMD have suggested a model with only four instead of six TMDs in the second cluster of membrane-spanning domain (MSD-2).[61]

The human MRP2 is encoded by human ABCC2 gene. The gene is on chromosome 10q23-24 and has 32 coding exons spanning 65 kb.[53] MRP2 is a 192kDa protein that is highly expressed on the apical membrane of polarized cells such as hepatocytes[59,62], enterocytes[63] and proximal tubule of the kidney[64]. The localization of this transporter in the apical side of the membrane of various polarized cells enables it to serve as a major detoxification protein.[62,65] In the kidney proximal tubule it enables the secretion of endotoxins and exotoxins into the urine. In the intestine it transports from intestinal epithelial cells back into the intestinal lumen and in the liver it facilitates transport of various toxins and xenobiotic conjugates of glucuronides and sulfates from the hepatocytes into the bile for elimination.

There are numerous drugs that are substrates, inhibitors and inducers of this transporter. This particular transporter plays a crucial role in development of cancer chemotherapy resistance. The overexpression of this transporter in cancer cells decreases their sensitivity to chemotherapy and there is ample data in literature in support such a function. MRP2 transfected cells are shown to be

resistant to vinca alkaloids (e.g. vincristin), anthracyclines (e.g. etoposide), camtothecins (e.g. irinotecan active metabolite SN-38) and methotrexate. [66-69]

Over 200 polymorphisms in this gene have been identified.[61] Polymorphism in this gene have been associated with altered pharmacokinetics of pharmaceuticals as well as causing Dubin-Johnson syndrome. Although a comprehensive assessment on the functional influence of polymorphisms of this gene on drug transport is beyond the scope of this dissertation, I will discuss the subject in the context a few polymorphisms that I discovered in the rhabdomyolysis cases.

2.3.6. BCRP Overview

The BCRP efflux transporter, also known as breast cancer resistance protein or mitoxantrone resistance-associated protein, is an ABC transporter coded by the ABCG2 gene. The gene encodes a 72.6 kDa membrane transporter with 655 amino acids. P-gp[70] and MRP1[71] were the first two ABC-transporters that were known to contribute to cancer cell drug resistance, but these two transporters could not explain the observed resistance in MCF-7 cell lines to mitoxantrone, doxorubicin and daunorubicin. This particular transporter was first discovered in 1998 in efforts to understand the mechanisms of chemotherapy resistance to mitoxantrone, doxorubicin and daunorubicin in MCF-7 multi-drug resistant human breast cancer cells, hence the name.[72] The involvement of BCRP in chemotherapy resistance was further proven when inhibition of BCRP by GF120918 increased cell sensitivity to mitoxantrone.[73]

Structurally this transporter has one MSD with six TMDs and one ATP-binding region and it is thought for it to be functionally active, it will have to form a homodimer.[74] BCRP is expressed in the apical side of bile canalicular membrane, enterocytes, trophoblast cells in placenta and the lactiferous duct in the mammary gland.[75] As a transporter it has known endogenous and exogenous substrates as well as inducers and inhibitors however the list of such compounds is not as extensive yet. BCRP, in addition to conferring resistance to cancer chemotherapy agents, is shown to transport rosuvastatin[76], pitavastatin[77] and cerivastatin[10]. A 421C>A polymorphism, the most prevalent polymorphism in Chinese and Japanese with a minor allele frequency of 35%, is shown to increase AUC of rosuvastatin by almost 2 fold in healthy volunteers[76]. There are at least 40 different SNPs identified in ABCG2 gene.[78-81]

2.3.7 HMG-CoA Reductase Overview

HMG-CoA reductase (HMGCR) is the rate limiting enzyme in the cholesterol synthesis pathway and it is targeted for inhibition by our favorite cholesterol lowering drugs, the statins. Thus far in this dissertation I have not mentioned my reasoning for selecting this enzyme as a sequencing target. As noted in the first chapter the exact mechanism of cholesterol synthesis pathway is not well known but there are data showing that downstream intermediary products in synthesis of ubiquinone and isoprenylated proteins (Chapter 1 - Figure 1.3) , not inhibition of cholesterol synthesis, are the primary cause of statin induced myotoxicity.[82] Given the importance of this pathway as established by the significance of the downstream products other than cholesterol in cell

physiology as well as the high sequence conservation between the two classes of this enzyme[83], one would expect any deleterious mutations in this enzyme to be detrimental to cell survival. Since rhabdomyolysis is an extreme response, I took a somewhat expansive step in search of an explanation. In my efforts I was attempting to identify any changes in coding sequence that may have led to a decrease in the function of the enzyme where subsequent exposure to cerivastatin could lead to further depletion of critical downstream products resulting in the observed adverse reaction.

HMGCRs form a large family of proteins that are divided into two classes. Class I protein is found in archaea and eukaryotes and class II is found in bacteria.[84] In eukaryotes HMGCR has eight TMD with a catalytic domain anchored in the endoplasmic reticulum (ER).[85] The human HMGCR consists of 888 amino acids. Of these 888 amino acids, 339 are bound to the endoplasmic reticulum membrane connected by a 10-amino acid linker to the rest of the amino acids that also include the catalytic domain in the cytoplasm.[83] HMGCR is among the most highly regulated enzymes known.[86]

Although I have not been able to identify in literature polymorphisms in the coding region of the enzyme, several polymorphisms in the non-coding regions have been associated with lipid and lipoprotein abnormalities in coronary heart disease in Chinese.[87]

2.4. Patient Selection, Consent and DNA Sequencing[88,89]

The study recruited subjects with cerivastatin-associated rhabdomyolysis through attorneys whose plaintiffs had settled their cases with the manufacturer. Seventeen United States based attorneys and one attorney from Canada helped recruit rhabdomyolysis case participants. The attorneys made initial contact with potential participants by mailing letters describing the study to their clients. The attorneys telephoned each letter recipient to obtain permission to release the clients' contact information to study staff. Study staff then contacted the clients who released their contact information to explain the study, answer questions and obtain consent. Buccal mouthwash samples were extracted using either a Qiagen or Puregene extraction kit. The DNA was aliquoted and stored at -70°C. Participating subjects provided written consent to obtain copies of their medical records from attorneys, doctors, and hospitals.

Trained abstracters used the medical records to validate the rhabdomyolysis event. Eligible rhabdomyolysis was defined as muscle pain or weakness associated with creatine kinase levels greater than 10 times the upper limit of laboratory normal. Subjects who would have likely met the definition of rhabdomyolysis had they been tested when their symptoms were most acute were also included in the analysis. Buccal cell DNA from study subjects was collected using a swish and spit mouthwash kit. One-hundred and twenty-six of the case subjects who consented to participate and provided a usable DNA sample had their DNA sequenced. From these, 118 were white, four black, one Asian, and three of unknown race. For a more detailed description of the patient

characteristics I refer the reader to our paper by Marciante et al[89] published in April of 2011.

2.5. PCR Method

DNA samples were PCR amplified using the primers listed in Appendix 1 representing all the amplicons designed to target coding regions, the intron-exon boundaries, promoter and the UTR regions of the targeted genes. PCR primers were designed using Primer 3 (http://frodo.wi.mit.edu). There are three general PCR conditions and they are as follows for all the amplicons:

2.5.1. P601X35

4 ng of genomic DNA (1 μl of water) was incubated in a 10 μl reaction composed of 1 μl of Buffer, 0.7 μl of $MgCl_2$ (50mM), 0.4 μl of dNTP (2.5mM), 0.03 μl of Taq polymerase, 4.87 μl of water, 1 μl of Forward primer (1μM), 1 μl of Reverse primer (1μM) (Buffer, and enzyme are from Invitrogen Corporation, Carlsbad, CA) with cycling conditions of denaturation at 95°C for 2 min followed by 35 cycles of 92°C for 10 sec, annealing at 60°C for 20 sec, and extension at 72°C for 1 min. At the end of the 35 cycles, the reaction mixture was held at 72°C for 10 min before being cooled to 4°C until the next step.

2.5.2. Betaine P601X35

4 ng of genomic DNA (1 μl of water) was incubated in a 10 μl reaction composed of 1 μl of Buffer, 0.42 μl of $MgCl_2$ (50mM), 0.16 μl of dNTP (2.5mM), 0.03 μl of Taq polymerase, 0.41 μl betaine (Sigma-Aldrich, St. Louis, MO), 2.14

μl of water, 1 μl of Forward primer (1μM), 1 μl of Reverse primer (1μM) (Buffer, and enzyme are from Invitrogen Corporation, Carlsbad, CA) with cycling conditions similar to P601X35.

2.5.3. Q56 or Q601X35

8 ng of genomic DNA was incubated in a 10 μl reaction composed of 1 μl of Buffer, 2 μl of Q-mix, 0.4 μl of dNTP (2.5mM), 0.06 μl of Qiagen Taq polymerase, 2 μl of Forward primer (1μM), 2μl of Reverse primer (1μM) (Buffer, Q-Mix, and enzyme are from Qiagen hot start kit (QIAGEN, Inc., Valencia, CA)) with cycling conditions of denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 1 min, annealing at 56 or 60°C for 1 min, and extension at 72°C for 1 min. At the end of the 35 cycles, the reaction mixture was held at 72°C for 10 min before being cooled to 4°C until the next step.

2.6. PCR Cleanup and Sequencing Reaction

A 10 μl PCR product from every PCR reaction was purified by incubation with 0.4 μl of PCR Clean-up Reagent (PerkinElmer Life Sciences, Inc., Boston, MA) and 3.6 μl of PCR Clean-Up Dilution Buffer (PerkinElmer) at 37°C for 1 hour followed by enzyme inactivation at 90°C for 15 minutes. The purified PCR product was sequenced using ABI PRISM BigDye terminator sequencing Version 3.1 on an ABI Prism 3730xl DNA analyzer (Applied Biosystems, Inc., Foster City, CA). The 12 μl sequencing reaction was composed of 2.5 μl of purified PCR product, 4.5 μl of sequencing primer (1μM), 1 μl BigDyeV3.1, 2 μl of 5X buffer, and 2 μl water.

Cycling conditions were 96°C for 2 min, 25 cycles of 96°C for 15 sec, 50°C for 1 sec, 60°C for 4 minutes.

2.7. Data Analysis

After sequencing, the DNA sequence files were imported into and aligned with SEQUENCHER 4.6 (Gene Codes Corporation, Ann Arbor, MI) for variant analysis. The sequences were further scanned for polymorphisms using Mutation Surveyor V2.51 (Softgenetics LLC, State College, PA). Haplotypes for *SLCO1B1* and *ABCC2* were inferred using PHASE and fastPHASE programs.

2.8. Results

2.8.1 All Variants Discovered in the Patient Population

For the pharmacogenetics aim seven genes were sequenced. Table 2.2 summarizes the total bases pairs (BP's) and numbers of samples that were sequenced for each gene. A total of 95 amplicons were designed and optimized to sequence 49.8 kbs.

Table 2.2: Sequencing summary

Gene	Bases Sequenced	Samples Sequenced
CYP2C8	**6967**	126
UGT1A1	**4987**	126
UGT1A3	**2193**	126
SLCO1B1	**10816**	126
ABCC2	**10584**	92
ABCG2	**6062**	95
HMGCoAR	**8225**	33
	49834	<<Total BPs sequenced

Table 2.3 summarizes the sequencing results of all the genes in terms of the total number of SNPs identified. A total of 203 SNPs were identified in these samples and of these only 52 were in the coding region. Of these 52 SNPs, 17 were synonymous (Syn) and 35 were non-synonymous (NS). Of the 17 synonymous SNPs only four were novel. Of the 35 non-synonymous SNPs, 16 were novel SNPs. The majority of the SNPs were in the non-coding region with a total of 151 SNPs and of these 54 were novel.

Table 2.3: Total SNP summary for all genes

SNP Summary for ALL Genes

	SNPs	Coding	NonCoding	Syn SNP	NS SNPs	Novel NonCoding	Novel Syn SNP	Novel NS SNPs
UGT1A1	17	2	15	1	1	4	1	1
UGT1A3	19	11	8	4	7	3	1	4
CYP2C8	40	7	33	1	6	9	0	3
SLCO1B1	54	10	44	3	7	18	0	3
ABCC2	42	18	24	8	10	15	2	3
ABCG2	19	4	15	0	4	3	0	2
HMGCR	12	0	12	0	0	2	0	0
	203	52	151	17	35	54	4	16

2.8.2 *CYP2C8* Sequencing Results in Cases

A total of 40 SNPs were found in our cases with 32 non-coding and seven coding SNPs. Of the seven coding SNPs, three were novel non-synonymous coding SNPs discovered in exons 1, 8 and 9 (Table 2.4). The two non-synonymous SNPs in exons 1 and 8 (both A>G SNPs) caused amino acid changes of N56S and M426V, respectively. The third novel coding SNP was a frame-shift mutation in exon 9 that changed the last 22 amino acids in the C-terminus of the CYP2C8 gene. Each of these SNPs was discovered once, as

heterozygous in three different cases. We also identified three novel promoter SNPs. The two SNPs that form the CYP2C8*3 alleles were in perfect LD with each other.

Table 2.4: CYP2C8 coding variants and known haplotypes in our cases and their comparison to published minor allele frequencies.

SNP	Location	Location in Gene / Protein	Allele	Cases		Minor Allele Frequency (MAF) from Published Data			
				Black (n=4)	White(n=118)	YRI	Japanese	Chinese	White
ss179319940	Ex 1	167 A>G / N56S	---	0	0.004	---	---	---	---
rs11572080	Ex3	2,130 G>A / R139K	CYP2C8*3	0	0.098	0	0	0	0.108/0.13[2]/0.153[3]
rs1058930	Ex5	11,041 C>G / I264M	CYP2C8*4	0	0.042	0	0	0	0/0.075[1]
rs10509681	Ex8	30,411 A>G / K399R	CYP2C8*3	0	0.098	0	0	0	0.117/0.150[1]/0.153[3]
ss86217921	Ex 8	30,491 A>G / M426V	---	0	0.004	---	---	---	---
ss86217920	Ex9	32,219 / A>- / V472 FS	---	0	0.004	---	---	---	---
rs7909236	Pro	-271 C>A	CYP2C8*1B	0.25	0.174	0	0.089	0.1	0.233/0.24[3]
rs17110453	Pro	-370 T>G	CYP2C8*1C	0.125	0.114	0.008	0.333	0.289	0.119/0.22[3]

MAF values for comparison were taken from HAPMAP White (CEU: Caucasian European), Black (YRI,Yoruba in Ibadan), Japanese (JPT: Japanese in Tokyo), and Chinese (CHB: Han Chinese Beijing) or literature --- no data available.

The minor allele frequency (MAF) that was found in cases was compared to what was found in literature and HapMap (Table 2.4). SNPs that formed the CYP2C8*3, CPY2C8*1B, and CYP2C8*4 had a slightly lower MAF than what is published for the Caucasians. Interestingly CYP2C8*1C and CYP2C8*1B promoter alleles had a significantly higher MAF in the black population than in compared to Yorubans, albeit the source of these numbers are just four cases.

2.8.3. *UGT1A1* Sequencing Results in Cases

Sequencing the *UGT1A1* promoter and exon 1 in cases we found four SNPs in the samples. Of the four SNPs, two were novel coding SNPs of which

one was a synonymous and the other a non-synonymous SNP (Table 2.5). The non-synonymous SNP is characterized by an amino-acid change of N276Y. As mentioned in the introduction for the UGT cluster, all genes in this cluster have a unique promoter and first exon that is spliced to exons 2-5 encoding the C-terminal portion of all 13 UGT1A enzymes (Figure 2.3). [20,21] Sequencing exons 2-5 in this cluster for the patients we found only 13 non-coding SNPs of which only three were novel in our cases.

Table 2.5: Selected UGT1A1 variants in cases

SNP	Location	Location in Gene / Protein	Allele	Cases		MAF from HAPMAP			
				Black (n=4)	White (n=118)	Yoruba	Japanese	Chinese	White
rs34526305	Ex1	**146 C>T / I47I**	---	0	0.001	---	---	---	---
ss86217916	Ex1	**A>T /** N276Y	---	0	0.004	---	---	---	---
rs5839491 + 5 more	Pro	**-53(TA)6>7**	UGT1A1*28	0	0.065	---/0.37[94]	---	---	---/0.24[93]/0.38[94]

The most well-known SNP in this gene is the UGT1A1*28 allele. This is a TATA box polymorphism with the reference sequence having six TA repeats, $A(TA)_6TAA$, while the low active polymorphism, UGT1A1*28, has seven TA inserts, $A(TA)_7TAA$.[30] This polymorphism is known to decrease glucuronidation of substrates such as L-thyroxine[32] and SN-38[32,33] as well as leading to elevated serum bilirubin in Gilbert and Crigler-Najjar syndrome[95]. All rhabdomyolysis cases with this polymorphism where white and heterozygous for this polymorphism. Although there is no report of the allele frequency of this polymorphism in the HapMap population, there are other studies that do report

the MAF of the UGT1A1*28 genotype (Table 2.5). In our study the allele frequency was 6.5%, which is less than 24-38% observed in other studies.[93,94]

In a study comparing the glucuronidation rate of SN-38, the active metabolite of irinotecan, the 6/6, 6/7 and 7/7 (referring to number of TAs in TATA box with 6 being the reference) genotype of UGT1A1*28 allele had a SN-38 glucuronidation ratio of 9.28, 4.04 and 2.41 (P=0.001).[96] Furthermore, patients carrying this polymorphism experienced a significant trend towards lower absolute neutrophil count (ANC) with genotypes 6/7 and 7/7 and more severe grades of diarrhea and neutropenia.[96] However, glucuronide formation is considered a minor clearance pathway for statins. For cerivastatin particularly the CYP2C8 and CYP3A4 mediated metabolism has a Cl_{int} of 20.8 µL/min/mg protein compared to UGT-mediated metabolism that has an intrinsic clearance (Cl_{int}) of 2.9 µL/min/mg.[97] The P-450 mediated metabolic rate is 7.2 to 71 fold higher than the UGT mediated rate for tested statins except for pitavastatin where P-450 and UGT mediated Cl_{int} values are 2.5 and 3.1 µL/min/mg, respectively.[97]

More than 60 UGT1A1 variants have been discovered that contribute to Gilbert's and Crigler-Najjar syndromes Type I and II.[20,22,31] In my data, I only have one of the 60 SNPs and that is the UGT1A1*28 polymorphism.

2.8.4. *UGT1A3* Sequencing Results in Cases

UGT1A3 gene polymorphisms have the least amount of data in terms of pharmacogenetics in the literature while at the same time it has, for the cases,

the most number of novel non-synonymous SNPs. A total of 19 SNPs were identified in promoter and exon 1 of the cases. Of these 11 coding SNPs all were found in exon 1 (Table 2.6). Of the 11 coding SNPs, seven caused a change in protein and of these four were novel. Interestingly there are four SNPs that have MAF in white cases ranging from 5.1-40% and yet there is no reported MAF in the HapMap Caucasians for these SNPs. Specifically the R11W and E27E have MAF's of 38.3% and 40% in the white population of cases and despite the fact that values are reported in Asians for both SNPs and in Yorubans for the E27E polymorphism, none are reported for Caucasians.

Table 2.6: *UGT1A3* gene coding SNPs in cases

SNP	Location*	Location in Gene / Protein	Cases		MAF from HAPMAP			
			Black (n=4)	White (n=118)	Yoruba	Japanese	Chinese	White
ss86217911	**Ex 1**	**30G>A/P10P**	0.000	0.009	---	---	---	---
rs3821242	Ex 1	**31T>C/R11W**	0.188	0.383	---	0.676	0.011	---
rs6706232	Ex 1	**81G>A/E27E**	0.313	0.400	0.908	0.314	0.357	---
rs6431625	Ex 1	**140T>C/V47A**	0.250	0.322	---	---	---	---
rs17868336	Ex 1	**234A>G/T78T**	0.000	0.026	0	0	0	0.025
rs61764029	**Ex 1**	**insC/**F146L.STOP	0.000	0.004	---	---	---	---
rs13406898	Ex 1	**431C>T/T144I**	0.063	0.000	0.025	0	0	0
rs61764030	**Ex 1**	473C>T/A158V	0.000	0.004	---	---	---	---
rs7574296	Ex 1	**477A>G/A159A**	0.375	0.474	0.9	0.273	0.205	0.417
rs45449995	**Ex 1**	808A>G/M270V	0.063	0.051	---	---	---	---
rs61764031	**Ex 1**	**523A>T/N175Y**	0.000	0.009	---	---	---	---

*the Ex 1s that are bold mark the novel coding SNPs discovered.

2.8.5. *SLCO1B1* Sequencing Results in Cases

In sequencing the *SLCO1B1* gene we identified a total of 54 SNPs. Of these 10 were coding while 44 were non-coding. Of the coding SNPs, seven were non-synonymous SNPs and of these three were novel (Table 2.7). The two

novel non-synonymous SNPs in exons 3 and 9 caused amino acid changes of R58Q and T345M, respectively. The third novel coding polymorphism was an insertion of GT in exon 9 that caused a frame-shift polymorphism changing amino acid sequences in the latter half of the *SLCO1B1* gene (Table 2.7b). Each of these SNPs is a singleton, found heterozygous in the three different white cases.

Table 2.7: SLCO1B1 Sequencing Summary. A)*SLCO1B1* gene coding and a promoter SNPs in cases; B)protein changes due to GT insertion in exon 9

A)

SNP	Location	Location in gene / protein	Cases		MAF from HAPMAP				Non-gemfibrozil users	
			Black (n=4)	White (n=118)	Yoruba	Japanese	Chinese	White	Black (n=4)	White (n=118)
rs61760183	**Ex 3**	**170G>A / R58Q**	0	0.004	---	---	---	---	**0**	**0**
rs2306283	Ex 5	**388A>G / N130D**	0.625	0.360	**0.810**	**0.651**	**0.837**	**0.403**	**0.33**	**0.49**
rs11045818	Ex 5	**411G>A / S137S**	0	0.119	**0.008**	**0**	**0.011**	**0.155**	**0**	**0.38**
rs11045819	Ex 5	**463C>A / P155T**	0.250	0.119	**0.058**	**0**	**0.011**	**0.150**	**0**	**0.38**
rs4149056	Ex 6	**521T>C / V174A**	0	0.212	**0.008**	**0.100**	**0.156**	**0.161**	**0**	**0.66**
rs4149057	Ex 6	**571T>C / L191L**	0.875	0.441	**0.008**	**0.100**	**0.156**	**0.161**	**0.50**	**0.48**
rs2291076	Ex 6	**597C>T / F199F**	0.500	0.394	**0.567**	**0.364**	**0.567**	**0.392**	**0.50**	**0.47**
ss86217931	**Ex 9**	**C>T / T345M ins GT > Y338V**	0	0.004	---	---	---	---	**0**	**0**
rs113495867	**Ex 9**	**FS**	0	0.004	---	---	---	---	**0**	**0**
rs34671512	Ex 15	**1929A>C / L643F**	0.125	0.034	**0.067**	---	---	**0.058**	**0**	**0.25**
rs4149015	Pro	-11187G>A	0	0.034	---	---	---	---	0	**0.25**

*the bold exons mark the novel coding SNPs discovered.

B) FFQSFKSILTNPLYVMFVLLTLLQVSSYIGAFTYVFKYVE (without insertion)

FFGSFKSILTNPLCMLCLCFSTOP

The GT insertion causes a frame shift change in protein and premature termination of protein synthesis. The protein synthesis terminates at amino acid 344; 247 amino acids short of the expected protein. Undoubtedly, the transporter

would not be functional if this copy of the gene was being expressed and this certainly would be a risk factor for rhabdomyolysis secondary to elevated systemic levels of cerivastatin.

A haplotype analysis of all the OATP1B1 coding SNPs revealed common (frequency >1%) known and novel haplotypes. Table 2.8 lists the haplotypes and their corresponding frequencies based on the two ethnic groups in our cases.

Table 2.8: *SLCO1B1* gene/protein haplotypes in cases. The amino acid combination making the haplotype are marked in red.

Haplotype	Amino Acids	White	Black	Non-gemfibrozil users Black (n=4)	White (n=118)
Reference		0.554	0.250	0.5	0.489
*1b	**388A>G / N130D** **463C>A /** P155T **521T>C /** V174A	0.098	0.375	0.5	0.61
*15	**388A>G / N130D** **521T>C / V174A**	0.157	0.000	0	0.65
*17	-11187G>A **388A>G / N130D** **521T>C / V174A**	0.013	0.000	0	0.67
*5	**388A>G /** N130D **521T>C / V174A**	0.0720	0.000	0	0.47
Novel 1 (N1)	**388A>G / N130D** **463C>A / P155T**	0.119	0.250	0	0.43
Novel 2 (N2)	**388A>G / N130D** **1929A>C / L643F**	0.034	0.125	1.00	0.25
Novel 3 (N3)	**170G>A / R58Q** **388A>G / N130D** **463C>A / P155T**	0.004	0.000	0	0

A more comprehensive coverage of the *SLCO1B1* variants on the pharmacokinetics of statins and their clinical implication is the subject of Chapter 3. Suffice to say that the N130D, P155T and V174A variants and the *15 and *5

haplotypes are the most commonly described variants in the literature. The V174A variant and *5, *15 and *17 haplotypes were predicted to cause a reduction in the function of OATP1B1 transporter. Since the V174A variant is present in all the functionally disadvantaged haplotypes mentioned, one suspects the alanine substitution for valine in this polymorphism to be culpable.

Although we only sequenced four black subjects, none of them have the known deleterious haplotypes mentioned above. The black cases in our study are protected from the V174A polymorphism because none are carriers, furthermore, the MAF for this polymorphism in Yorubans is reportedly rare (0.8%). Niemi et al.[43] in a recent review of OATP1B1 transporter, reported that the minor allele frequency of this polymorphism for Sub-Saharan African/African American to be in the range of 1-8% and that of Europeans to be between 8-20%.

2.8.6. *ABCC2* Sequencing Results in Cases

In sequencing the *ABCC2* gene in 92 cases of rhabdomyolysis we found a total of 42 coding and non-coding SNPs. Of these 18, 10 and 3 are coding SNPs, non-synonymous and novel non-synonymous SNPs, respectively. Table 2.9 presents all the non-synonymous SNPs identified in the cases along with the MAF in three blacks and 86 white sequenced. In the table2.9 there are three SNPs that don't have rs numbers assigned to them. This is because the novel SNPs found

Table 2.9: *ABCC2* gene non-synonymous SNPs

SNP	Location	Location in Gene /Protein	Cases		MAF from HAPMAP			
			Black (n=3)	White (n=86)	Yoruba	Japanese	Chinese	White
rs56131651	Ex 7	981G>A:S281N	0	0.013	---	---	---	---
rs2273697	Ex 10	1388G>A:V417I	0.500	0.195	0.217	0.089	0.078	0.233
rs17222617	Ex 19	2685T>G:L849R	0	0.024	---	---	---	---
-------	Ex 20	C>A:R915S	0	0.006	---	---	---	---
rs41318029	Ex 21	2900G>A:G921S	0.167	0.019	---	---	---	---
-------	Ex 21	G>A:E944K	0	0.006	---	---	---	---
rs45441199	Ex 23	3246T>C:I1036T	0	0.006	---	---	---	---
-------	Ex 23	C>T:R1066X	0	0.013	---	---	---	---
rs17222723	Ex 25	3702T>A:V1188E	0	0.070	0.058	0	0	0.067
rs8187710	Ex 32	4683G>A:C1515Y	0.167	0.073	0.108	0	0	0.059

in *ABCC2, ABCG2* and *HMGCR* have not been submitted to dbSNP, whereas the novel SNPs discovered in *CYP2C8*, *UGT1A1, UGT1A3* and *SLCO1B1* have all been submitted to dbSNP.

We identified a novel stop codon in exon 23 that was present as a heterozygote in two white cases. It is very likely that this particular polymorphism if expressed would lead to a non-functional MRP2 efflux transporter. In addition to this we identified two additional novel missense SNPs in exons 20 and 21 both of which were present as a heterozygote in two white cases (Table 2.9). Table 2.10 lists the six MRP2 haplotypes common (frequency>1%) to these patients. We identified three novel haplotypes, N4, N5 and N6 with frequencies of 1.3% for N5 and N6 for whites and 1.9% and 17% for N4 in whites and blacks, respectively. There are large differences in haplotype frequencies between the two population groups.

Table 2.10: ***ABCC2*** **gene/protein haplotypes in cases. The amino acid combinations making the haplotype are marked in red.**

Haplotype	Amino Acids	White	Black	Non-gemfibrozil users Black (n=4)	White (n=118)
Reference		0.554	0.250	0.50	0.49
*1b	388A>G / N130D 463C>A / P155T 521T>C / V174A	0.098	0.375	0.5	0.61
*15	388A>G / N130D 521T>C / V174A	0.157	0	0	0.65
*17	-11187G>A 388A>G / N130D 521T>C / V174A	0.013	0	0	0.67
*5	388A>G / N130D 521T>C / V174A	0.0720	0	0	0.47
Novel 1 (N1)	388A>G / N130D 463C>A / P155T	0.119	0.250	0	0.43
Novel 2 (N2)	388A>G / N130D 1929A>C / L643F	0.034	0.125	1.00	0.25
Novel 3 (N3)	170G>A / R58Q 388A>G / N130D 463C>A / P155T	0.004	0	0	0

Of the SNPs that I have identified in the cases, the V417I, V1188E and C1515Y are the three that are cited in the literature. The V417A(1294G>A) allele is reported to increase the transporter function by Haenisch et al.[99] causing lower oral bioavailability of talinolol as measured by AUC. In that study the AUC for talinolol was 3420±708, 2910±485 and 1750±695 ng.h/ml, respectively, for GG, GA and AA genotypes of V417A polymorphism and there was an increase in residual clearance (Cl_{res}) with GG and GA having Cl_{res} of 180 and 169 compared to 226 ml/min for AA genotype. This presents evidence for a gene-dose related lower oral bioavailability as well as increased Cl_{res} with talinolol in patients. The

AA polymorphism has shown increased gastrointestinal toxicity to methotrexate in rheumatoid arthritis patients.[100] However, in a case control study of tenofavir, the presence of this SNP was associated with tenofovir induced renal proximal tubulopathy (OR 6.11[95% CI, 1.20-31.15]) due to lower tubular secretion of tenofavir via MRP2, suggesting that the activity of the transporter is low.[101] The data are not in agreement in regards to the functional effect of this polymorphism.

The V1188E-C1515Y haplotype was associated with anthracycline-induced cardiotoxicity secondary to doxorubicin therapy in German non-Hodgkin lymphoma patients (OR, 2.3;95% CI, 1.0-5.4).[98] The frequency of this haplotype is 7% in white cases only (Table 2.10). Unfortunately I am not aware of the function of this haplotype or any of the polymorphisms identified for MRP2 in regards to cerivastatin pharmacokinetics and or adverse drug reactions.

2.8.7. *ABCG2* Sequencing Results in Cases

The last transporter gene sequenced was the *ABCG2* gene that encodes the BCRP transporter. We sequenced 88 white and two black rhabdomyolysis cases for this transporter to find only 19 SNPs of which only four were coding and non-synonymous and two of these four were novel. Table 2.11 presents the information on these SNPs. We identified 15 non-coding SNPs of which three were novel.

Table 2.11: *ABCG2* gene non-synonymous SNPs

SNP	Location	Location in Gene / Protein	Cases		MAF from HAPMAP			
			Black (n=2)	White (n=88)	Yoruba	Japanese	Chinese	White
rs2231137	Ex 2	34G>A:V12M	0.25	0.078	0.05	0.193	0.289	0.017
rs2231142	Ex5	421C>A:Q141K	0	0.291	0	0.307	0.289	0.117
----	Ex 5	C>T:A145T	0	0.011	---	---	---	---
----	Ex 10	A>G:V536A	----	0.023	---	---	---	---

The minor allele frequencies for the two known SNPs, V12M and Q141K, are quite different between white cases and the Caucasians in HapMap. Of these SNPs the 421C>A (Q141K) polymorphism has been associated with increased AUC and decreased Cl/F for rosuvastatin in healthy volunteers.[76] Volunteers with the CA and AA polymorphisms had an AUC of 62.2±23.5 ng.h/ml, which was almost twice as high as CC volunteer, 34.9±11.9 ng.h/ml. The same volunteers had a Cl/F value in 421CA and 421AA groups that was lower than the 421CC group (384.7±161.2 vs. 674±297.6 l/h, P=0.043).[76] However a study of this same polymorphism in 38 healthy volunteers taking pitavastatin did not result in any alteration in pitavastatin pharmacokinetics.[102] The 421AA polymorphism has been further linked to reduced expression of the transporter protein in placenta and the expression in a gene-dose dependent manner.[78] Mizuarai et al.[103] showed in *in vitro* assays that LLC-PK1 cells transfected with V12M and Q141K had 1/10th of the drug resistance to topoisomerase I inhibitor as well has higher intracellular concentration and decreased efflux compared to reference. Confocal microscopy in this paper revealed a potential mechanism for the phenotype observed with the V12M

polymorphism. It showed that this polymorphism disturbed membrane localization of the BCRP transporter.

These data show that both of the polymorphisms in exons 2 and 5 are associated with decreased transporter function and that the decrease seems to be substrate dependent at least for rosuvastatin and pitavastatin. There are no data assessing the function of these variants on cerivastatin transport, but data showing a decrease in elimination of the cerivastatin would not be an unexpected finding.

2.8.8. *HMGCR* Sequencing Results in Cases

HMGCR is the last of the 7 genes sequenced. Due to DNA sample limitation I only sequenced the coding regions of 33 rhabdomyolysis cases. I identified 12 non-coding SNPs of which two were novel.

2.9. Final Discussion Point

In April of this year our seminal paper on the rhabdomyolysis project was published.[89] In this paper Kristin Marciante, the first author and a collaborator, acquired all the SNP data that I had for *CYP2C8, UGT1A1, UGT1A3* and *SLCO1B1* and did a permutation test to see if there was any association between the SNPs and rhabdomyolysis. The result of this analysis suggested an association between cerivastatin induced rhabdomyolysis and variants in *SLCO1B1* (P=0.002), but no significant association with any variants in *CYP2C8* and *UGT*s. It is very exciting to learn that the V174A polymorphism in the OATP1B1 uptake transporter was the only SNP associated with the risk of

rhabdomyolysis in cases with an odds ratio of 1.89 (95%, CI:1.40-2.56). This particular polymorphism was identified in a genome wide association study (GWAS) conducted by the Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) collaborative group and reported in a 2009 publication linking it to simvastatin induced myopathy in patients with a history of myocardial infarction on 80mg of simvastatin ($P=4x10^{-9}$).[104]

Our group also conducted a GWAS study on 185 cases of rhabdomyolysis that were collected over the life time of this study. These data, which are also presented in our paper, do not identify the OATP1B1 variant to be associated with cerivastatin rhabdomyolysis. Instead the intronic variant, rs2819742, in ryanodine receptor 2 gene (*RYR2)* is identified to be significantly associated with our phenotype ($P=1.7x10^{-7}$).

As mentioned the permutation studies were done in the aforementioned four genes. I have been very interested in repeating this analysis with all the SNPs that I have, including the *ABCC2, ABCG2* and *HMGCR.* However, despite repeated efforts, I have not been unable to acquire access to additional information needed to conduct such analysis. My repeated efforts to obtain a drug history on cases, their use of gemfibrozil and disease status have also been denied. Unfortunately, this situation further limits our analysis of data in this and subsequent chapters. The only information that I have on the cases is their ethnicity; White (n=118), Black (n=4), Unknown (n=3) and Asian (n=1).

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CHAPTER 3
OATP1B1 and cerivastatin uptake

3.1. Introduction

There is significant inter-individual variation in response to statins in terms of lipid-lowering and clinical outcome despite the benefit of statins in prevention of coronary heart disease. It is well recognized that genetic variations contribute to drug disposition and response. However, the origin of this variation is not clearly understood and is under intense investigation. The source of drug response differences can be due to variants in a variety of candidate genes: 1) genes encoding drug transporters and enzymes that influence the pharmacokinetics of drugs, 2) genes encoding targets and pathways influencing the pharmacodynamics of the drug, and 3) genes that influence the underlying disease. The interplay between these three sources of inter-individual variation is further complicated by the reported diversity in genetic variation in different populations. This chapter describes a study that addresses the first source of variation by an *in vitro* evaluation of genetic polymorphisms identified in the sequencing of the *SLCO1B1* gene in cerivastatin (CER) induced rhabdomyolysis patients. However, by way of introducing the interplay of these three sources of variation, I will start the chapter by discussing rosuvastatin pharmacogenetics.

3.2. Rosuvastatin Pharmacogenetics

The package insert of Crestor® (Rosuvastatin), the latest in the HMG-CoA reductase inhibitor class of drugs, carries the following updated statement:

"Results of a large pharmacokinetic study conducted in the US demonstrated an approximate 2-fold elevation in median exposure in Asian subjects (having either Filipino, Chinese, Japanese, Korean, Vietnamese or Asian-Indian origin) compared with Caucasian control group. This increase should be considered when making rosuvastatin dosing decisions for Asian patients"

"the risk of myopathy during treatment with rosuvastatin may be increased in circumstances which increase rosuvastatin drug levels (see clinical pharmacology, race and renal insufficiency"

Due to the observed 2-fold increase in systemic exposure in Asians[1] AstraZeneca recommends that the starting dose of rosuvastatin be 5 mg a day for Asian patients instead of the recommended 10 mg per day. This recommendation was made based on the observed pharmacokinetic data but the exact etiology for this ethnic difference in exposure, i.e. Area Under the Concentration-Time Curve (AUC), is not yet understood.

We do know that metabolic transformation plays only a minor role in rosuvastatin clearance with 90% of orally administered dose recovered as unchanged drug in the feces. The absolute bioavailability of rosuvastatin in Japanese is 29% and in whites it is 20% [1,2]. Based on this it is unlikely that ethnic difference in drug metabolism would contribute to the observed data. A number of studies focusing mostly on genetic variants in the OATP1B1 hepatic membrane transporter have been conducted in order to explain the observed discrepancies in statin kinetics among different ethnic groups.

Choi *et al.*[3] conducted a study in Koreans to explore the potential association between the OATP1B1 c.388A>G and c.521T>C SNPs to the observed pharmacokinetics of rosuvastatin. In thirty healthy Koreans, divided in groups based on their genotype of the SNPs in question, it was determined that AUCs of rosuvastatin in group 1 (*1a/*1a, *1a/*1b, *1b/*1b), group 2 (*1a/*15,*1b/*15) and group 3 (*15/*15) were 111, 126 and 191ng.h ml^{-1}, respectively. This observed difference in AUC was statistically significant between groups. Lee et al.[1] in a study comparing plasma exposure of rosuvastatin in whites and Asians (Chinese, Malay and Asian-Indian) subjects reported the AUC of rosuvastatin to be 2.31 fold higher in Chinese, 1.63-fold higher in Asian-Indians and 1.91 fold higher in Malay subjects compared to white subjects in their study. These differences were all statistically significant. Interestingly, in this study the investigators reported a significant effect of 521T>C genotype on AUC among white subjects only. AUC was higher in CC homozygous, than in TC and TT homozygotes. The AUC difference between TC and TT homozygotes was not statistically significant. There was only a marginal effect of the 388A>G genotype on C_{max} with higher C_{max} in 388G homozygotes. What is interesting about this study is that there was no effect of the 521T>C genotype on systemic exposure to rosuvastatin in Chinese, Malay and Asian-Indian subjects. Also the A388T>C genotype had no association with AUC or C_{max} in any of the Asian groups. *SLCO1B1* genotypes ***did not account*** for the observed differences.

The data from Lee et al.[1] could not fully explain the inter-individual differences of rosuvastatin pharmacokinetics and therefore prompted investigators to look for other possible explanations. Zhang et al.[4] identified a polymorphism (421C>A) in Breast Cancer Resistance Protein (BCRP) membrane transporter, which excretes rosuvastatin from hepatocytes into the bile, to be associated with the pharmacokinetics of rosuvastatin. In their study they reported lower AUC and C_{max} values in Chinese males homozygous for the CC variant compared to CA and AA Chinese subjects. So polymorphism in OATP1B1 along with BCRP may contribute to rosuvastatin observed pharmacokinetic variations, however, there is not a study that looks for both variants in the population simultaneously.

HapMap data reports the minor allele frequency of 521C in Japanese, Chinese and Caucasians to be 0.1, 0.156 and 0.161, respectively. The same database reports the minor allele frequency for the 388G in Japanese, Chinese and Caucasians to be 0.636, 0.844 and 0.392. The differences in allele frequencies between Asians and Caucasians for the 388G allele are far more striking than the differences in the 521C allele. However, studies have associated *5 and *15 allele of the OATP1B1 more with increased exposure of the drug. It is not clear if the frequency of the *15 allele in whites is different from the Asian subjects. Judging from the reported HapMap allele frequencies, these data do not support the OATP1B1 mediated pharmacokinetic observation of higher exposure rate of rosuvastatin in Asians compared to Caucasians. One would expect similar kinetics between these groups since the allele frequency of

521C, the culprit SNP, is similar. Therefore genetic variation in OATP1B1 might be one of many factors contributing to the observed pharmacokinetic difference of rosuvastatin in Asians and Caucasians.

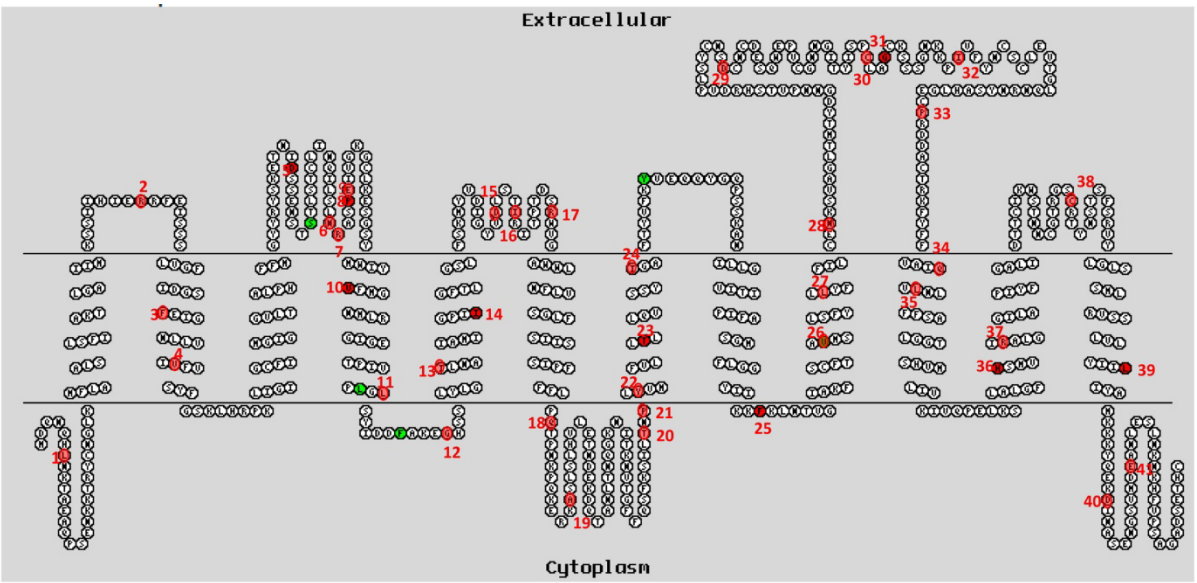
The observation that pharmacokinetic differences for rosuvastatin in Asians and Caucasians lead to a dosing recommendation is a significant step for the field of pharmacogenomics. But the lack of convincing explanation for the observation demonstrates the complicated nature of the sources of variation. It would seem for rosuvastatin that involvement of other sources of variation might lessen the impact of reduced OATP1B1 function in the Chinese, Malay and Asian-Indian subjects. It is also interesting to note that observed differences in pharmacokinetics in association with genotype for different statins has not led to a change in clinical recommendations for this class of drugs. This attests to the problems and challenges of translating pharmacogentics to the clinics at this stage of development.

3.3. Pharmacogenetics: *SLCO1B1* Variants and Functional Studies with Statins

SNPs in OATP1B1 are common and, importantly, a number of SNPs have been shown to markedly affect the pharmacokinetics of statins. Figure 3.1 depicts the positions of 41 known non-synonymous SNPs for OATP1B1.[5] The two common SLCO1B1 SNPs, the subject of numerous *in vitro* and *in vivo* studies for various statins, atorvastatin[6,7], CER[6,8] rosuvastatin[3], pitavastatin[9], simvastatin[10,11] and pravastatin[12-16], are c.388A>G (Asn130Asp) and c.521T>C (Val174Ala). Together these two SNPs define four haplotypes: OATP1B1*1a

(Asn130Val174), OATP1B1*1b (Asp130Val174), OATP1B1*5 (Asn130Ala174) and OATP1B1*15 (Asp130Ala174). Although an extensive review of these polymorphisms in the context of statins is beyond the scope of this thesis, I will discuss a selected few. Of the mentioned polymorphisms and haplotypes, based on my review of the literature, the presence of the 521C allele is associated with reduced transporter function phenotype. The reduced transporter function may be due to intracellular accumulation and reduced expression of the V174A variant.[6]

Figure 3.1: Predicted TM structure of OATP1B1 with 41 non-synonymous SNPs[5]



1. T10A	2. R57Q	3. F73L	4. V82A
5. N130D	6. N151S	7. L152K	8. P155T
9. E156G	10. V174A	11. L193R	12. G203E
13. I211M	14. I222V	15. D241N	16. I245V
17. R253Q	18. Q281K	19. A292V	20. T334S
21. P336R	22. Y338N/D	23. T345M	24. I353T
25. F400L	26. V416L	27. L424F	28. N432D
29. D462G	30. C485F	31. G488A	32. I499V
33. P525S	34. Q541L	35. L543W	36. H575L
37. R580X	38. C613R	39. L643P	40. N655G
41. E667G			

In a pharmacokinetic study of pravastatin in 23 healthy Japanese volunteers, Nishizato et al. [13] identified the OATP1B1*15 allele (Asp130Ala174) to be associated with reduced total and non-renal clearance of pravastatin compared with individuals with OATP1B1*1b allele (Asp130Val174); non-renal clearance values in *1b/*1b, *1b/*15 and *15/*15 subjects were 2.01 ± 0.42 L/kg/hr, 1.11 ± 0.34 L/kg/h and 0.29 L/kg/hr respectively. Based on these kinetic results it is clear that the presence of OAPT1B1*15 haplotype leads to altered kinetics of pravastatin such that its clearance is decreased. Niemi *et al.*[16] reported reduction in cholesterol synthesis with 40 mg of pravastatin in Caucasian heterozygous carriers of the SLCO1B1*17 (containing -11187G, 388A and 521C) haplotype compared with non-carriers, suggesting that this polymorphism decreased the uptake of pravastatin into hepatocytes. A single in vivo study demonstrated a non-significant trend towards lower pravastatin AUC in subjects carrying the OATP1B1*1b haplotype compared to the wild type.[17] Whereas expression of the *5 and *15 alleles are associated with reduced hepatic uptake consistently, there is discrepancy in the effect of the *1b allele. Mwinyi *et al*.[17] results indicate that the expression of *1b haplotype leads to accelerated pravastatin uptake. Further work is needed to understand the role of this polymorphisms in the pharmacokinetics of statins.

Chung et al.[9] studied the effect of these two OATP1B1 variants in 24 healthy Koreans divided in three groups based on their genotypes and measured the pitavastatin AUC for group 1 (*1b/*1b), group 2 (*1a/*1a or *1a/*1b) and group 3 (*1a/*15 or *1b/*15) to be 38.8, 54.4 and 68.1 $ng.h.ml^{-1}.mg^{-1}$,

respectively; a difference that was statistically significant. Additionally, these authors reported a statistically significant difference between carriers and non-carriers of the *15 allele.

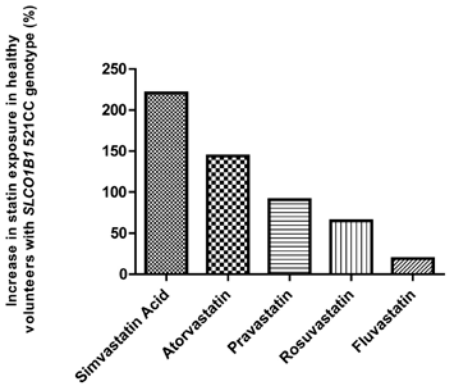
OATP1B1 polymorphism has also been demonstrated to have a marked effect on plasma pharmacokinetics of simvastatin acid but not its lactone metabolite.[10] In a study of 32 Caucasian volunteers taking a single dose of 40 mg simvastatin the mean AUC and C_{max} of the simvastatin acid were about 3 fold higher in individuals carrying the 521CC genotype (i.e. Homozygous Ala:Ala) compared to the 521TT (i.e. homozygous Val:Val) individuals. The lack of a significant difference in pharmacokinetics of the lactone metabolite of simvastatin between the groups suggests that this metabolite is able to penetrate the hepatocyte plasma membrane via passive diffusion or alternatively via an uptake transporter other than OATP1B1. Incidentally, the observation of lack of change in pharmacokinetics of glucuronide metabolite for subjects carrying various haplotypes holds true for rosuvastatin, pravastatin and pitavastatin as well.

It should be realized that not all statins are equally affected by OATP1B1 and its variants. Kameyama *et al.*[6] demonstrated a substrate dependent effect on uptake of pravastatin, atorvastatin, cerivastatin and simvastatin in transiently transfected HEK293 cells with reference (*1a), N130D (*1b), V174A (*5) and *15 variants of *SLCO1B1*. The variants that exhibit the strongest effects are the *5 and *15 on pravastatin followed by atorvastatin and CER and no effect of the transporter and variants on simvastatin.[6] Such *in vitro* data may be explained based on the statin's physiochemical properties. Molecular size, lipophilicity and

charge are the major determinant of membrane permeability. Generally compounds with high Log P (octinol/water partition coefficient) values are more lipophilic and can readily cross membranes by passive diffusion and transporters would play a smaller role in uptake. The log P values for pravastatin, atorvastatin, cerivastatin and simvastatin are -0.47, 1.53, 2.32 and 4.4 respectively.[6] These values explain why OATP1B1 transporter *in vitro* would have the highest effect on pravastatin and no effect on simvastatin. In this paper the authors did not clarify whether by simvastatin they were referring to the lactone form of the drug or the active acid form. The lactone forms of pravastatin, atorvastatin, CER and simvastatin have log P value of 2.42, 4.2, 5.2, and 4.4, respectively.[18] They were most likely testing the lactone form of simvastatin. Evidence for this comes from a several sources two of such studies are mention in the next paragraph.

Pasanen et al.[10] showed that OATP1B1 variants had an effect on simvastatin acid and not on the parent simvastatin lactone. Figure 3.2 is based on plasma concentrations of statins in healthy volunteers homozygous for the 521CC allele of *SLCO1B1* gene. It clearly demonstrates the differential exposure to different statins as well as simvastatin acid as a substrate for OATP1B1.[5]

Figure 3.2: Effects of 174AA (521CC) polymorphism on exposures of different statins in healthy volunteers. [5]

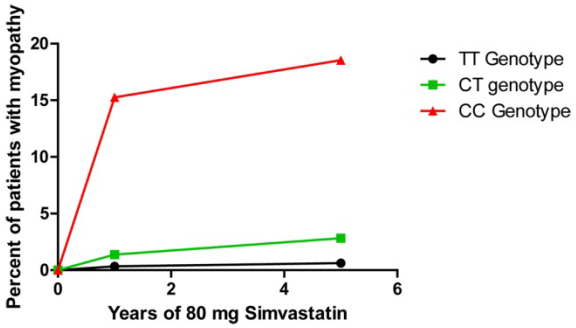


3.4. Pharmacogenetics: Clinical Implication

The clinical implication of *SLCO1B1* variants is best exemplified by their effects on statins. In Chapter 1 we discussed that statin-induced toxicity is a concentration-dependent effect. The most reproducible data that we have in terms of SLCO1B1 pharmacogenetics on statins are the presence of the 521C allele. Figure 3.2 shows that the 521CC allele increases the exposure of patients to statins. It stands to reason that this polymorphism is associated with risk of adverse reaction with statins. This was indeed confirmed. The 521C allele was identified in a GWAS conducted by the SEARCH collaborative group to simvastatin induced myopathy in patients with a history of myocardial infarction on 80mg of simvastatin ($P=4x10^{-9}$).[11] They identified the odds ratio for myopathy to be 4.5 per copy of the 521C allele (95% CI, 2.6-7.7) and 16.9 in 521CC allele (95% CI, 4.7 to 61.1). Furthermore, more than 60% of the myopathy in cases was attributed to this C allele.[19]

Of the patients that had the 521CC allele, the cumulative risk of myopathy in six years among patients on 80 mg of simvastatin daily was 18%, with 15% of myopathy occurring primarily during the first year of treatment. The cumulative risk of myopathy for CT and TT alleles were 3% and 0.6% (Figure 3.3).[11] Pasanen *et al.*[10] measured the plasma concentration of a single dose of 40 mg simvastatin in 31 healthy Caucasian volunteers genotyped for 521T>C polymorphism.[10] The AUC of simvastatin in this study is about 3 fold higher in subjects with 521CC allele compared to 521TT reference genotype. These data not only confirmed that simvastatin acid is a substrate of OATP1B1 but we also see a correlation between the risk of myopathy secondary to elevated plasma concentration of simvastatin in 521CC allele.

Figure 3.3: Effect of 521C allele on cumulative incidence of myopathy in SEARCH trial[11]



Permutation analysis of SNPs from *SLCO1B1 gene* data acquired by sequencing CER induce rhabdomyolysis cases suggest an association between CER induced rhabdomyolysis and the 521C allele of *SLCO1B1* (P=0.002) with an odds ratio of 1.89 (95%, CI:1.40-2.56).[8] Our results extend the influence of 521T>C variant to CER associated rhabdomyolysis.

Since the 521C allele of the SLCO1B1 gene has been associated with reduced uptake of statins, it is hypothesized that this particular variant is associated with reduced cholesterol lowering response due to statin therapy.[16] Tachibana *et al.*[20] retrospectively analyzed the response of 66 Japanese patients to atorvastatin (n=11), pravastatin (n=22) and simvastatin (n=33). They found that in heterozygous 521CT subjects the change in total cholesterol was less than the 521TT homozygotes (-16.5% vs. -22.3%; P<0.05). Although a trend in attenuated LDL reduction in TC groups (-12.4% vs. 29%; P =0.094) was observed, the difference was not statistically significant.

The genotypic difference did not lead to changes in HDL and TG,[20] but they did not have any homozygous CC patients in their study. Igel *et al.*[21] found no significant change in lipid lowering efficacy of 40 mg pravastatin for 3 weeks between the *SLCO1B1* *15 and *17 haplotypes (both include the 521T>C SNP), and controls despite the variant groups having elevated plasma concentration of statins. Takane *et al.*[22] assessing the contribution of genetic variants in the *SLCO1B1* gene to the variability of pravastatin efficacy in 33 hypercholesterolemic patients found that in the initial phase of pravastatin treatment (8 weeks), heterozygous carriers of the *SLCO1B1**15 allele had poor

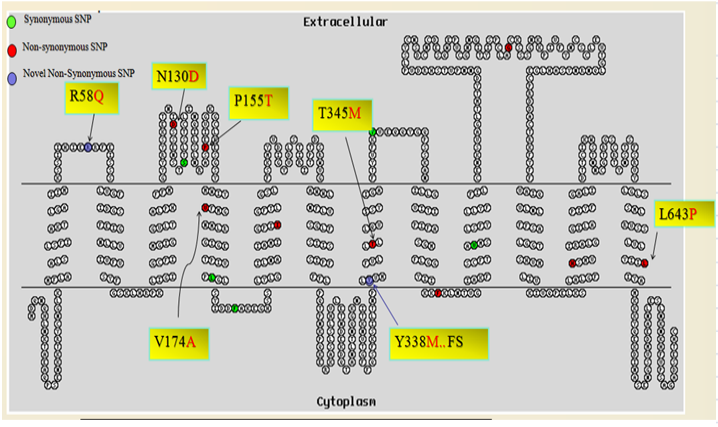
LDL reduction relative to non-carriers (percent reduction: -14.1 vs. -28.9%); however, the genotype-dependent difference in the cholesterol-lowering effect disappeared after 1 year of treatment.[22] Despite strong evidence for *SLCO1B1* polymorphisms impacting statin pharmacokinetics and myopathy, the data on pharmacodynamic influence[11,20-22] are mixed.

3.5. Pharmacologic Aim

With numerous studies pointing to the functional influence of some *SLCO1B1* variants on the statins, we further evaluate the functional significance of genetic variants identified in rhabdomyolysis cases by performing several *in vitro* assays. Figure 3.4 shows the location of all polymorphisms of interest in the OATP1B1 protein and lists the variants and haplotypes I studied in terms of CER trafficking across the cell membrane.

Figure 3.4: SLCO1B1 polymorphisms and haplotypes of interest for functional characterization. A) the location of these polymorphism in protein (Source: PMT website) B) list of all non-synonymous SNPs and haplotypes.

A)



B)

SNP	Location	Location in gene / protein	Allele
rs61760183	Ex 3	170G>A / R57Q	
rs2306283	Ex 5	388A>G / N130D	OATP1B1*1b
rs11045819	Ex 5	463C>A / P155T	OATP1B1*4
rs4149056	Ex 6	521T>C / V174A	OATP1B1*5
ss86217931	Ex 9	C>T / T345M	
rs113495867	Ex 9	ins GT > Y338V FS	
rs34671512	Ex 15	1929A>C / L643F	
Haplotypes			
Reference			
OATP1B1*15	Ex 5	388A>G / N130D	
	Ex 5	521T>C / V174A	
Novel 1 (N1)	Ex 5	388A>G / N130D	
	Ex 5	463C>A / P155T	
Novel 2 (N2)	Ex 5	388A>G / N130D	
	Ex 15	1929A>C / L643F	

3.6. Materials and Methods

3.6.1 *SLCO1B1* Plasmid

The *SLCO1B1* reference cDNA, containing exons 2-15 and 3 bases in 3'UTR, was cloned from human liver tissue and inserted into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and subsequently inserted into pcDNA5/FRT vector (Invitrogen). The plasmid was a gift from the Kroetz lab.

3.6.2 Construction of *SLCO1B1* Reference and Variant Expressing Plasmids

Plasmid containing the variants and haplotypes was constructed by site-directed mutagenesis using a QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The primer sequences (Table 3.1) for the site-directed mutagenesis were designed using QuickChange Primer Design Program (www.stratagene.com/sdmdesigner/default.aspx) from Stratagene. PCR for the site-directed mutagenesis (SDM) was performed according to the following conditions: 30 sec at 95°C for denaturation/activation followed by 15 cycles of denaturation at 95°C for 30 sec, 1 min annealing at 55°C and extension at 68°C for 14.5 minutes.

The SDM product was digested by addition of 1 μl of *Dpn* I enzyme and incubated for 1 hour at 37°C to remove the methylated reference plasmid. The *Dpn* I digested product was transformed via XL1-Blue Supercompetent cells from Strategene and each transformation reaction was plated on LB-ampicillin (100 μg/ml ampicillin) agar plates and incubated overnight at 37°C. Colonies were

selected and purified plasmid obtained by QIAfilter Plasmid Midi Kit (QIAGEN, Inc., Valencia, CA) was sequenced using ABI PRISM BigDye terminator sequencing Version 3.1 on an ABI Prism 3730xl DNA analyzer (Applied Biosystems, Inc., Foster City, CA) to verify the insertion of the variant. After sequencing, the DNA sequence files were imported into and aligned with SEQUENCHER 4.8 (Gene Codes Corporation, Ann Arbor, MI) for variant analysis.

Table 3.1: SDM primers

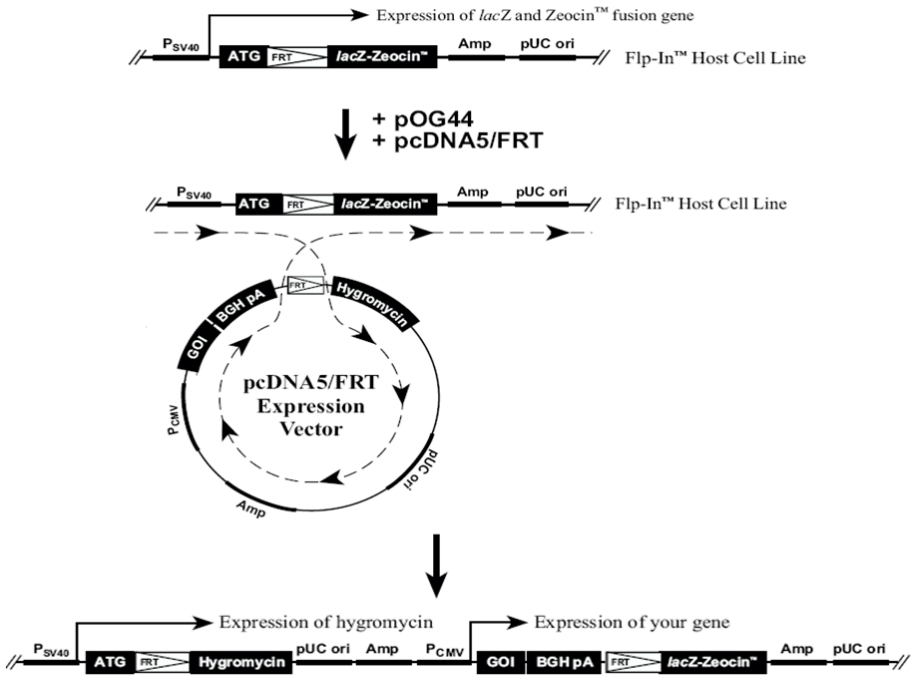
Primer Name	Sequence-Sense	Sequence-Anti-Sense
170G>A / R57Q	ATCATTCATATAGAACAGAGATTTGAGATATCC	TAGTAAGTATATCTTGTCTCTAAACTCTATAGG
388A>G / N130D	AAAGAAACTAATATCGATTCATCAGAAAATT	TTTCTTTGATTATAGCTAAGTAGTCTTTTAA
463C>A / P155T	CTCAATAGAGCATCAACTGAGATAGTGGGAA	GAGTTATCTCGTAGTTGACTCTATCACCCTT
521T>C / V174A	ACATGTGGATATATGCGTTCATGGGTAATAT	TGTACACCTATATACGCAAGTACCCATTATA
C>T / T345M	TGTTTGTGCTTTTGATGTTGTTACAAGTAAG	ACAAACACGAAAACTACAACAATGTTCATTC
ins GT > Y338V FS	TCCTTACTAATCCCCTGTGTATGTTATGTTTGTGCTT	AGGAATGATTAGGGGACACATACAATACAAACACGAA
1929A>C / L643F	TTTATATATTATATTCATTTATGCCATGAAG	AAATATATAATATAAGTAAATACGGTACTTC

3.6.3. Construction of Stable Human *SLCO1B1* Expressing Cell Lines

Human embryonic kidney epithelial Flp-In (HEK293/FRT) cells (Invitrogen) were stably transfected with pcDNA5/FRT (empty vector), pcDNA5/FRT/*SLCO1B1* (reference) and pcDNA5/FRT/*SLCO1B1*-variant vectors using FuGENE 6 transfection reagent (Roche Applied Sciences, Mannheim, Germany). Briefly, on the day before transfection, 1.5 x 10^5 HEK293/FRT cells were seeded in a BD multiwell 24-well plate (BD Biosciences Discover Labware, Bedford, MA) and incubated for 24 hr in 250 μl of Dulbecco's Modified Eagle Medium 4.5% glucose (DMEM-H-21; UCSF Cell Culture Facility, San Francisco, CA) and 10% heat inactivated fetal bovine serum (UCSF Cell Culture Facility)

growth media. The next day cells were transfected with a DNA:FuGENE 6 complex containing 80 ng of vector, 720 ng pOG44, a Flp-recombinase expression vector (Invitrogen), 2.4 μl FuGENE 6 and 17.6 μl Opti-MEM (UCSF Cell Culture Facility). The cells were incubated at 37°C, >95% relative humidity and 5% CO_2 for 24 hr before they were split. Selection media containing 150 μg/ml of Hygromycin (Invitrogen), 100 μg/ml Zeocin (Invitrogen), 1% penicillin and streptomycin (UCSF Cell Culture Facility) and 89% DMEM-H21 was added 48 hr post transfection. Colonies were isolated and screened for the expression of *SLCO1B1*.

Figure 3.5: Flp-In expressing cell line (Source: invitrogen)



3.6.4. RT-qPCR Expression Assay

Total RNA was isolated from each individual colony using RNeasy Plus Micro Kit (Qiagen) per manufacturer's protocol. The isolated RNA was used to make cDNA via iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA) per manufacturer's protocol followed by quantitative PCR (qPCR). The qPCR protocol started with 2 μl of cDNA incubated in a 11 μl total reaction composed of 0.5 μl each of the Forward (5'-TCTTCTCTTGTTGGTTTTATTGACG-3') and Reverse primers (3'-TCCCATAATGAAACAACCGATTC-5') (both at 1 μM), 5 μl of Power SYBR Green PCR Master Mix (Applied Biosystems) and 3 μl of distilled nuclease free water. The qPCR reaction was transferred to Applied Biosystems Prism7900HT Real-Time PCR Systems (Applied Biosystems) with cycling conditions of denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec denaturation, annealing/extension at 60°C for 1 min for data collection. The qPCR data was analyzed by 7900HT Version 2.3 Sequence Detection Systems (Applied Biosystems).

3.6.5. Functional Cellular Assay

Stably transfected HEK293/FRT cells expressing the empty vector, SLCO1B1 reference, variants and haplotypes were plated onto poly-D-lysine-coated 24-well plates (BD Biosciences Discover Labware). Cerivastatin (CER) cellular accumulation studies were performed 24 hr post cell seeding. The accumulation study started with first aspirating the media and washing the cells two times followed by 15 min incubation with warm Krebs-Henseleit buffer (UCSF Cell Culture Facility). Following the removal of the buffer, cells were incubated

with either 5 nM [^{3}H]-cerivastatin (CER) (American Radiolabeled Chemicals, St. Louis, MO) or 20 nM [^{3}H]-estrone-3-sulfate (ES) for a 5 min accumulation study at 37°C, >95% relative humidity and 5% CO_2 incubator.

Accumulation was stopped by removing CER and washing the cells three times with ice-cold Krebs-Henseleit buffer. The cells were lysed by addition of 500 μl/well of lysis buffer composed of 0.1 N NaOH and 0.1% sodium dodecyl sulfate. The intracellular concentration of CER was measured via liquid scintillation counting by transferring 400 μl of the lysate to 2 ml Ecolite scintillation fluid (MP Biomedicals, Irvine, CA) in a Mini-Scintillation vial (Denville Scientific, Metuchen, NJ). The disintegration per minute (dpm) value for each sample, measured by LS-6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Brea, CA), was normalized to the sample protein concentration measured using a BCA protein assay kit (Pierce Biotechnology Inc, Rockford, IL).

3.6.6. Data and Statistical Analysis

The estimated OATP1B1 rate of uptake for each variant was determined by subtracting the rate of uptake for a variant from that of the mean uptake of the substrate in empty vector cells followed by normalization to uptake value of the reference cells. All values are expressed relative to OATP1B1 Reference and are shown as mean ± standard deviation S.D. of replicates measured in three separate experiments. The transport rate for CER or ES is than plotted using GraphPad Prism version 5.04 (GraphPad Software, Inc). Significant differences

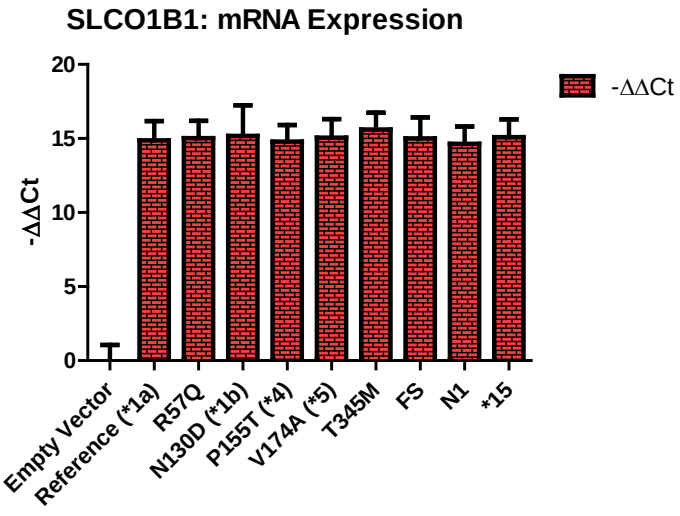
were detected by one-way ANOVA followed by Bonferroni correction for multiple testing and post hoc multiple comparison testing

3.7. Results

3.7.1. Expression of SLCO1B1 Gene in HEK293/FRT Cells

Level of OATP1B1 mRNA expression in HEK293/FRT stably transfected cells was analyzed by RT-qPCR. As shown in Figure 3.6 the levels of mRNA were equal among OATP1B1 reference and variants for all the cell lines.

Figure 3.6: RT-qPCR analysis of mRNA expression

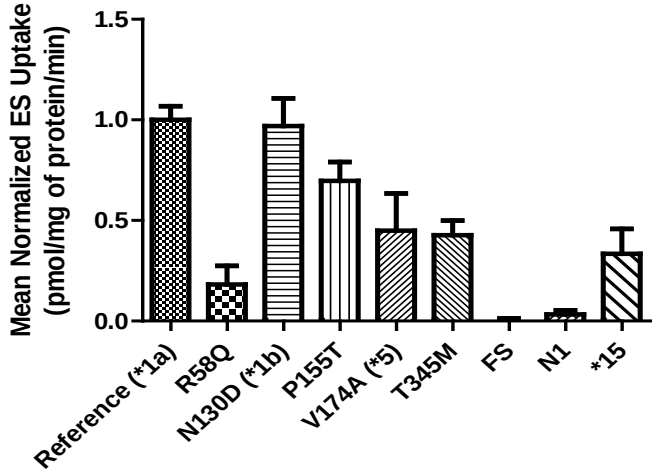


We intended to create and examine the effect of 12 stable cell lines, seven variants, three haplotypes, reference and pcDNA/5 empty vector. Figure 3.6 shows the data for 10 cell lines. The results for the 1929A>C / L643F and its corresponding haplotype, N2, are missing because despite numerous attempts to make a stable cell line with this variant and its N2 haplotypes, we were never successful.

3.7.2. Transport Activity of SLCO1B1 Variants and Haplotypes on ES

The data for uptake of estrone-3-sulfate (ES), a prototypical substrate of the OATP1B1 transporter, with OATP1B1 in stable HEK293/frt cells expressing the reference and variant transporter are shown in Figure 3.7. ES, our positive control, was used as means of confirming the assay success. The transporter activity for N130D did not differ from the reference ($P>0.05$) while all of the remaining variants did have a significant decrease in uptake compared to reference ($P<0.001$) (Table 3.2). The variants with mean rate of uptake (pmol/mg of protein/min) that were significantly different from reference ($P<0.001$) in order of smallest to largest were, frame shift polymorphism, FS, (0.00042±0.012), N1 (0.033±0.019), R57Q (0.18±0.09), *15 (0.34±0.12), T345M (0.43±0.07), V174A (0.45±0.19), and P155T (0.70±0.09). The extent of decrease in function was greatly different among these variants ranging from 70% to baseline. Transport of ES by the OATP1B1 reference cells was on average 15.6 fold higher than the empty vector cells (1.00 ± 0.06 vs. 0.06 ± 0.03; $p < 0.001$), indicating a high affinity for OATP1B1-specific transport (data not shown).

Figure 3.7: OATP1B1 uptake of ES



3.7.3. Transport Activity of SLCO1B1 Variants and Haplotypes on CER

The effect of SLCO1B1 variants and haplotypes on OATP1B1 uptake of CER was measured in stable HEK293/FRT cells expressing the reference and variant transporter (Figure 3.8). The transporter activity for two variants, N130D and T345M did not differ significantly from the reference (P>0.05). However the transporting activities for CER uptake by OATP1B1*15 (0.06±0.18), N1 (0.02±0.06), FS (0.03±0.18), R57Q (0.18±0.11), V174A (0.32±0.18), and P155T (0.72±0.21) decreased significantly compared to reference (P<0.001) (Table 3.2). Transport of CER by the OATP1B1 reference cells was on average 2.7-fold higher than the empty vector cells (1.01 ± 0.12 vs. 0.38 ± 0.12; p < 0.001), indicating OATP1B1-specific transport (data not shown). These data clearly identify 7 OATP1B1 variants that lead to a significant reduction (P<0.001) in ES uptake.

Figure 3.8: OATP1B1 Uptake of CER

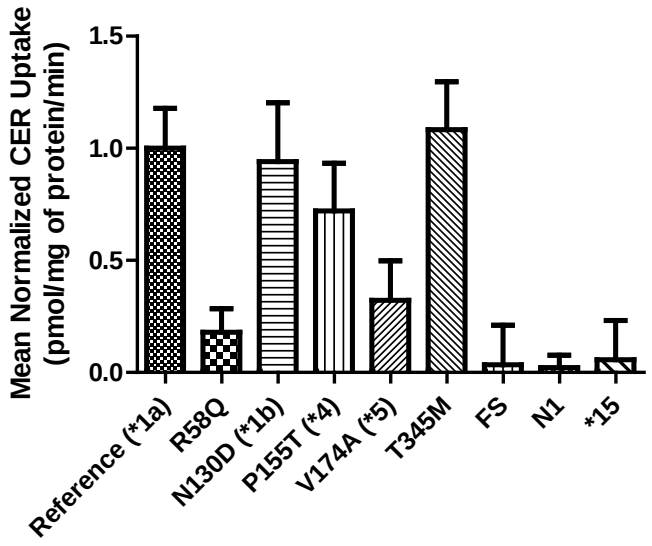


Table 3.2: Summary of means and standard deviations for OATP1B1 uptake of ES and CER

	Estrone-3-sulfate (ES)			Cerivastatin (CER)		
Cell line	Mean	SD	Reference (*1a) vs Variants Significance?	Mean	SD	Reference (*1a) vs Variants Significance?
Reference (*1a)	1.00	0.07		1.00	0.18	
R58Q	0.18	0.09	P<0.001	0.18	0.11	P<0.001
N130D (*1b)	0.97	0.14	P>0.05	0.94	0.26	P>0.05
P155T	0.70	0.09	P<0.001	0.72	0.21	P<0.001
V174A (*5)	0.45	0.19	P<0.001	0.32	0.18	P<0.001
T345M	0.43	0.07	P<0.001	1.08	0.21	P>0.05
FS	0.0004	0.01	P<0.001	0.03	0.18	P<0.001
N1	0.03	0.02	P<0.001	0.02	0.06	P<0.001
*15	0.34	0.12	P<0.001	0.06	0.18	P<0.001

3.7.4. Metabolism of CER by Recombinant CYP2C8

In Chapter 2 we described the polymorphisms that were found by sequencing CYP2C8 in CER induced rhabdomyolysis cases. Rüdiger Kasper,

our collaborator in the Department of Medicinal Chemistry at the University of Washington, created recombinant CYP2C8 in several strains of *Escherichia coli* expressing the discovered variants to study the change in its catalytic function on CER metabolism by measuring the formation of the two major CER metabolites; M-1 and M-23.[23] Table 3.3 summarizes his findings.

Table 3.3: Kinetic evaluation of recombinant CYP2C8 towards M-23 and M-1 metabolite formation

	M-23			M-1			Cl_{int}(M-1 + M-23)	
Variant	K_m (μmol/l)	V_{max} (nmol M-23/nmol P450/min)	Cl_{int} (μl/min/nmol)	K_m (μmol/l)	V_{max} (nmol M-1/nmol P450/min)	Cl_{int} (μl/min/nmol)	(μl/min/nmol)	Ratio Cl_{int}M-23/M-1
CYP2C8.1	23 ± 2	0.22 ± 0.01	9.6	24 ± 3	0.57 ± 0.02	23.8	33.4	0.40
CYP2C8.2	14 ± 1	0.23 ± 0.01	16.4	14 ± 1	0.49 ± 0.01	35.0	51.4	0.47
CYP2C8.3	13 ± 2	0.61 ± 0.03	46.9	13 ± 2	1.47 ± 0.06	113.1	160.0	0.41
CYP2C8.4	9 ± 1	0.20 ± 0.01	22.2	9 ± 1	0.50 ± 0.09	55.6	77.8	0.40
CYP2C8 (N56S)	66 ± 3	0.58 ± 0.01	8.8	61 ± 3	1.59 ± 0.03	26.1	34.9	0.34
CYP2C8 (M426V)	43 ± 3	0.97 ± 0.03	22.6	42 ± 2	0.95 ± 0.02	22.6	45.4	1.00
CYP2C8 (V472*f*sL494)	37 ± 7	0.31 ± 0.03 low heme incorporation	8.4	36 ± 7	0.58 ± 0.04	16.1	23.5	0.52

Based on this data the metabolic clearance as measured by intrinsic clearance ($Cl_{int} = V_{max}/K_m$) was either similar to or higher than reference, CYP2C8.1. Both novel variants, N56S and M426V, had similar combined Cl_{int} values to that of the wildtype. The sum of Cl_{int} value for CYP2C8.3 and CYP2C8.4 were 6-fold and 2.5 fold higher than the reference, CYP2C8.1. The V472fs polymorphism, is expected to have low metabolic activity due to replacement of the last 22 amino acids of the C-terminus end of the protein leading to poor heme incorporation.

3.8. Discussion

The re-sequencing of *SLCO1B1* gene in our unique patient populations that experienced rhabdomyolysis identified six non-synonymous SNPs (including two novel single-base variants and one novel frame-shift mutation, see Table 2.7, Chapter 2), and five non-synonymous coding haplotypes (of which three were novel, see Table 2.8, Chapter 2). We constructed HEK293/FRT stable cell lines expressing all the variants except for the L643F variant and its corresponding N2 haplotype. Although we were able to select colonies of cell lines based on similar expression of cytoplasmic mRNA for our functional assay, we were not able to compare OATP1B1 protein cell membrane expression level across variants via western protein assay despite repeated efforts using two different commercially available antibodies. Therefore it is difficult to conclude whether the decrease in function of the transporter compared to reference is due to decreased expression of OATP1B1 in the cell membrane or decreased function of the transporter variants.

Nevertheless, we were able to identify a significant decrease in uptake of CER and ES in a number of variants and haplotypes. The changes in function followed a similar pattern for both substrates with the exception of two cell lines. For T345M we observed a reduction in ES uptake (0.43±0.07) compared to reference while for CER there was no change (1.08±0.21) compared to reference indicating a substrate dependent effect for this polymorphism. For the OATP1B1*15 haplotypes, although both substrates demonstrated a reduction in uptake, the extent of reduction was different. In regards to ES, this

polymorphism still exhibited the presence of reduced transporter activity (0.34±0.12) compared to reference while for CER the uptake was not different than that for empty vector (P<0.001). The substrate dependent effect that is observed with respect to OATP1B1*15 is not novel. Kameyama *et al.*[6] reported a similar pattern of substrate dependent uptake for this allele when comparing ES and CER.

For the remaining variants, the changes in uptake for the two substrates followed a similar pattern of transport. Our finding confirms that the presence of 521T>C allele in SLCO1B1*5 and *15 leads to significant reduction in uptake. We also identified the functional effect of two novel variants, R57Q and FS, as well as a novel haplotype, N1 to be that of a reduce transport. The uptake pattern for N130D, V174A, *15 is similar to that reported by Kameyama *et al*.[6] for both substrates.

Earlier in this and Chapter 2, evidence was presented in support of increased risk of occurrence of rhabdomyolysis in cases with higher plasma concentration of CER or other statins. A consequence of carrying *SLCO1B1* polymorphism causing a reduction in uptake can be elevated plasma concentration secondary to reduced metabolism of CER. The importance of this transporter in CER metabolism is highlighted by a paper that was published by Liao *et al.*[24] from Millennium Pharmaceutical, Inc. In that paper, using a sandwich-cultured human hepatocyte model, they showed that OATP1B1 inhibition by siRNA led to a 20-30% reduction in total uptake of cerivastatin into

human hepatocytes causing a 50% reduction in formation of M-1 cerivastatin metabolite with no change in M-23 formation. It is worth noting that formation of M-23 accounts only for 10% of total metabolites formed in cultured hepatocytes.[24] Furthermore, in a randomized, double-blind crossover study, of 10 healthy volunteers who took 600 mg of gemfibrozil after three days of treatment with 0.3 mg daily dose of CER, the AUC of CER and its M-1 metabolites were 559% and 435% higher than placebo controls respectively, while the AUC of M-23 metabolite decreased by 22%.[25]

Dr. Leslie Z. Benet's lab was one of the first labs to hypothesize transporter-enzyme interplay.[26,27] Mück *et al.*[28] presented data specific to CER showing the importance of OATP1B1 transporter on CER plasma concentration. In the paper they present data showing that the plasma AUC of CER in kidney transplant patients receiving cyclosporine, a known OATP1B1 inhibitor, was 3 to 5-fold higher than CER in healthy volunteers on CER. In our *in vitro* study all the reduced-function variants lead to decreased CER uptake that ranged greater than 20% with three showing no signs of uptake at all (FS, N1, *15). The presence of these variants can pose a serious risk of rhabdomyolysis secondary to a significant reduction in CER metabolism.

In light of this transporter-enzyme connection we can now explain for a subset of cases why the CYP2C8 variants that showed either no reduction or an increase in transport would not be protective. If cases have reduced-function polymorphisms for OATP1B1, the presence of a functioning CYP2C8 enzyme,

would still pose a risk since the drug would have trouble reaching the enzyme in the first place. Table 3.4 categorizes the CYP2C8 SNPs found in cases, based on them having OATP1B1 reduction-function polymorphism.

Table 3.4: CYP2C8 polymorphisms with OATP1B1 reduction-function polymorphisms

	A>G / N56S	CYP2C8.3			CYP2C8.4			A>G / M426V	V472 FS	CYP2C8.1				Total
# of subjects	1	22			9			1	1	87				118
OATP1B1 reduction function polymorphism	*1a/*15	*1a/*15	*15/*15	*1a/*4	*1a/*5	*1a/*15	*1a/*4		R57Q/N1	*1a/*15	*1a/*4	*1a/*15	*1a/FS	
# of subjects with above reduction function polymorphism	1	4	1	3	1	3	2	0	1	25	22	11	1	75
% of subjects with reduction function polymorphism	100	36			67			0	100	68				64

Overall 75 out of 118 rhabdomyolysis cases, 64%, carry a copy of the *SLCO1B1* polymorphism that we have identified to have a reduction in uptake for CER. For example the sum of Cl_{int} value for CYP2C8.3 is 6-fold higher than reference, CYP2C8.1. Looking at Table 3.4 we see that 22 subjects have this polymorphism. Of these 22 cases, 8 or 36% (4 cases- *1a/*15, 1 case- *15/*15; 3 cases - *1a/*4 genotype for *SLCO1B1* gene) have genotypes of *SLCO1B1* that we have shown to lead to a reduction of OATP1B1 mediated uptake with respect to CER transport.

Evaluation of these potential genetic risk factors would require appropriate control groups, which we do not have. However, with our OATP1B1 data we are able to explain the risk of CER associated rhabdomyolysis in a significant number of cases. Of the 118 White cases, 57 subjects (48%), and of the four

Black cases, two subjects (50%), were carriers of reduced-function *SLCO1B1* polymorphism. Furthermore with the OATP1B1 transporter data we are able to establish genotypes in *SLCO1B1* gene as a risk factor for rhabdomyolysis in 64% of subjects regardless of the CYP2C8 metabolic function.

3.9. References

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CHAPTER 4

Cerivastatin and OATP1B1 mediated drug-drug interaction

4.1. Introduction

In this chapter we turn our attention from drug-gene interaction, the subject of the previous chapter, to drug-drug interactions as a cause of cerivastatin associated rhabdomyolysis in our cases. The therapeutic and unwanted effects of a drug arise from the drug's concentration at the site of its action. This concentration of the drug at the site of action is dependent on the administered dose of the drug as well as the final plasma concentration of the drug, which is dependent on the absorption, distribution, metabolism and elimination (ADME) properties of the drug. Drug elimination occurs primarily either by metabolism in the liver and/or gut mucosa or by excretion into the urine by the kidneys and/or into the bile by the liver. In the liver, metabolism occurs mainly via one or more CYP450 enzymes. Factors that alter hepatic metabolism include status of enzyme and transporter function, disease state, food and other concomitant drugs.

While enzyme mediated pathways of metabolism have been known for a long time and US Food and Drug Administration (FDA) has established guidelines for conducting drug interactions with metabolizing enzymes, the role of transporters in metabolism, at least from the FDA regulatory perspective, is not well defined. The recommendations of the International Transporter Consortium (ITC) published in 2010, is an unprecedented authoritative report to

first establish guidelines for determining transporter mediated influence on the ADME, safety and drug interaction profile of drugs.[1]

Adverse drug reactions are a major cause of morbidity and mortality. There are numerous studies reporting various numbers on this subject,[2,3] all stressing the importance of these effects. For example, In 1998 Lazarou et al. published that in 1994 2.2 million hospitalized individuals experienced serious adverse drug reactions (ADRs), defined as those adverse events that required hospitalization, were permanently disabling or resulted in death.[4] The mean fatal ADR incidence among the 2.2 million hospitalized individuals was 106,000. The latter number ranks ADR associated deaths between the fourth and sixth leading cause of death in the United States.[4]

The paper divided serious ADRs in two broad categories a) those that were the cause of admission and b) those that occurred during a hospitalized admission. Based on this categorization, 4.7% were serious ADRs responsible for admission and 2.1% of serious ADRs were cases that occurred during an admission with an overall fatality rate of 0.32%. Results such as these, which may have a certain degree of bias introduced by mining various databases or heterogeneous collection of studies, should be viewed with a certain degree of circumspection, yet the inevitable conclusion is that ADRs are an important issue in clinical practice. Furthermore, the true incidence of ADRs is not known, because not every ADR is documented, not every ADR causes significant harm to patients, and not every ADR requires hospitalization.[5]

There are three kinds of ADRs. The first is an ADR where the exact mechanism is unknown. HMG-CoA reductase inhibitors and bisphosphonate-associated rhabdomyolysis are examples where one cannot explain the occurrence of this ADR based on current knowledge of pharmacology. The second kind, the subject of the previous chapter, is an ADR due to a drug-gene interaction where genetic polymorphisms increase the risk of drug associated ADR. The third kind is an ADR due to a drug-drug interaction where the interactions between one or more co-administered drugs alter the effectiveness or toxicity of one or more of the co-administered drugs. This cause of ADR is much more predictable and much is known about it. We have entire databases devoted to collecting and documenting such information. In the case of statins, an example of drug-drug interaction associated ADR is where the combination of gemfibrozil with statins increased the relative reporting rate of rhabdomyolysis by a factor of 725 times (Table 1.3, Chapter 1).[6]

Incidentally, it is important to mention that not every drug-drug interaction is metabolism based, but can be due to changes in absorption (e.g. calcium carbonate decreases absorption of ciprofloxacin), and excretion (e.g. probenecid decreasing the excretion of penicillin) (Aside: We do not include distribution because protein displacement interactions are shown to be test-tube phenomenon and not clinically relavent[7]). Furthermore, not every drug-drug interaction is pharmacokinetically based. Some interactions are based on pharmacodynamic effects such as alcohol combined with medications that alter sedation (e.g., benzodiazepines or opioids) that results in additive and unwanted

sedation.[5] Finally, not every drug interaction is undesirable (e.g., combination of carbidopa with levodopa in Sinemet™).

4.2. Pharmacoepidemiologic Based Aim

The goal is to assess the influence of other concomitant drugs identified by means of pharmacoepidemiology on OATP1B1 mediated uptake of CER via an *in vitro* assay and to determine if any of the *in vitro* findings are clinically significant.

4.3. Pharmacoepidemiologic Study

James Floyd, MD, an epidemiologist at Cardiovascular Health Research Unit, University of Washington in Seattle designed a case-control study to discover new drug-drug interactions in cerivastatin associated rhabdomyolysis cases. He reviewed prescription medication use restricted to subjects with relevant medical conditions from 72 rhabdomyolysis cases and compared these to that of 287 controls from Cardiovascular Health Study[8] who were on atorvastatin from 1998 to 2001 (manuscript in preparation). The ideal control group to be used as a basis for comparison to determine the frequency of use of another medication would be, which we don't have, individuals on cerivastatin that did not develop rhabdomyolysis.

Since atorvastatin and cerivastatin are metabolized by CYP3A4 and CYP2C8, respectively, positive findings from the case control study were further evaluated using FDA's Adverse Event Reporting System (AERS) database for prevalence of use of the positive finding with cerivastatin and atorvastatin. Using

logistic linear regression adjusted for age and gender, odds ratios and 95% confidence intervals for each medication were estimated.

4.4. Pharmacoepidemiologic Results

For the drug-drug interaction, a total of 37 prescription medications were identified that were being used by at least 4% of the cases (Appendix 2). The results of his analysis identified the use of the following medications associated with an increased risk of rhabdomyolysis: gemfibrozil (prevalence 32% in cases vs. 0% in controls; odds ratio (OR) 95% CI, 25.0-infinity), fluoxymesterone (prevalence 8% in cases vs. 0% in controls; OR 95% CI, 4.4-infinity), clopidogrel (OR 29.6; 95% CI, 6.1-143), rosiglitazone (OR 19.8; 95% CI, 1.0-402), lansoprazole (OR 5.7; 95% CI, 1.3-24.0), rofecoxib (OR 4.9; 95% CI, 1.1-20.8), and propoxyphene (OR 4.8; 95% CI, 1.7-13.9). Excluding gemfibrozil users from the analysis, the OR associated with clopidogrel increased from 29.6 to 47.8 (95% CI, 12.5-182). Of these seven drugs, both gemfibrozil and clopidogrel use were associated with rhabdomyolysis in cerivastatin users in AERS. (ref: James Floyd)

To verify the above finding, the AERS database was searched. In the AERS database there were 594 reported cases of cerivastatin and 75 reported cases of atorvastatin associated rhabdomyolysis from 1998 to 2001 (Appendix 3). The odds ratio for concomitant gemfibrozil use in cerivastatin users compared to atorvastatin users was 24.6 (95% CI, 8.1-74.5), which is not surprising. However, the prevalence of concomitant clopidogrel use was 17% in cerivastatin users and 0% in atorvastatin users (OR 95% CI, 2.6-infinity) with no evidence

supporting the association of fluoxymesterone, lansoprazole, propoxyphene, rofecoxib and rosiglitazone with CER or atorvastatin associated rhabdomyolysis.

This analysis further established the role of gemfibrozil as a risk factor for cerivastatin associated rhabdomyolysis and furthermore identified clopidogrel from two different data bases as a new drug-drug interaction with cerivastatin. From the list of drugs identified in this analysis we selected 19 drugs that included clopidogrel but excluded gemfibrozil, for *in vitro* drug-drug interaction study with both OATP1B1 (my work) and CYP2C8 (Rüdiger Kaspera).

4.5. Materials and Methods

4.5.1. Compounds

[^{3}H]-Cerivastatin (CER) (50 µM) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]-Estrone-3-sulfate ammonium salt (ES) was purchased from PerkinElmer Life Science, Inc. (Boston, MA). Clopidogrel hydrogen sulfate, and celecoxib were purchased from Sigma-Aldrich (St. Louis, MO). Irbesartan, rofecoxib, pioglitazone hydrochloride, montelukast sodium, verapamil, diltiazem, glyburide, amlodipine, clopidogrel thiolactone and lansoprazole were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Rosiglitazone-KCl was purchased from Cayman Chemical (Ann Arbor, MI)

4.5.2. Lessons of Science

Although various aspects of this project proved challenging, the problems that I faced in my efforts to complete the third aim of the project defied expectations and as a results they were particularly painful and educational. The problems and the educational points are divided in three parts: 1) solubility,

2) cell effects, and 3) degradation of labeled [^{3}H]-cerivastatin (CER). I will discuss each briefly.

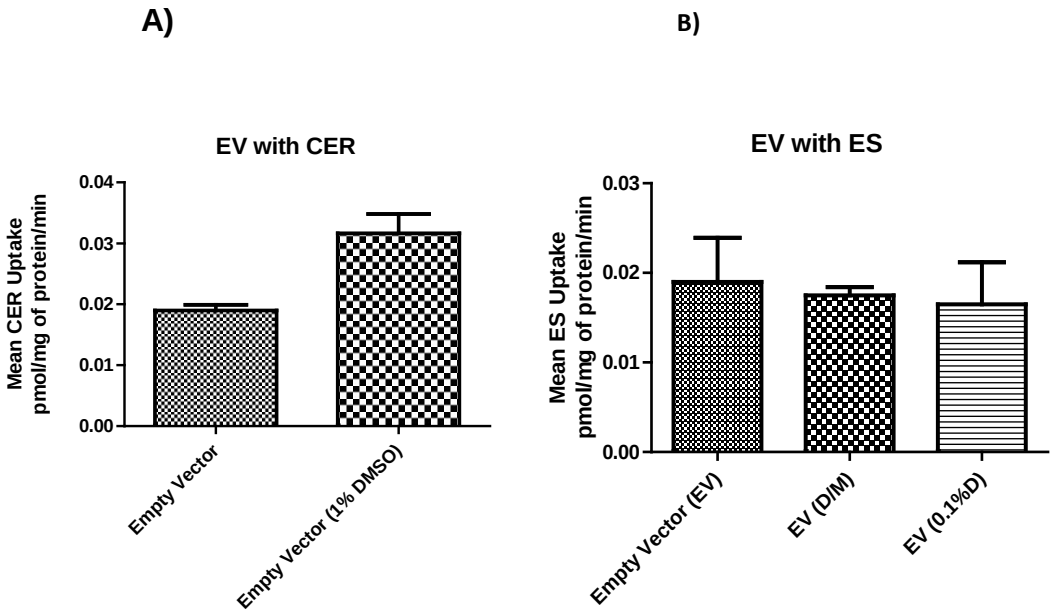
4.5.2.1. Solubility

There were 19 different compounds that we had identified for drug-drug interaction testing. I was naively under the expectation that since I already had a well established assay for OATP1B1 mediated uptake of CER, to do a drug-drug interaction (DDI) study I would simply need to mix these compounds at various concentrations with CER and measure the CER uptake. However, I envisioned the term mix very loosely without really knowing what I was talking about. The term solubility was a textbook concept that hitherto I had never fully appreciated until I tried to dissolve my first drug, pioglitazone.

The manufacturer recommends dissolving pioglitazone in DMF or DMSO. That sounds simple enough. To make a 100 µM final concentration for the interaction study, a 10 mM DMSO stock was made and this stock was further diluted into Krebs-Henseleit buffer; a 1 to 100 dilution resulting in a final concentration of 100 µM pioglitazone and 1% DMSO. However, I found that 1% DMSO altered significantly membrane permeability ($P = 0.03$) increasing passive diffusion of CER into HEK293/FRT cells (Figure 4.1A). Changing the final concentration of DMSO in buffer to 0.1% did not alter the uptake of estrone-3-sulfate (ES) into HEK293/FRT (Figure 4.1B) but pioglitazone, precipitated out of buffer at 0.1% DMSO. This was a problem. We finally found, after weeks of trying different combinations of solvents, that making a 100 mM stock concentration of pioglitazone in DMSO and further diluting it with methanol to a

concentration of 10 mM (10% DMSO/90% MeOH) followed by a 1:100 dilution of this into Krebs-Henseleit buffer successfully dissolved the drug. Furthermore, this concentration of DMSO and methanol did not have an effect on the uptake assay (Figure 4.1B). In fact all the compounds, regardless of their solubility differences, were prepared for experimentation using this recipe.

Figure 4.1: Solvent effect. A) CER uptake B) ES uptake. D/M is 0.1% DMSO and 0.9% Methanol. 0.1% D is 0.1% DMSO



4.5.2.2 Cell Effects

The cell problem was two-fold. The first was mentioned in our solubility discussion. Whereas MDCKII cells tolerated 1% DMSO in buffer (Data from CMFDA assay for MRP2 functional study. MRP2 functional data not presented in this dissertation) without any functional changes, HEK293/FRT cells did not.

The second cell problem had to do with a passage effect (by passage I mean the number of times a cell line is split).

In our efforts to study the impact of *SLCO1B1* variants on OATP1B1 mediated uptake of CER as described in Chapter 2 we used young cell lines. The data collection went relatively smoothly. The main challenge of that particular step was constructing stable cell lines. In order to carry out the experiments for OATP1B1 mediated DDI, new cells were thawed out. These cells were stocks prepared after the initial successful transfection of *SLCO1B1* gene. Cryopreserved cells thawed for these experiments, were cells of low passage typically 2 to 3. These cells are considered young and consequently transporter function should be well preserved; at least one hopes so.

In these studies, however, a number of the cell lines had a significant reduction in OATP1B1 activity at passage numbers less than 10. This was different than my previous experience. While collecting the uptake data for OATP1B1 variants, in the set of experiments described in Chapter 3, I had reference cell lines that still had significant OATP1B1 function at passage number 15. However, after thawing the new batch of cells for the DDI assays, cell lines had a significant reduction in OATP1B1 activity at passage numbers less than 10. The problem at this point was that when experiments failed to give results with seemingly normal cells, initially one does not consider cells as the culprit. By the process of elimination, which took weeks, I discovered the source of the initial problem to be the cells. I endured a few such cycles until I came to

the realization that the problem could be resolved only by using the cells for no more than 3 or 4 passages after thawing a new batch.

4.5.2.3. Degradation of [^{3}H]-CER

The first [^{3}H]-CER was purchased from American Radiolabeled Chemicals (ARC) in May of 2009. The uptake assay was developed and completed for all the variants of OATP1B1 in March of 2010. I used the same batch of [^{3}H]-CER for the initial DDI experiments. There was much inconsistency with the [^{3}H]-CER data, while there was no such variation with ES data. These data indicated that there was a problem with [^{3}H]-CER. When the manufacturer was contacted, they stated that the stability of [^{3}H]-CER was under investigation but they suspected that the integrity of the label was short lived. They, however, were surprised that our first purchase had lasted for almost a year. Left with no choice at the time, we made a second purchase.

Although initially the data improved immediately with our newly purchased [^{3}H]-CER, over a short period of time we began to see a decrease in the transporter effect (Table 4.1). The ratio of reference to empty vector (EV) uptake is a good indication of the OATP1B1 transporter function. The first experiment that was done on 9/8/2010, the first day after the arrival of the new batch of [^{3}H]-CER, had an incredible reference to EV ratio of 4.1. This was the first time I had ever observed such a high ratio. Typically the numbers were around 2.7 to 3.5 fold different. However, as time went on, the ratio continued to decrease (Table 4.1). My initial reaction to this observation was to suspect the transfected cells

losing their transporter function overtime. Indeed, testing the cells with ES revealed that OATP1B1 had lost function in HEK293/FRT cells despite the cells being in passage number 8 (goes back to cell effect). New cells where thawed and these cells had normal function with respect to ES but when CER was tested the reference to EV ratio was below 2. This again indicated that the problem was CER.

Table 4.1: Ratio of reference to empty vector (EV) for [^{3}H]-CER uptake

Date	Uptake Ratio Reference/EV
9/8/2010	4.1
10/5/2010	3.3
10/14/2010	2.69
10/14/2010	2.65
10/19/2010	1.9
10/22/2010	1.47

The scientists at ARC were contacted regarding this change in [^{3}H]-CER. They confirmed that [^{3}H]-CER had lost its label and that we had to purchase a new batch. At this time they stated that the compound is stable for maybe the first month or two, although the package insert that comes with the compound states that "stability is under investigation". Left with no choice, we purchased in early December of 2010 a new and final batch of [^{3}H]-CER thinking that with this new batch we would collect the remaining DDI study data. The first 5 sets of DDI studies with the newly purchased [^{3}H]-CER gave us reference to EV ratios of less than 2! The data was similar to that of degraded compound. The manufacturer was contacted immediately at that point and they claimed that the [^{3}H]-CER was

fine and the problem is most definitely my assay. Testing the cells again with ES further proved that the problem had to be [^{3}H]-CER. With manufacturer refusing to help and experiments still waiting to be completed we decided in consultation with Dr. Benet to purify the [^{3}H]-CER using HPLC.

4.6. HPLC Method

HPLC was performed with an Agilent 1100 system using a binary pump and a series 1050 diode array detector. The stationary phase was a Beckman Ultrasphere 5 µm, 100 Å, 4.6 x 250 mm C8 column, maintained at 25 °C. The mobile phase was a mixture of 30 mM pH 7.4 $NH_4CH_3CO_2$ buffer and acetonitrile (ACN), pumped at 1.0 ml/min according to the following gradient: 0-9 min, linear ramp from 20% ACN to 100% ACN; 9-11 min, 100% ACN; 11-12 min, linear ramp from 100% ACN to 20% ACN; 12-16 min, 20% ACN. Under these conditions the retention of CER was 7.9 min. UV detection was at 280 nm with a 10-nm bandwidth, using a reference of 550 nm with a 100-nm bandwidth. Tritium was measured by collecting 30 or 60-sec fractions of column eluate and counting using a LS-6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Brea, CA).

4.7. Solid Phase Extraction

To remove the mobile phase and concentrate the [^{3}H]-CER peak collected, we used solid phase extraction (SPE) by Oasis® columns (Waters, Milford, MA). Prior to loading sample, the cartridge is conditioned with 2.5 ml of 100% methanol followed by 2.5 ml of 20% methanol:water. In order to increase the binding of [^{3}H]-CER to the column, the ACN was diluted down from an

estimated 64% at the time of peak elution to 10%. The sample was loaded onto an Oasis cartridge. The cartridge was washed with 3 ml of 20% methanol:water and washed again with 1 ml of 10% ethanol:water to remove the more toxic methanol from the cartridge. The cartridge was rinsed with 0.2 ml of ethanol to elute most of the water from the cartridge, and the cartridge was transferred to a clean 13 x 100 mm falcon tube. The column was eluted with 0.5 ml of ethanol and aliquots of all fractions eluted from the cartridge were counted using a LS-6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Brea, CA). The eluted ethanol sample was evaporated to the desired volume under nitrogen gas. The compound was stored at -20°C.

4.8. Testing the Effect of Various Compounds on OATP1B1 Mediated Uptake of CER

Stably transfected HEK293/FRT cells expressing the empty vector and SLCO1B1 reference were plated onto poly-D-lysine-coated 24-well plates (BD Biosciences Discover Lab ware). [^{3}H]-CER cellular accumulation studies were performed 24 hr post cell seeding. The accumulation study started with first aspirating the media and washing the cells two times followed by a 15 min incubation with warm Krebs-Henseleit buffer (UCSF Cell Culture Facility). Following the removal of the buffer, cells were incubated with 100, 50, 10, 1 and 0.1 µM concentrations of various compounds mixed with either 5 nM [^{3}H]-CER (American Radiolabeled Chemicals, St. Louis, MO) or 20 nM [^{3}H]-estrone-3-sulfate (ES) for a 5 min accumulation study in a 37°C, >95% relative humidity and 5% CO_2 incubator.

Accumulation was stopped by removing [^{3}H]-CER and washing the cells three times with ice-cold Krebs-Henseleit buffer. The cells were lysed by addition of 500 μl/well of lysis buffer composed of 0.1 N NaOH and 0.1% sodium dodecyl sulfate. The intracellular concentration of [^{3}H]-CER was measured via liquid scintillation counting by transferring 400 μl of the lysate to 2 ml Ecolite scintillation fluid (MP Biomedicals, Irvine, CA) in a Mini-Scintillation vial (Denville Scientific, Metuchen, NJ). The disintegration per minute (dpm) value for each sample, measured using a LS-6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Brea, CA), were normalized to the sample protein concentration measured using a BCA protein assay kit (Pierce Biotechnology Inc, Rockford, IL).

4.9. Data Analysis

The estimated OATP1B1 rate of uptake with and without concomitant drugs was determined by subtracting the rate of uptake of [^{3}H]-CER from that of the mean uptake in empty vector cells followed by normalization to the uptake value of the reference cells. All values are expressed relative to OATP1B1 control (no concomitant substrate) with replicates measured in two separate experiments for CER only (No replicate were done for ES). The transport rate for CER or ES is than plotted using GraphPad Prism version 5.04 (GraphPad Software, Inc). IC50 values were determined using the one site –Fit logIC50 equation in GraphPad Prism version 5.04. Plots of logarithm of the concentration of the unlabeled compound and transporter rate (pmol/mg of protein/min) normalized to no-compound were created using the same program. Note that

this analysis assumes that there is only one site and that binding to that site is reversible and at equilibrium.

Table 4.2: Compounds selected for DDI study

	Drug	Oral Dose (mg)	Oral Dose (μmol)	Cmax (ng/ml)	Cmax (g/ml)	MW (g/mol)	Cmax (uM)	fu	I in, max (μM)	Fa
1	Amlodipine	10	24.46	18	1.80E-08	408.9	0.044	0.07	0.04	1
2	Celecoxib	100	262.19	705	7.05E-07	381.4	1.85	0.03	----	1
3	Clopidogrel	75	233.06	31	3.10E-08	321.8	0.10	0.02	0.10	1
		300	932.26	70.8	7.08E-08	321.8	0.22	0.02	0.22	1
		600	1864.51	**38**	3.80E-08	321.8	0.12	0.02	0.12	1
4	Clopidogrel Thiolactone	600	1780.42	0.0488	4.88E-11	337	1.45E-04	0.02	0.00	1
5	Clopidogrel Acid	75	244.30	2780	2.78E-06	307	9.06	0.06	----	----
6	Clopidogrel Acyl-glucuronide	600	1239.85	----	----	483.93	----	----	----	----
7	Diltiazem	120	289.51	151	1.51E-07	414.5	0.36	0.22	0.36	1
8	Fluoxymesterone	100	297.27	19	1.90E-08	336.4	0.06	0.04	----	----
9	Glyburide	5	10.12	105	1.05E-07	494	0.21	0.002	0.21	1
10	Irbesartan	50	116.69	1300	1.30E-06	428.5	3.03	0.1	----	----
11	Lansoprazole	15	40.61	248	2.48E-07	369.4	0.67	0.01	----	----
12	Levothyroxine	0.6	0.75	607.13	6.07E-07	798.86	0.76	0.01	0.76	1
13	Montelukast	10	17.06	542	5.42E-07	586.2	0.92	0.01	0.92	1
14	Pioglitazone	30	84.18	1500	1.50E-06	356.4	4.21	0.01	4.21	1
15	Propoxyphene	65	191.46	100	1.00E-07	339.5	0.29	0.2	----	----
16	**Rifampin (positive Control)**	600	729.08	10000	1.00E-05	822.95	12.15	0.11	12.15	1
17	Rofecoxib	25	79.52	320	3.20E-07	314.4	1.02	0.13	1.02	1
18	Rosiglitazone	8	16.89	598	5.98E-07	473.52	1.26	0.002	1.26	1
19	Verapamil	120	263.97	272	2.72E-07	454.6	0.60	0.1	0.60	1

4.10. Prediction of Clinical DDI Interaction

To predict whether the *in vitro* data is clinically significant, the decision tree for OATP interactions proposed in the white paper was used (Figure 4.2).[9] The fraction unbound, fu, and maximum plasma concentration, Cmax, values for each compound were found in literature (Table 4.2). Based on the algorithm if

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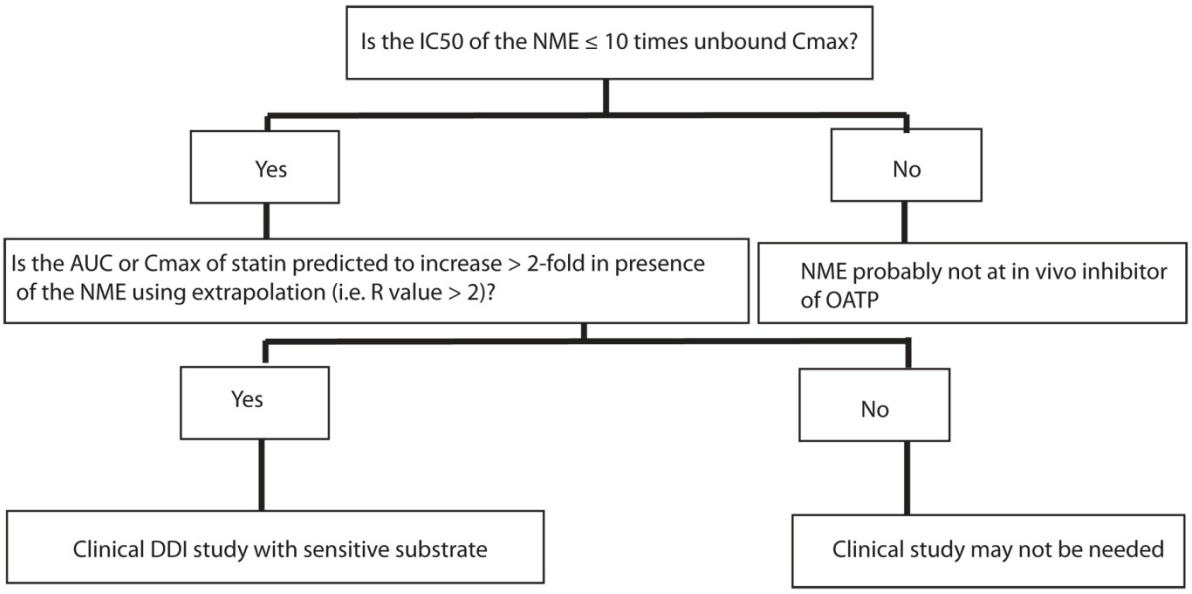
then we calculate the R value, which is an extrapolation of *in vitro* to *in vivo,* representing the ratio of the uptake clearance in the absence and presence of the inhibitor

where fu is the protein unbound fraction of the inhibitor and I in, max is the estimated maximum inhibitor blood concentration at the inlet to the liver and is calculated using the following equation:

Fa is the fraction of the dose of the inhibitor that is absorbed from the intestine,Ka is the absorption rate constant of the inhibitor at the intestine and Q_h is the hepatic blood flow (1500 ml/min). Fa was set to 1 and Ka was set at 0.1 ml^{-1}.[10] For R values greater than 2, the *in vitro* DDI finding may be clinically significant and a clinical DDI study is recommended.

Figure 4.2: Decision tree[9] different colors

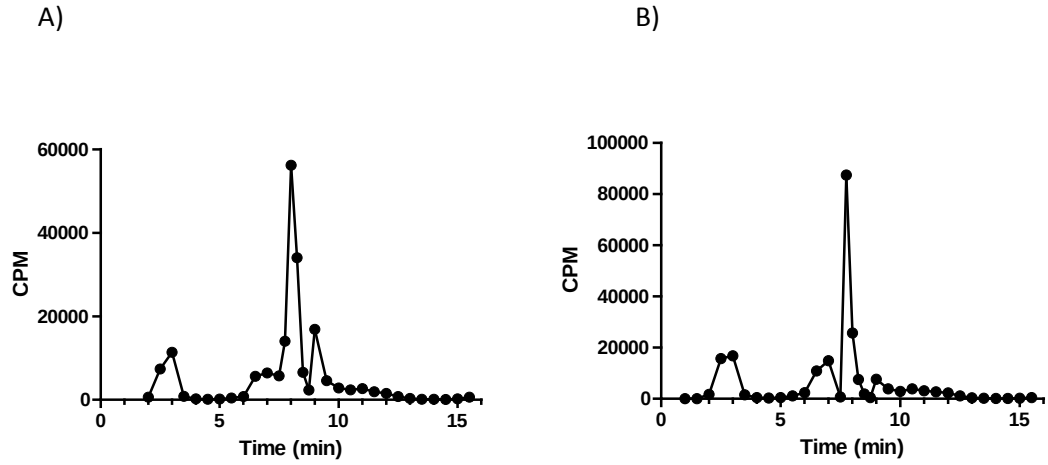


4.11. Results

4.11.1 [^{3}H]-CER Chromatographs

Figure 4.3 illustrates chromatograms obtained by loading two different sources of [^{3}H]-CER on an HPLC column. The first source (Figure 4.3A) was the [^{3}H]-CER purchased in September 2010 and the second chromatogram is for our latest purchase made in December of 2010. It is clear that the chromatograms obtained from these two sources of [^{3}H]-CER are very similar and furthermore that the HPLC assay is able to separate the main peak of CER that is eluted at the 8-minute time point from other impurities. Although peaks other than the major [^{3}H]-CER peak are minor, the extent of the effect of these peaks on the uptake assay is not known. All we know is that collecting and concentrating the [^{3}H]-CER peak with SPE improved the uptake data considerably.

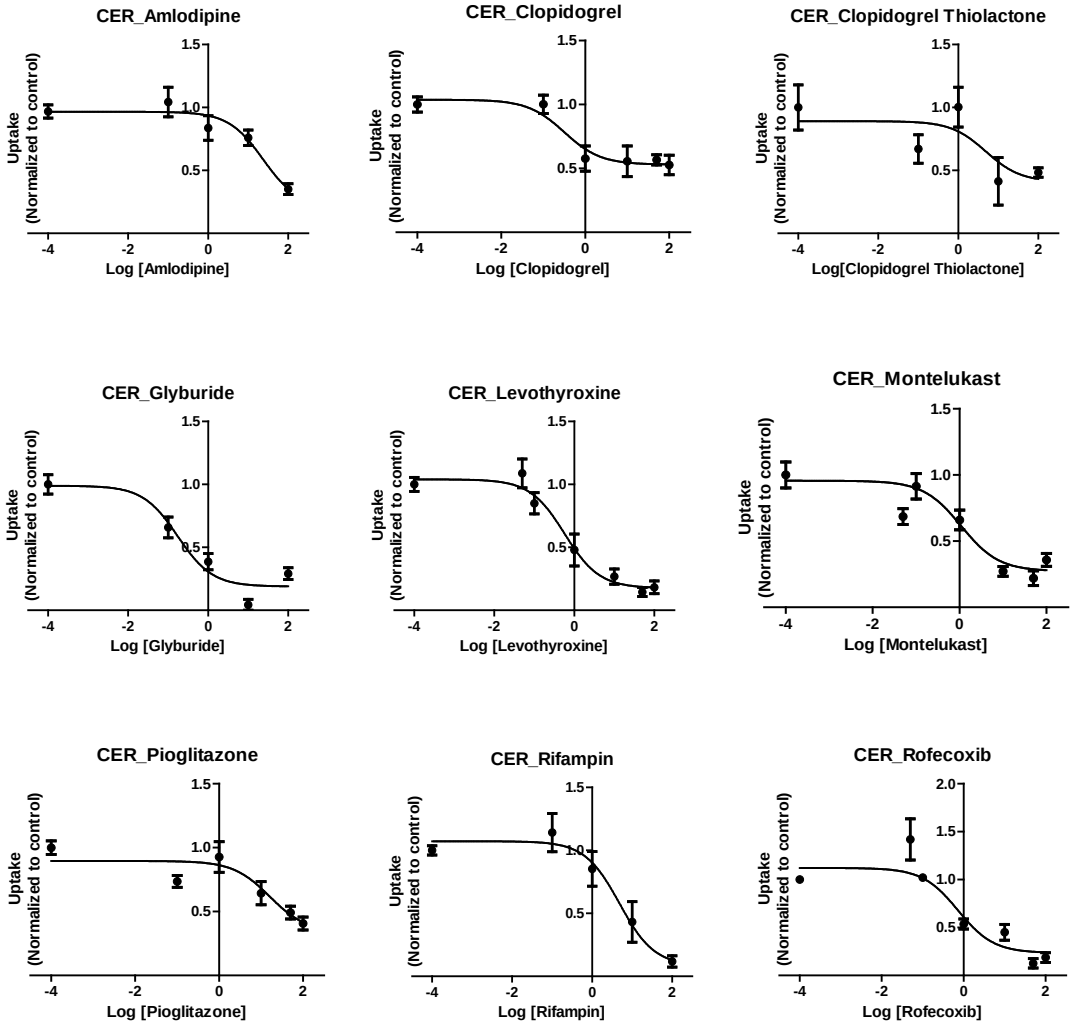
Figure 4.3: HPLC chromatograms for [^{3}H]-CER purchased in A) September 2010 and B) December 2010

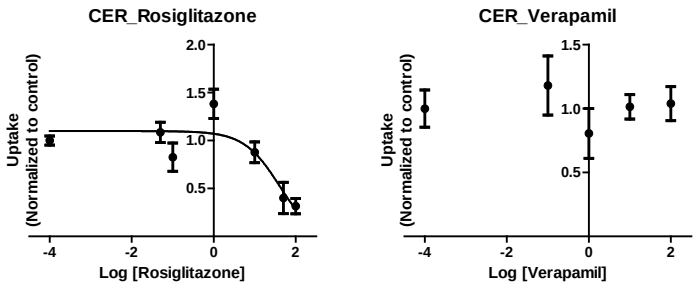


4.11.2. Inhibitory Effects of Various Compounds on OATP1B1 Mediated uptake of CER and ES

The inhibitory effects of various drugs on the uptake of [^{3}H]-CER and [^{3}H]-ES in HEK293/FRT cells stably expressing the reference OATP1B1 are plotted in Figure 4.4 and Figure 4.5, respectively. We tested a total of 19 compounds, and of these celecoxib, clopidogrel acid, clopidogrel acyl-glucuronide, diltiazem, irbesartan and lansoprazole did not inhibit OATP1B1 mediated uptake for both CER and ES at 100 μM concentrations (data not shown). Fluoxymesterone, limited by solubility, was tested at concentrations up to 3 μM and no inhibitory effects on OATP1B1 mediated uptake of CER and ES were seen. Propoxyphene was not studied due to its availability in ACN only; the affect of which on our cell system was not determined. The effects of all the compounds on OATP1B1 mediated uptake of CER and ES were comparable with regards to the compound's inhibitor or non-inhibitory effect, except for verapamil. Verapamil did not inhibit CER uptake at all concentration tested, while it inhibited ES uptake to 32% of control at the 100 μM concentration only.

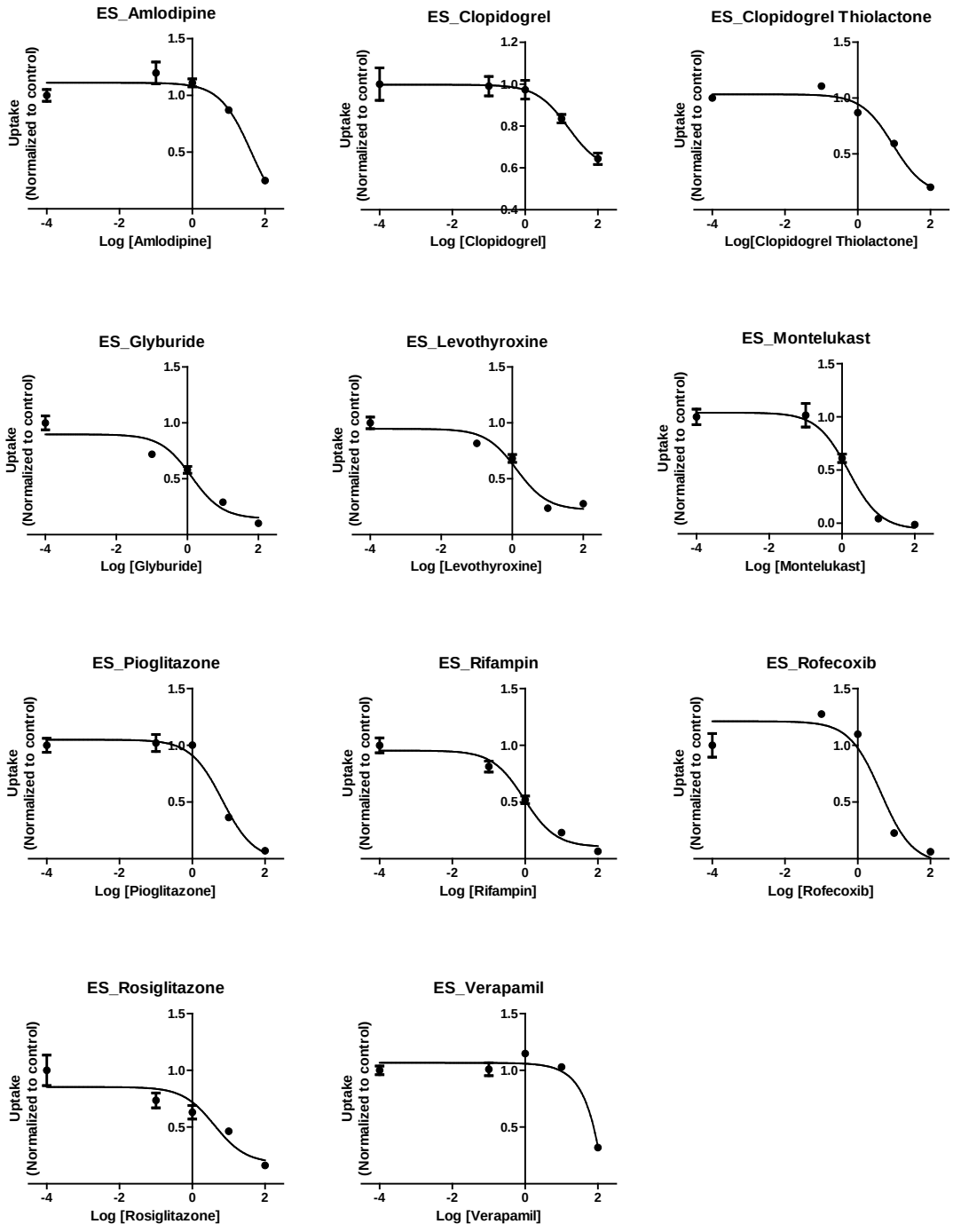
Figure 4.4: The inhibitory effects of various compounds on OATP1B1 mediated uptake of CER (5nM). All values are expressed relative to OATP1B1 control (no inhibitor) and are shown as mean ± SEM of 8 replicates measured in two separate experiments. If there is no visible vertical bar, SEM is contained within the limits of the point marking the mean.





We calculated the maximum concentration of each drug at the inlet to the liver (I in, max) using Cmax and fu values that were obtained from literature (Table 4.2). The experimental IC50 values and their 95% confidence interval (CI) for the compound inhibiting uptake of CER and ES are calculated and presented in Table 4.3. Next we proceeded to use the algorithm from the white paper (Figure 4.2) to determine the clinical significance of our findings. With respect to CER the ratio of unbound Cmax to IC50 was greater than 0.1 for all experimental IC50s except for rifampin and rofecoxib (Table 4.3). However, the same calculation for ES complemented the results from CER whereby the unbound Cmax to IC50 ratio was less than 0.1 for all except rofecoxib. According to the algorithm any number less than 0.1 would not be an *in vivo* inhibitor. However, despite this we proceeded with a calculation of the R value.

Figure 4.5: The inhibitory effects of various compounds on OATP1B1 mediated uptake of ES (20nM). All values are expressed relative to OATP1B1 control (no inhibitor) and are shown as mean ± SEM of 4 replicates measured in 1 experiment. If there is no visible vertical bar, SEM is contained within the limits of the point marking the mean.

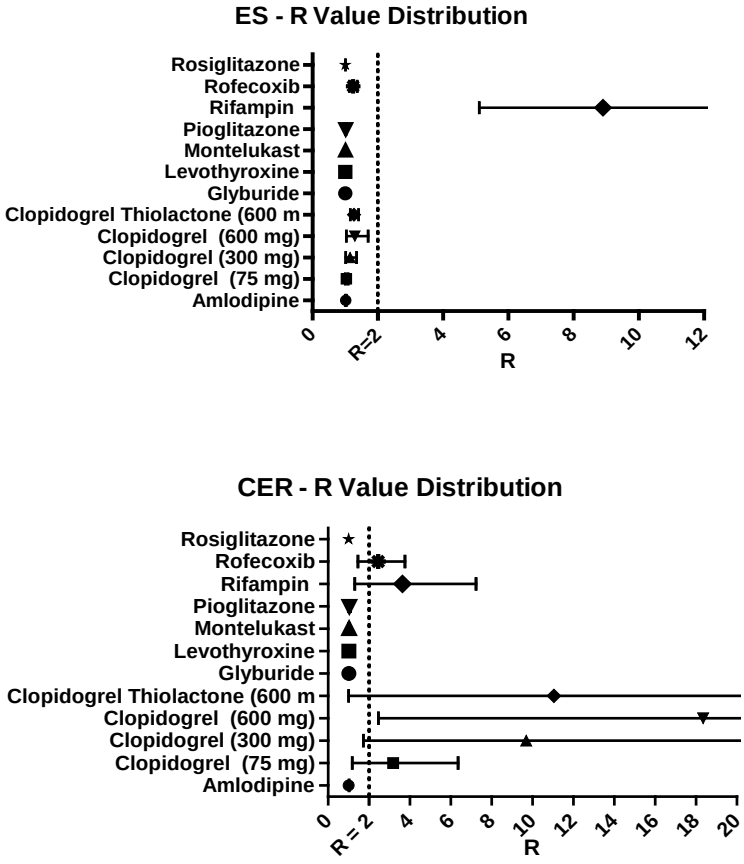


The calculated $I_{in, max}$ and experimental IC50 values were used for calculation of the R value. Table 4.3 summarizes these R values while Figure 4.6 in two separate forest plots, one for the R values of each drug, shows the upper and lower bounds of the calculated R value for CER and ES. For the drugs that we tested only the R values for clopidogrel (Plavix™), rifampin (our positive control) and rofecoxib (Vioxx™) were greater than 2 for CER while for ES the R values reached significance only for rifampin. For all other drugs, despite *in vitro* inhibition, the R values did not achieve significance.

Table 4.3: Experimental IC50 values for CER and ES and their 95% CI followed by unbound concentration (I(u)) to IC50 ratio and corresponding R values.

				Experimental IC50 Values (µM)									
	Drug	Oral Dose (mg)	I in, max (µM)	CER IC50 (µM)	95% CI IC50 of CER		ES IC50 (µM)	95% CI IC50 of ES		CER I(u)/IC50	ES I(u)/IC50	CER R Value	ES R Value
1	Amlodipine	10	1.67	23.61	4.02	138.60	42.2	17.56	101.4	0.0001	0.0001	1.00	1.00
2	Celecoxib	100	----	>100	----	----	>100	----	----	----	----	----	----
3	Clopidogrel	75	15.63	0.32	0.06	1.71	15.14	3.54	64.82	0.0061	0.0001	**1.99**	1.02
		300	62.37	0.32	0.06	1.71	15.14	3.54	64.82	0.0139	0.0003	**4.95**	1.08
		600	124.42	0.32	0.06	1.71	15.14	3.54	64.82	0.0075	0.0002	**8.89**	1.16
4	Clopidogrel Thiolactone	600	118.69	4.88	0.08	297.20	9.33	5.74	15.16	5.94E-07	3.10E-07	1.49	1.25
5	Clopidogrel Acid	75	----	>100	----	----	>100	----	----	----	----	----	----
6	Clopidogrel Acyl-glucuronide	600	----	5.60	----	----	27.39	----	----	----	----	----	----
7	Diltiazem	120	19.66	>100	----	----	>100	----	----	----	----	----	----
8	Fluoxymesterone	100	----	>3	----	----	>3	----	----	----	----	----	----
9	Glyburide	5	0.89	0.17	0.06	0.50	1.30	0.60	2.84	0.0024	0.0003	1.01	1.00
10	Irbesartan	50	----	>100	----	----	>100	----	----	----	----	----	----
11	Lansoprazole	15	----	>100	----	----	>100	----	----	----	----	----	----
12	Levothyroxine	0.6	0.81	0.57	0.22	1.47	2.12	1.36	3.31	0.0133	0.0036	1.01	1.00
13	Montelukast	10	2.06	1.09	0.38	3.11	1.52	0.83	2.79	0.0085	0.0061	1.02	1.01
14	Pioglitazone	30	9.82	6.54	1.10	38.80	6.64	3.71	11.87	0.0064	0.0063	1.02	1.01
15	Propoxyphene	65	----	NA	----	----	NA	----	----	----	----	----	----
16	Rifampin (positive Control)	600	60.76	4.83	1.07	21.81	0.93	0.54	1.63	**0.2766**	**1.4300**	**2.38**	**8.15**
17	Rofecoxib	25	6.32	0.73	0.30	1.80	3.74	2.21	6.34	**0.1810**	0.0354	**2.12**	1.22
18	Rosiglitazone	8	2.39	16.10	3.48	74.42	4.04	0.82	19.82	0.0002	0.0006	1.00	1.00
19	Verapamil	120	18.20	>100	----	----	14.66	----	----	----	----	NA	1.12

Figure 4.6: Forest plots for R.



4.12. Clopidogrel and Gemfibrozil Use in Cases

Tables 4.4A and 4.4B tabulates the frequency of non-gemfibrozil and non-clopidogrel users in CER induced rhabdomyolysis cases according to OATP1B1 genotype and haplotype. The lack of proper controls with OATP1B1 genotype information and CER use makes it impossible to determine if the observed frequencies in cases associated with a given genotype and phenotype is statistically significant. But the use of these drugs for subset of cases in the absence of a transporter effect still presents a risk factor for rhabdomyolysis

occurrence. For example, with OATP1B1*1b haplotype which based on our *in vitro* uptake data has not affect on the uptake of CER, the use of clopidogrel in 30% of whites and 50% of blacks and gemfibrozil in 50% of black and 39% of whites presents as risk factor for CER induced rhabdomyolysis. Applying similar analysis to OATP1B1*15 haplotype which we know leads to a significant reduction in CER uptake, explains why 65% and 61% of whites that were not on gemfibrozil and clopidogrel, respectively, would be at risk of rhabdomyolysis. For the same OATP1B1*15 polymorphism, one can speculate for cases that are on gemfibrozil and/or clopidogrel have both a drug-gene and a drug-drug interaction that are not favorable.

Table 4.4: OATP1B1 A) polymorphisms and B) haplotypes and the frequency of non-clopidogrel and non-gemfibrozil users in CER induced rhabdomyolysis cases

A)

SNP	Location	Location in gene / protein	Cases		Non-gemfibrozil users		Non-clopidogrel users	
			Black (n=4)	White (n=118)	Black (n=4)	White (n=118)	Black (n=4)	White (n=118)
rs61760183	**Ex 3**	**170G>A / R58Q**	0	0.004	**0**	**0**	**0**	**0**
rs2306283	Ex 5	**388A>G / N130D**	0.625	0.360	**0.33**	**0.49**	**0.67**	**0.72**
rs11045818	Ex 5	**411G>A / S137S**	0	0.119	**0**	**0.38**	**0**	**0.85**
rs11045819	Ex 5	**463C>A / P155T**	0.250	0.119	**0**	**0.38**	**1.00**	**0.85**
rs4149056	Ex 6	**521T>C / V174A**	0	0.212	**0**	**0.66**	**0**	**0.66**
rs4149057	Ex 6	**571T>C / L191L**	0.875	0.441	**0.50**	**0.48**	**0.50**	**0.38**
rs2291076	Ex 6	**597C>T / F199F**	0.500	0.394	**0.50**	**0.47**	**0.50**	**0.76**
ss86217931	**Ex 9**	**C>T / T345M**	0	0.004	**0**	**0**	**0**	1.00
rs113495867	**Ex 9**	**ins GT > Y338V FS**	0	0.004	**0**	**0**	**0**	**0**
rs34671512	Ex 15	**1929A>C / L643F**	0.125	0.034	**0**	**0.25**	**1.00**	**0.88**
rs4149015	Pro	-11187G>A	0	0.034	0	**0.25**	**0**	**1.00**

B)

Haplotype	Amino Acids	White	Black	Non-gemfibrozil users Black (n=4)	Non-gemfibrozil users White (n=118)	Non-clopidogrel users Black (n=4)	Non-clopidogrel users White (n=118)
Reference		0.554	0.250	0.50	0.49	0.50	0.82
*1b	388A>G / N130D 463C>A / P155T 521T>C / V174A	0.098	0.375	0.5	0.61	0.50	0.70
*15	388A>G / N130D 521T>C / V174A	0.157	0	0	0.65	0	0.61
*17	-11187G>A 388A>G / N130D 521T>C / V174A	0.013	0	0	0.67	0	1.00
*5	388A>G / N130D 521T>C / V174A	0.0720	0	0	0.47	0	0.88
Novel 1 (N1)	388A>G / N130D 463C>A / P155T	0.119	0.250	0	0.43	1.00	0.75
Novel 2 (N2)	388A>G / N130D 1929A>C / L643F	0.034	0.125	1.00	0.25	0	0.88
Novel 3 (N3)	170G>A / R58Q 388A>G / N130D 463C>A / P155T	0.004	0	0	0	0	0

4.13. Discussions

To determine the risk of other concomitant medication as a mechanism for CER induced rhabdomyolysis, we performed a drug-drug interaction study using our rhabdomyolysis cases and matched controls. Medications taken concurrently with CER were identified in patients who developed rhabdomyolysis. The risk of association of these medications to CER-induced rhabdomyolysis with or without gemfibrozil was determined. Despite limitations of not having the perfect controls (controls were atorvastatin users), 6 medications were identified to be significantly associated with CER induced rhabdomyolysis (Appendix 2). Of all the drugs studied, the use of clopidogrel was strongly associated with CER-induced rhabdomyolysis with gemfibrozil (OR 29.6; 95% CI, 6.1-143) and without gemfibrozil (OR 47.8; 95% CI, 12.5-182).

This finding was replicated by analyzing separate rhabdomyolysis cases reported in the FDA AERS database (Appendix 3). The results of this epidemiology study suggest that there is an adverse reaction between CER and clopidogrel. At the same time concomitant use of fluoxymesterone , lansoprazole, rofecoxib, propoxyphene and rosiglitazone also increased the risk of rhabdomyolysis in case-control study that were not replicated in rhabdomyolysis cases in AERS database. To my knowledge with the exception of rosiglitazone reducing the OATP1B1 mediated uptake of ES in *Xenopus* oocytes to 60% of control,[11] no prior work has demonstrated that these 5 drugs are inhibitors of OATP1B1.

We selected 15 drugs that were either significant or had borderline lack of association with rhabdomyolysis in the case-control study for *in vitro* screening of OATP1B1 inhibition in HEK293/FRT stable transfected cell lines. Since clopidogrel is a prodrug, we also selected three of its inactive metabolites, clopidogrel acid, clopidogrel thiolactone and clopidogrel acyl-glucuronide for testing. Rifampin, a known inhibitor of OATP1B1, was added in as a positive control, bringing the total number of compounds tested to 19. All these compounds were screened for their inhibitory effects on OATP1B1 mediated uptake for CER and ES. Of the 19 compounds tested, 10 inhibited the uptake of CER while 11 inhibited the uptake of ES. Verapamil, known to inhibit OATP1B1 (Ki=51.6),[10] did not inhibit CER mediated uptake but it did inhibit ES uptake (IC50 = 14.66 µM; 95% CI, very wide) at the highest concentration tested (100 µM). A significant interaction between verapamil and atorvastatin has been reported with

atorvastatin leading to elevated verapamil level in healthy male Korean volunteers without testing or implicating OATP1B1.[12]

While clopidogrel has been the subject of much attention recently because of drug-gene interaction with CYP2C19 and its variants[13] as well as controversial drug-drug interaction with omeprazole,[14] a proton-pump inhibitor, there is no data to date in support of clopidogrel inhibiting OATP1B1. Our finding is the first to show that clopidogrel inhibits OATP1B1 and that this interaction may be clinically significant requiring further clinical drug-drug interaction investigation with a sensitive OATP1B1 substrates such as pravastatin, pitavastatin or rosuvastatin. Clopidogrel at dose of 75 mg had an R value of 1.99, just under 2, with a lower limit of 1.18 and an upper limit of 6.36 while at doses of 300 and 600 mg the R values were 4.95 (lower & upper limits, 1.72 & 22.4) and 8.89 (lower & upper limits, 2.45 & 43.68). Although there has been some evidence in the literature suggesting a potential interaction with statins that are metabolized by CYP3A4 (e.g. atorvastatin, simvastatin) and clopidogrel there is no strong clinical evidence to stop the co-administration of any statin with clopidogrel.[15,16] However, in those studies CER was never included, which leaves the question of interaction at least between CER and clopidogrel open. Of the three inactive metabolites of clopidogrel tested, only clopidogrel thiolactone metabolite inhibited OATP1B1 for both CER and ES with R values of 1.49 (lower & upper limits, 1 & 30.6) and 1.25 (lower & upper limits, 1.41 & 1.16).

It is not clear to me at this time how to correctly interpret the range associated with a given R value. For example if the 95% confidence interval of

an odds ratio includes 1, than we consider the null hypothesis of no-difference to be true. Does applying the same principle in reverse, given that the R value of 2 is significant than if the interval of an R contains 2 would we consider the value significant? As an example in case of clopidogrel thiolactone, both R values for CER and ES are below 2 but the upper and lower boundaries of this value are different. The CER interval includes 2 while for ES, it does not. Following our algorithm we know that the ES effect is not clinically significant, but I am not certain about the conclusion that can be drawn for CER. In the available literature and even in the white paper, the R value is reported without any error analysis.

Increased risk of rhabdomyolysis was identified in the case-control study to be associated with fluoxymesterone , lansoprazole, rofecoxib, propoxyphene and rosiglitazone as well. Propoxyphene was not tested. Lansoprazole at concentrations up to 100 µM, and fluoxymesterone at concentrations of up to 3 µM did not inhibit OATP1B1 uptake of ES and CER. Both rosiglitazone and pioglitazone, which have been both shown previously to inhibit OATP1B1 mediated uptake,[11] inhibited CER and ES uptake but their R values did not reach significance. Rofecoxib, as well inhibited OATP1B1 mediated uptake of both CER and ES, a novel finding, but the R value was only significant for CER (2.12; lower & upper limits, 1.46 & 3.76).

Rifampin, a known inhibitor of OATP1B1, was used as a positive control and it inhibited the uptake of both CER and ES with R values that were greater than 2 for both drugs. This confirms the known significant impact that rifampin

plays in *in vivo* inhibition of OATP1B1 mediated uptake of drugs such as glyburide.[17] Montelukast, levothyroxine, glyburide and amlodipine, the newly identified *in vitro* inhibitors of OATP1B1, had R values less than 2, indicating a clinically insignificant drug interaction.

4.14. REFERENCES

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FINAL COMMENTS

In this project we used a candidate gene approach in search of genetic polymorphism in drug transporter genes and enzymes to identify genetic variants that would lead to accumulation of systemic CER. Although 7 candidate genes were selected for sequencing in cases, functional studies were successfully completed for CYP2C8 by Rüdiger Kaspera, a collaborator in University of Washington at Seattle, and OATP1B1 by me. It is worth noting that 24 stable cells lines (including empty vector), 10 for MRP2, 4 for BCRP and 9 for OATP1B1 representing polymorphisms and haplotypes found in rhabdomyolysis cases were successfully constructed. However, despite repeated efforts we were not able to measure efflux function successfully.

In a published case-control analysis[1] of polymorphisms we found in CYP2C8, OATP1B1, UGT1A1 and UGT1A3 genes, the V174A SNP was identified to be significantly associated with CER induced rhabdomyolysis with an odds ratio of 1.89 (95%, CI:1.40-2.56). The V174A SNP along with R57Q, P155T, FS and OATP1B1*15 and N1 haplotypes were shown in *in vitro* assays to be associated with significant reduction (P>0.001) in CER uptake (32%, 17.9%, 72%, 3.4%, 2.1% and 5.7% of reference, respectively). This reduction-function in OATP1B1 mediated uptake of CER was further confirmed by *in vitro* ES uptake. Since CER induced rhabdomyolysis, and more generally, statin induced rhabdomyolysis is associated with elevated plasma concentration of statins and their active metabolites, the reduction in uptake of satins due to OATP1B1 leads to elevated plasma concentration. Although it is difficult to say

whether the *in vitro* data would translate to *in vivo,* based on other available *in vivo* data one can certainly generate some plausible and testable hypothesizes.

As discussed in Chapter 3, the V174A polymorphism has been associated with significant risk factor for simvastatin induced rhabdomyolysis. Furthermore, this polymorphism has shown to increase plasma concentration of simvastatin and pravastatin. The *in vitro* cellular uptake data reported for this polymorphism is comparable to ours in terms of polymorphism associated reduction in CER uptake. Based on such data, I believe that one can hypothesize that the R57Q and FS polymorphisms as well as OATP1B1*15 and N1 haplotypes can certainly increase the plasma concentration of CER and other statins such that carriers of these variations would be at a significant risk for rhabdomyolysis.

We also studied the occurrence of CER induce rhabdomyolysis by studying the contribution of other concomitant drugs that could lead to elevated plasma CER level. As discussed in Chapter 4, Clopidogrel was epidemiologically the only drug, both in our case-control study as well as a case-control analysis using FDA's AERS data, associated with CER induce rhabdomyolysis. The *in vitro* analysis of clopidogrel-CER interaction proved to be a significant interaction and one worthy of further clinical study.

In this dissertation, the two main mechanisms of adverse event occurrence namely drug-gene and drug-drug interaction were studied to identify the cause of CER induced rhabdomyolysis. The Ph.D. that I am about to receive in regards to this work carries the title of Pharmaceutical Sciences and

Pharmacogenetics. The major question is how does the work presented in this thesis contribute to the advancement of the field in such a way that can benefit the public health? The short, cautious and very orthodox answer to this (and most other pharmacogenetics studies of this caliber) is that further testing of these findings are needed. While I agree that certain aspects of this study need further research, I am more cavalier in translatability of some of our data.

In a recent publication by Krishna Prasad[2] the obstacles that prevent the use of pharmacogenetics data are well described (Table 1). Although I acknowledge and agree with the presented challenges as stated by Krishna Prasad, I believe that we can work on clinical application of our findings without filling all the necessary steps.

Table 2.[2] Possible factors limiting pharmacogenetic testing.

A. Factors intrinsic to PGx information
   1. Consistency of observations/results?!!
   2. Size of population affected
   3. Complexity of the test
   4. Impact on practice?!!
      - Regulatory issues
      - Prescription impact
      - Availability & commercialization of tests
      - Socio economic impact
   5. Best way to convey / enforce the information

B. Approach to Clinical trials—"A MINDSET"

C. Factors extrinsic to PGx Information
   1. Awareness of Medical profession/ physician workforce
   2. PGx education at professional education (medical/pharmacy)
   3. integration of PG knowledge into practice,
   4. Effect of PGx on healthcare.

Current medical practice relies on data generated from clinical trials that were themselves limited in size of the patients and controls as well as phenotypic characteristics. CER story is certainly one that shows that these trials are

inadequate in detecting significantly rare but dangerous adverse events. Our analysis, though limited, combined with what is already known about this class of drugs and OATP1B1 role can be employed to avert disasters through improving patient safety.

The OATP1B1 facts limited to statins so far are: First, we know that V174A polymorphism is associated with a reduction in OATP1B1 function such that it leads to elevated plasma levels of simvastatin and pravastatin consistently in *in vitro* and *in vivo* studies. Second, in the SEARCH trial this polymorphism was associated with simvastatin induced rhabdomyolysis. Lastly, in our case-control analysis this polymorphism is associated with CER induced rhabdomyolysis. Although this finding is not backed by clinical trial I believe that there is enough data to place a safety warning on this polymorphism in the package insert of statins. At the very least the genotype of OATP1B1 polymorphism should be considered in statin therapy. Although this polymorphism is not tested in the context of therapy initiation, closer monitoring of patients should be warranted which should include drug level monitoring.

Furthermore I believe that R57Q, P155T, FS and OATP1B1*15 and N1 haplotypes which are functionally far more deficient relative to V174A polymorphism as shown in *in vitro* data should be genotyped in patients. This information can be used as a basis for further and closer monitoring of patients including measuring statin plasma levels. Based on current standards of clinical trials which need statistical significance for effect in a comparator population, evidence linking rare polymorphisms such R57Q and FS to rhabdomyolysis will

never achieve statistical significance. Instead of cataloging this data in a library I believe the use of this data can be not only be cost effective but can and will improve patient safety. I am of the belief that when it comes to patient care it is better to be “complete and safe rather than sorry”.

The knowledge of OATP1B1 genotype can be even more important when integrated with information regarding a potential drug-drug interaction. Given that clopidogrel-CER interaction is real, then a patient on CER, carrying a reduction in function polymorphism of OATP1B1 such as V174A while on clopidogrel should be particularly at risk of CER induced rhabdomyolysis. Although the concomitant use of these drugs is often necessary combining the information available on drug-drug interaction with that of the drug-gene interaction can, despite lack of clinical trial support, again be used as a caution and recommendation for careful monitoring of patients. This would include measuring plasma drug level. I think our threshold for adopting safety related pharmacogenetics information such as ours that is consistent and plausible should be lower. By this I do not mean to imply proceeding without lack of sufficient clinical data. On the contrary data is the guiding light of science but safety of patients is of paramount.

Muscle, which is the target of statin associated rhabdomyolysis, was not discussed in this dissertation. Since the exact mechanism of rhabdomyolysis is not known and statin induced rhabdomyolysis is linked to drug level, a pharmacokinetic approach was deemed more relevant. However, data published in 2010 showed the expression of OATP2B1 transporter in sarcolemmal

membrane of human skeletal muscle fiber to mediate exposure and toxicity of statins including CER.[3] A closer look at this and other transporters in the muscle may identify other pathways and risk factors with statin induced rhabdomyolysis.

References

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2. Prasad, K. Role of regulatory agencies in translating pharmacogenetics to the clinics. *Clinical Cases in Mineral and Bone Metabolism* **6**, 29-34 (2009).
3. Knauer, M.J. et al. Human skeletal muscle drug transporters determine local exposure and toxicity of statins. *Circulation Research* **106**, 297-306 (2010).

APPENDIX

Appendix 1

Primers and PCR conditions used for sequencing CYP2C8, UGT1A3, ALXO1B1, ABCC2, ABCG2 and HMGCoA Reductase Genes

Region	Primer sequence for *CYP2C8* gene P1	P2	Amplicon Size (bp)	PCR Condition
Pro,3'UTR	GCACCAGGACCACAAAAGGT	GCCAGCTGTGGTGTAAGTGG	1040	P60 1X35
Ex 1, 3'UTR	CACACACTAAATTAGCAGGGAGTG	TTCAGAGGGAGTATTTTGCTTTACAA	379	P60 1X35
Ex 2, 3	GGCACATCACAGGCCATCTA	CCTACACCCTATGAACCAACACA	890	P60 1X35
Ex 4	GGTCCCCAACTTTTCTCTTCC	ACCCCTTGCACTTCTGATGG	795	P60 1X35
Ex 5	TTTCCCTTCAAAATGGACATGA	TGAAACCTTTCTTCTGTTCCACA	743	P60 1X35
Ex 6	ATGAAGGCCATTGCCAGAAG	TGCTGGCTCTCCTTACCACA	846	P60 1X35
Ex 7	TGGCTGGTTGTACTTCTGGAC	TGCATGAACATGTTAAGTCTTTCC	651	P60 1X35
Ex 8	CTTCAAATGTGATTGGAAAGCTC	GGAGCTCTTGGGTGCCTTAG	623	P60 1X35
Ex 9, 5'UTR	ATGGAAACTCAAAATGGCAAAA	TCCTCACCCTCTTCTCCTTTG	1000	P60 1X35
		Total bases sequenced>>>	**6967**	

Region	Primer sequence for *UGT1A1* gene P1	P2	Amplicon Size (bp)	PCR Condition
Pro, 5'UTR,Ex 1	GCCTTCTGTTTAATTTCTGGAAAAG	GGGAACAGCCAGACAAAAGC	765	Q56 1X35
Ex 1-1	TGCTGTGTTCACTCAAGAATGTG	CCATGAGCTCCTTGTTGTGC	633	Betaine P601X35
Ex 1-2	GATTCTTTCCTGCAGCGTGT	TGCCAAAGACAGACTCAAACC	720	P60 1X35
Ex 2	CTGGATTTTGCATCTCAAGGA	GGCAGGGAAAAGCCAAATCT	483	P60 1X35
Ex3, 4	CACGTAGTGCATACACCCTTGTAA	TGAAACAACGCTATTAAATGCTACG	898	P60 1X35
Ex 5, 3'UTR	AAGTTTGGAAAATCTGGTAGTCTTC	TTTAAAGCACTCTGGGGCTG	542	Betaine P601X35
Ex 5, 3'UTR	TGGCTACCGGAAATGCTTGGGGA	TGCATGCACACGCAATGAAGGCG	946	P60 1X35
		Total bases sequenced>>>	**6967**	

Region	Primer sequence for *UGT1A3* gene P1	P2	Amplicon Size (bp)	PCR Condition
Pro	GCTGTGCCTGCTACATTTGC	AGACTGGAGCCTTCGGCATT	827	P60 1x35
Ex 1-1	GGAGGGCACTCTGTCTTCCA	TCAGGGCCTCATTATGTAGTAGCTC	544	Betaine P601x35
Ex 1-2	CAGTGGTCCTCACCCCAGAG	TGACAAGGAGAAGCAGAAATGAA	822	P60 1x35
		Total bases sequenced>>>	**2193**	

Region	Primer sequences for *SLCO1B1* gene P1	P2	Amplicon Size (bp)	PCR Condition
Pro	TTTTGCTGCAACCATATCAACA	CCTTACTTTTGGGAATGGCTTTT	730	P60 1X35
Pro + Ex1	CCAGGTGGTATCTCCAGTCTCC	GCTCTGTGTCTTCCACACGC	598	P60 1X35
5'UTR, Ex 2	CAGCATTGACCTAGCAGAGTGG	TTCCTAAATATGTCGTGATCAATCC	586	P60 1X35
Ex 3	ACCAAATTAGAAATGATGCTTTATCAG	AAGTATGACATGGCGTTAGTTTGC	677	P60 1X35
Ex 4	TGTCTTGGACTCTATTTGCATCC	CACTTAGTGGGTATCTTCTCAAAAGG	348	P60 1X35
Ex 5	TTTACCCATCACATCTCTTAAAACACA	CTGTGTTGTTAATGGGCGAAC	468	P60 1X35
Ex 6, Ex 7	GAGTCCATTAGACCCTTTTCCTTT	TCAACATCCAAGCCACCATC	1017	P60 1X35
	Seq primer for Ex 6,7 Amplicon>>>>	TCAAAAGTAGACAAAGGGAAAGTGA		
Ex 8	TTCATACCATTATTTCCCTGAACC	TTTGAACTCTCCCAGTTTAGACCA	634	P60 1X35
Ex 9	AAGCTGTGAACAGCCTGTGG	TGAGTCTTGATTTCAAAGTCCATAAA	686	P60 1X35
Ex 10	GACTGTTGAGGGGTGGGAAG	TGCTATACATGGTAATCATGGAAAAGT	1139	P60 1X35
Ex 11	TGCCATTTCGTCATCATCAA	GGCTTTAGTCAAATGAGGTGCTT	654	P60 1X35
Ex 12	TTCCTCCTCAGGGCATGTCT	TTGTCAGTCTGTGTCTTCAGATTCTT	834	P60 1X35
Ex 13	TGATGCATTGAATAAATAAGGGAATAA	CGAATTCTCCTTTAGGTCCATCA	513	P60 1X35
Ex 14	TCCCATATTAACCAACATAACTTCCA	GACATGAGGAGAGTTTTGGAAACA	779	P60 1X35
Ex 15, 3'UTR	TTTCTATGGCTTCATAACCCTATTACA	CTGAATCAATGCAATGCTGTTT	465	P60 1X35
Ex 15, 3'UTR	TGGGGCAGATAGTGAAACACA	GGGCCCACTGGAAACTTAAC	688	P60 1X35
		Total bases sequenced>>>	**10816**	

Region	Primer sequences for *ABCC2* gene P1	P2	Amplicon Size (bp)	PCR Condition
Ex1	TGCATCTAGGCAAGGTTAACGA	TGACCACCCTAAGTTAACTAACTACCA	283	P60 1X35
Ex2	AATGCATGTATGCAACAATCC	GCACCAAGGAATTAGAGTTCA	256	P60 1X35
Ex3	TATCCATCACCGGAAACCAT	AAAAGACAGATGACCTTGGAATTA	315	P60 1X35
Ex4	CTGACATCCTTCTCCCCTCA	TTAGAGGTTGCCCCATGGTA	260	P60 1X35
Ex5	ATCATAGGCTTTAATCACAAG	ATGAGATGGGCATATTTAGAA	231	P60 1X35
Ex6	GTCCCATGAAGTTCCTGTCTC	TGACACTTTCAGAGGAGTGAGAG	176	P60 1X35
Ex7	AGTGGTGGAGATAGCCTCTGA	ATCGCCATGATGCTGATGTA	370	P60 1X35
Ex8	CTGCTCAGGCCAGTAACAGA	AGCTCCTGCTTGCATACACA	305	P60 1X35
Ex9	GGCTTTGGACAATTCTGGTC	TCACATTTTCCTCAACCTTGG	390	P60 1X35
Ex10	CATATGGAGCACATCCTTCC	AAGTCTTCCACCAGCTTTGC	478	P60 1X35
Ex11	AGCCACAAAGTAGCAGTGAGG	TTACCCACAGAGAGCCACCTA	198	P60 1X35
Ex12	GGCAATCATGTGAGCTGTATT	AGATGCCAGCTAGTCTATCAA	271	P60 1X35
Ex13	AATGCTGCTTGGTCCCTTTTA	TTACCAGCAAAAGTTAGACATGG	301	P60 1X35
Ex14	TTAGGAGATGCCAGCTGTGG	TCCACACTCTCTCTCAGTTTGC	304	P60 1X35
Ex15	TCCTAGGAGCTGATGGAGAAA	GCTCATCTCTCCAGGGGTTT	317	P60 1X35
Ex16	CCAGACTTCATGGAGACTCAGA	AGTGAGGCAAAAGCACGAAG	287	P60 1X35
Ex17	AGTCATCCTGATGCACAGTTATT	CCCCTGTGTAGTTCTTCACCAC	389	P60 1X35
Ex18_19	TCTTCCTTTTACCCCTCCCTA	GGATACTGAGCAGTTCAGGAATTA	649	P60 1X35
Ex20	GTACATCTGGGATCCCTTGC	CCATGGTGTTAACCCTCACA	346	P60 1X35
Ex21	ACCATGGACACTCCAAGCTC	TTGCTCCTGTAAGTATGCGTTC	424	P60 1X35
Ex22_23	GCTACCTGCTACCCATGCTC	CAGTGTTGTCTAGGGGGACA	730	P60 1X35
Ex24	CTGGGAACACACAGAATCCA	GAGAGAAGATTCTCAGGAAAGAGG	341	P60 1X35
Ex25	CATCAAGGGGAAGAATGCTG	TGTGTGTGGCCAGAGTGAAT	414	P60 1X35
Ex26	TGCGGCCCGATCAAGTCAAAC	GGGTTTAAGAAGGAATCAAGAGC	340	P60 1X35
Ex27	TGTCCTTGTGGTTTGAGTGG	ATGTTGTTTTTGCCTTGTTCC	220	Q601x35
Ex28	TGCAGAGGCCATTAGGTAGC	CTTTCCCTCCATCCAAATGA	380	P60 1X35
Ex29	GCCAGTCACTGCCTCTTACC	CCGAGTAGACCGTGGAATTG	383	P60 1X35
Ex30	CTCAGGCCAGTCCTATCCAC	GGAATCAGACCTGGATGAAAA	380	P60 1X35
Ex31	CTGATCTGGAACATGAAAATGG	CCTTCTGCCATCAGGTGTTT	373	P60 1X35
Ex32	GCCTAGACTTGAGATGCTGCT	CAGAAATGAAAGGCAAAGGA	473	P60 1X35
		Total bases sequenced>>>	**10584**	

Region	Primer sequence for *ABCG2* gene P1	P2	Amplicon Size (bp)	PCR Condition
Ex1 noncoding				
Ex2	GTATGGGCCATTCATTGGAAAT	TGAGGTTCACTGTAGGTAAA	415	P60 1X35
Ex3	AAGAGTTGGTTTGTGCTTGTGTTC	GTGGCTTTTAAAAGCACATT	254	P60 1X35
Ex4	TTGGATTCAAAGTAGCCATGAGA	TGGAAGCACATTGAACTATCAGC	311	Q601X35
Ex5	GCAGAACTGCAGGTTCATCATT	TCCAGGTTAATTTCCACGTTCA	468	P60 1X35
Ex6	GCACACGTTAGGAGCTCACC	CCCTACACCCTCATCACAGACA	438	P60 1X35
Ex7	GACAAAGTCAGGCTGAACTAGAGC	TGGAACAAACACATTTTGAAGTGA	405	P60 1X35
Ex8	CCTCCCTCACCCTCTGAAAG	GCCACATTTTAATTAGCCACCA	267	P60 1X35
Ex9	GCATCCAAGAAAGGGTTCACA	AATTGGAAGGGTGGGTAGAAGA	434	P60 1X35
Ex10	ATTTTCTCCCAGGGACGTTG	CTGACTCATCCTACCCTCAAT	371	P60 1X35
Ex11	GCTTCCCTGTTCCAACCAGA	GTAATCCTCCGGATCCCATC	340	P60 1X35
Ex12	GCTTGTTCAACCCCTGCTGT	TGTTTCCTTATCTCATGGTTTGG	357	P60 1X35
Ex13	TGCACAATAAGCAATCCCAAA	GATGGCCTTAAGTAAAGCAGAGC	358	P60 1X35
Ex14	TGCACATGCAGAGGAGAAGA	GGGAGTTTCTGGATGGGAGA	386	P60 1X35
Ex15	TGGTTTGGTGAGACAAAGACTG	GCCCAGTAGCCTTTATACATCACA	295	P60 1X35
Ex16	AAAGGCTTGGTTCAATTTTAGGC	TGCAACAGTGTGATGGCAAG	367	P60 1X35
		Total bases sequenced>>>	**6062**	

Region	Primer sequence for *HMGCR* gene P1	P2	Amplicon Size (bp)	PCR Condition
Ex1	ACCCTGCGTTTTCCCTCAC	AGCTGCCACACCGACAAAG	678	P60 1X35
Ex2	TCAAATAGTCATATGTATGGGACA	AGCATTTCCAGTGCATTCCA	487	P60 1X35
Ex3_4	CAGTGGTTTGATTAAAAGTTGGATTT	ATTTCCCCTTATTTTCTTAACTTTTGA	784	P60 1X35
Ex5	GCTTTGAGGATGCCAAAGAAA	TGGAGACCAGTCTTGGGACTT	404	P60 1X35
Ex6	AAGCCATAGGATGCTCTAACATTT	TGCTGCTAAGTTTCCACCAA	359	P60 1X35
Ex7_8	TCCACCTGCAGGAAGACAAA	GGAAGAAGGGGGTGCAATATC	544	P60 1X35
Ex9	GGCTGTCCAGTTGCTAGGGATA	TGACAGCCAGAAGGAGAGCTAA	421	P60 1X35
Ex10_11	TCCCTCTGGCAGTTTTATCTCTC	TGAAAAATGCCACATTGGAGT	718	P60 1X35
Ex12	GCTTAGCATTGTTTGTATTAAATGGTT	TGCACAAAATTTTATCTCAGCTTG	620	P60 1X35
Ex13_14	CAAGCTGAGATAAAATTTTGTGCATT	TTCTGTTATGTGCTCTTTAGCAGGT	656	P60 1X35
Ex15	GCATAATCAACACAAATGATAACCA	TCAGGATATGTGTAATGCCAAGTC	690	P60 1X35
Ex16	ACAAAGAACTTGGTTTTCATACCG	ACACAGACAGATGGGCAAGG	558	P60 1X35
Ex17_18	TGGGGCCACATCTAAACTTTG	CATGCTTATTTGGACACAATGGA	652	P60 1X35
Ex19_20	TGTCAGGTGTGGCTGTCAAG	CCAAAAACCAAGTGGCTGTC	654	P60 1X35
		Total bases sequenced>>>	**8225**	

Appendix 2

		Cases†	Controls†	Gemfibrozil Users Included			Gemfibrozil Users Excluded		
	Restrictio	n=72	n=287	OR	95% CI	P	OR	95% CI	P
Alendronate	-	8%	7%	1.3	0.5-3.4	0.653	1	0.3-3.1	0.986
Allopurinol	-	6%	2%	3.5	0.8-14.9	0.09	2	0.2-20.0	0.554
Amitriptyline	-	6%	2%	2	0.5-9.0	0.343	2	0.3-12.6	0.465
Amlodipine	HTN	28%	15%	2.3	0.9-5.5	0.074	1.9	0.7-5.0	0.199
Aspirin	CVD	60%	71%	0.6	0.3-1.3	0.179	0.8	0.3-1.9	0.579
Atenolol	HTN	24%	11%	1.9	0.7-4.8	0.208	2.2	0.8-5.9	0.125
Celecoxib	-	7%	7%	1.3	0.4-3.8	0.683	1.6	0.5-4.9	0.384
Clopidogrel	CVD	50%	8%	29.6	6.1-143	$2.4x10^{-5}$	47.8	12.5-182	$1.6x10^{-6}$
Digoxin	AF, CHF	35%	26%	1.3	0.4-4.9	0.688	0.3	0.1-1.7	0.169
Diltiazem	HTN	10%	6%	2.2	0.5-9.3	0.276	2.4	0.5-10.8	0.254
Enalapril	HTN	4%	5%	1.2	0.2-7.0	0.861	1.4	0.3-8.3	0.689
Furosemide	CHF	20%	42%	0.7	0.1-3.2	0.629	0.7	0.0-9.5	0.755
Gemfibrozil	-	32%	0%	inf	25.0-inf‡	0	-	-	-
Glimepiride	DM	21%	12%	3.4	0.5-22.5	0.206	2	0.3-14.6	0.508
Glipizide	DM	29%	19%	3.7	0.7-19.7	0.12	2.4	0.3-22.9	0.44
Glyburide	DM	36%	41%	0.2	0.0-1.1	0.061	0.4	0.1-2.6	0.349
Fluoxymesterone	-	8%	0%	inf	4.4-inf‡	0.0002	inf	5.8-inf‡	0.0001
HCTZ	HTN	24%	21%	1.2	0.5-2.7	0.71	1.1	0.5-2.8	0.799
Irbesartan	HTN	6%	1%	4.8	0.8-29.8	0.094	4.4	0.6-34.6	0.156
Lansoprazole	-	11%	3%	5.7	1.3-24.0	0.018	4.7	1.3-17.5	0.02
Levothyroxine	-	26%	20%	1.7	0.9-3.2	0.1	1.5	0.7-3.2	0.251
Lisinopril	HTN	12%	19%	0.9	0.3-2.9	0.861	0.7	0.2-2.6	0.638
Losartan	HTN	6%	6%	0.9	0.2-4.2	0.905	0.9	0.2-5.2	0.925
Metformin	DM	29%	31%	0.6	0.1-3.3	0.553	0.2	0.0-3.3	0.242
Metoprolol	HTN	24%	18%	1.4	0.6-3.1	0.476	1.3	0.5-3.2	0.556
Montelukast	Asthma	9%	3%	2.8	0.3-25.0	0.36	inf	0-176‡	1
Omeprazole	-	4%	7%	0.5	0.2-1.6	0.263	0.6	0.1-2.2	0.404
Pioglitazone	DM	7%	0%	inf	0.0-inf‡	0.836	inf	0.1-inf‡	0.543
Propoxyphene	-	8%	2%	4.8	1.7-13.9	0.003	2.7	0.6-11.2	0.174
Ramipril	HTN	4%	2%	0.9	0.2-3.8	0.848	1.1	0.2-4.5	0.938
Ranitidine	-	7%	3%	2	0.6-7.0	0.295	1.3	0.3-5.9	0.736
Rofecoxib	-	13%	4%	4.9	1.1-20.8	0.033	5.1	1.2-22.1	0.031
Rosiglitazone	DM	29%	5%	19.8	1.0-402	0.052	26.2	0.7-989	0.078
Triamterene	HTN	8%	5%	1.4	0.3-5.9	0.657	0.8	0.2-4.7	0.851
Valsartan	HTN	6%	5%	1.8	0.3-9.4	0.501	1.2	0.2-7.3	0.849
Verapamil	HTN	4%	4%	1.3	0.4-4.6	0.655	1.1	0.2-6.6	0.904
Warfarin	AF	29%	39%	0.5	0.1-2.4	0.372	0.2	0.0-1.2	0.08

Medication Use and Association with Rhabdomyolysis (Source - James Floyd).

AF = atrial fibrillation, CHF = congestive heart failure, CVD = cardiovascular disease, DM = diabetes mellitus, HTN = hypertension, inf = infinity.

† Prevalence of medication use in the restricted population. ‡ Exact methods used to obtain 95% confidence intervals and P values because there were no medication users in the control group.

Appendix 3

	Cerivastatin Users		Atorvastatin Users							
	n=594		n=75		Gemfibrozil Users Included			Gemfibrozil Users Excluded		
	Count	Percent	Count	Percent	OR	95% CI	P	OR	95% CI	P
Clopidogrel	101	17%	0	0%	inf	2.6-inf	0.0003	inf	5.2-inf	0
Clopidogrel†	41	29%	0	0%	Inf	1.2-inf	0.03	Inf	2.1-inf	0.004
Gemfibrozil	284	48%	5	7%	24.6	8.1-74.5	$1.1x10^{-8}$	-	-	-
Fluoxymesterone	0	0%	0	0%	-	-	-	-	-	-
Lansoprazole	21	4%	1	1%	3.3	0.3-37.0	0.336	inf	0.4-inf	0.334
Propoxyphene	9	2%	1	1%	1	0.2-4.9	1	2.3	0.4-12.0	0.316
Rofecoxib	5	1%	1	1%	0.2	0.0-1.6	0.147	0.5	0.1-2.9	0.412
Rosiglitazone	14	2%	1	1%	0.7	0.1-6.1	0.772	0.6	0.1-7.3	0.715

Medication Use in Rhabdomyolysis Cases from Food and Drug Administration Adverse Event Reporting System (source - James Floyd)

†Restricted to aspirin users only.

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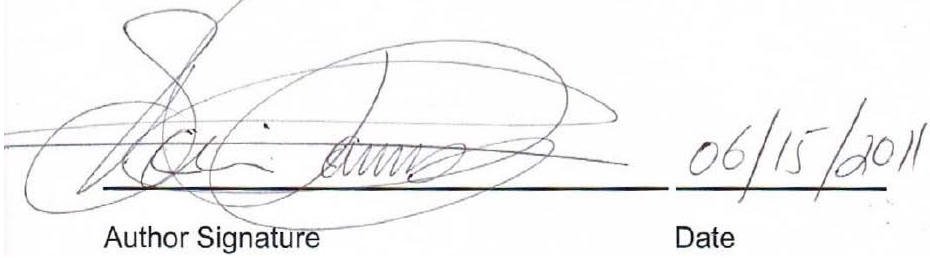

06/15/2011

Author Signature Date