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Downregulation of Organic Anion Transporting Polypeptide (OATP) 1B1 Transport Function by Lysosomotropic Drug Chloroquine: Implication in OATP-Mediated Drug-Drug Interactions

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Abstract

Organic anion transporting polypeptide (OATP) 1B1 mediates the hepatic uptake of many drugs including lipid-lowering statins. Decreased OATP1B1 transport activity is often associated with increased systemic exposure of statins and statin-induced myopathy. Antimalarial drug chloroquine (CQ) is also used for long-term treatment of rheumatoid arthritis and systemic lupus erythematosus. CQ is lysosomotropic and inhibits protein degradation in lysosomes. The current studies were designed to determine the effects of CQ on OATP1B1 protein degradation, OATP1B1-mediated transport in OATP1B1-overexpressing cell line, and statin uptake in human sandwich-cultured hepatocytes (SCH). Treatment with lysosome inhibitor CQ increased OATP1B1 total protein levels in HEK293-OATP1B1 cells and in human SCH as determined by OATP1B1 immunoblot. In HEK293-FLAG-tagged OATP1B1 stable cell line, co-immunofluorescence staining indicated that intracellular FLAG-OATP1B1 is colocalized with lysosomal associated membrane glycoprotein (LAMP)-2, a marker protein of late endosome/lysosome. Enlarged LAMP-2-positive vacuoles with FLAG-OATP1B1 protein retained inside were readily detected in CQ-treated cells, consistent with blocking lysosomal degradation of OATP1B1 by CQ. In HEK293-OATP1B1 cells, without pre-incubation, CQ concentrations up to 100 μM did not affect OATP1B1-mediated [^{3}H]$E_2$17G accumulation. However, pre-incubation with CQ at clinically

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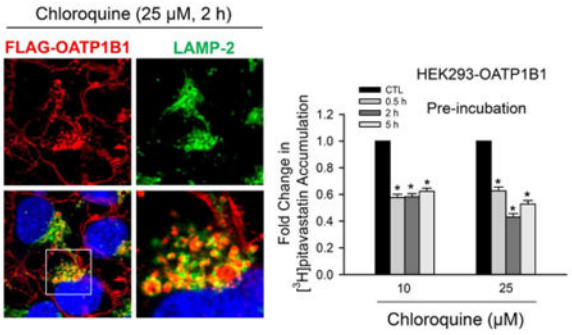
Characterization of HEK293-FLAG-OATP1B1 stable cell line, colocalization of FLAG-OATP1B1 with LAMP-2, treated with CQ (100 μM, 5 h) and CTL, and cytotoxicity in HEK293-OATP1B1 cells and human SCH (PDF)

Notes: The authors declare no competing financial interest.

relevant concentration(s) significantly decreased [^{3}H]$E_2$17G and [^{3}H]pitavastatin accumulation in HEK293-OATP1B1 cells and [^{3}H]pitavastatin accumulation in human SCH. CQ pretreatment (25 μM, 2 h) resulted in ~1.9-fold decrease in V_{max} without affecting K_m of OATP1B1-mediated [^{3}H]$E_2$17G transport in HEK293-OATP1B1 cells. Pretreatment with monensin and bafilomycin A1, which also have lysosome inhibition activity, significantly decreased OATP1B1-mediated transport in HEK293-OATP1B1 cells. Pharmacoepidemiologic studies using data from the U.S. Food and Drug Administration Adverse Event Reporting System indicated that CQ plus pitavastatin, rosuvastatin, and pravastatin, which are minimally metabolized by the cytochrome P450 enzymes, led to higher myopathy risk than these statins alone. In summary, the present studies report novel findings that lysosome is involved in degradation of OATP1B1 protein and that pre-incubation with lysosomotropic drug CQ downregulates OATP1B1 transport activity. Our *in vitro* data in combination with pharmacoepidemiologic studies support that CQ has potential to cause OATP-mediated drug–drug interactions.

Graphical abstract



Keywords

organic anion transporting polypeptide (OATP); chloroquine (CQ); human sandwich-cultured hepatocytes (SCH); lysosomotropic drug; drug–drug interactions (DDIs); FDA Adverse Event Reporting System (FAERS)

Introduction

Drug–drug interactions (DDIs), which often lead to adverse drug effects, are a major concern in patients receiving multidrug therapy.[1] The organic anion-transporting polypeptides (OATPs) belong to the solute carrier organic anion (SLCO) transporter superfamily.[2] OATP1B1 is predominantly expressed on the basolateral membranes of hepatocytes and mediates the hepatic uptake of many drugs such as HMG-CoA reductase inhibitor statins, rifampicin, and paclitaxel as well as endogenous compounds.[3,4] Decreased transport activity of OATP1B1 due to genetic variation [e.g., the single nucleotide polymorphism (SNP) V174A of OATP1B1] and DDIs is often associated with increased systemic exposure of statins and statin-induced myopathy.[5–7] OATP1B1 has been recognized as an important determinant of transporter-mediated DDIs.[8]

Chloroquine (CQ), a 4-aminoquinoline class of drug, is a lysosomotropic agent that accumulates preferentially within the lysosome. It increases the intralysosomal pH,[9] and inhibits lysosomal degradation of proteins including many transport proteins such as glutamate transporter[10] and glucose transporter (GLUT) 1 and 2.[11,12] The effect of CQ on OATP1B1 protein degradation has not been reported. CQ interacts with several transporters as substrate and/or inhibitor. CQ is a substrate and inhibitor of multidrug and toxin extrusion (MATE) 1.[13] It also inhibits substrates transport mediated by P-glycoprotein (P-gp)[14] breast cancer resistance protein (BCRP)[14] and OATP1A2.[15] In rat sandwich-cultured hepatocytes (SCH), pretreatment with 10 μM CQ for 48 h significantly decreased total accumulation of rosuvastatin in cells and bile canaliculi.[16] Since rosuvastatin is a substrate of multiple transporters including OATP1B1,[17] we hypothesize that CQ pretreatment may modulate the transport function of OATP1B1.

In addition to being used for treatment of malaria and amebic infection,[18] CQ is also used for long-term treatment of rheumatoid arthritis and systemic lupus erythematosus.[19–22] It is currently in clinical trials for cancer therapy either alone[23–25] or in combination with chemotherapy drugs.[26,27] Considering the widespread use of statins in cardiovascular diseases, concurrent administration of statins and CQ is likely. To the best of our knowledge, the effects of CQ on systemic exposure of statins and statin-associated myopathy have not been reported in clinical studies. The FDA Adverse Event Reporting System (FAERS) is a database that contains information on adverse events and medication error reports submitted to the FDA.[28] This database is designed to support the FDA's postmarketing safety surveillance program for drug and therapeutic biologic products. The FAERS has been widely used for pharmacoepidemiologic studies including assessing statin-related myopathy risk due to DDIs.[29,30] The current studies were designed to determine the effects of CQ on OATP1B1 protein degradation, OATP1B1-mediated transport in OATP1B1-overexpressing cell line, and statin uptake in human SCH. The myopathy risk was also compared in patients concomitantly taking CQ and statins vs statins alone using data from the FAERS.

The effects of CQ on OATP1B1 protein degradation and transport activity were determined in HEK293 stable cell lines overexpressing OATP1B1 (HEK293-OATP1B1) or FLAG-tagged OATP1B1 (HEK293-FLAG-OATP1B1). Pitavastatin and estradiol 17β-D-glucuronide ($E_2$17G), which are sensitive OATP1B1 substrates for studying the inhibition of OATP1B1 transport activity *in vitro*,[31] were used as OATP1B1 probe substrates in HEK293-OATP1B1 and -FLAG-OATP1B1 stable cell lines. The effect of CQ on hepatic uptake of therapeutic drug pitavastatin was also determined in the physiologically relevant human SCH model. Pitavastatin, pravastatin, and rosuvastatin are OATP1B1 substrates[32] and are minimally metabolized by the cytochrome P450 enzymes.[33–35] These three statins were selected in current pharmacoepidemiologic studies to determine whether CQ plus statins (pitavastatin, rosuvastatin, and pravastatin) leads to a higher risk for myopathy than these statins alone, using data from the FAERS.

Experimental Section

Materials

Estradiol 17β-D-glucuronide ($E_2$17G), chloroquine diphosphate, IGEPAL (NP-40), Hanks balanced salt solution (HBSS), dexamethasone, dimethyl sulfoxide (DMSO), Triton X-100, Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), trypsin–EDTA solution, antibiotic antimycotic solution, Dulbecco's phosphate-buffered saline (DPBS), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Monensin sodium salt was purchased from Santa Cruz (Dallas, TX). Bafilomycin A1 was purchased from LC Laboratories (Woburn, MA). Insulin, minimum essential medium (MEM) nonessential amino acids (NEAA), L-glutamine, and penicillin–streptomycin were purchased from Life Technologies (Grand Island, NY). Matrigel and insulin/transferrin/selenium (ITS^+ premix) were purchased from BD Biosciences (Bedford, MA). Cultrex poly-L-lysine was purchased from Trevigen, Inc. (Gaithersburg, MD). Geneticin was purchased from Life Technology (Grand Island, NY). Complete protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Indianapolis, IN). [^{3}H]Pitavastatin (specific activity 5 Ci/mmol) and unlabeled pitavastatin were purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]$E_2$17G (specific activity 41.4 Ci/mmol) was purchased from PerkinElmer Life Science (Waltham, MA). Bio-Safe II liquid scintillation mixture was obtained from Research Products International (Mt. Prospect, IL). All other reagents were purchased from VWR International (Radnor, PA).

Estimation of Maximum Unbound Concentration of CQ at the Inlet to Liver

Maximum concentration of CQ at the inlet of the liver ($I_{in,max}$) was estimated according to eq 1, described previously.[36]

$$I_{\mathrm{in,max}}=C_{\max}+(k_{\mathrm{a}}DF_{\mathrm{a}}F_{\mathrm{g}}/Q_{\mathrm{h}}) \quad (1)$$

The values for the unbound fraction of CQ in the plasma (f_u), absorption rate constant (k_a), and hepatic blood flow (Q_h) used in eq 1 are 0.45,[18] 0.1 min^{-1},[37] and 1500 mL/min,[38] respectively. The maximum concentration of CQ in the systemic circulation (C_{max}) and associated dose (D) were obtained from the literature and are summarized in Table 2. Since CQ has high bioavailability and almost complete absorption after oral administration,[18] the fraction of CQ absorbed from the gastrointestinal tract intact (F_aF_g) was set at 1.[18,39] Unbound concentration of $I_{in,max}$ ($I_{u,in,max}$) was calculated as $f_uI_{in,max}$.

Cell Culture

The human embryonic kidney (HEK) 293 stable cell line overexpressing OATP1B1 (HEK293-OATP1B1) was provided by Dr. Dietrich Keppler.[3] A HEK293 stable cell line overexpressing FLAG-tagged OATP1B1 (HEK293-FLAG-OATP1B1) was established by transfection of a mammalian plasmid expression vector encoding C-terminus FLAG-tagged OATP1B1 (pCMV6-FLAG-OATP1B1), which was custom constructed through Origene (Rockville, MD). Both cell lines were maintained in DMEM medium containing 10% FBS,

1% antibiotic and antimycotic solution, and 600 μg/mL Geneticin (Life Technologies, Grand Island, NY). Cells were cultured in a humidified atmosphere (95% O_2, 5% CO_2) at 37 °C.

Human Sandwich-Cultured Hepatocytes (SCH)

Human hepatocytes were purchased from Triangle Research Laboratories, LLC (Research Triangle Park, NC). Demographics of the donors are listed in Table 1. Human SCH were cultured as described previously.[40] In brief, on day 0, cells were plated at 3.5×10^5 cells per well in 24-well Biocoat culture plates in phenol red free DMEM containing 5% (v/v) FBS, 1% (v/v) MEM NEAA, 2 mM L-glutamine, 100 units of penicillin G sodium/mL, 100 g/mL of streptomycin sulfate, 1 μM dexamethasone, and 4 μg/mL insulin. Cells were cultured at 37 °C in a humidified incubator (95% O_2, 5% CO_2). At 6 h after seeding, when the cells were attached to the plate, cells were overlaid with Matrigel at a final concentration of 0.25 mg/mL in phenol red free DMEM supplemented with 2 mM L-glutamine, 1% (v/v) MEM NEAA, 100 units/mL of penicillin G sodium, 100 g/mL of streptomycin sulfate, 0.1 μM dexamethasone, and 1% (v/v) ITS^+ premix. Culture medium was replaced every 24 h. Uptake experiments were conducted on day 2 of culture.

Immunoblotting

Immunoblotting was conducted similarly to those described previously.[41] In brief, cells were seeded at a density of 1.1×10^5 cells per well in 24-well culture plates precoated with poly-L-lysine and cultured for 48 h. Two cell lysis methods (with or without trypsinization) were compared to optimize the OATP1B1 immunoblot. After aspirating culture media and rinsing once with DPBS, whole cell lysates (WCL) were prepared by adding ice cold lysis buffer, which contained 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.5% Na-deoxycholate, and Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), either directly onto the cells in culture plate (without trypsinization) or to cell pellets collected after trypsinization with 1× Trypsin/EDTA (Sigma-Aldrich, St. Louis, MO) and subsequent washing with DPBS (with trypsinization). Whole cell lysates (WCL) (50 μg) were resolved on 10% SDS–PAGE (Bio-Rad, Hercules, CA). Proteins were then transferred to nitrocellulose membranes and subsequently probed with the following primary antibodies: rabbit polyclonal OATP1B1 antibody (Fisher Scientific Company, LLC, Denver, CO) custom-generated according to a previous publication (1:1000 dilution),[43] rabbit polyclonal anti-FLAG antibody (1:4000 dilution) and mouse monoclonal anti-β-actin antibody (1:5000 dilution) (Sigma-Aldrich, St. Louis, MO), mouse monoclonal anti-GAPDH antibody (1:2000) (Santa Cruz Biotechnology, Inc., Dallas, TX), and anti-alpha 1 sodium potassium ATPase antibody (1:8000 dilution) (Abcam, Cambridge, MA). After incubation with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Dallas, TX), signals were detected by Supersignal West Duro (Pierce, Rockford, IL) using a Bio-Rad ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA). Image Lab v4.1 software (Bio-Rad Laboratories, Hercules, CA) was used to perform the densitometry analysis.

Immunofluorescence Staining

Experiments were conducted similarly to those published previously, with slight modifications.[44] In brief, HEK293-FLAG-OATP1B1 cells were grown to confluence on

poly-L-lysine coated coverslips in 24-well plates. Cells were fixed for 20 min with methanol, permeabilized with Triton X-100 (0.25%) in DPBS for 10 min at room temperature, and blocked with 5% BSA in DPBS for 1 h at room temperature. For co-immunofluorescence staining of FLAG-OATP1B1 and LAMP-2, cells were co-incubated with the rabbit polyclonal anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO, 1:100 dilution) and mouse monoclonal antibody of LAMP-2 (Clone H4B4, Santa Cruz Biotechnology, Inc., Dallas, TX, 1:50 dilution) at 37 °C for 2 h. After washing 3 times with DPBS, cells were co-incubated with secondary antibodies Alexa Fluor 488-conjugated goat anti-mouse IgG (Life Technologies, Grand Island, NY, 1:200 dilution) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Life technologies, Grand Island, NY, 1:200 dilution) at 37 °C for 1 h. Both primary and secondary antibodies were diluted in DPBS containing 5% BSA. Diamidino-2-phenylindole (DAPI) (300 nM) was used for counterstaining nuclei. After washing, the coverslips were mounted on glass slides with ProLong gold antifade reagent (Life Technologies, Grand Island, NY). Images were taken using Leica SP2 MP (Leica Microsystems, Inc., Buffalo Grove, IL) or Olympus FV1000 (Olympus Scientific Solutions Americas Corp., Waltham, MA) confocal microscopes as indicated in the figure legends.

Transport Studies

Transport studies in HEK293 stable cell lines and in human SCH were conducted similarly to those described previously.[41,42] HEK293-OATP1B1, -FLAG-OATP1B1, and -Mock cells were seeded at a density of 0.8–1.1 × 10^5 cells/well in 24-well culture plates precoated with poly-L-lysine and were cultured for 48–72 h. Cells from stable cell lines or human SCH were either pretreated with culture medium containing CQ, monensin, bafilomycin A1, or vehicle control for the designated times or not pretreated. 0.1% DMSO was used as vehicle control for monensin and bafilomycin A1 treatment. As CQ stock (50 mM) was resolved in water, CQ-free fresh medium was used as vehicle control for CQ pretreatment. To determine the effects of CQ on OATP1B1-mediated transport after prolonged treatment, HEK293-OATP1B1 cells were preincubated with CQ-free (CTL) or 25 μM CQ-containing medium for 2 h. At the end of pre-incubation, the culture medium was aspirated and CTL cells were cultured in CQ-free medium and cells pretreated with 25 μM CQ were cultured in medium containing 1.5 μM CQ, for the indicated time, up to 24 h.

At the time of uptake experiments, after rinsing with prewarmed (37 °C) HBSS buffer (pH 7.4) three times, cells were incubated with HBSS containing [^{3}H]$E_2$17G (1 μM, 2 min) or [^{3}H]pitavastatin (1 μM, 0.5 min) in the absence or presence of testing drugs (CQ or rifampicin). At the end of incubation, the buffer was aspirated rapidly, and the cells were rinsed with ice-cold HBSS three times and then lysed with Triton X-100 (0.5% v/v) in DPBS. An aliquot of the lysate was subjected to liquid scintillation counting (LS6500 scintillation counter, Beckman Coulter, Brea, CA). Substrate accumulation was normalized to protein concentration determined by BCA assay (Pierce Chemical, Rockford, IL) and corrected for nonspecific binding of the substrate by including a non-overlaid poly-L-lysine coated blank plate for uptake studies in stable cell lines and a Matrigel overlaid blank plate for human SCH, respectively.

To determine [^{3}H]$E_2$17G transport kinetic parameters, the maximal transport velocity (V_{max}) and the affinity constant (K_m), [^{3}H]$E_2$17G accumulation (0.1–40 μM, 2 min) was determined in HEK293-OATP1B1 and -Mock cells following pretreatment with CQ(25 μM) or CTL. Values of [^{3}H]$E_2$17G accumulation in Mock cells were subtracted from those in HEK293-OATP1B1 cells. The V_{max} and K_m values of $E_2$17G transport were estimated by fitting the Michaelis–Menten equation (eq 2) to the data using Phoenix WinNonlin, v6.3 (Certara, St. Louis, MO), where ν is $E_2$17G transport velocity and S is $E_2$17G concentration.

$$\nu = \frac{V_{max} S}{K_m + S} \quad (2)$$

Lactate Dehydrogenase (LDH) Cytotoxicity Assay

Experiments were conducted similarly to those published previously.[45] After treatment with CQ, monensin, bafilomycin A1, or respective vehicle control, cell culture medium was assayed for LDH activity with a cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Triton X (2%) treated and non-treated cells served as the 100% cytotoxicity positive control and negative control, respectively.

Pharmacoepidemiologic Studies Using the FDA Adverse Event Reporting System (FAERS)

Studies using the FAERS database were similar to those published previously.[29] All of the adverse event case reports from quarter 1 of 2004 to quarter 3 of 2012 were used for data analysis (n = 6.47 millions). The myopathy adverse event was defined as published previously.[29] It includes all the mild or severe myopathy symptoms. The drug names and their synonyms were mapped based on their DrugBank[46] drug names. Our drug interaction hypothesis was whether CQ plus statins (pitavastatin, rosuvastatin, and pravastatin) leads to higher myopathy risk than these statins alone. A similar hypothesis was posed to test the interactions between sulfonylureas and statins in our previous work.[47] The interaction between CQ and statins was further tested in different subpopulations defined by age and gender.

Data Analysis

For the statistical analysis in Figures 4, 5, 6 and 7B, fold changes and associated standard errors (SEs) were estimated by generalized linear mixed models with the log link function, a fixed effect (treatment time or group), and a random effect (experiment date or hepatocyte donor), adjusting for treatment time/group-specific overdispersion. In the case of multiple comparisons, p-values were adjusted based on Bonferroni's method. The Chi-square test and Student's t test were used for statistical analysis in Table 3 and the Figure 8 inset, respectively. A two-sided p-value of <0.05 defines statistical significance. SAS software (version 9.3, Cary, NC) was used for statistical analyses.

Results

Estimation of the Maximum Unbound CQ Concentration at the Inlet to the Liver

The $I_{u,in,max}$ of CQ was estimated to be 18.3–23.4, 28.2, and 56.8 μM based on data in rheumatoid arthritis patients chronically treated with CQ at a daily dose of 191 mg[48] and after a 300 and 600 mg single dose of CQ in healthy volunteers,[39,49] respectively (Table 2). CQ concentrations of 2.5–25 μM, which are clinically relevant, were used in subsequent experiments.

CQ Treatment Increased Total Protein Levels of OATP1B1 in HEK293-OATP1B1 Cells and in Human SCH

Initially, we tested the specificity of our custom-generated OATP1B1 antibody and optimized OATP1B1 immunoblot protocol by comparing two cell lysis methods, directly lysing the cells in the culture plate without trypsinization and lysing the cell pellets collected after trypsinization. As shown in Figure 1, OATP1B1 antibody specifically detected OATP1B1 in HEK293-OATP1B1 stable cell line but not in negative control HEK293-Mock (Mock) cells in whole cell lysates prepared with both methods. In cells that were lysed directly in the culture plates without trypsinization, the molecular weight of OATP1B1 detected by the OATP1B1 antibody was ∼90 kDa. A lower molecular weight band of OATP1B1 (∼64 kDa) was prominent in cell lysates prepared from trypsinized HEK293-OATP1B1 cells, while this band was negligible in cells lysed directly without trypsinization. Since the molecular weight of OATP1B1 (∼90 kDa) that resulted from the direct lysis method is similar to that published previously,[3] this method was used to prepare whole cell lysates for immunoblot in subsequent experiments.

To determine the involvement of lysosome in degradation of OATP1B1, total protein levels of OATP1B1 were compared in HEK293-OATP1B1 cells treated with CQ or CTL by OATP1B1 immunoblotting. Treatment with 25 μM CQ for 2 and 5 h and 100 μM CQ for 5 h increased OATP1B1 total protein levels to 1.2 ± 0.1, 1.5 ± 0.1 and 1.5 ± 0.3 fold of control, respectively (Figure 2A).

Increased total protein levels of Na,K-ATPase following lysosome inhibition by CQ treatment were reported previously.[50] In the current studies, we used Na,K-ATPase as a positive control for increased total protein levels following lysosome inhibition. As shown in Figure 2B, treatment with CQ (25 μM) for 2 and 5 h increased Na,K-ATPase total protein levels to 1.2 ± 0.1 and 1.4 ± 0.2 fold of control, respectively.

Total protein levels of endogenous OATP1B1 were also compared in human SCH treated with CQ or vehicle control. As shown in Figure 2C, treatment with 10 μM CQ for 5 h increased OATP1B1 protein levels to 1.7 ± 0.5 fold of control in human SCH (ranging from 1.2–2.1-fold of control, $n = 3$ donors). In human SCH, a major OATP1B1 band at ∼64 kDa was observed (Figure 2C), similar as published previously.[51]

Colocalization of Intracellular FLAG-OATP1B1 with LAMP-2 in HEK293-FLAG-OATP1B1 Stable Cell Line

olocalization of OATP1B1 with LAMP-2, a late endosome/lysosome marker protein,[52] was determined by immunofluorescence staining. Since our custom-generated OATP1B1 antibody is not suitable for immunofluorescence staining (data not shown), a stable cell line overexpressing FLAG-tagged OATP1B1 was established in HEK293 cells to overcome this limitation. Expression and transport function of FLAG-OATP1B1 in HEK293-FLAG-OATP1B1 cells were confirmed by immunoblotting and [^{3}H]$E_2$17G accumulation, respectively. Both OATP1B1 and FLAG antibodies specifically detected the FLAG-tagged OATP1B1 in HEK293-FLAG-OATP1B1 cells, but not in the negative control HEK293-Mock cells (Figure S1A). The [^{3}H]$E_2$17G accumulation (1 μM, 2 min) in HEK293-FLAG-OATP1B1 cells was ~70-fold higher than in the HEK293-Mock cells (Figure S1B).

FLAG immunofluorescence staining showed specific staining in HEK293-FLAG-OATP1B1 cells but not in HEK293-Mock cells (data not shown). In both CQ-treated and vehicle control treated cells, in addition to the plasma membrane localization of FLAG-OATP1B1, intracellular localization of FLAG-OATP1B1 was also observed, which had a punctuated staining pattern reminiscent of vesicular compartments (Figure 3G–O, red). Some intracellular FLAG-OATP1B1 was detected in LAMP-2 positive vacuoles and colocalized with LAMP-2 (Figure 3J–O, as indicated by arrows and yellow coloring in merged images). Enlarged LAMP-2-positive vacuoles with FLAG-OATP1B1 protein retained inside were readily detected in cells treated with 25 μM CQ (Figure 3K,L,N,O) and 100 μM CQ (Figure S2H), consistent with inhibition of lysosomal degradation of FLAG-OATP1B1 by CQ.

Pretreatment Effects and Direct Inhibition of CQ on OATP1B1-Mediated Transport

To determine the pretreatment effects of CQ on OATP1B1-mediated transport, HEK293-OATP1B1 or -FLAG-OATP1B1 was preincubated with CQ or vehicle CTL at indicated concentrations for 0.5–5 h. After washing, accumulation of [^{3}H]$E_2$17G (1 μM, 2 min) or [^{3}H]pitavastatin (1 μM, 0.5 min) was determined in the absence of CQ (Figure 4A–C, pre-incubation). In HEK293-OATP1B1 cells, at all CQ concentrations (2.5–100 μM) and time points determined (0.5–5 h), pretreatment with CQ significantly decreased [^{3}H]$E_2$17G accumulation (all $p < 0.001$ vs control, Figure 4A). CQ pretreatment (10 and 25 μM) for 0.5–5 h also significantly decreased [^{3}H]pitavastatin accumulation (all $p < 0.001$ vs control, Figure 4B). In HEK293-FLAG-OATP1B1 cells, [^{3}H]$E_2$17G accumulation was significantly decreased following pre-incubation with CQ (10–100 μM) for 0.5–5 h (all $p < 0.05$ vs control, Figure 4C).

To determine the direct interaction of CQ on OATP1B1-mediated transport, experiments were conducted in HEK293-OATP1B1 cells without CQ pre-incubation. [^{3}H]$E_2$17G accumulation (1 μM, 2 min) was determined in the presence of CQ at indicated concentrations, vehicle CTL, or positive control rifampicin (Figure 4D, co-incubation). As shown in Figure 4D, co-incubation with 25 μM rifampicin, a potent inhibitor of OATP1B1,[53] significantly decreased [^{3}H]$E_2$17G accumulation to 0.02 ± 0.021 fold of control (adjusted $p < 0.01$). However, there is no significant difference in [^{3}H]$E_2$17G accumulation in the presence of CQ (5–100 μM) vs CLT (all adjusted $p > 0.05$).

The effect of CQ on OATP1B1-mediated [^{3}H]$E_2$17G accumulation was further compared between two scenarios, a pre+co-incubation scenario, when [^{3}H]$E_2$17G accumulation (1 μM, 2 min) was determined in the presence of 100 μM CQ after pre-incubation with CQ (100 μM, 5 h), and a co-incubation scenario as described above. For comparison purposes, 100 μM CQ co-incubation data in Figure 4D was replotted in Figure 4E. Under pre+co-incubation conditions, [^{3}H]$E_2$17G accumulation decreased to 0.432 ± 0.02 fold compared to values measured under the co-incubation condition ($p < 0.001$, Figure 4E). The LDH assay showed negligible toxicity after 5 h treatment with 100 μM CQ in HEK293-OATP1B1 cells (Figure S3A).

Effects of Monensin and Bafilomycin A1 on OATP1B1-Mediated Transport

Three classes of compounds are frequently used to elevate the pH in endosome and other acidic organelles, which include lysosomotropic weak bases (e.g., chloroquine[54]), carboxylic ionophores (e.g., monensin[55]), and inhibitors of vacuolar H^+-ATPase (e.g., bafilomycin A1[56]) (reviewed by Huotari[57]). In addition to CQ, monensin and bafilomycin A1 are also reported to inhibit endosome–lysosome system acidification and lysosome activity.[58,59] We determined whether pretreatment with monensin or bafilomycin A1 also affects OATP1B1-mediated [^{3}H]$E_2$17G accumulation (1 μM, 2 min) in HEK293-OATP1B1 cells. Pretreatment with monensin (5 μM) for 0.5–2 h significantly decreased [^{3}H]$E_2$17G accumulation ranging from 0.356 ± 0.036 to 0.512 ± 0.047 fold of CTL (all $p < 0.0001$ vs. CTL) (Figure 5A). Pretreatment with bafelomycin A1 (0.5 μM) for 1 and 5 h significantly decreased [^{3}H]$E_2$17G accumulation to 0.60 ± 0.083 to 0.40 ± 0.19 fold of CTL, respectively (all $p < 0.01$ vs. CTL) (Figure 5B). The LDH assay showed negligible toxicity after monensin and bafilomycin A1 treatment in HEK293-OATP1B1 cells (Figure S3A).

Effects of CQ on OATP1B1-Mediated Transport after Prolonged Treatment

CQ is administered daily for long-term treatment of rheumatoid arthritis and lupus.[22] In patients who had been taking CQ for at least six months, the dose normalized average unbound steady state concentration (C_{ss}) of CQ is ~1.7 μM.[48] The effect of CQ on OATP1B1-mediated transport after prolonged treatment was determined in HEK293-OATP1B1 cells. After 2 h treatment with CQ-free medium or medium containing 25 μM CQ, cells were subsequently cultured in CQ-free medium or 1.5 μM CQ-containing medium, respectively. Values of [^{3}H]$E_2$17G accumulation in CQ-treated cells were compared to CQ-free treatment (CTL) at indicated times. A similar trend as in Figure 4A, CQ pretreatment (25 μM, 2 h) significantly decreased [^{3}H]$E_2$17G accumulation to 0.373 ± 0.014 fold of control (Figure 6, 0 h). At each time point of 6, 18, and 24 h, [^{3}H]$E_2$17G accumulation in 1.5 μM CQ-containing medium was significantly decreased compared to CTL (all $p < 0.0001$ vs CTL) (Figure 6). The LDH assay showed negligible toxicity following 24 h incubation with 1.5 μM containing medium in HEK293-OATP1B1 cells pretreated with CQ(25 μM, 2 h) (Figure S3B).

Pretreatment with CQ Significantly Decreased [^{3}H]-Pitavastatin Accumulation in Human SCH

The effects of CQ on OATP1B1 transport function was further determined in the physiologically relevant human SCH model with [^{3}H]pitavastatin[60] as a probe substrate.

[^{3}H]Pitavastatin (1 μM) accumulation in human SCH was linear at least up to 5 min (Figure 7A). An incubation time of 0.5 min was used for subsequent uptake experiments in human SCH. Pretreatment with CQ (10 μM, 5 h) significantly decreased [^{3}H]pitavastatin accumulation to 0.65 ± 0.084 fold of control ($p < 0.01$ vs control, Figure 7B). The LDH assay showed negligible toxicity after 5 h treatment with CQ at 10 μM (Figure S3C).

DDIs Associated with Increased Risk of Myopathy

To determine whether CQ plus statins (rosuvastatin, pravastatin, and pitavastatin) leads to higher myopathy risk than these statins alone, pharmacoepidemiological studies were conducted using data from the FAERS database. As shown in Table 3, among all patient data, CQ plus statins led to 17.2% myopathy risk compared to statins alone, 9.2%. The relative risk (RR) is 1.87 with a p-value of 0.05. It has been reported that the female gender and advanced age are associated with higher risk for myopathy.[61] Therefore, the interaction effect between CQ and statins was further analyzed within the subpopulations. The only sub-population that showed significant CQ and statin DDIs was women. In women, CQ plus statins significantly increased the myopathy risk from 9.6% to 21.9% (Relative Risk = 2.28, $p < 0.05$ vs. statins alone). In other subpopulation analyses, men, >50 years, and <50 years, the CQ plus statins all showed increased myopathy risk, but were not statistically significant.

Effects of CQ on Transport Kinetics of [^{3}H]$E_2$17G in HEK293-OATP1B1 Cells

As shown in Figure 8, CQ pretreatment (25 μM, 2 h) significantly decreased the maximum rate of [^{3}H]$E_2$17G uptake (V_{max}) values compared to vehicle CTL treatment (57.15 ± 7.65 vs 30.38 ± 5.22 pmol/mg protein/min, $p < 0.05$), without affecting the K_m values (7.20 ± 0.57 vs 6.30 ± 0.70 μM).

Discussion

Understanding the mechanism(s) underlying altered transport activity of OATP1B1 has significant relevance in predicting potential OATP1B1-mediated DDIs. The current study demonstrates that the lysosome pathway is involved in degradation of OATP1B1, and that pre-incubation with lysosomotropic drug CQ downregulates OATP1B1-mediated transport. Pharmacoepidemiologic studies using data from the FAERS indicated that CQ plus statins (pitavastatin, rosuvastatin, and pravastatin) led to a greater myopathy risk than these statins alone.

Plasma membrane proteins are constitutively subjected to endocytosis.[57] A portion of the internalized cargo is transported via late endosome for degradation by lysosomes.[57] Treatment with CQ increased the total protein levels of OATP1B1 expressed exogenously in HEK293-OATP1B1 cells and endogenously in human SCH (Figure 2A,C). Increased total protein levels of membrane proteins following CQ treatment was reported previously for bone morphogenic protein type II receptor (BMPR-II),[62] the transmembrane protein β-site amyloid precursor protein cleaving enzyme (BACE),[63] and Na,K-ATPase.[50] Increased Na,K-ATPase total protein levels following CQ treatment was also detected in current studies (Figure 2B), consistent with previous report.[50] Plasma membrane localization of OATP1B1 has been detected by immunofluorescence staining in several reports.[3,53,64]

However, association of intracellular OATP1B1 with subcellular organelles has not been reported. Current studies demonstrated that, in addition to plasma membrane localization, the intracellular FLAG-OATP1B1 proteins were localized to LAMP-2 positive late endosomes/lysosomes in HEK293-FLAG-OATP1B1 cells without CQ treatment and under lysosome inhibition following CQ treatment (Figure 3 and Figure S2 merged images). Overall, increased total protein levels of OATP1B1 upon CQ treatment and localization of OATP1B1 with LAMP-2 suggest that the lysosome pathway is involved in the degradation of OATP1B1 proteins.[65]

In the current studies, we focused on determining whether clinically relevant concentrations of CQ affect OATP1B1 transport activity and statin uptake in human SCH. The estimated $I_{u,in,max}$ of CQ is 18.3–56.8 μM (Table 2). The unbound C_{ss} of CQ in the plasma is ~1.7 μM in rheumatoid arthritis patients treated with CQ daily for long-term.[48] In HEK293-OATP1B1 cells, pretreatment with CQ at clinically relevant concentrations (2.5–25 μM) significantly decreased OATP1B1-mediated transport of [^{3}H]$E_2$17G (Figure 4A,C) and [^{3}H]pitavastatin (1 μM, 0.5 min) (Figure 4 B). CQ pretreatment (10 μM, 5 h) also significantly decreased [^{3}H]pitavastatin accumulation (1 μM, 0.5 min) in human SCH (Figure 7B). Additionally, in HEK293-OATP1B1 cells preincubated for 2 h with 25 μM CQ (a concentration relevant to the $I_{u,in,max}$ of CQ), and subsequently treated for up to 24 h with 1.5 μM CQ (a concentration relevant to the unbound C_{ss} of CQ), values of [^{3}H]$E_2$17G accumulation were significantly less than those in the CQ-free treatment CTL (Figure 6). These findings suggest that pretreatment with CQ at clinically relevant concentrations can downregulate OATP1B1-mediated transport in OATP1B1-overexpressing cell lines and accumulation of pitavastatin in human SCH. Pitavastatin is a substrate of multiple OATP transporters, including OATP1B1, 1B3, and 2B1.[60,66] Currently, there is no known specific probe substrate or inhibitor of OATP1B1 available. This is a technical challenge to specifically study the transport function of endogenous OATP1B1 in primary human hepatocytes. Therefore, from our current study, we cannot draw a conclusion regarding whether transport function of endogenous OATP1B1 is downregulated by CQ in human SCH. The current study does not exclude the possibility that CQ may affect transport activity of other transporters in human SCH. Decreased pitavastatin accumulation in human SCH following CQ pretreatment (10 μM, 5 h) may represent a net effect of CQ on hepatic transport of pitavastatin. Further studies are warranted to determine whether other OATP transporters, including the closely related family member OATP1B3, can also be regulated by CQ.

The inhibitory effects of CQ toward OATP1B1 transport activity at clinically relevant concentrations led to our hypothesis that concurrent use of CQ and statins may have a higher myopathy risk than statins alone in patients. Pharmacoepidemiologic studies using data from the FAERS was utilized to test our hypothesis. Since CQ is a substrate and inhibitor of multiple CYP enzymes,[67,68] to minimize potential CYP450 mediated DDIs, we selected three statins, pitavastatin, rosuvastatin, and pravastatin, which are minimally metabolized through CYP450 enzymes,[34,35,69] in our pharmacoepidemiologic studies. Increased systemic exposure of these statins has been reported in subjects bearing the V174A polymorphism of OATP1B1 (reviewed by Niemi[32]). Our data indicated that CQ plus these three statins is associated with increased risk of myopathy compared to these statins alone

(Table 3). This result is consistent with the *in vitro* inhibitory effects of CQ toward OATP1B1-mediated transport and pitavastatin accumulation in human SCH following pretreatment at clinically relevant concentrations. Taken together, combination of our data from *in vitro* and pharmacoepidemiologic studies supports that potential OATP-mediated DDIs caused by CQ are likely.

A higher myopathy risk in women than men was observed in both the statins alone group and the CQ plus statins group (Table 3). The relative risk of myopathy (CQ plus statins vs statins alone) reaches a statistically significant level only in women, but not in men (Table 3). These findings are consistent with what we have observed in the medical record database[76] and reports from others[61,77] that females have higher risk for myopathy than males. Smaller vascular volumes and reduced muscle mass in females, which may result in greater tissue drug exposure per statin dose, are possible reasons of higher myopathy risk in women.[61] Higher systemic exposure of statins in women was observed for pitavastatin,[78] while there is no gender difference in systemic exposure of pravastatin.[79] For rosuvastatin, one study reported higher AUC and C_{max} in women than in men,[80] while no gender difference was observed for rosuvastatin pharmacokinetics in another study.[81] The gender difference in pharmacokinetics of CQ and the effects of CQ on pharmacokinetics of statins have not been reported. A clinical DDI study determining the effects of CQ on systemic exposure of statins in women and men may help to elucidate the potential mechanism underlying the higher myopathy risk in patients in whom CQ and statins are administered concurrently compared with statins alone.

Recently, the effects of pretreatment on OATP1B1-mediated transport were reported for several OATP1B1 inhibitors. Pretreatment with cyclosporine,[70] saquinavir, ritonavir,[71] simeprevir, asunaprevir, or daclatasvir[72] significantly decreases OATP1B1-mediated transport. Pretreatment with cyclosporine decreased the apparent IC_{50} values toward OATP1B1 inhibition.[31,73] In current studies, without CQ pre-incubation, CQ at concentrations 5–100 μM did not affect [^{3}H]$E_2$17G accumulation (1 μM, 2 min) in HEK293-OATP1B1 cells (Figure 4D). This result is consistent with a recent report that 10 μM CQ did not affect OATP1B1-mediated estrone-3-sulfate transport.[15] However, when [^{3}H]$E_2$17G accumulation was determined in the presence of CQ following CQ pre-incubation, CQ, an apparent non-inhibitor of OATP1B1 determined without pre-incubation (Figure 4D), significantly decreased OATP1B1-mediated transport (Figure 4E). The current study reports a novel inhibitory effect of lysosomotropic drug CQ toward OATP1B1-mediated transport, where an apparent noninhibitor CQ “gained” its inhibitory effect toward OATP1B1 after pre-incubation. In clinical conditions, a perpetrator drug may be administered prior to the victim drug. A perpetrator drug and a victim drug may coexist in the body for a period of time that is longer than the *in vitro* incubation time used to determine the perpetrator/substrate interaction (a few minutes in many cases). Our results together with others[31,73,74] highlight the importance of introducing a pre-incubation step when assessing the OATP1B1-mediated DDI potential of an investigational drug *in vitro*. The current approach of using the OATP1B1 inhibition tree and *R*-value to assess OATP-mediated DDIs *in vitro* assumes competitive transporter inhibition by investigational drugs.[75] Our data support that mechanism(s) other than competitive inhibition of OATP1B1 transport activity may also be important in causing OATP1B1-mediated DDIs.

The finding that CQ pretreatment increased total OATP1B1 protein levels, but decreased OATP1B1 transport activity, may seem paradoxical at first. However, transporter function is not always correlated with the total protein levels of the transporters. For example, following PKC activation, the total protein levels of OATP1B3,[41] OATP1A2,[82] and OAT1 and OAT4[83,84] were not affected while their transport function was significantly decreased. CQ pretreatment decreased V_{max} without affecting the K_m values of OATP1B1-mediated [^{3}H]$E_2$17G transport (Figure 8). A similar trend of decreased V_{max} and unaffected K_m was reported for OATP1A2[82] and OAT1,3 and 4,[83–85] dopamine transporter,[86] and sodium-glucose transporter (SGLT1)[87] upon PKC activation. In these previous studies, the decreased V_{max} of substrate transport was associated with decreased surface levels for OATP1A2,[82] OAT1, 3 and 4,[83–85] increased phosphorylation for dopamine transporter,[86] or decreased turnover rate but unaltered surface levels for SGLT1.[87] CQ has been reported to affect cell surface levels of several plasma membrane proteins such as the tumor necrosis factor receptor (TNF-R),[88] the α-macroglobulin-protease (αM-P) surface receptors,[89] and BMPR-II.[62] Short-term treatment with monensin and bafilomycin A1, which have lysosome inhibition activity, also resulted in significantly decreased OATP1B1-mediated transport (Figure 5). In addition to inhibiting lysosomal activity, CQ, monensin, and bafilomycin A1 have other effects such as affecting trafficking of the membrane protein.[90] The exact mechanism(s) through which CQ, monensin, and bafilomycin A1 downregulate transport function of OATP1B1 remain unknown and warrant further investigation.

In conclusion, the present studies report novel findings that CQ inhibits lysosome degradation of OATP1B1 protein; pre-incubation with CQ downregulates OATP1B1-mediated transport in stable cell lines, and decreases pitavastatin uptake in human SCH. Our *in vitro* data in combination with pharmacoepidemiologic studies support that CQ has potential to cause OATP-mediated DDIs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

$E_2$17G	estradiol 17β-D-glucuronide
ABC	ATP-binding cassette
DDIs	drug–drug interactions
HBSS	Hanks balanced salt solution

DMEM	Dulbecco's modified Eagle medium
BSA	bovine serum albumin
MEM	minimum essential medium
NEAA	nonessential amino acids
DPBS	Dulbecco's phosphate-buffered saline
HEK293	human embryonic kidney 293
LDH	lactate dehydrogenase
OATP	organic anion transporting polypeptide
OATs	organic anion transporters
SLCO	solute carrier organic anion

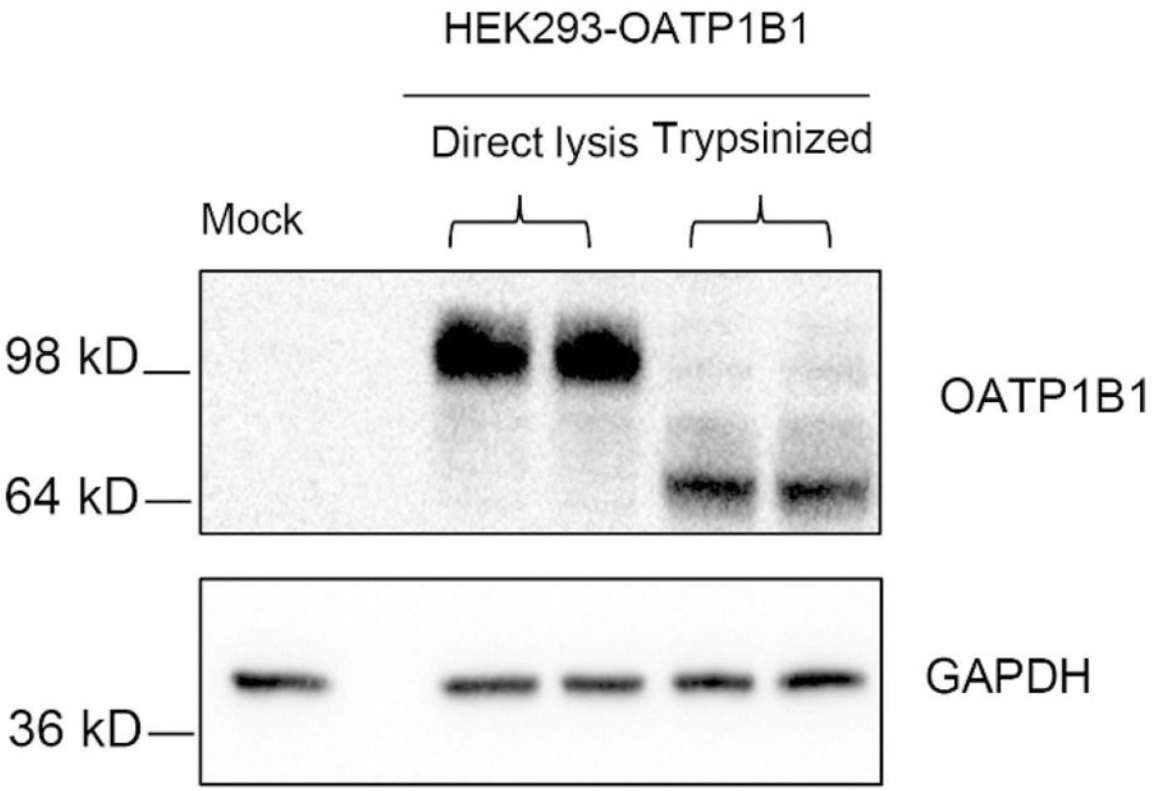


Figure 1.

Characterization of OATP1B1 antibody and optimization of OATP1B1 immunoblot protocol. HEK293-OATP1B1 and -Mock cells were seeded at 1.1×10^5 cells/well in 24-well plates and cultured for 48 h prior to being harvested for immunoblot. Whole cell lysates (WCL) of HEK293-OATP1B1 cells were prepared either by adding ice-cold lysis buffer directly onto the culture plate after aspirating culture medium without trypsinization or by lysing the cells after trypsinization and subsequent washing. Immunoblot of OATP1B1 was performed with the custom-generated OATP1B1 antibody. GAPDH was used as the loading control. Representative images from $n = 3$ experiments are shown.

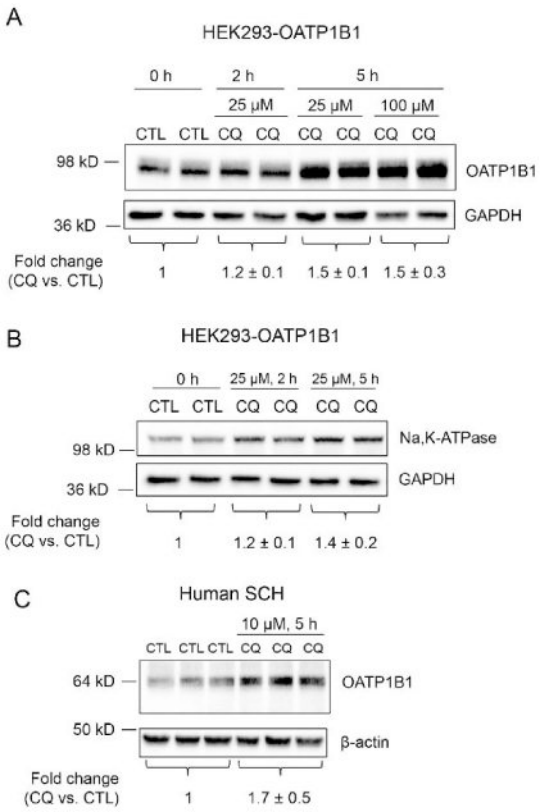


Figure 2.

Effects of CQ on total protein levels of OATP1B1 and Na,K-ATPase in HEK293-OATP1B1 cells and on OATP1B1 total protein levels in human SCH. HEK293-OATP1B1 cells were seeded in 24-well plates at 1.1×10^5 cells/well, and were cultured for 48 h prior to treatment. Human SCH were cultured as described in the Experimental Section. WCL was prepared by directly lysing the cells in the culture plate without trypsinization. Representative immunoblot images of OATP1B1 (A) and Na,K-ATPase (B) in WCL of HEK293-OATP1B1 pretreated with CQ (25 or 100 μM) or CTL for indicated times are shown. GAPDH were used as the loading control for A and B. OATP1B1 and Na,K-ATPase protein levels determined by densitometry were normalized to levels of GAPDH. Fold changes of total protein levels of OATP1B1 in A and Na,K-ATPase in B (CQ vs CTL) are expressed as mean ± SD (n = 4 for both A and B). (C) Immunoblot of OATP1B1 and β-actin in WCL of human SCH pretreated with CQ (10 μM) or CTL for 5 h. OATP1B1 protein levels determined by densitometry were normalized to levels of β-actin. Fold changes of total protein levels of OATP1B1 (CQ vs CTL) are expressed as mean ± SD of n = 3 donor hepatocytes. Representative images are shown.

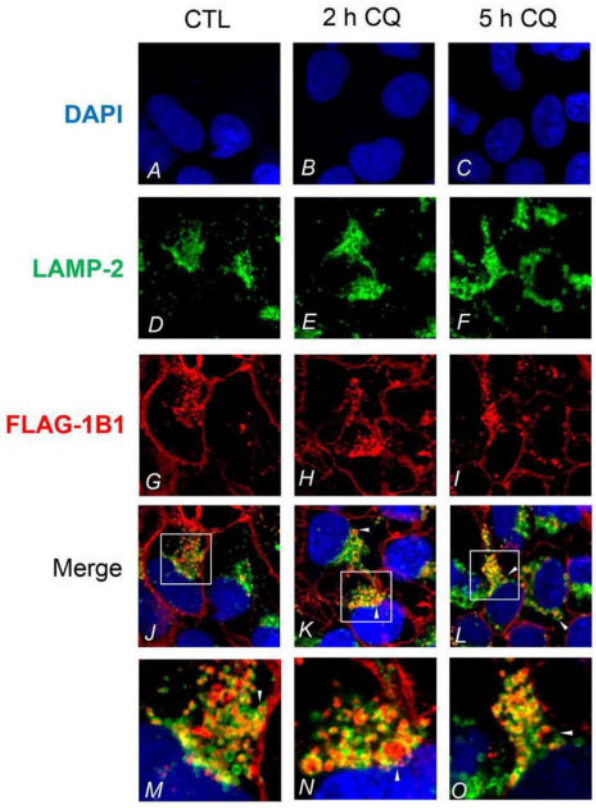


Figure 3.

Colocalization of FLAG-OATP1B1 and LAMP-2 in HEK293-FLAG-OATP1B1 cells. Coimmunofluorescence staining of FLAG-OATP1B1 (red) and LAMP-2 (green) was performed in HEK293-FLAG-OATP1B1 cells treated with vehicle CTL or 25 μM CQ for 2 and 5 h as described in the Experimental Section. White arrow heads indicate the accumulation of FLAG-OATP1B1 inside the LAMP-2-positive vacuoles. Nuclei were counterstained with DAPI (blue). Images were taken using Olympus FV1000 confocal microscope. Representative images from the same experiments are shown (3 and 2 separate experiments for 2 h and 5 h treatment, respectively).

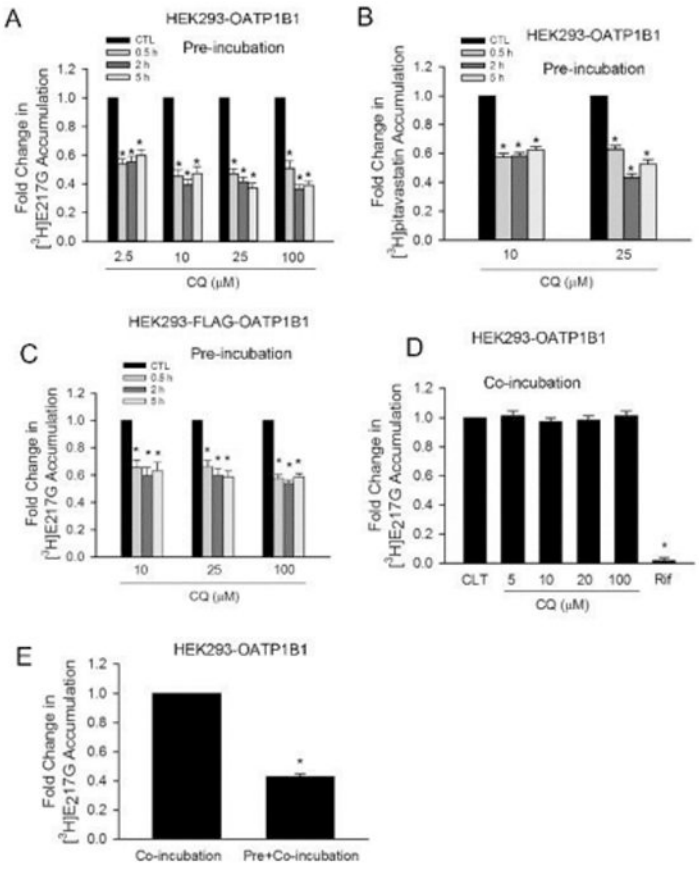


Figure 4.

Pretreatment effect and direct inhibition of CQ on OATP1B1-mediated transport. Cells were seeded at 1.1×10^5 cells/well in 24-well plates and cultured for 48 h (A, B, C, and E) or 72 h. (D) Accumulation of [^{3}H]$E_2$17G (1 μM, 2 min) or [^{3}H]pitavastatin accumulation (1 μM, 0.5 min) was determined in HEK293-OATP1B1 or -FLAG-OATP1B1 cells, as indicated in the figures. (A) Model-estimated fold change and associated SE in [^{3}H]$E_2$17G accumulation vs CTL treatment in HEK293-OATP1B1 cells at each indicated pretreatment concentration and time (pre-incubation). (B) Model-estimated fold change and associated SE in [^{3}H]pitavastatin accumulation vs CTL treatment in HEK293-OATP1B1 cells at each indicated pretreatment concentration and time (pre-incubation). (C) Model-estimated fold change and associated SE in [^{3}H]$E_2$17G accumulation vs CTL treatment in HEK293-FLAG-OATP1B1 cells at each indicated pretreatment concentration and time (pre-incubation). (D) Model-estimated fold change and associated SE in [^{3}H]$E_2$17G accumulation in the presence of 5–100 μM CQ or 25 μM rifampicin (Rif) vs CTL in HEK293-OATP1B1 cells without CQ pretreatment (co-incubation). (E) Model-estimated fold change and associated SE in [^{3}H]$E_2$17G accumulation vs co-incubation control. Following pretreatment in culture medium containing 100 μM CQ (pre+co-incubation) or vehicle control (co-incubation) for 5 h, HEK293-OATP1B1 cells were rinsed three times with HBSS, and the [^{3}H]$E_2$17G accumulation was determined in the presence of 100 μM CQ. A generalized linear mixed model as described in the Experimental Section was fit to data in A–E (n = 3 in triplicate for all panels of A–E). To account for multiple comparisons, p-values were adjusted based on

Bonferroni's method. * indicates a statistically significant difference (adjusted $p < 0.05$) vs CTL.

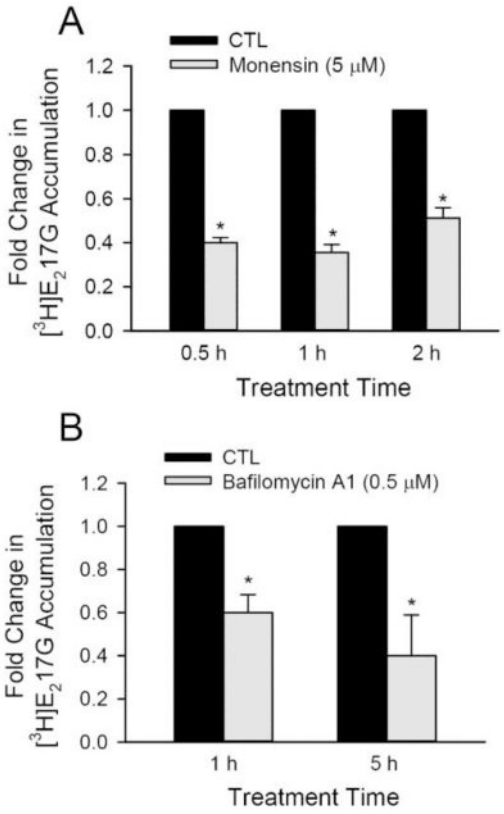


Figure 5.

Effects of monensin and bafilomycin A1 on OATP1B1-mediated [^{3}H]$E_2$17G transport. HEK293-OATP1B1 were seeded at 1.1 × 10^5 cells/well in 24-well plates for up to 72 h. (A) Model-estimated fold change and associated SE in [^{3}H]$E_2$17G accumulation vs CTL at each indicated monensin (5 μM) pretreatment time. (B) Model-estimated fold change and associated SE in [^{3}H]$E_2$17G accumulation vs CTL at each indicated bafilomycin A1 (0.5 μM) pretreatment time. A generalized linear mixed model as described in the Experimental Section was fit to data (n = 3 in triplicate for both A and B). * indicates a statistically significant difference ($p < 0.05$) vs CTL.

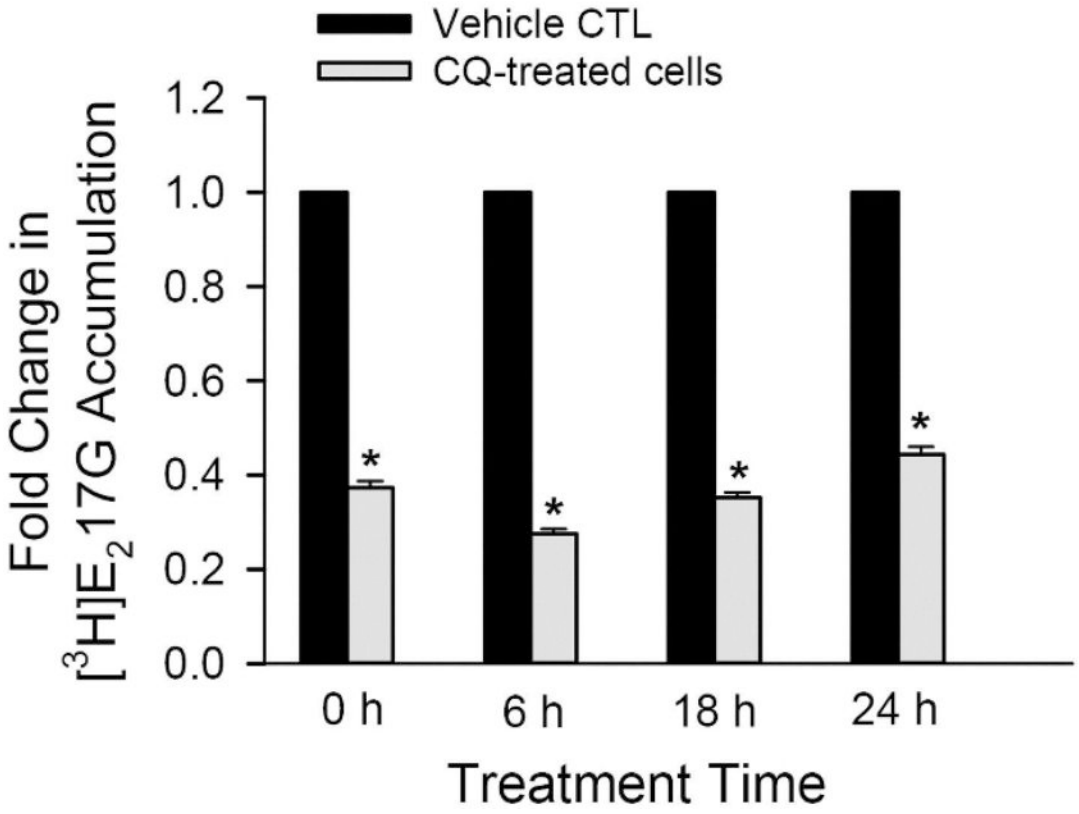


Figure 6.

Effects of CQ on OATP1B1-mediated [^{3}H]$E_2$17G transport after prolonged treatment in HEK293-OATP1B1 cells. HEK293-OATP1B1 cells were seeded at 0.8×10^5 cells/well in 24-well plates, and cultured for 48 h prior to treatment. Cells were preincubated with CQ-free (CTL) or 25 μM CQ-containing medium for 2 h. At the end of pre-incubation, the culture medium was removed. CTL medium preincubated cells were cultured in CQ-free medium (CTL, black bar) and 25 μM CQ-preincubated cells were cultured in medium containing 1.5 μM CQ (grey bar), for indicated times up to 24 h. [^{3}H]$E_2$17G accumulation (1 μM, 2 min) was determined at indicated time points ($n = 4$ in triplicate). Fold change and SE in [^{3}H]$E_2$17G accumulation vs CTL at each indicated time points were estimated by generalized linear mixed models, as described in the Experimental Section. * indicates a statistically significant difference ($p < 0.05$) vs CTL.

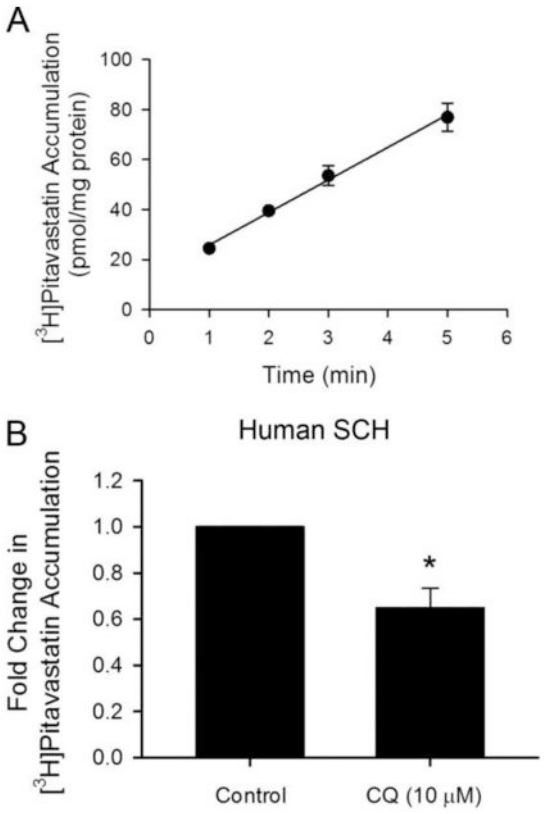
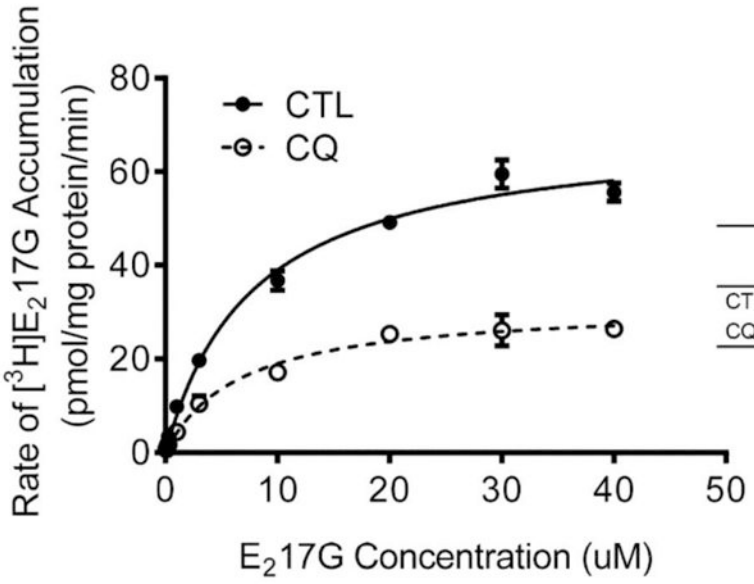


Figure 7.

Effect of CQ pretreatment on the accumulation of [^{3}H]pitavastatin in human SCH. (A) Time-dependent accumulation of [^{3}H]pitavastatin (1 μM) in human SCH. Data represent the mean ± SD in triplicate from a single hepatocyte donor. (B) Model-estimated fold change and associated SE in [^{3}H]pitavastatin accumulation vs CTL in human SCH pretreated with 10 μM CQ or CTL for 5 h. Following pretreatment in culture medium containing CQ (10 μM) or control, human SCH were rinsed three times with HBSS, and [^{3}H]pitavastatin accumulation (1 μM, 0.5 min) was determined. Fold change and SE were estimated by a generalized linear mixed model, as described in the Experimental Section (n = 3 in triplicate). * indicates a statistically significant difference ($p < 0.05$) vs CTL.



	V_{max} pmol/mg protein/min	K_m μM
CTL	57.15 ± 7.65	7.20 ± 0.57
CQ	30.38 ± 5.22*	6.30 ± 0.70

Figure 8.

Effects of CQ on kinetic parameters of OATP1B1-mediated [^{3}H]$E_2$17G transport. HEK293-OATP1B1 and -Mock cells were seeded at 1.1 × 10^5 cells/well in 24-well plates and were cultured for 48 h prior to experiment. The concentration-dependent accumulation of [^{3}H]$E_2$17G (0.1–40 μM, 2 min) was determined in HEK293-OATP1B1 and -Mock cells pretreated with CTL or CQ (25 μM, 2 h). Values of [^{3}H]$E_2$17G accumulation in Mock cells were subtracted from those in HEK293-OATP1B1 cells. V_{max} and K_m values were determined as described in the Experimental Section. Solid and dashed lines represent the best fits of the Michaelis–Menten equation to the data of CTL (black circles) and CQ (25 μM, 2 h) pretreatment (white circles), respectively. Representative graph of three independent experiments in triplicate is shown. Student's *t* test was conducted to compare the V_{max} and K_m values between CQ and CTL pretreatment. * indicates a statistically significant difference ($p < 0.05$; CQvs CTL).

Table 1

Demographics of Human Hepatocyte Donors

donors	age	gender	race	BMI[a]	smoking	alcohol use	experiments
GC4008	69	M	Caucasian	24.7	no	no	Figures 2C, 7B
HUM 4059	17	F	Caucasian	25.5	no	no	Figure 7B
HUM 4089	36	M	Caucasian	30	no	no	Figure 7A
HUM 4110	56	M	Caucasian	26	yes	8–10 beers/week	Figure 7B
HUM 4130	64	M	African American	23.6	quit 3 months ago	none reported	Figure 2C
HUM 4140	21	M	Caucasian	32	yes	rare	Figure 2C

[a]Body mass index.

Table 2

Estimation of CQ Maximum Unbound Concentration at the Inlet to the Liver ($I_{u,in,max}$)

variable			
dose of CQ (mg)[a]	191[48]	300[39]	600[49]
C_{max} (μM)	0.72–12.18[48]	0.24[39]	1.2[49]
$I_{u,in,maxb}$[b] (μM)	18.3–23.4	28.2	56.8

[a] CQ doses were converted to CQ base.

[b] Estimated on the basis of eq 1.

Table 3

DDI-Myopathy Analysis Adjusted for Age and Sex

population	drugs	*N*[a]	*M*[a]	risk[b]	relative risk/*P*-value[c]
all	statins[d] alone	88,682	8,149	9.2%	1.87/0.05
	CQ and statins[d]	58	10	17.2%	
women	statins[d] alone	44,212	4,234	9.6%	2.28/0.03*
	CQ and statins[d]	32	7	21.9%	
men	statins[d] alone	44,123	3,743	8.5%	1.35/0.48
	CQ and statins[d]	26	3	11.5%	
>50 years[e]	statins[d] alone	50,322	5,347	10.6%	1.89/0.06
	CQ and statins[d]	40	8	20.0%	
<50 years[e]	statins[d] alone	33,413	2,732	8.2%	1.35/0.65
	CQ and statins[d]	18	2	11.1%	

[a] *N*: Number of patients taking drug. *M*: Number of myopathies. *N* and *M* represent sums of counts for each individual statin.

[b] Risk is calculated as $M/N \times 100\%$.

[c] Relative risk is calculated as risk (CQ and statins)/risk (statins alone).

Statistical analysis was performed with the Chi-square test.

[*] indicates a statistically significant difference ($p < 0.05$) (CQ and statins vs statins alone).

[d] Statins: rosuvastatin, pravastatin, and pitavastatin.

[e] Age.