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Cerivastatin *in vitro* metabolism by CYP2C8 variants found in patients experiencing rhabdomyolysis

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Abstract

Cerivastatin, an HMG-CoA reductase inhibitor withdrawn from the market due to serious adverse effects, is metabolized primarily by CYP2C8. The occurrence of associated myotoxicity and rhabdomyolysis were attributed to altered cerivastatin pharmacokinetics due to gemfibrozil-inhibition or genetic variations in CYP2C8 and drug transporters involved in cerivastatin clearance. However, the effect of CYP2C8 genetic variation on cerivastatin metabolism has not been fully elucidated. In this study, patients (n=126) with confirmed cases of rhabdomyolysis following cerivastatin administration had their CYP2C8 gene resequenced and the metabolism of cerivastatin by the discovered CYP2C8 variants was investigated. In this unique patient population, twelve novel SNPs were discovered of which six were exclusively found in patients not using gemfibrozil. Three rare exonic variants resulted in amino acid substitutions and a frame shift deletion (V472fsL494 generating a defective mostly heme-free CYP2C8 protein). A particular promoter located deletion (-635_-634delTA) was tightly linked to CYP2C8*3. Seven exonic variants were heterologously expressed in *E.coli* to assess their influence on cerivastatin metabolism. Recombinant CYP2C8.3 and CYP2C8.4 displayed an increase in cerivastatin metabolic clearance up to six fold compared to the wild type enzyme. Similarly, an independent sample of microsomes from human livers carrying the CYP2C8*3 and CYP2C8*4 alleles exhibited a 2 to 14-fold increase in normalized cerivastatin intrinsic clearance, compared to microsomes from livers carrying only the wild type allele. This gain or loss of catalytic function

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Conflict of interest: Currently, Psaty serves on a data safety and monitoring committee for a clinical trial of a device funded by Zoll-Lifecor, and it is unrelated to this work on cerivastatin. In 2002-3, Psaty served as an expert witness for plaintiffs who had used cerivastatin.

Supplementary digital content (SDC) is available online.

could certainly alter cerivastatin pharmacokinetics and may influence, at least in part, susceptibility to the development of myotoxicity.

Keywords

pharmacogenetics; CYP2C8 variants; *CYP2C8*4*; *CYP2C8*3*; promotor linkage disequilibrium; non-functional variants

1. Introduction

The HMG-CoA reductase inhibitors, or statins, are well established cholesterol lowering drugs commonly used due to their effectiveness in reducing the risk of cardiovascular disease [1]. Cerivastatin, a synthetic statin, was introduced into clinical practice in 1997, but was withdrawn shortly afterwards in 2001 due to an unacceptable high risk of myotoxicity. Statistical evaluations reported a 16-80 times higher rate of fatal rhabdomyolysis cases (total 31) for cerivastatin compared to other statins [2,3].

Reasons behind the higher occurrence of myotoxicity cases related to cerivastatin administration are complex and likely involve genetic variations in oxidative metabolism mediated by cytochrome P450 (CYP) enzymes as well as drug uptake transporters. Metabolic drug-drug interactions involving cerivastatin were initially believed to be less likely because of a desirable dual oxidative metabolism pathway mediated by both CYP2C8 and CYP3A4 [4,5]. CYP2C8 accounts for up to 60% of cerivastatin's oxidation to 6-hydroxyl- (M-23) and O-desmethyl- (M-1) cerivastatin, while CYP3A4 contributes to about 40% [1,6] of mostly M-1 formation (figure 1). Additionally, UDP-glucuronosyl transferase facilitates the generation of a lactone after glucuronidation, and compared to the free acid, cerivastatin lactone is metabolized faster by CYP-enzymes [7] (figure 1). However, glucuronide formation is considered to be a minor clearance pathway due to a 10-fold lower rate of glucuronidation compared to CYP-mediated oxidation of the free acid [1,7].

Changes in oxidative metabolism can be caused by CYP inhibition, induction or genetic polymorphisms. Combination therapies pairing cerivastatin with fibrates such as gemfibrozil, taken by 12 patients of the 31 death cases reported to the FDA [2], demonstrated a significant increase in cerivastatin plasma concentrations. Gemfibrozil not only inhibits CYP2C8 [8] and UGT-mediated glucuronidation of cerivastatin, but its glucuronide (gemfibrozil glucuronide) is also an effective mechanism based inhibitor of CYP2C8 [9,10]. Nonetheless, co-administration of gemfibrozil provides only a partial explanation to the increased myotoxicity observed with cerivastatin.

At least 17 genetic variants in *CYP2C8* with variable effects on drug metabolism have been described (<http://www.cypalleles.ki.se/cyp2c8.htm>). *CYP2C8* variants also seem to be ethnic specific [11]. For example, *CYP2C8*2* and *CYP2C8*5* are exclusively found in African-Americans and Japanese, respectively. *CYP2C8*3* has been detected in Caucasians and African-Americans (at low frequency) [12], and *CYP2C8*4* is found only in Caucasians [13]. Other rare variants have been reported in a Japanese population [14]. Of relevance, a rare frame-shift deletion *CYP2C8*5*, which introduces an early stop codon and results in complete loss of enzyme function, was discovered in a single Japanese patient suffering from rhabdomyolysis and was correlated to high cerivastatin blood concentration [15].

The aim of this study was to assess the functional consequences of genetic variation in *CYP2C8* on the oxidative metabolism of cerivastatin. For this purpose, the *CYP2C8* gene of a unique population of patients that had been treated with cerivastatin and subsequently

suffered from rhabdomyolysis was re-sequenced in an effort to determine both the frequency of known CYP2C8 variants and to identify novel variants. CYP2C8 wild type and coding region variants were subsequently engineered and corresponding enzymes were evaluated for their metabolic activity towards cerivastatin. To corroborate these results, the contribution of common CYP2C8 variants to cerivastatin metabolism was measured in microsomes derived from human livers with pre-determined CYP2C8 genotypes.

2. Experimental section

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated, and used without further purification. Cerivastatin sodium salt ((3R,5S,6E)-7-[4-(4-Fluorophenyl)-5-(methoxymethyl)-2,6-bis(1-methylethyl)-3-pyridinyl]-3,5-dihydroxy-6-heptenoic Acid Sodium Salt), hydroxy cerivastatin (M23, 6-[(1S)-2-hydroxy-1-methylethyl]-metabolite), desmethyl cerivastatin (M1, 5-(hydroxyl-methyl)-metabolite), and desmethyl hydroxy cerivastatin (M24, 5-(hydroxymethyl)-6-[(1S)-2-hydroxy-1-methylethyl]-metabolite) sodium salt were purchased from Toronto Research Chemicals (North York, ON, CA). Fluvastatin was a gift from Professor W. Trager (University of Washington), L- α -dilauryl-sn-glycero-3-phosphocholine (DLPC) was from Avanti Polar Lipids Inc., (Alabaster, AL, USA). Human liver samples were obtained from the University of Washington School of Pharmacy Human Tissue Bank (Seattle, WA) [16,17]. Emulgen 911 was a gift from KAO chemicals (Tokyo, JP), Ni-NTA resin was from Qiagen (Valencia, CA, USA), isopropyl- β -D-1-thiogalactopyranoside (IPTG) was from Gold Biotechnologies (St. Louis, MO), the pGRO7 plasmid was from Takara Bio Inc. (Otsu, Shiga, JP, through Fisher, Rockville, MD). CYP Supersomes[®] were purchased from BD Bioscience (San Jose, CA).

Patient selection, consent and DNA sequencing

The study recruited subjects with cerivastatin-associated rhabdomyolysis through attorneys whose plaintiffs had settled their cases with the manufacturer (see supplementary digital content (SDC), p. 3 <http://links.lww.com/FPC/A201>). Participating subjects provided written consent to obtain copies of their medical records from attorneys, physicians, 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 normal laboratory values. Subjects who would have likely met the definition of rhabdomyolysis had they been tested when their symptoms were acute were also included in the analysis after consultation with a physician (5 of 126). 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 (see SDC for detailed methods, p. 3.). From these, 118 were Caucasian, 4 African American, 1 Asian, and 3 of unknown race. Of the 126 patients, 51 were non-users of gemfibrozil, 46 of these were Caucasian and 2 African American. Asians and subjects of unknown race were not used in data evaluation.

Expression of cytochrome P450 reductase, cytochrome b5 and CYP2C8 wild type and variants

Rat cytochrome P450 reductase and cytochrome b5 were expressed using previously established protocols [18,19]. CYP2C8.1 (wild type), which was modified as described earlier [20], was used as a template to introduce the novel variants of CYP2C8 using the site directed mutagenesis kit Quikchange (Stratagene, La Jolla, CA) and designed primers to mutate or delete the relevant nucleotides (see SDC, table S3). CYP2C8 protein was expressed as previously described [20]. P450 CO difference spectra, gel electrophoresis,

pyridine hemochromogen analysis and Lowry protein determination assay were used to evaluate CYP-enzyme quality and quantity [21,22].

Metabolism of cerivastatin by recombinant CYP2C8, CYP3A4, and Human Liver Microsomes

DLPC micelles (2.5 mM) were prepared by repeated sonication. CYP2C8 was reconstituted in a 1:2:1 ratio with cytochrome P450 reductase and cytochrome b5 [23], respectively, and 100 μ M DLPC micelles for 20 min on ice. The mixture was diluted with potassium phosphate buffer (100 mM KPi, pH 7.4) to reach final assay concentration of 100 pmol/mL for recombinant CYP2C8.1, M426V, N56S, A471C, 50 pmol/mL for CYP2C8.2, 5 pmol/mL for CYP2C8.3, and 20 pmol/mL for CYP2C8.4. $V_{\max}/K_m$ determination was performed under linear conditions with respect to time and protein. Human liver microsomal protein (0.1–0.4 mg/mL), CYP3A4 and CYP2C8 supersomes (20 pmol/mL), or reconstituted enzymes were diluted in KPi buffer (100 mM, pH 7.4), supplemented with cerivastatin sodium (in buffer, final concentration 0.5–100 μ M) and preincubated for 5 min in a shaking water bath set at 37° C. The reaction was initiated with NADPH (1 mM final concentration) and allowed to proceed for 5 min. Reactions were quenched by adding ice-cold 10% trichloroacetic acid (10 μ L), vortexed and 10 μ L of the internal standard solution (2.5 μ M fluvastatin) was added. Samples were vortexed again and centrifuged for 10 min at 3,500 rpm. Calibration standards were performed under assay conditions with heat inactivated recombinant CYP2C8 or microsomes. Lactone formation was not observed in incubations with liver microsomes under conditions used.

To determine apparent K_m and $V_{\max}$ values for metabolite formation, liver microsomes as well as CYP enzyme preparations were incubated with the following cerivastatin concentrations in duplicate: 0, 0.5, 1, 2, 5, 10, 20, 50, 100 μ M. Heat-denatured microsomes and incubation mixtures without NADPH served as negative controls.

Quantification of cerivastatin and its metabolites

Supernatants were transferred into shallow 96 well plates (Nunc, Roskilde, DK) and kept in the temperature-controlled autosampler at 4°C until analysis. Cerivastatin, and metabolites, were quantified using HPLC in tandem with an electrospray-Mass Spectrometer using a Hewlett-Packard series HP1100 liquid chromatograph, a G1367A autosampler, a G1312A binary pump, a G1322A degasser and a G1948A mass selective detector (all components Hewlett-Packard, Palo Alto, CA). The HPLC/MS system was connected to ChemStation software version 10.02 (Hewlett-Packard) which was used for data analysis. The mobile phase of the analytical column consisted of aqueous phase A: 10 mM ammonium acetate (pH 5.5) and organic phase B: 10 mM ammonium acetate in methanol. 20 μ L of sample was injected directly onto an Agilent Zorbax XDB C8-column (2.1 μ m, 5 cm), and analyzed using the following gradient: mobile phase B: 0–1 min, 30%; 1–2 min, 30 to 70%, 2–4 min 70 to 100%, 4–6.5 min 100%, 6.5–6.6 min 100–30%. The column was re-equilibrated at initial conditions for 1.4 min. The flow rate was 0.3 ml/min. The mass selective detector was adjusted to the following parameters (nomenclature according to the ChemStation software): nebulizer gas nitrogen 25 p.s.i.; drying gas nitrogen, flow 10.0 L/min; 350°C; capillary voltage (V_{cap} positive), 5000 V; capillary exit (fragmentor) voltage, 150V for fluvastatin and cerivastatin, 200V for M-1, and 250V for M-23 and cerivastatin lactone. Cerivastatin and metabolites were detected under ES positive ionization as [M+1]⁺ 460.2 (cerivastatin), 476.2 (M-23), 446.2 (M-1), 462.2 (desmethyl hydroxyl cerivastatin), 412.2 (fluvastatin), 442.2 (cerivastatin lactone). The dwell time for each ion was 95 ms.

The assay had the following specifications: limit of quantitation, 5 nM (cerivastatin alcohol, M-23, and desmethyl, M-1) or 10 nM (for desmethyl hydroxyl cerivastatin); linearity, 5 nM to 500 nM (r^2 0.990); intraday variability (tested for three concentrations, each $n=3$) 2–6%

with an accuracy of $\pm 2\%$, n was reduced to 2. All samples were analyzed within 24 h (max. duration between first and last sample).

Data analysis—Haplotypes were inferred by PHASE 2.0 [24,25] using selected tagSNPs [26] and novel SNPs of high abundance. Because of the uncertainty inherent in the estimation of haplotypes from unphased genotype data in unrelated individuals, subjects could have more than one possible haplotype pair. The probability associated with each haplotype pair was used as a weight in estimating minor allele frequency. Haplotypes observed at a frequency of less than 2.5 percent were grouped into a single rare haplotype category. Linkage disequilibrium was assessed using r^2 using the genome variation server website (<http://gvs.gs.washington.edu/GVS/>).

Apparent Michaelis-Menten constants K_m and V_{max} were derived after nonlinear regression analysis of the kinetic data using a simple enzyme kinetics model in SigmaPlot 2004 windows version 9.0 (Systat Software, Chicago, IL). Kinetic data is reported as the mean $\pm$ S.D. (computer generated). Statistical significance of median differences (Figure 4) was assessed using Mann-Whitney test (GraphPad Prism version 4.0, GraphPad Software Inc., San Diego, CA).

Determination of CYP2C8 genotype in HLMs and localization of amino acid changes in variant CYP2C8 protein—The genotype of *CYP2C8*1*, *CYP2C8*2*, *CYP2C8*3* and *CYP2C8*4* in HLMs was determined and CYP content was measured as previously described [27]. The novel SNP ss86217920 in the 5'-flanking region was genotyped as described in the SDC p.8. Amino acid changes were located in the CYP2C8 crystal structure 2NNI [28] and visualized in Protein Workshop 2005.

3. Results

CYP2C8 variants discovered in the patient population

A total of 40 SNPs were found in the CYP2C8 gene (8 exonic and 32 intronic or promotor, table 1 and SDC S2, p. 6) from 122 samples used for analysis. Of the 40 SNPs, six were restricted to patients not concomitantly using gemfibrozil (2 exonic and 4 intronic). Two of the eight exonic SNPs were synonymous, three were non-synonymous: rs11572080 and rs10509681, associated with *CYP2C8*3* and in expected high linkage disequilibrium $r^2=0.91$ [28], and rs10509681 for *CYP2C8*4*. Since a suitable control group, of cerivastatin users who did not develop rhabdomyolysis, was not available the minor allele frequencies (MAF) of the SNPs found in the patient population were compared to general published Caucasian populations (table 1) [13,29]. Here, SNPs of genotype *CYP2C8*3*, *CYP2C8*1C* (haplotype C), and also, but less pronounced, *CYP2C8*4* indicated slightly lower MAF in the patient population., Particularly the MAF for rs10509681 (*CYP2C8*4*) (0.04 in patient population) can be as high as 0.09 in Caucasian (HAPMAP).

Three novel coding region variants were discovered on exons 1, 8, and 9. Two SNPs (exon 1 and 8, both C>T) resulted in amino acid changes N65S and M426V, respectively. SNP 32,219delA on Exon 9 led to a frame shift in protein sequence (V472fsL494), changing the sequence of the last 22 amino acids of the CYP2C8 C-terminus and introducing three additional amino acids. Each of these three variants were observed only once in the study population, and ss86217920 (V472fsL494) and ss179319940 (N65S) were restricted to patients not using gemfibrozil (table 1). The coding region variants of CYP2C8 were visualized in the available crystal structures [28,30]. Apart from M426V, all variants seemed to be distant from the CYP2C8 active site (Figure 2).

The CYP2C8 promoter region contains three common SNPs (*CYP2C8*1B*, *CYP2C8*1C*, and rs7912549), of which *CYP2C8*1C* and *CYP2C8*1B* seem to have a lower MAF in the gemfibrozil non-users in the patient population (table 1). Three previously not described promoter SNPs were also found. ss86217925 is of particular interest, because it displays a deletion of two base pairs -635_-634delTA and is in high linkage disequilibrium ($r^2=0.81$) with SNPs associated with the *CYP2C8*3* haplotype (rs1050968 and rs11572080).

To investigate further the linkage between ss86217925 and rs1050968 (or rs11572080) the human liver tissue bank (n=56) (available at University of Washington, School of Pharmacy) was genotyped for these SNPs as an alternative population. The results indicated a congruent linkage and MAF of the promoter -TA deletion and genotypical *CYP2C8*3* SNPs ($r^2=1$ for n= 14) (see SDC, Figure S1, p.5).

Recombinant CYP2C8.3 and CYP2C8.4 have increased activity towards cerivastatin

CYP2C8 wild type and coding region variants (including CYP2C8.2) were expressed in *E.coli* to characterize metabolic activities and evaluate any alteration in cerivastatin intrinsic clearance. Only the major metabolites M-23 and M-1 were investigated since the secondary metabolite, desmethyl hydroxy cerivastatin (M-24) was only formed in trace amounts under experimental conditions used. Single enzyme kinetics were observed for all enzymes examined (in the range of 0-100 μ M cerivastatin, Figure 3). Apparent K_m values for CYP2C8 variants decreased for CYP2C8.2 and CYP2C8.3 with CYP2C8.4 having the lowest K_m (about 0.5) in comparison to CYP2C8.1 for both M-23 and M-1 metabolites (table 2). V_{max} values for CYP2C8.1 were similar to CYP2C8.2 and CYP2C8.4 while CYP2C8.3 exhibited a V_{max} that was approximately 3 fold higher for M-23 and M-1. These data translate into a more than four fold increase in metabolic clearance ($Cl_{int}= V_{max} / K_m$) for CYP2C8.3 for both metabolites and a 2-3 fold higher Cl_{int} for CYP2C8.4 for M-23 (table 2). The sum of M-1 and M-23 formation clearance was almost 6 fold higher for CYP2C8.3 and about 2.5 times higher for CYP2C8.4 compared to CYP2C8.1.

Both novel variants, N56S and M426V, demonstrated approximately 2-3 times higher K_m values, but V_{max} values were also slightly higher in both mutants for M-1 and M-23, leading to similar Cl_{int} compared to CYP2C8.1. Interestingly, M426V variant formed almost identical amounts of M-1 and M-23 due to a higher Cl_{int} for M-23, whereas CYP2C8 usually forms M1 in a 2-3-fold excess over M-23 (table 2). For the CYP2C8 V472fsL494 variant, the expressed protein incorporated only 8% heme into the apoprotein compared to wild type based on CO difference spectra and pyridine hemochromogen analysis. Repeated expression confirmed this variant characteristic and although the active protein displayed similar Cl_{int} in comparison to wild type (table 2), this variant is most likely defective *in vivo* compared to CYP2C8.1 due to expression of mostly apoprotein.

The metabolism of cerivastatin by CYP3A4, the second most important isozyme in its clearance, was investigated and compared to CYP2C8 wild type using Supersomes®. Although a direct comparison between K_m and V_{max} in the different expression systems is not possible, the K_m for M-23 is higher than for CYP2C8 using supersomes (K_m 3.5 ± 0.4 μ M for M-23 and 5.0 ± 0.6 μ M for M-1) and in agreement with previously reported data (see Baycol, Labeling Revision 01/04/1999 003, drugs@FDA) Formation of M-23 by CYP3A4 was detectable but the Cl_{int} for the CYP2C8 selective metabolite M-23 was almost seven-times lower for CYP3A4 than for CYP2C8. As indicated above, the ratio of Cl_{int} for M-23 to M-1 is much lower for CYP3A4 (see SDC, figure S2).

Human livers carrying *CYP2C8*3* and *CYP2C8*4* alleles have increased cerivastatin clearance

To validate the differences in metabolic activity observed in recombinant CYP2C8 variants, liver microsomes from the University of WA human tissue bank ($n = 54$) were used. These livers have previously been phenotyped for CYP2C8, and CYP3A4 content by immunoblotting and genotyped for CYP2C8 genetic variants (*CYP2C8*3* and **4*), and were screened for metabolic activity towards cerivastatin at 10 μ M (apparent K_m of CYP2C8 wild type) (figure 4). To control for interindividual variability in CYP2C8 expression, cerivastatin turnover was normalized to CYP2C8 protein content. Further, the K_m and V_{max} constants of exemplary HLMs carrying the *CYP2C8*1/*3*, **3/*3* and **1/*4* genotype were determined and compared to livers carrying the *CYP2C8*1/*1* genotype. HLMs with the *CYP2C8*1/*1* and *CYP2C8*1/*3* genotype possessing similar contents of CYP2C8 and CYP3A4 were chosen for determination of Cl_{int} (figure S3 and S4, table 3). For the *CYP2C8*3/*3* and *CYP2C8*1/*4* genotypes, only a limited set of samples were available and the investigation was further complicated by a low CYP2C8 and CYP3A4 content of these livers [27].

Screening livers with different genotypes demonstrated higher turnover (normalized to CYP2C8 content) for *CYP2C8*3/*3* and *CYP2C8*1/*4* towards the formation of M-23, whereas *CYP2C8*1/*3* was slightly more active than *CYP2C8*1/*1*, but did not reach statistical significance (figure 4 A). The ratio of M-23 to M-1 significantly increased with liver samples carrying the more active *CYP2C8*3* allele (figure 4 B). In comparison, normalization to CYP3A4 did not show any metabolic differences between CYP2C8 genotypes, indicating, that the differences in figure 4A are solely due to CYP2C8 genetic polymorphism (figure S5, SDC).

In a set of selected livers, HLMs carrying one or two *CYP2C8*3* alleles exhibited a higher clearance for both M-23 and M-1 (similar K_m , but higher V_{max} values compared to *CYP2C8*1/*1*) (figure S3 and S4, SDC). HLMs carrying *CYP2C8*1/*4* genotype showed slightly higher K_m values resulting in a decrease in Cl_{int} for M-1, and similar to lower clearance for M-23 in comparison to HLMs carrying the *CYP2C8*1/*1* genotype. Normalization of Cl_{int} values to CYP2C8 content demonstrated a clear discrepancy between livers carrying *CYP2C8*1/*1* and *CYP2C8*1/*3* or **3/*3* genotype, with up to a 14-fold higher Cl_{int} for M1 and up to a 6-fold higher Cl_{int} for the M-23 metabolite, which is almost solely formed by CYP2C8 (HLM 119 and 153, table 3). Interindividual variation was remarkably large masking the difference between livers of *CYP2C8*1* and *CYP2C8*3* genotypes. Due to low CYP2C8 content, increased Cl_{int} of HLMs carrying the *CYP2C8*1/*4* genotype was only apparent after normalization.

A closer look at HLM 169 and HLM 153, both carrier of *CYP2C8*3/*3* genotype, clearly depict the metabolic contribution of CYP3A4. HLM 169 formed M-1 twice as effective as HLM 153, but even without the contribution of CYP3A4 in HLM 169, M-23 clearance is about 5- to 7-fold higher than in HLMs carrying *CYP2C8*1/*1*, and even more pronounced with CYP3A4 contribution (HLM 153). Total CYP2C8 and CYP3A4 clearance show an up to 6 fold difference between livers carrying *CYP2C8*1/*1* and *CYP2C8*1/*3*, whereas Cl_{int} of *CYP2C8*1/*4* is similar to *CYP2C8*1/*1*.

Haplotype D contains a novel tightly linked promoter SNP

Haplotypes within the patient population were determined using Phase 2.0. Haplotype D (which contains both mutations of *CYP2C8*3*), previously defined [29,31], contains not only the novel promoter SNP ss86217925, but also two intron SNPs all in very high linkage ($r^2 > 0.8$, figure 5, and table S4). For haplotype C which includes *CYP2C8*4*, two additional

SNPs at the 3'-end close to the UTR-region could also be included, but the linkage to SNP rs17110453 (*CYP2C8*1C*) is not as tight as for the promoter SNP in haplotype D (figure 4). Because some tagSNPs used in previous haplotype characterizations were not sequenced in the study population, the overall haplotype determinations varied slightly from previous classifications [29]. Apart from the lower abundance of haplotype D (7.6%) and C2 (including *CYP2C8*4*, 3.8%, see SDC, table S4) in the patient population compared to Rodriguez-Antona and coworkers [29] signifying a lower MAF of *CYP2C8*3*, the overall haplotype distribution in this patient population displays a similar pattern as previously shown in other Caucasian populations.

4. Discussion

The re-sequencing of *CYP2C8* in this unique patient population experiencing rhabdomyolysis revealed known genetic variants and additional twelve novel variants (3 exonic, 6 intronic, 3 promoter SNPs). One of the novel exonic *CYP2C8* variants, V472fsL494 which results in a modification of the C-terminus, can be expected to exhibit low metabolic activity *in vivo* because of poor heme incorporation and thus a defective protein. Consequently, carriers of this variant might be expected to experience higher cerivastatin plasma concentrations, as previously reported for the truncated *CYP2C8*5* variant [32] - which was not found in this study. Allele frequencies of SNPs for *CYP2C8*3* and *CYP2C8*4* indicated a slightly lower prevalence in the patient population compared to published MAFs or haplotype frequencies in Caucasian populations, but a statistical analysis could not be performed due to the lack of an appropriate control group. However, corresponding recombinant proteins (*CYP2C8.3* and *CYP2C8.4*) or liver microsomes carrying the *CYP2C8*3* or *CYP2C8*4* genotypes exhibited a significantly higher clearance (normalized) in comparison to wild type. This strong variation in metabolic clearance can be anticipated to affect plasma drug concentration [1]. Interindividual differences in *CYP2C8* protein content aggravate a significant correlation between genotype and metabolic clearance, but a higher clearance in the *CYP2C8*3/*3* and *CYP2C8*1/*4* genotype may be underestimated due to a very small sample size with low protein content. The non-functional but rare frame shift (V472fsL494) variant and the higher activity of genotypes *CYP2C8*3* and *CYP2C8*4* will invariably contribute to alteration in cerivastatin pharmacokinetics.

The significance of the novel -635_-634delTA promoter SNP with respect to metabolism cannot be explained on the basis of these results. This SNP was highly linked to both *CYP2C8*3* coding SNPs in the University of Washington liverbank, thus the LD seems not to be restricted to the patient population. This SNP was sorted into the haplotype D, and occurrence in the patient population seems lower in comparison to other studies [26]. So far, only *CYP2C8*1B* increases transcription factor binding, promoter activity and subsequently paclitaxel 6 α -hydroxylation activity, whereas *CYP2C8*1C* was associated with lower CYP activity *in vivo* [26]. Interestingly, all of these promoter SNPs are not close to known regulatory regions [33]. Further, comparison of *CYP2C8*3* and *CYP2C8*4* genotypes within the liverbank [27] did not show significantly higher mRNA, protein levels or higher metabolic activity which would result from an increased promoter activity. Quite the opposite, livers carrying the *CYP2C8*3* and *CYP2C8*4* genotypes exhibited lower mRNA and CYP protein levels in heterozygous and even lower levels in homozygous genotypes in comparison to wild type [27] thus suggesting that the novel -635_-634delTA promoter SNP and *CYP2C8*1C* indeed may have repressor function.

Altered drug metabolism by *CYP2C8*3* was previously reported for several drugs and clinical data has been recently reviewed [26]. For example, patients carrying the *CYP2C8*3* allele displayed a shorter elimination half-life of rosiglitazone and an increase in plasma concentration of its desmethyl metabolite [34], a higher clearance for repaglinide [35], and is

suggested to enhance the clearance of arachidonic acid as measured by dihydroxy eicosatrienoic acids formation in urine [36]. In contrast, decreased activity is reported for amiodiarone *N*-de-ethylation [37], paclitaxel-6 α -hydroxylation [13,29,38], and arachidonic acid epoxidation by CYP2C8.3 [12,20] *in vitro*. In this study, both recombinant enzymes and human liver microsomes demonstrated a higher metabolic activity of CYP2C8.3 towards cerivastatin. This seemingly substrate dependent metabolism of CYP2C8.3 is the subject of ongoing studies.

Rationalization of altered cerivastatin metabolism by CYP2C8 variants is hampered by an incomplete molecular understanding of the tertiary structure of this cytochrome P450 enzyme during catalysis. The described CYP2C8 variants N56S, R139K, I264M, K399R found in the patient population are located outside the active site in the published protein crystal structures and have a minor effect on K_m . They may, however, increase cerivastatin turnover by affecting the conformation of substrate access channels. It is also likely that they affect interactions with cytochrome b5 or the FMN domain of cytochrome P450 reductase responsible for electron transfer on the proximal site of the heme (such as R139K) [39]. If the active site is distorted, different metabolites could be favored (e.g. M426V), or activity could be abolished if insufficient heme is incorporated (e.g. SNP causing a frameshift V472fsL494).

The Cl_{int} ratio of M-23 to M-1 ranges from 0.4-1.0, and the tendency of a decreased ratio through low CYP2C8 activity aligns with the *in vivo* ratio observed in patients [15]. The ratio of CYP2C8 to CYP3A4 content seems to correspond to the ratio of M-23 to M-1, consistent with a negligible M-23 formation by CYP3A4 [32]. *In vivo*, M-23 is about 3 times more abundant than M-1 in plasma [5], thus changes in overall clearance through M-23 might be more significant than presented here. Further, investigations geared towards the understanding of metabolite toxicity would be important to resolve the increase in cerivastatin toxicity compared with other statins.

Despite all efforts, a genetic variation in the *CYP2C8* gene that would identify patients with higher susceptibility towards developing rhabdomyolysis could not be found. Further, lower MAF for *CYP2C8**3 and *CYP2C8**4 in the patient population could not be statistically tested. However, a combination of interindividual variation of CYP2C8 and CYP3A4 content, together with other limited or non-functional *CYP2C8*-genes might have pharmacokinetic consequences and implications with regard to a low effective concentration of cerivastatin [5] and to the about 10 million prescriptions of cerivastatin prior to market withdrawal [2]. This might contribute statistically to a higher number of adverse side effects, independent from the co-administration of gemfibrozil. The evaluation of these potential genetic risk factors will require an appropriate control group.

In addition to *CYP2C8* genetic polymorphism and inhibition, genetic polymorphism in transport pathways may also play a significant role in the alteration of cerivastatin pharmacokinetics [40], and will be the subject of a separate investigation (Marciante et al., manuscript under review).

In conclusion, the results of this study suggest that observed *CYP2C8* genetic variations found in this population alter cerivastatin metabolism *in vitro* and can affect cerivastatin metabolism *in vivo*. Inheriting the inactive V472fsL494 frameshift variant could certainly increase susceptibility for adverse effects, whereas inheriting one or more allele of *CYP2C8**3 or *CYP2C8**4 might actually be associated with a lower risk for this complication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Scheme of cerivastatin acid metabolism.

Figure 2.

CYP2C8 crystal structure (2NNI) depicting CYP2C8 residues involved in genetic variation. I269 (I269F corresponding to CYP2C8.2) is located in the middle of the A helix, R139 (R139K corresponding to CYP2C8.3) in the hinge region between the C and D helix, K399 (K399R corresponding to CYP2C8.3) faces outwards in the turn after the β 2 strand and K'-helix, I264 (I264M corresponding to CYP2C8.4) is displayed in the beginning of the H helix. N56 (N56S variant) appears at the beginning of the A helix and M426 (M426V variant) is situated close to the heme proximal side before the L-helix. Image was rendered in Protein Workshop 2005 [41].

Figure 3.

Rates of cerivastatin metabolite formation catalyzed by (A) CYP2C8.1, (B) CYP2C8.2, (C) CYP2C8.3, (D) CYP2C8.4, (E) CYP2C8 N65S, (F) CYP2C8 M426V.

Figure 4. Cerivastatin metabolite formation normalized to CYP2C8 content (pmol/mg) and categorized according to liver bank genotype (A: M23 formation, B: ratio of M23- and M1- formation; the bar displays the median of each data set, assays performed at 10 μM cerivastatin and 0.2 mg mL^{-1} protein concentration and 5 min incubation time).

Figure 5.

Location of SNPs representing haplotype C and D of Caucasian patient population (r^2 value for LD below the mutation referring to rs3216029 for haplotype C, and rs11572082 for haplotype D).

Table 1

Variants identified by re-sequencing *CYP2C8* from patients with rhabdomyolysis compared to available published MAF.

Polymorphism ^a	Location	Location in gene ^b /protein	Allele	MAF of study population			Non-gemfibrozil. users			MAF	
				Total			Published data				
				African-American (n=4)	Caucasians (n=118)	African-American (n=2)	Caucasians (n=46)	African-American	Caucasians		
rs11572080	Exon 3	2,130 G>A / R139K	CYP2C8*3	0	0.10	0	0.10	-	0.15 [27], [29]		
rs11572081	Ex3	2,194 G>A / K160K	synonymous	0	0.004	0	0	0.091	-		
rs1058930	Ex5	11,041 C>G / I264M	CYP2C8*4	0	0.04	0	0.04	-	0.06 [26], [13], [29]		
rs11188150	Ex7	26,423 C>T / H353H	synonymous	0	0.004	0	0	-	-		
rs10509681	Ex8	30,411 A>G / K399R	CYP2C8*3	0	0.10	0	0.10	-	0.15 [26], [13], [29]		
rs7909236	Promoter	-271 C>A	CYP2C8*1B	0.25	0.17	0	0.13	-	0.24 [29]		
rs17110453	Pro	-370 T>G	CYP2C8*1C	0.13	0.11	0.25	0.09	0.008	0.22 [29]		
rs7912549	Pro	-411 T>C		0.500	0.18	0.25	0.19	-	0.11 [29]		
Novel CYP2C8 variants:											
ss179319940	Ex 1	167 A>G / N56S		0	0.004	0	0.011				
ss86217921	Ex 8	30,491 A>G / M426V 32,219delA / V472fsL494 forms		0	0.004	0	0				
ss86217920	Ex 9	...nttatpkglftchphtrxsasslsee* c		0	0.004	0	0.011				
ss86217923	Pro	-105 T>G		0	0.004	0	0.011				
ss86217924	Pro	-576 T>A		0.13	0	0	0				
ss86217925	Pro	-635_-634delTA		0	0.09	0	0.09				

^aNovel SNPs were submitted to dbSNP and CYPallele databases.

^bThe *CYP2C8* genomic DNA sequence NG_007972 was used as reference. MAF values from the cited references for Caucasians, - no data available, or HAPMAP data taken for African American from (YRI, Yoruba in Ibadan) did not show any abundance of corresponding SNP.

^cC-terminus of *CYP2C8*.1 is ...nttavtkgvsfpssycicfpv*.

Table 2
Kinetic evaluation of recombinant CYP2C8 and CYP3A4 proteins towards M23 and M1 metabolite formation.

Variant	M-23				M-1				Ratio Cl_{int} M-23/M-1
	K_m [μ M]	V_{max} [nmol M23 nmol P450 ⁻¹ min ⁻¹]	Cl_{int} [μ L min ⁻¹ nmol ⁻¹]	K_m [μ M]	V_{max} [nmol M1 nmol P450 ⁻¹ min ⁻¹]	Cl_{int} [μ L min ⁻¹ nmol ⁻¹]	Cl_{int} [μ L min ⁻¹ nmol ⁻¹]	Cl_{int} (M-1+M-23)	
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 (V472fsL494)	37 ± 7	0.31 ± 0.03	8.4	36 ± 7	0.58 ± 0.04	16.1	23.5		0.52
CYP3A4 ^I	<i>Low</i> 36 ± 7	<i>heme incorporation</i> 0.05 ± 0.004	1.4	18.3 ± 0.3	1.2 ± 0.1	65.6	67.0		0.02

^ISupersomes[®].

Table 3
Kinetic evaluation of human liver microsomes towards M-23 and M-1 metabolite formation.

Liver	Genotype	CYP content pmol per mg liver microsomes,	M-23		M-1		Cl_{int}^2 (M-1 + M-23) [$\mu\text{L min}^{-1} \text{mg}^{-1}$]
		CYP2C8	CYP3A4	Cl_{int} [$\mu\text{L min}^{-1} \text{mg}^{-1}$] per CYP2C8 content ¹ [$\mu\text{L min}^{-1} \text{pmol}^{-1}$].	Cl_{int} [$\mu\text{L min}^{-1} \text{mg}^{-1}$] per CYP2C8 content ¹ [$\mu\text{L min}^{-1} \text{pmol}^{-1}$].	Cl_{int} [$\mu\text{L min}^{-1} \text{mg}^{-1}$] per CYP2C8 content ¹ [$\mu\text{L min}^{-1} \text{pmol}^{-1}$].	
HLM-102	(*1/*1)	61.2	89.2	16.9	18.5	0.30	35.4
HLM-111	(*1/*1)	59.8	95	9.8	12.9	0.22	22.7
HLM-119	(*1/*3)	43.3	105.7	36.6	92.7	2.14	129.3
HLM-168	(*1/*3)	68.7	75.5	34.2	34.8	0.51	69.0
HLM-153	(*3/*3)	24.6	52.8	23.9	73.0	2.97	96.9
HLM-169	(*3/*3)	18.0	5	14.7	26.2	1.44	40.9
HLM-115	(*1/*4)	22.7	51.9	8.2	18.5	0.81	26.7
HLM-118	(*1/*4)	15.4	20.1	3.7	7.6	0.49	11.3