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OATP1B1-related drug–drug and drug–gene interactions as potential risk factors for cerivastatin-induced rhabdomyolysis

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Objective **Genetic variation in drug metabolizing enzymes and membrane transporters as well as concomitant drug therapy can modulate the beneficial and the deleterious effects of drugs. We investigated whether patients exhibiting rhabdomyolysis who were taking cerivastatin possess functional genetic variants in *SLCO1B1* and whether they were on concomitant medications that inhibit OATP1B1, resulting in accumulation of cerivastatin.**

Methods **This study had three components: (a) resequencing the *SLCO1B1* gene in 122 patients who developed rhabdomyolysis while on cerivastatin; (b) functional evaluation of the identified *SLCO1B1* nonsynonymous variants and haplotypes in in-vitro HEK293/FRT cells stably transfected with pcDNA5/FRT empty vector, *SLCO1B1* reference, variants, and haplotypes; and (c) in-vitro screening of 15 drugs commonly used among the rhabdomyolysis cases for inhibition of OATP1B1-mediated uptake of cerivastatin in HEK293/FRT cells stably transfected with reference *SLCO1B1.***

Results **The resequencing of the *SLCO1B1* gene identified 54 variants. In-vitro functional analysis of *SLCO1B1* nonsynonymous variants and haplotypes showed that the V174A, R57Q, and P155T variants, a novel frameshift insertion, OATP1B1*14 and OATP1B1*15 haplotype were associated with a significant reduction ($P<0.001$) in cerivastatin uptake (32, 18, 72, 3.4, 2.1 and 5.7% of reference, respectively). Furthermore, clopidogrel and seven other drugs were shown to inhibit OATP1B1-mediated uptake of cerivastatin.**

Conclusion **Reduced function of OATP1B1 related to genetic variation and drug–drug interactions likely contributed to cerivastatin-induced rhabdomyolysis. Although cerivastatin is no longer in clinical use, these findings may translate to related statins and other substrates of OATP1B1. *Pharmacogenetics and Genomics* 23:355–364 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme-A reductase, are the most potent cholesterol lowering agents. Generally, statins are well tolerated and have acceptable safety profiles. Adverse drug reactions associated with statins include asymptomatic elevation of hepatic transaminases, extremely rare cases of hepatitis, and skeletal muscle-related complaints that in very rare cases can progress to rhabdomyolysis, the most serious side effect of these drugs. Cerivastatin (Baycol), a potent synthetic inhibitor of 3-hydroxy-3-methylglutaryl coenzyme-A reductase, was removed from the US market in August 2001, only 3 years after approval, because of its high reported incidence of rhabdomyolysis compared with existing statins [1].

Although the mechanism of rhabdomyolysis associated with statins is not yet elucidated, data support an increase in the relative risk of rhabdomyolysis when statins are administered concomitantly with drugs that inhibit their metabolism [2–4] or in the presence of the 521T > C variant of *SLCO1B1* that reduces transporter function [5,6].

The epidemiologic arm of a recent case–control analysis published by our group identified the use of seven medications (gemfibrozil, fluoxymesterone, clopidogrel, rosiglitazone, rofecoxib, lansoprazole, and propoxyphene) to be significantly associated with an increased risk of cerivastatin-induced rhabdomyolysis. Furthermore, these data identified clopidogrel use to be strongly associated with cerivastatin-induced rhabdomyolysis both in the presence [odds ratio (OR) 29.6; 95% confidence interval (CI), 6.1–143] and absence (OR 47.8; 95% CI, 12.5–182) of gemfibrozil use [7]. The clopidogrel finding was further

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replicated using US Food and Drug Administration Adverse Event Reporting System data (OR ∞; 95% CI, 2.6–∞) and supported by in-vitro data demonstrating inhibition of CYP2C8-mediated and CYP3A4-mediated metabolism of cerivastatin by clopidogrel or its metabolites [7].

An association analysis of single nucleotide polymorphisms identified by sequencing *SLCO1B1*, *CYP2C8*, *UGT1A1*, and *UGT1A3* in the same cerivastatin-induced rhabdomyolysis cases identified the *SLCO1B1* 521T > C polymorphism as associated with the risk of rhabdomyolysis (OR 1.89; 95% CI 1.40–2.56) [6]. Furthermore, in-vitro cellular uptake data showed a 40% reduction in cerivastatin uptake with this polymorphism [6]. This particular polymorphism was identified in a genome-wide association study of myopathy in patients with a history of myocardial infarction who used simvastatin at a daily dose of 80 mg [5].

Here, we report the functional consequences of additional nonsynonymous *SLCO1B1* gene variants identified in 122 individuals who developed rhabdomyolysis while taking cerivastatin. In addition, we conducted an in-vitro drug–drug interaction screen of 15 medications identified in the epidemiologic study to identify potential inhibitors of OATP1B1-mediated cerivastatin uptake.

Methods

Compounds

[^{3}H]-Cerivastatin sodium salt (CER) (1 mCi/ml; specific activity 5 Ci/mmol) was purchased from American Radiolabeled Chemicals (St Louis, Missouri, USA) and was purified by high-performance liquid chromatography to remove degradation products. [^{3}H]-Estrone-3-sulfate ammonium salt (ES) (1 mCi/ml; specific activity 50 Ci/mmol) was purchased from PerkinElmer Inc. (Boston, Massachusetts, USA). Clopidogrel hydrogen sulfate, rifampin, and celecoxib were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Irbesartan, rofecoxib, pioglitazone hydrochloride, montelukast sodium, verapamil, diltiazem, glyburide, amlodipine, clopidogrel thiolactone, and lansoprazole were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Rosiglitazone-potassium salt was purchased from Cayman Chemical (Ann Arbor, Michigan, USA).

DNA samples, PCR, and sequencing

DNA from 126 individuals confirmed to have rhabdomyolysis while on cerivastatin was used for sequencing the *SLCO1B1* gene. A detailed description of the cases and the recruitment process is found in our previous reports [6–8]. A detailed description of PCR and sequencing of our samples method is also previously reported [6]. Data from four individuals of Asian, mixed, or unknown ethnicity were excluded from the current analysis.

Construction of *SLCO1B1* reference and variant plasmids

The *SLCO1B1* reference cDNA, containing exons 2–15 and three bases in the 3′-untranslated region, was cloned from human liver tissue and inserted into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, California, USA) and subsequently inserted into pcDNA5/FRT vector (Invitrogen). Plasmids containing the variants and haplotypes were constructed by site-directed mutagenesis (SDM) using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA) according to the manufacturer's protocol. The primer sequences (primer sequences available upon request) for the SDM were designed using QuickChange Primer Design Program (*http://www.stratagene.com/sdmdesigner/default.aspx*) from Stratagene. PCR for the SDM was performed under the following conditions: 30 s at 95°C for denaturation/activation followed by 15 cycles of 95°C for 30 s, 1 min at 55°C, and 68°C for 14.5 min.

The SDM product was digested with *Dpn* I and incubated for 1 h at 37°C to remove the methylated reference plasmid. The *Dpn* I digested product was transformed into XL1-blue supercompetent cells (Stratagene). Plasmids were purified with QIAfilter Plasmid Midi Kits (Qiagen Inc., Valencia, California, USA) and sequenced using ABI PRISM BigDye terminator sequencing version 3.1 on an ABI Prism 3730xl DNA analyzer (Applied Biosystems, Foster City, California, USA) to verify the insertion of the variant.

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 plasmids using FuGENE 6 transfection reagent (Roche Applied Sciences, Mannheim, Germany). Briefly, on the day before transfection, 1.5×10^5 HEK293/FRT cells were seeded in a 24-well plate (BD Biosciences Discover Labware, Bedford, Massachusetts, USA) and incubated for 24 h in Dulbecco's modified Eagle's medium 4.5% glucose (DMEM-H-21; UCSF Cell Culture Facility, San Francisco, California, USA) and 10% heat inactivated fetal bovine serum (UCSF Cell Culture Facility). The next day cells were transfected with a DNA:FuGENE 6 complex containing the SLCO1B1 plasmids, pOG44, a Flp-recombinase expression vector (Invitrogen), FuGENE 6, and Opti-MEM (UCSF Cell Culture Facility). The cells were incubated at 37°C, greater than 95% relative humidity, and 5% CO_2, and selection media containing 150 μg/ml of hygromycin B (Invitrogen), 100 μg/ml zeocin (Invitrogen), 1% penicillin and streptomycin (UCSF Cell Culture Facility), and 89% DMEM-H-21 was added 48 h after transfection. Colonies were isolated and screened for the expression of SLCO1B1 using reverse-transcription quantitative PCR (RT-qPCR).

RT-qPCR expression assay

Total RNA was isolated from each individual cell 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, California, USA) per manufacturer's protocol followed by quantitative PCR (qPCR). A singleplex qPCR reaction mix for *SLCO1B1* gene included cDNA, the forward (5′-TCTTCTCTTGTTGG TTTTATTGACG-3′), and reverse (3′-TCCCATAATGAA ACAACCGATTC-5′) primers both at 1 μmol/l and Power SYBR Green PCR Master Mix (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was the internal control gene in a separate singleplex qPCR reaction for the same cDNA. Real-time qPCR for each variant was repeated four times. The qPCR reaction was analyzed using an Applied Biosystems Prism7900HT Real-Time PCR System with cycling conditions of denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The qPCR data was analyzed by 7900HT version 2.3 Sequence Detection Systems software (Applied Biosystems).

The PCR efficiency (E) was measured using standard curves generated by serial dilutions of RNA and calculated by the formula:

$$E=10^{1/-\text{slope}}-1.$$

To analyze the data from RT-qPCR experiments the $2^{-\Delta\Delta C_t}$ method was used to assess the relative changes in gene expression. Details of this method have been previously described [9].

Functional cellular assays

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 cellular accumulation studies were performed 24 h after cell seeding. The accumulation study started with incubation with either 5 nmol/l [^{3}H]-CER or 20 nmol/l [^{3}H]-ES for 5 min at 37°C. Concentrations of substrate and reaction times were selected to be in the linear range of transport. The cerivastatin concentration is also clinically relevant [10,11].

Accumulation was stopped by removing the media and washing the cells three times with ice-cold Krebs–Henseleit buffer. The cells were lysed by addition of 0.1 N NaOH and 0.1% sodium dodecyl sulfate. The intracellular concentration of cerivastatin was measured by liquid scintillation counting. The disintegration per minute value for each sample, measured on a LS-6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Brea, California, USA), was normalized to the sample protein concentration measured using a BCA protein assay kit (Pierce Biotechnology Inc., Rockford, Illinois, USA). The OATP1B1-mediated uptake for each variant was determined by subtracting the rate of uptake in empty vector cells from the uptake in cells expressing OATP1B1 reference or variant transporters. Specific transport rates were then expressed relative to reference values. Transport assays were performed in quadruplicate and repeated a minimum of two times.

Analysis of potential drug–drug interactions used conditions identical to those of the accumulation assays described above, with the exception that 100, 50, 10, 1 and 0.1 μmol/l concentrations of various compounds were included in the reactions. All uptake values are corrected for background and expressed relative to no inhibitor. IC_{50} values were extrapolated from curves fitted to the experimental data by GraphPad Prism software, version 5.04 (GraphPad Software Inc., San Diego, California, USA).

Statistical analysis

Significant differences in ES or cerivastatin uptake between reference and variant OATP1B1 transporters and in the presence and absence of potential inhibitors were detected by one-way analysis of variance followed by Bonferroni's correction for multiple testing and post-hoc multiple comparison testing. Haplotypes for non-synonymous *SLCO1B1* genotype data of rhabdomyolysis cases were inferred using PHASE and fastPHASE (University of Washington, Washington, USA) software programs [12].

Prediction of clinical drug–drug interactions

To predict whether an in-vitro finding of reduced uptake is clinically significant, the decision tree for OATP interactions proposed in the International Transporter Consortium (ITC) paper by Giacomini *et al.* [13] was followed. The fraction unbound, f_u, and maximum plasma concentration, C_{max}, values for each compound were obtained from the literature. If

$$\frac{\text{unbound}\,C_{max}}{IC_{50}} \geq 0.1, \text{ where unbound}\,C_{max}=f_u\times C_{max},$$

then we calculated the R value, representing the ratio of the uptake clearance in the absence and presence of the inhibitor:

$$R=1+\frac{f_u\times I_{in,max}}{IC_{50}},$$

where f_u 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:

$$I_{in,max}=C_{max}+\left(F_a\times\text{dose}\times\frac{K_a}{Q_h}\right),$$

where F_a is the fraction of the dose of the inhibitor, dose, that is absorbed from the intestine, K_a is the absorption

Table 1 ***SLCO1B1*** **coding SNPs identified in rhabdomyolysis cases**

			Minor allele frequencies							
			All cases		HAPMAP[b]		1000 genome		Pasanen *et al.* [16][c]	
SNP ID	Location	Location in gene/ protein	Black ($n=4$)	White ($n=118$)	YRI ($n=90$)	CEU ($n=90$)	YRI ($n=118$)	CEU ($n=120$)	YRI ($n=22$)	Europe ($n=150$)
rs61760183	Exon 3	170G>A/R57Q	–	0.004	–	–	–	–	–	–
rs2306283	Exon 5	388A>G/N130D	0.63	0.36	0.81	0.39	0.08	0.38	0.91	0.41
rs11045818	Exon 5	411G>A/S137S	–	0.12	0.03	0.14	0.01	0.16	0	0.16
rs11045819	Exon 5	463C>A/P155T	0.25	0.12	0.06	0.15	0.07	0.16	0.02	0.17
rs4149056	Exon 6	521T>C/V174A	–	0.21	0.01	0.16	–	0.16	0.05	0.18
rs4149057	Exon 6	571T>C/L191L	0.88	0.44	0.15	0.67	0.19	0.63	0.14	0.61
rs2291076	Exon 6	597C>T/F199F	0.50	0.39	0.03	0.47	0.03	0.47	0.66	0.42
rs113495867	Exon 9	974insGT[a]	–	0.004	–	–	–	–	–	–
rs61760243	Exon 9	1034C>T/T345M	–	0.004	–	–	–	–	–	–
rs34671512	Exon 15	1929A>C/L643F	0.13	0.03	0.07	0.06	0.05	0.07	0.05	0.05

The minor allele frequencies in cases are compared with respective populations reported in HAPMAP, 1000 genome, and Pasanen *et al.* [16] publication.
SNP, single nucleotide polymorphism.
[a]A two base pair insertion in exon 9 results in a frameshift (FS) that encodes for a truncated 334 amino acid OATP1B1 transporter.
[b]HAPMAP notations are YRI (Yoruba in Ibadan, Nigeria) and CEU (Utah resident with Northern and Western European ancestry from the CEPH collection).
[c]Pasanen *et al.* [16] is the source of this data. See publication for description of European population.

Table 2 **Common** ***SLCO1B1*** **haplotypes based on nonsynonymous SNPs in rhabdomyolysis cases**

	SNPs				All cases		1000 genome		Pasanen *et al.* [16]		
Haplotype	388A>G	463C>A	521T>C	1929A>C	White ($n=118$)	Black ($n=4$)	CEU ($n=85$)	YRI ($n=88$)	Europe ($n=151$)	North Africa ($n=29$)	Sub-Saharan ($n=105$)
*1a	A	C	T	A	0.56	0.38	0.58	0.16	0.56	0.24	0.21
*1b	G	–	–	–	0.07	0.25	0.04	0.7	0.26	0.48	0.77
*5	–	–	C	–	0.07	–	0.01	0	0.02	0.02	–
*15	G	–	C	–	0.14	–	0.13	0.01	0.16	0.16	0.02
*14	G	A	–	–	0.11	0.25	0.2	0.05	–	–	–
*35	G	–	–	C	0.03	0.13	0.03	0.06	–	–	–

The haplotype frequencies in cases are compared with Pasanen *et al.* [16] publication reporting and 1000 genome.
*1a is the reference haplotype.
SNP, single nucleotide polymorphism.

rate constant of the inhibitor at the intestine, and Q_h is the hepatic blood flow (1500 ml/min). F_a was set to 1 and K_a was set at 0.1/min [14]. For R values greater than 2 (ITC recommendation [13]) or greater than or equal to 1.25 (Food and Drug Administration – Center for Drug Evaluation and Research recommendation [15]), the in-vitro interaction finding may be clinically significant and a clinical study is recommended [13].

Results

SLCO1B1 sequencing in cerivastatin rhabdomyolysis cases

In sequencing the *SLCO1B1* gene we identified a total of 54 variants, which we have previously listed in supplementary material of Marciante *et al.* [6] publication. Of these, 10 were found in exons, whereas 44 were outside of the exons. Of the coding variants, seven were nonsynonymous (Table 1). rs113495867 resulted in insertion of GT in exon 9 that caused a frameshift (FS) in the latter half of the *SLCO1B1* gene and introduction of a stop codon at amino acid 344 leading to premature termination and a transporter that is 347 amino acids shorter than the reference. The variants rs61760183, rs61760243, and rs113495867 were singletons, found as heterozygous changes in three different individuals.

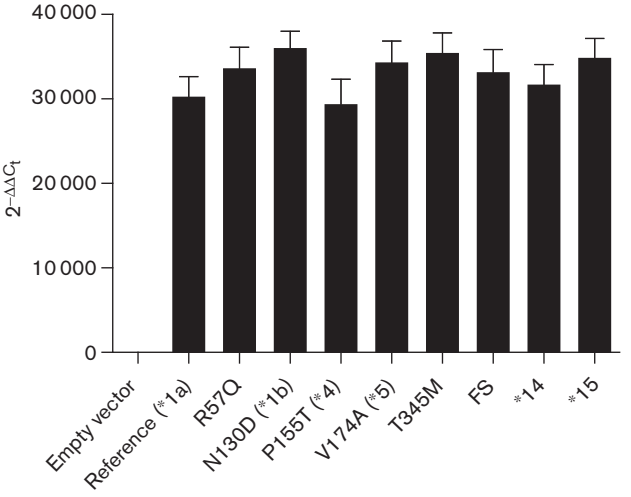
A haplotype analysis of all the *SLCO1B1* nonsynonymous coding variants revealed common (frequency > 1%) known and novel haplotypes. Table 2 lists the common haplotypes, the nonsynonymous nucleotides, and their corresponding frequencies on the basis of the two ethnic groups in our cases.

ES uptake transport by *SLCO1B1* variants and haplotypes

Ten SLCO1B1 stable HEK293/FRT cell lines were created for this study. They expressed six variants, two haplotypes, the reference sequence, and empty vector. Numerous attempts to create stable cells lines expressing 1929A > C variant and its corresponding haplotype, *35 were not successful. The level of SLCO1B1 mRNA expression was analyzed by RT-qPCR and found to be similar among the reference and variant cell lines (Fig. 1). Uptake of ES, a prototypical substrate of OATP1B1, by reference and variant transporters is shown in Fig. 2. Transport of ES by cells containing the OATP1B1

reference sequence was on average greater than 13-fold higher than that by cells with the empty vector (0.2 ± 0.01 vs. 0.01 ± 0.006 pmol/mg of protein/min; $P < 0.001$), indicating a high capacity for OATP1B1-specific transport. The transporter activity for the N130D variant did not differ from the reference, whereas all of the remaining variants showed a significant decrease in uptake compared with reference ($P < 0.001$). ES transport by FS, *14, and R57Q was reduced 100, 97, and 82%, respectively, compared with transport by the reference OATP1B1 ($P < 0.001$). A significant loss of function was also noted for the *15 haplotype and the V174A, T345M, and P155T variants [66, 55, 57, and 31% decrease ($P < 0.001$), respectively].

Fig. 1



RT-qPCR analysis of mRNA expression of *SLCO1B1* in stably transfected HEK293/FRT cells. The mean±SD of $2^{-\Delta\Delta C_t}$ for empty vector and variants is calculated and plotted representing the fold change in *SLCO1B1* gene expression in transfected cells and empty vector normalized to internal control gene (*GAPDH*). FS, frameshift; RT-qPCR, reverse-transcription quantitative PCR.

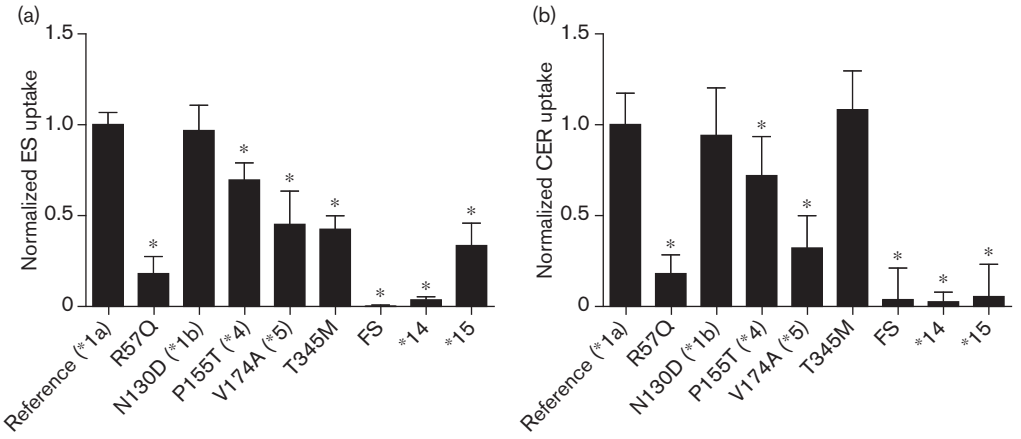
Cerivastatin uptake transport by SLCO1B1 variants and haplotypes

Uptake transport of cerivastatin by HEK293/FRT cells expressing OATP1B1 reference sequence was on average 2.5-fold higher than the empty vector cells (0.07 ± 0.01 vs. 0.02 ± 0.01 pmol/mg of protein/min; $P < 0.001$), indicating OATP1B1-specific transport. With the exception of the T345M variant, in general, the effect of SLCO1B1 variants on cerivastatin transport was similar to the effect on ES transport (Fig. 2). Transport of cerivastatin by the FS, *14, and *15 transporters was barely detectable. Significant reductions in cerivastatin transport were also found for the R57Q, P155T, and V174A variants [82, 28, and 68% decrease ($P < 0.001$), respectively]. The T345M variant demonstrated a substrate-dependent effect with significant reduction in ES transport, whereas cerivastatin transport remained unchanged.

Inhibitory effects of various drugs on OATP1B1-mediated uptake of cerivastatin and ES

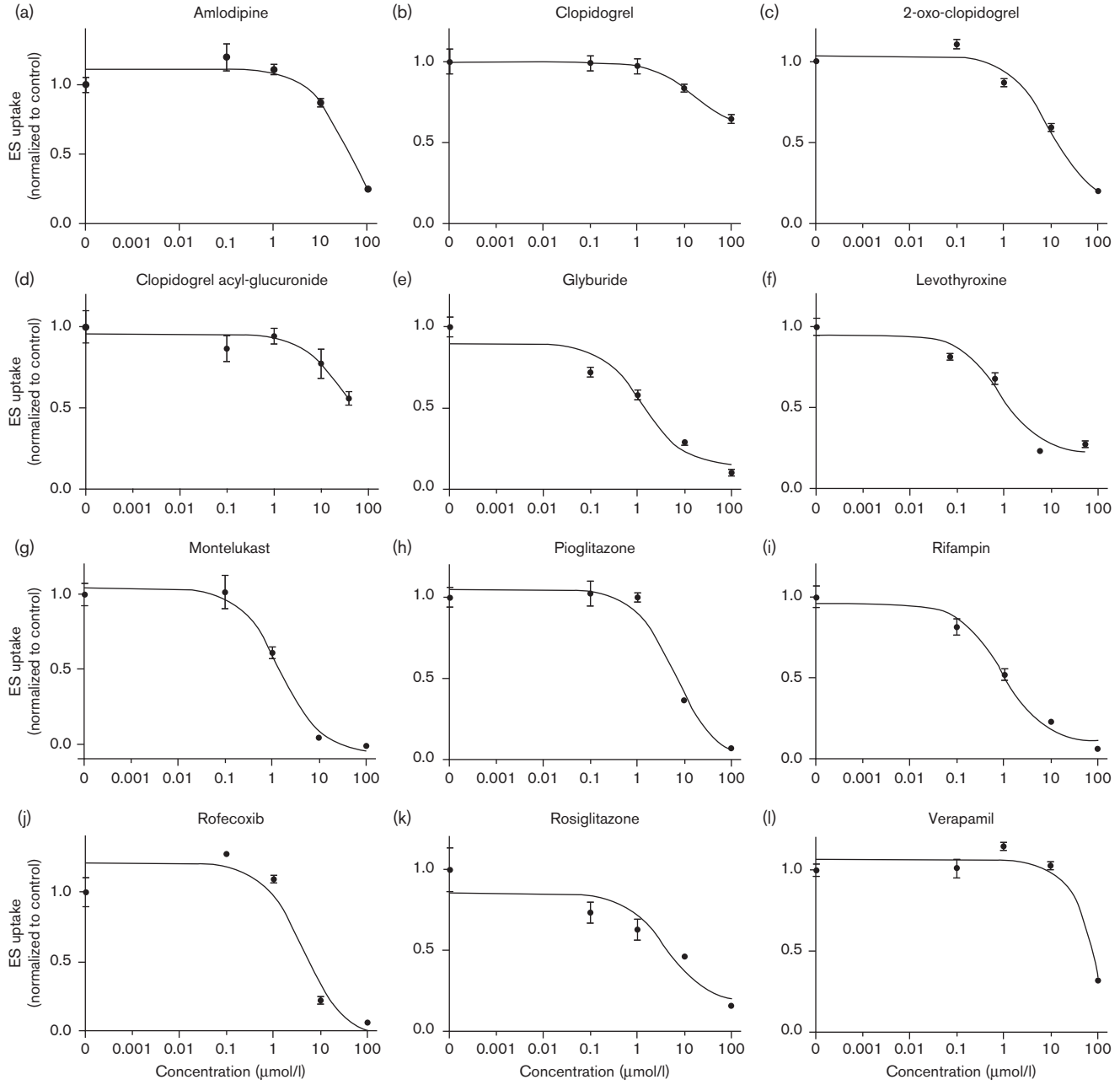
To determine whether concomitant medication use might decrease OATP1B1-mediated uptake of cerivastatin, 15 drugs that were commonly used among the rhabdomyolysis cases were screened for in-vitro inhibition of OATP1B1 function. The inhibitory effects of these compounds on the uptake of [^{3}H]-ES and [^{3}H]-CER by HEK293/FRT stably transfected with reference SLCO1B1 are shown in

Fig. 2



Effect of *SLCO1B1* variants on estrone-3-sulfate (ES) and cerivastatin (CER) uptake transport. Intracellular accumulation of 20 nmol/l [^{3}H]-ES (a) and 5 nmol/l [^{3}H]-CER (b) was measured in HEK293/FRT cells stably expressing *SLCO1B1* reference and variants. Transport was expressed as pmol/min/mg of protein. Data are normalized relative to OATP1B1 reference and are shown as mean±SD of results from three separate experiments. Significant transport differences relative to reference were detected by one-way analysis of variance followed by post-hoc multiple comparison testing (*$P < 0.001$). FS refers to two base frameshift insertions (974insGT). FS, frameshift.

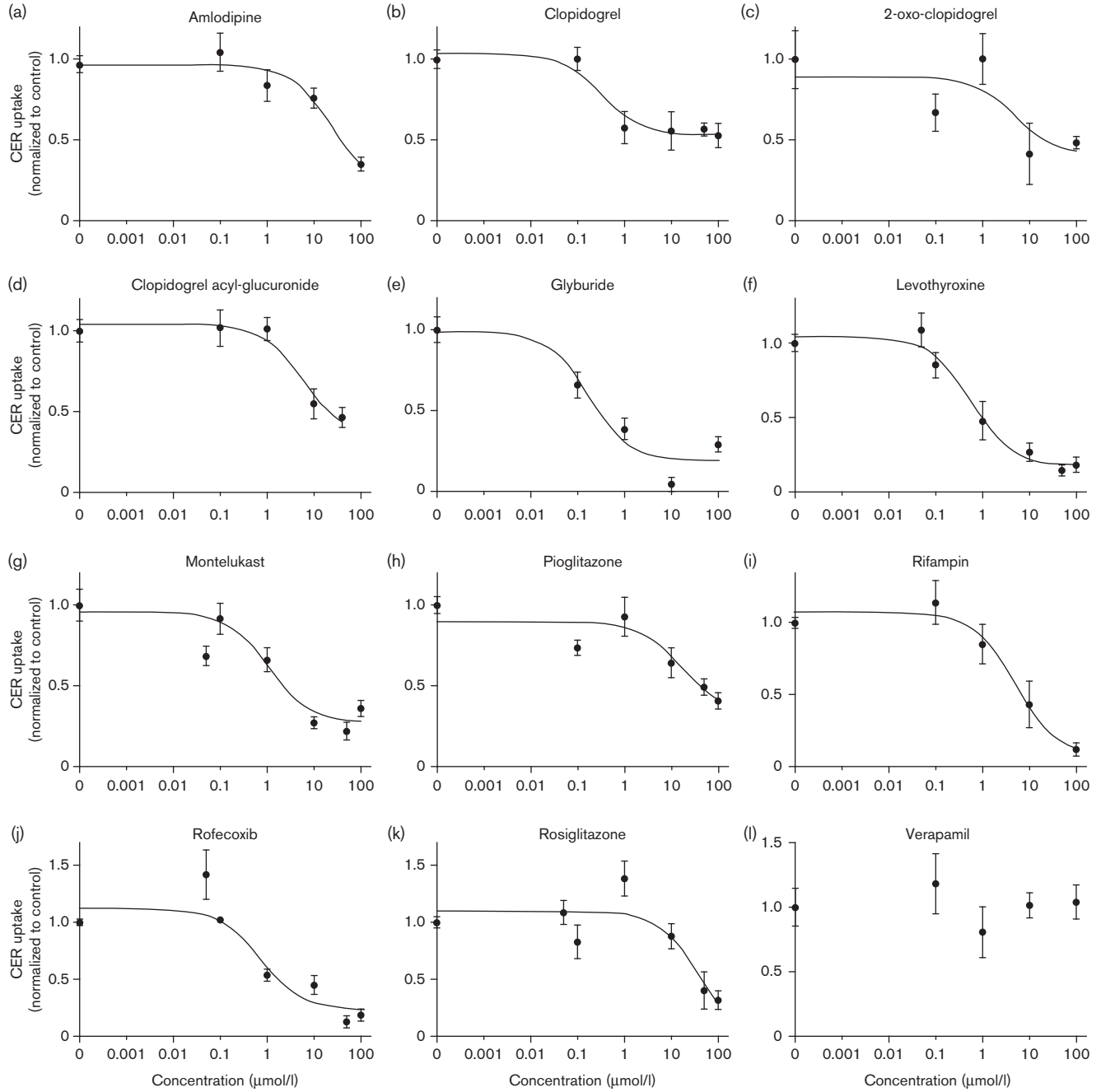
Fig. 3



Inhibitory effects of various pharmaceuticals on the OATP1B1-mediated uptake of estrone sulfate (ES). The OATP1B1-mediated uptake of 20 nmol/l [^{3}H]-ES was measured in HEK293/FRT cells stably expressing *SLCO1B1* reference in the presence and absence of (a) amlodipine, (b) clopidogrel, (c) 2-oxo clopidogrel, (d) clopidogrel acyl glucuronide, (e) glyburide, (f) levothyroxine, (g) montelukast, (h) pioglitazone, (i) rifampin, (j) rofecoxib, (k) rosiglitazone, and (l) verapamil. All values are expressed relative to OATP1B1 control (no inhibitor) and are shown as mean±SEM of a representative experiment. If there is no visible vertical bar, SEM is contained within the limits of the point marking the mean.

Figs 3 and 4, respectively. As clopidogrel is a prodrug, we also selected three of its inactive metabolites, clopidogrel acid, 2-oxo clopidogrel, and clopidogrel acyl glucuronide for testing. Rifampin, a known inhibitor of OATP1B1, was tested as a positive control. Of the 19 compounds tested (15 drugs, three metabolites of clopidogrel and rifampin), 11 inhibited the uptake of cerivastatin and 12 inhibited the uptake of ES (Table 3). Celecoxib, clopidogrel acid, diltiazem, irbesartan, and lansoprazole did not inhibit OATP1B1-mediated cerivastatin or ES uptake even at concentrations as high as 100 μmol/l (data not shown). Fluoxymesterone, limited by solubility, was tested at concentrations up to 3 μmol/l and no inhibitory effects on OATP1B1-mediated uptake of cerivastatin and ES were

Fig. 4



Inhibitory effects of various pharmaceuticals on the OATP1B1-mediated uptake of cerivastatin (CER). The OATP1B1-mediated uptake of 5 nmol/l [^{3}H]-CER was measured in HEK293/FRT cells stably expressing *SLCO1B1* reference in the presence and absence of (a) amlodipine, (b) clopidogrel, (c) 2-oxo clopidogrel, (d) clopidogrel acyl glucuronide, (e) glyburide, (f) levothyroxine, (g) montelukast, (h) pioglitazone, (i) rifampin, (j) rofecoxib, (k) rosiglitazone, and (l) verapamil. All values are expressed relative to OATP1B1 control (no inhibitor) and are shown as mean±SEM of a representative experiment. If there is no visible vertical bar, SEM is contained within the limits of the point marking the mean.

observed (data not shown). Amlodipine, clopidogrel, and its two metabolites, 2-oxo clopidogrel and clopidogrel acyl glucuronide, glyburide, levothyroxine, montelukast, pioglitazone, rofecoxib, and rosiglitazone inhibited OATP1B1-mediated uptake of cerivastatin and ES. Verapamil did not inhibit cerivastatin uptake at concentrations of up to 100 μmol/l, whereas it decreased ES uptake by 68% at the concentration of 100 μmol/l only (Figs 3 and 4).

For the tested drugs, the *R* value calculated using extrapolated IC_{50} was greater than 2 only for rifampin

Table 3 Inhibitor IC_{50} and *R* values for OATP1B1-mediated uptake of cerivastatin

Drug name	Oral dose (mg)	C_{max}[a] (ng/ml)	f_u	$I_{in,\ max}$ (μmol/l)	CER IC_{50} (μmol/l)	ES IC_{50} (μmol/l)	*R* (CER)	*R* (ES)
Amlodipine	10	18	0.07	1.67	35.5	39.6	1.00	1.00
Clopidogrel	75	2	0.02	14.54	3.95	>100	1.10	–
	600	31.4	0.02	124.40	3.95	>100	1.63	–
2-Oxo clopidogrel	600	0.05	0.02	118.69	8.18	8.97	1.29	1.26
Clopidogrel acyl glucuronide	600	–	–	–	10.9	33.5	–	–
Glyburide	5	105	0.002	0.89	0.26	1.40	1.01	1.00
Levothyroxine	0.6	591	0.01	0.79	0.88	2.04	1.01	1.00
Montelukast	10	542	0.01	2.06	2.04	1.40	1.01	1.01
Pioglitazone	30	1500	0.01	9.82	39.6	6.80	1.00	1.01
Rifampin[b]	600	10000	0.11	60.76	5.65	0.88	2.18	8.56
Rofecoxib	25	320	0.13	6.32	1.17	5.65	1.70	1.15
Rosiglitazone	8	598	0.002	2.39	43.4	4.27	1.00	1.00
Verapamil	120	272	0.1	18.2	>100	75.7	NA	1.02

Pharmacokinetic parameters, extrapolated IC_{50} values for OATP1B1-mediated uptake of CER and ES and prediction of clinical significance of the drug–drug interaction (*R*) are shown for drugs tested as OATP1B1 inhibitors.
CER, cerivastatin; ES, estrone sulfate.
[a]C_{max} source(s): amlodipine [17], celecoxib [17], clopidogrel [17–19], 2-oxo clopidogrel [19], glyburide [17], levothyroxine [20], montelukast [17], pioglitazone [17], rifampin [21], rofecoxib [17], rosiglitazone [17], and verapamil [17,22].
[b]Positive control.

(our positive control), with both cerivastatin and ES as substrates. Rifampin inhibition of cerivastatin and ES uptake produced *R* values of 2.18 and 8.56, respectively (Table 3). For all other drugs, despite in-vitro inhibition, the *R* values did not achieve the threshold for potential clinical significance of 2. However, the *R* values did achieve the threshold of 1.25 for clopidogrel, 2-oxo clopidogrel, and rofecoxib for cerivastatin (Table 3). The *R* value for 2-oxo clopidogrel was 1.26 with ES as a substrate (Table 3). Among all the drugs screened for interaction with cerivastatin, rofecoxib had the highest *R* value of 1.7.

Discussion

The resequencing of *SLCO1B1* in our unique population of 122 individuals that experienced rhabdomyolysis while on cerivastatin identified two rare and four common (> 1% minor allele frequency) nonsynonymous variants and one rare FS polymorphism, which we have previously reported [6]. Three of these variants, the FS polymorphism as well as the common haplotype *14, significantly reduced OATP1B1-mediated cerivastatin uptake. Our findings confirm the well-established in-vivo and in-vitro effect of the 521T > C allele in *SLCO1B1**5 and *SLCO1B1**15 haplotypes that is associated with a significant reduction in uptake of atorvastatin [23,24], cerivastatin [6,23], rosuvastatin [24], pitavastatin [25], simvastatin [5,26], and pravastatin [27–31]. We cannot rule out the possibility that decreased transport function is a result of decreased expression of OATP1B1 on the cell membrane. Attempts to quantify OATP1B1 levels in the stable cell lines were not successful.

In general, the effect of nonsynonymous variation followed a similar pattern for cerivastatin and ES uptake, with the T345M variant being the only exception. The T345M variant leads to a reduced ES uptake (0.43 ± 0.07 of reference) with no effect on cerivastatin uptake, indicating a substrate-dependent effect on activity for this variant. Interestingly, the *SLCO1B1**15 haplotype significantly reduced ES uptake, whereas the effect on CER uptake was less dramatic, an observation similar to that reported by Kameyama *et al.* [23].

As cerivastatin-induced rhabdomyolysis, and more generally statin-induced rhabdomyolysis, is associated with elevated plasma concentration of statins and their active metabolites [10], the reduction in uptake of statins by OATP1B1 variants is expected to lead to higher cerivastatin exposure and an increased risk of rhabdomyolysis. The V174A variant has been associated with an increased risk of simvastatin-induced [5] myopathy and cerivastatin-induced [6] rhabdomyolysis. Given that the R57Q, P155T, and FS variants, as well as OATP1B1*15 and *14 haplotypes, were shown to have reduced uptake transport *in vitro*, they might increase the plasma concentration of cerivastatin and perhaps other statins, so that individuals with these variants could be at increased risk for rhabdomyolysis. However, this hypothesis requires confirmation in a clinical study.

A consequence of carrying *SLCO1B1* variants that cause a reduction in hepatic uptake of cerivastatin is reduced metabolism of cerivastatin. The importance of the OATP1B1 transporter in cerivastatin metabolism is highlighted in a recent publication showing that OATP1B1 inhibition by siRNA led to a 20–30% reduction in total uptake of cerivastatin into human hepatocytes, 50% reduction in formation of M-1 cerivastatin metabolite, and no change in M-23 formation [32]. Transporter–enzyme interplay is now well recognized as an important factor influencing drug metabolism [33,34]. With respect to cerivastatin, exposure to this statin in kidney transplant patients receiving cyclosporine, a known OATP1B1 and CYP3A4 inhibitor, was three- to five-fold higher than healthy volunteers on cerivastatin alone [35].

Furthermore, OATP1B1 was recently shown to be the rate limiting step in atorvastatin hepatic clearance at a subtherapeutic microdose [36]. This suggests that in the subset of cerivastatin-induced rhabdomyolysis cases reported by Kaspera *et al.* [8] to have 'normal' or overactive CYP2C8 function, reduced function of OATP1B1 might be a significant risk factor for toxicity, thus mitigating any protective effect from normal or increased CYP2C8 activity. In fact, 67 of 118 white rhabdomyolysis cases (57%) (data not shown) reported by Kaspera *et al.* [8] to have no change or increase in CYP2C8-mediated cerivastatin metabolism carry at least one copy of a *SLCO1B1* polymorphism that we have identified in our in-vitro experiments to have reduced cerivastatin uptake. However, in the absence of information about a control group, the contribution of these variants to the risk of clinical events remains uncertain. Furthermore, the impact of these variants needs to be validated in a clinical pharmacokinetic study.

We also explored potential drug–drug interactions resulting in decreased OATP-mediated drug transport. Our in-vitro data identified clopidogrel, 2-oxo clopidogrel, and clopidogrel acyl glucuronide as modest inhibitors of OATP1B1. Although our epidemiological study identified a clinically significant clopidogrel–cerivastatin interaction further confirmed by in-vitro metabolism assays [7], the clinical significance of our OATP1B1 cellular data requires further investigation. The ITC [13] suggests that R values greater than 2 should trigger clinical follow-up studies, whereas the Food and Drug Administration – Center for Drug Evaluation Research [15] recommendation sets the threshold at R values greater than 1.25. In fact neither cutoff is validated and the choice of cutoff value is controversial. Depending on the cutoff selected for determining the potential clinical significance of our in-vitro data either none or three (clopidogrel, 2-oxo clopidogrel, and rofecoxib) of the drugs/metabolites should be considered for clinical evaluations according to these guidelines. The R values calculated for clopidogrel ($R = 1.1$ and 1.63 at clopidogrel doses of 75 and 600 mg, respectively) and 2-oxo clopidogrel ($R = 1.29$ at clopidogrel dose of 600 mg) depending on the selected cutoff may or may not reach the threshold for clinical follow-up but are consistent with our epidemiological findings. Given the potential for drug interaction and high concomitant use of statins and clopidogrel an in-vivo follow-up might be given further consideration.

It is important to note that for most compounds tested, we are not able to obtain data for a full IC_{50} curve, because the inhibition of OATP1B1 at the highest tested concentration was not complete. In the case of clopidogrel, the maximal OATP1B1 inhibition of cerivastatin uptake was 52.7%. Although our data support clopidogrel and its two tested metabolites as OATP1B1 inhibitors, the curves are insufficient to determine a formal IC_{50} value.

Although clopidogrel has been the subject of much attention recently because of drug–gene interactions with CYP2C19 and its variants [37] as well as controversial drug–drug interactions with omeprazole, [38] the current data are the first to identify clopidogrel as an inhibitor of OATP1B1. Although there is some evidence suggesting a potential interaction of clopidogrel with statins that are metabolized by CYP3A4 (e.g. atorvastatin, simvastatin), this has received limited clinical attention [39,40].

To our knowledge, these data are the first to identify amlodipine, rofecoxib, levothyroxine, and montelukast as inhibitors of OATP1B1 transport. Verapamil [41], glyburide [42], rosiglitazone [43], and pioglitazone [43] were previously identified as inhibitors of OATPB1. With the exception of rifampin, none of the inhibitors tested achieved the ITC recommended R value of 2 for a clinically significant drug interaction.

Conclusion

Although we recognize that a proper evaluation of the potential genetic and environmental risk factors described in this paper would require a control group of cerivastatin users, the data in this study suggest that certain genetic variations in *SLCO1B1* and coadministered drugs found in our rhabdomyolysis cases can alter cerivastatin transport and subsequent metabolism *in vivo* such that they might increase the risk for adverse reactions. Although cerivastatin is no longer in clinical use, these findings may be translatable to related statins, most of which carry a reduced but measurable risk of a muscle toxicity, and other substrates of OATP1B1.

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Conflicts of interest

There are no conflicts of interest.

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