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OATP1B1/1B3 activity in plated primary human hepatocytes over time in culture

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ABSTRACT

Primary human hepatocytes are widely used as an *in vitro* model for evaluation of drug metabolism and transport. However, it has been shown that the gene expression of many drug-metabolizing enzymes and transporters change in culture. The aim of the present study was to evaluate the activity of organic anion-transporting polypeptide 1B1 (OATP1B1) and 1B3 (OATP1B3) in plated primary human hepatocytes over time in culture. The uptake kinetics of the OATP1B1/1B3 substrate [^{3}H]-estradiol-17β-D-glucuronide was determined in cells from five donors. An extensive and variable decrease in OATP1B1/1B3 activity and/or increase in passive diffusion was observed over time. Already after 6 h in culture, the OATP1B1/1B3 activity was not possible to determine in liver cells from one donor, while after 24 h, the uptake activity was not measurable in one additional donor. In the other three, the decrease in CL_{int} (V_{max}/K_m) values ranged from 15% to 86% after 24 h in culture compared to the values measured at 2 h. Visual examination of OATP1B1 protein expression by confocal microscopy showed localization to the plasma membrane as expected, and an extensive decrease in OATP1B1 expression over time in culture supported the decline in activity. The significant reduction in *SLCO1B1* and *SLCO1B3* gene expression over time determined by RT-PCR also supported the loss of OATP1B1/1B3 activity. In conclusion, plated primary human hepatocytes are useful as an *in vitro* model for OATP1B1/1B3-mediated uptake studies, but the culture time may substantially change the uptake kinetics.

1. Introduction

The propensity of new drugs to exhibit large variation in pharmacokinetic profiles and to be sensitive to drug–drug interactions are major concerns when developing new drugs. Therefore, the factors determining the pharmacokinetic profile of a compound should be described as early as possible in the drug discovery process. Membrane transporters are today recognized as a major determinant of pharmacokinetic variability of certain drugs, in addition to drug metabolizing enzymes [1]. Therefore, in the drug discovery and development process of new chemical entities, both uptake and efflux processes should be characterized in parallel with potential metabolism of drug candidates. Such studies require robust and human relevant *in vitro* models for reliable predictions of hepatic uptake, distribution, and excretion of drug candidates during preclinical testing.

The hepatic uptake transporter organic anion-transporting polypeptide 1B1 (OATP1B1) and 1B3 (OATP1B3) are expressed predominantly in the basolateral membrane of human hepatocytes [2–4] and serve as bidirectional facilitated diffusion transporters [5]. The OATP-mediated substrate uptake is pH dependent and generally accompanied by bicarbonate efflux [6]. OATP1B1 and OATP1B3 have an overlapping substrate spectrum. They are carriers of a variety of endogenous substances, including bile salts, hormone conjugates and steroids [7,8], and play a key role in the hepatic uptake of many drugs, e.g. HMG-CoA reductase inhibitors (statins), angiotensin II receptor antagonists, angiotensin-converting enzyme inhibitor and anti-cancer agents [9–14].

Several OATP1B1-mediated drug–drug interactions involving the agents mentioned above have been reported [15–19], e.g. substantially increased statin plasma levels during co-administration of the OATP1B1 inhibitor cyclosporine A [20,21]. Furthermore, several single nucleotide polymorphisms (SNPs) and haplotypes of *SLCO1B1*, the gene encoding OATP1B1 [22], have shown to affect drug disposition and drug response of OATP1B1 substrates in individuals carrying specific variants of *SLCO1B1* [23–25]. Polymorphisms in the *SLCO1B3* gene encoding OATP1B3 have also been

Abbreviations: OATP, organic anion-transporting polypeptide; SNP, single nucleotide polymorphism; E17βG, estradiol-17β-D-glucuronide; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Ct, cycle threshold; OCT1, organic cation transporter 1; OAT2, organic anion transporter 2; NTCP, sodium-taurocholate cotransporting polypeptide.[2]

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reported [26–28], but the knowledge on functional consequences of genetic variants in the *SLCO1B3* gene is limited [29].

Transfected (over-expressed) cell systems offers a convenient model for identification of OATP1B1 and OATP1B3-mediated transport, but *in vitro–in vivo* clearance extrapolation is challenging. Primary hepatocytes, plated or in suspension, represent a common *in vitro* model for evaluation of human hepatic drug transport, metabolism and clearance [30]. Primary hepatocytes express a complete set of enzymes and transporters involved in hepatic drug clearance. This might be favorable compared to transfected cell systems in terms of ability to predict relevant *in vivo* clearance parameters. Freshly isolated primary human hepatocytes have been extensively characterized with respect to expression and activity of drug-metabolizing enzymes and canalicular transporters [31–36]. However, the expression, localization and function of sinusoidal transporters, such as OATP1B1 and OATP1B3, have been less studied, especially with respect to a potential altered transport activity with time in culture. The aim of the present investigation was therefore to study uptake kinetics and expression of OATP1B1/1B3 in plated primary human hepatocytes over time in culture.

2. Materials and methods

2.1. Chemicals and reagents

[^{3}H]-estradiol-17β-D-glucuronide ([^{3}H]-E17βG) was obtained from Perkin Elmer (Boston, MA). Unlabeled estradiol-17β-D-glucuronide, $CaCl_2$, collagenase, DNase, EGTA, fetal bovine serum (FBS), L-glutamine (200 mM), $NaHCO_3$, saponin and trypsine inhibitor were all obtained from Sigma–Aldrich (Steinheim, Germany). Alexa fluor 488 conjugated goat anti-rabbit secondary antibody, Hanks' balanced salt solution (HBSS), HBSS without Ca^{2+}, Mg^{2+} and phenol red, HEPES, phosphate-buffered saline (PBS) and Williams' Medium E were obtained from Invitrogen (Carlsbad, CA). HCl and NaOH were obtained from Merck (Darmstadt, Germany). Sodium chloride was obtained from Fresenius Kabi (Bad Homburg, Germany). Rat tail collagen type I was obtained from BD Biosciences (Bedford, MA). Rabbit polyclonal antibody against human OATP1B1 was a kind gift from Dr. Bruno Stieger (University Hospital Zurich, Zurich). High-purity water was obtained from an ELGA purification system (ELGA, High Wycombe, UK). All other chemicals and reagents were of analytical grade and were available from commercial sources.

2.2. Human hepatocyte isolation

Adult human liver biopsies were obtained from Sahlgrenska Hospital (Göteborg, Sweden) and originated from patients undergoing partial hepatectomy for primary or secondary tumours. All tissues were obtained by qualified medical staff, with donor informed consent and the approval of the Local Ethics Committee at Sahlgrenska University Hospital. Donor information regarding age, sex, disease, size of resection, cell viability, yield of cells and genotype is summarized in Table 1. All donors were Caucasian.

The resections were flushed with ice cold physiological NaCl solution immediately after surgery and kept in Williams' Medium E (supplemented with 25 mM HEPES and 2 mM L-glutamine), pH 7.4 over night (max 18 h). Hepatocytes were isolated by a two-step collagenase perfusion through the existing vasculature [37–39]. The general buffer consisted of HBSS without Ca^{2+}, Mg^{2+} and phenol red supplemented with HEPES (10 mM) and $NaHCO_3$ (3.75 mM), pH 7.4. Washing buffer I contained general buffer supplemented with EGTA (0.5 mM). Digestion buffer contained general buffer supplemented with $CaCl_2$ (5 mM), collagenase (157 Units/ml) and trypsin inhibitor (68 mg/L). Washing buffer III contained general buffer supplemented with DNase (28 kunitz Units/ml). Collagenase, trypsin inhibitor and DNase were added to its respective buffer solutions directly before use. Washing buffer II and IV were the same as the general buffer.

Four cannulae were inserted into present vasculature, and the cut surfaces of the resection as well as the point of entry of the cannulae were coated with glue (Loctite 401 Prism Cyanoacrylate Adhesive, Henkel Corporation, Rocky Hill, CT). The tissue was perfused (7 ml/min/cannulae) with washing buffer I for 10 min, with washing buffer II for another 15 min, and finally with digestion buffer for 15 min. All steps were conducted at 37 °C. The digested tissue was transferred to washing buffer III, and cell dissociation was obtained by breaking the capsule with a sharp instrument and gently squeezing the tissue. After filtering the residual tissue and cell suspension through a fine nylon mesh, the cell suspension was centrifuged at 100 × *g* for 3 min. The cell pellet was resuspended in washing buffer III and centrifuged at 100 × *g* for 3 min. The cell pellet was resuspended in washing buffer IV and centrifuged at 100 × *g* for 3 min. The final cell pellet was resuspended in Williams' Medium E containing 25 mM HEPES and 2 mM L-glutamine, pH 7.4. Initial cell viabilities were determined using trypan blue exclusion. If the cells did not fully dissociate after collagenase perfusion, direct digestion incubation of the residual liver tissue was performed. The residual liver was placed in a tube with digestion buffer and incubated for 20 min at 37 °C in water bath. The content was filtered through a nylon mesh, and the suspension was centrifuged at 100 × *g* for 3 min. The cell pellet was resuspended in washing buffer III and further treated as the cell suspension from the collagenase perfusion.

Primary human hepatocytes were suspended in *InVitro*GRO™ CP Medium supplemented with *Torpedo*™ Antibiotic Mix (Celsis In Vitro Technologies, Baltimore, MD), and plated in 24-well plates pre-coated with rat tail collagen type I and in collagen type I coated 4-well culture slides (BD Biosciences) at a density of 0.25×10^6 cells/well. Based on a pilot experiment where cells were cultured for up to 48 h, the experimental set-up was chosen to allow cells to be cultured for 2, 6 and 24 h at 37 °C, 5% CO_2, and 90–95% humidity. At each time point OATP1B1 transport activity was determined and separate hepatocytes were harvested in RNAprotect Cell Reagent

Table 1
Human donor demographics.

Donor ID	Age (years)	Sex (F/M)	Weight (kg)	Disease	Size of resection (g)	Yield (viable hepatocytes/g)	Viability (%)	*SLCO1B1 521T>C* polymorphism
Donor # 1	66	M	83	Metastasis (GIST[a])	N.A.	N.A.	67	*T/T*
Donor # 2	60	M	89	Metastasis (colon)	26.5	7.6×10^6	88	*C/C*
Donor # 3	42	F	78	Metastasis (GIST[a])	39.0	7.2×10^6	85	*C/T*
Donor # 4	63	M	65	Metastasis (rectal)	30.6	14.7×10^6	85	*C/C*
Donor # 5	59	M	76	Metastasis (colon)	20.2	6.4×10^6	80	*C/T*

N.A., not applicable; M, male; F, female.
[a] Gastrointestinal stromal tumor.

(Qiagen, Hilden, Germany) for RNA isolation. Hepatocytes cultured in culture slides were fixated at the same time points for confocal microscopy (see method below) and frozen at −20 °C.

2.3. Transport studies

Culture medium was removed and the cells were rinsed twice with 700 μl/well buffer solution (HBSS containing 25 mM HEPES, pH 7.4) at 37 °C. Cells were pre-incubated in 250 μl/well buffer solution for 5 min in a plate incubator (THERMOstar, BMG LabVision, Stockholm) set to a temperature of 37 °C. 250 μl/well [^{3}H]-E17βG (0.07–50 μM, 1 μCi/ml) diluted in buffer solution was added, and the hepatocytes were incubated for 1 min at 37 °C. The cells were rinsed 3 times with ice-cold buffer solution and lysed with 0.2 M NaOH at 4 °C for 60 min. Cell lysates were neutralized with 0.2 M HCl, and accumulation of [^{3}H]-E17βG in the hepatocytes was determined by liquid scintillation spectroscopy (Wallac Win Spectral, 1414 Liquid Scintillation Counter, PerkinElmer). Total protein per well was quantified using the Thermo Scientific Pierce BCA Protein Assay Kit (Rockford, IL) according to the manufacturer's instructions. To determine the passive uptake, a parallel experiment was carried out at 4 °C following the same procedure as outlined above for the 37 °C incubation. To determine whether the decreasing temperature alter the passive diffusion of E17βG, uptake experiments was performed in HepG2 cells (1 μM, 1 μCi/ml, 1 min). To optimize the time of incubation and assure that the incubation time applied in the concentration dependent uptake studies was within the linear range of uptake, time dependent uptake assays were performed in the span of 0.5–20 min and the linear interval of uptake was determined. The relationship between the substrate concentration ([S]) and the initial transport velocity (v_0) was evaluated by the Michaelis–Menten equation (equation 1):

$$v_0 = \frac{V_{max} \times [S]}{K_m + [S]}$$

where K_m represents the Michaelis constant and V_{max} is the maximum initial uptake rate of the transporter-mediated uptake. The active transporter-mediated uptake was calculated as the difference in substrate accumulation at 37 °C and 4 °C, and K_m and V_{max} were estimated by fitting the calculated active uptake data to the Michaelis–Menten model (equation 1.4) without weighting (GraphPad Software, Prism 4 version 4.03). The results from the analyses were given as point estimates of the K_m and V_{max} values ±standard deviation (SD). Intrinsic clearance (CL_{int}) was calculated by the following equation (equation 2):

$$CL_{int} = \frac{V_{max}}{K_m}$$

using the K_m and V_{max} estimates.

2.4. Confocal laser scanning immunofluorescence microscopy

Primary human hepatocytes were fixed in 4% (w/v) paraformaldehyde for 15 min, and washed twice with PBS. Cells were permeabilized in 0.5% saponin in PBS for 10 min, washed 3 times with PBS, and allowed to dry. The cells were blocked in 10% FCS in PBS for 30 min. Blocking buffer was removed, and samples were incubated immediately with anti-OATP1B1 antibody diluted 1:100 in 1% FCS in PBS for 60 min. Samples were washed 3 times with PBS for 5 min, and subsequently incubated with fluorochrome-conjugated secondary antibody diluted 1:1000 in 1% goat serum in PBS. Samples were washed 3 times with PBS for 5 min, and rinsed briefly with distilled water. Hepatocytes were mounted onto glass cover slips and slides with ProLong Gold antifade reagent with DAPI (Moleculat Probes, Eugene, OR). Immunofluorescent images were detected using a Nikon Eclipse TE2000-E inverted research microscope (Nikon, Melville, NY). Confocal scanning was performed in the x–y field, and the laser power and PMT gain were held constant for each sample. Scan averages was set to 10 and the 60× magnification oil objective lens was used for all image acquisitions. Images were acquired digitally and processed using the EZ-C1 3.50 software (Nikon, Melville, NY). Negative control experiments showed no signal at the settings used to image specific fluorescence.

2.5. RNA isolation and cDNA synthesis

Extraction of total RNA from the cells was performed using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA was digested with DNase treatment on-column using RNase-free water (Qiagen). RNA quantity was determined spectrophotometrically using a GeneQuant pro RNA/DNA calculator (GE Healthcare Ltd., Buckinghamshire, England). 0.6 μg total RNA was reversed transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The pre-amplified cDNA was diluted with RNase-free water to a final concentration of 3 ng/μl.

2.6. Quantitative real-time PCR

Quantitative real-time PCR amplification reactions were carried out in an Applied Biosystems 7500 Real-Time PCR System. Gene expression was estimated using TaqMan® Gene Expression Assays (Applied Biosystems) according to the manufacturer's recommendations. Briefly, a 15 μl reaction mixture contained 5 μl cDNA, 7.5 μl Taqman® Universal Master Mix (2×), 0.75 μl TaqMan® Gene Expression Assays (20×) containing unlabeled PCR primers and TaqMan® FAM™ dye-labeled MGB probe, and 1.75 μl RNase free water. The thermal cycle had initial steps of 2 min at 50 °C for UNG activation and 10 min at 95 °C for initial denaturation of target DNA and activation of AmpliTaq Gold DNA polymerase, followed by 40 PCR cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing and extension. Each sample was analyzed in triplicate and data was captured using the 7500 Real-Time PCR System Sequence Detector Software v1.4.0 (Applied Biosystems). Gene product measured by QRT-PCR were *SLCO1B1* (Hs00272374_m1) and *SLCO1B3* (Hs00251986_m1). Expression levels were estimated using the ΔΔCt method and normalized against HPRT (Hs99999909_m1) using 2 h plated primary human hepatocytes as calibrator. ΔΔCt was transformed into fold-change by the formula: fold change = $2^{-\Delta\Delta Ct}$.

2.7. DNA isolation and genotyping

DNA was extracted with AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's instructions. All donors were screened for the single-nucleotide polymorphism *521T>C* (Val174Ala). The mutation was determined by a polymerase chain reaction–restriction fragment length polymorphism assay based on a previously reported method and nucleotide sequences of primers [40]. The DNA fragment patterns

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Table 2
Kinetic parameters for the uptake of ^{3}H-E17βG in plated primary human hepatocytes after 2, 6 and 24 h in culture. All data are shown as mean ± SD, $n = 3$.

Time in culture	2 h			6 h			24 h		
	K_m (μM)	V_{max} (pmol/min/mg protein)	CL_{int} (ml/min/mg protein)	K_m (μM)	V_{max} (pmol/min/mg protein)	CL_{int} (ml/min/mg protein)	K_m (μM)	V_{max} (pmol/min/mg protein)	CL_{int} (ml/min/mg protein)
Donor # 1	7.5 ± 3.9	454 ± 76	60.4 ± 19.7	N.A.	N.A.	N.A.	26.2 ± 19.8	224 ± 81	8.5 ± 4.1
Donor # 2	6.6 ± 1.4	155 ± 11	23.4 ± 8.3	4.2 ± 0.4	143 ± 4	34.1 ± 10.9	N.D.	N.D.	N.D.
Donor # 3	15.2 ± 1.0	342 ± 9	22.5 ± 9.0	10.5 ± 2.3	243 ± 19	23.0 ± 8.2	12.0 ± 0.8	177 ± 5	14.7 ± 5.5
Donor # 4	22.0 ± 3.8	566 ± 35	25.7 ± 9.3	18.6 ± 3.5	514 ± 33	27.6 ± 9.5	31.9 ± 3.8	698 ± 34	21.9 ± 8.9
Donor # 5	0.9 ± 0.7	28 ± 4	29.4 ± 5.3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.A., not applicable; N.D., not detectable.

generated after restriction enzyme digestion were analyzed by electrophoresis on a 3% agarose gel. Positive control was kindly supplied by Dr M. Nakajima, Division of Drug Metabolism, Kanazawa University, Kanazawa, Japan.

2.8. Statistical analysis

Triplicate experiments were performed, and the mean value ±SD was calculated for all parameters obtained from individual experiments. The significance of changes in kinetic parameter estimates (K_m and V_{max}) and the calculated intrinsic clearance (CL_{int}) of OATP1B1/1B3-mediated transport, changes in passive diffusion of [^{3}H]-E17βG, and changes in *SLCO1B1* and *SLCO1B3* mRNA expression was estimated using the Student's *t* test. *P*-values <0.05 were considered as statistically significant.

3. Results

3.1. Uptake of E17βG

To examine the activity of OATP1B1/1B3 in plated primary human hepatocytes over time in culture, concentration dependent uptake studies of [^{3}H]-E17βG at 37 °C and 4 °C was performed after 2, 6 and 24 h in culture. The estimated K_m, V_{max} and CL_{int} values at each time point are shown in Table 2. A concentration dependent active uptake was shown in all donors after 2 h in culture with an average CL_{int} value of 32.3 ± 15.9 ml/min/mg protein. The CL_{int} values estimated in donor # 2–5 were of similar magnitude (22.5–29.4 ml/min/mg protein), while donor # 1 had a higher CL_{int} value (60.4 ml/min/mg protein). The K_m values obtained from donor # 1–4 at 2 h ranged from 6.6 to 22 μM, and the respective V_{max} values ranged from 155 to 566 pmol/min/mg protein. Due to low active uptake in Donor # 5, an uncertain K_m estimate of 0.9 ± 0.7 μM and a low V_{max} (27 pmol/min/mg protein) were obtained.

After 6 h in culture, the OATP1B1/1B3 activity was not possible to determine in liver cells from donor # 5. After 24 h in culture, the OATP1B1/1B3 activity was not measurable in one additional donor (# 2). In the three donors (# 1, 3 and 4) that could be evaluated after 24 h in culture, the decrease in OATP1B1/1B3 activity ranged from 15% to 86% compared to the CL_{int} values assessed at 2 h. Concentration dependent uptake profile of plated primary human hepatocytes from donor # 3 at 2, 6 and 24 h are shown in Fig. 1. Compared to the V_{max} value at 2 h, the decrease in maximum initial uptake rate was 29% and 48% after 6 and 24 h, respectively in donor # 3. However, no change in CL_{int} was observed after 6 h, while after 24 h, there was a 35% decrease in OATP1B1/1B3 activity.

The average passive uptake of [^{3}H]-E17βG (50 μM) into hepatocytes (at 4 °C) after 6 and 24 h in culture compared to after 2 h is shown in Fig. 2. The average passive uptake increased by 28% ($p < 0.05$) and 90% ($p < 0.05$) after 6 and 24 h, respectively. The increase in passive uptake after 24 h ranged from 18% to 176%. Values of similar magnitude were observed at all concentrations tested (data not shown). The total uptake of [^{3}H]-E17βG (1 μM) into HepG2 cells at 37 °C was 1.6× higher than the uptake at 4 °C ($p = 0.18$).

3.2. Protein expression and localization of OATP1B1

Protein expression and cellular localization of OATP1B1 were determined by laser scanning confocal microscopy after incubation with OATP1B1 antibody followed by conjugation with a fluorochrome-labeled secondary antibody. Cell nuclei were stained with DAPI to determine the appearance of the hepatocytes. Examination of plated primary human hepatocytes cultured for 2 h before fixation showed that OATP1B1 was expressed and localized to the hepatocyte plasma membrane (Fig. 3, panels A and D). OATP1B1 was evenly distributed on the cell surface. Similar distribution pattern was observed in plated hepatocytes cultured for 2 and 6 h, but the expression of OATP1B1 was less pronounced after 6 h (Fig. 3, panels B and E). Examination of plated primary human hepatocytes cultured for 24 h before fixation showed an extensive decrease in OATP1B1 expression compared to 2 h (Fig. 3, panels C and F).

3.3. Gene expression of SLCO1B1 and SLCO1B3

The effect of culture time on *SLCO1B1* and *SLCO1B3* gene expression in plated primary human hepatocytes was determined by quantitative real-time PCR. Compared to the average gene expression level of *SLCO1B1* after 2 h in culture, there was a 13% reduction in gene expression level after 6 h ($p < 0.05$), and a 78% reduction after 24 h ($p < 0.001$) (Fig. 4A). The *SLCO1B3* gene expression level was reduced by 15% ($p < 0.05$) and 71% ($p < 0.001$) after 6 and 24 h in culture, respectively (Fig. 4B). The relative gene expression levels of *SLCO1B3* compared to *SLCO1B1* at 2, 6 and 24 h are shown in Fig. 4C. The average gene expression level of *SLCO1B3* was 48% ($p < 0.05$), 43% ($p < 0.05$) and 39% ($p < 0.05$) lower than the average *SLCO1B1* expression after 2, 6 and 24 h, respectively.

3.4. Genotyping of SLCO1B1

All donors were analyzed for the single-nucleotide polymorphism (SNP) *521T>C* in the *SLCO1B1* gene to investigate a possible relationship between uptake kinetics of ^{3}H-E17βG in plated human primary hepatocytes and the *SLCO1B1 521T>C* polymorphism. One donor was homozygous for *521T>C*, two donors were heterozygous for *521T>C*, while two donors did not carry the mutation (Table 1).

4. Discussion

To our knowledge, this is the first study to investigate the OATP1B1/1B3-mediated uptake kinetics in plated fresh primary human hepatocytes over time in culture. Overall, the results showed that plated primary human hepatocytes is a useful model

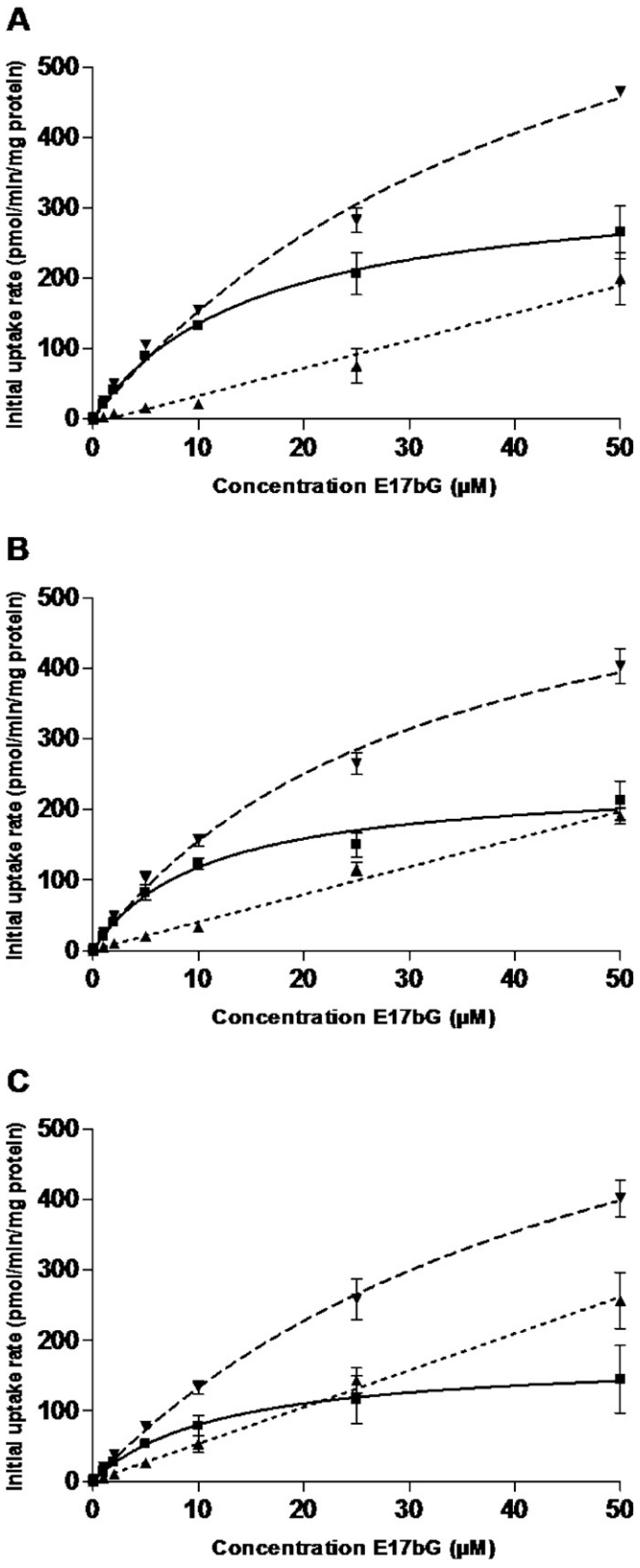

Fig. 1. Concentration profile of the uptake of ^{3}H-E17βG (0.07–50 μM) in plated primary human hepatocytes from donor # 3 after 2 h (A), 6 h (B) and 24 h (C) in culture. Transporter-mediated uptake (■) was calculated as the difference in substrate accumulation of ^{3}H-E17βG at 37 °C (▼) and 4 °C (▲). Data were fitted and kinetic parameters were estimated using non-linear regression. Each point represents the mean ± SD, $n = 3$. Where the vertical bars are not shown, the SD values are within the limits of the symbols.

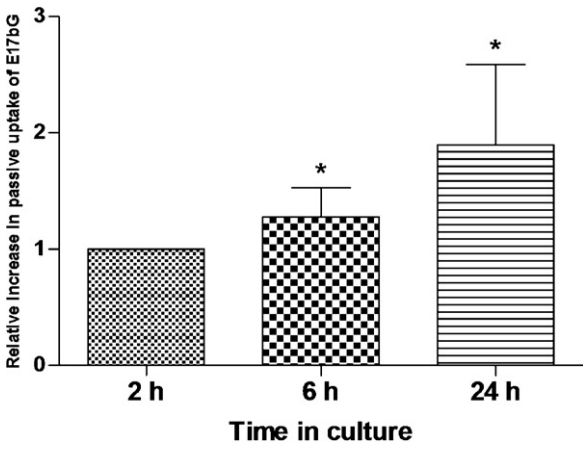

Fig. 2. Relative increase in passive uptake of ^{3}H-E17βG (50 μM) at 4 °C in plated primary human hepatocytes after 6 and 24 h in culture compared to after 2 h. Each bar represents the mean ± SD, $n = 4$–5. $^{*}p < 0.05$.

for *in vitro* studies of OATP1B1/1B3-mediated uptake, but the time in culture may dramatically change the OATP1B1/1B3 function. After 2 h in culture, an active OATP1B1/1B3-mediated uptake was observed in all donors, but an extensive decrease in OATP1B1/1B3 activity, along with an increased variability, was observed during longer culturing times. This is a major limitation with regard to the applicability of primary human hepatocytes as a screening model for OATP1B1/1B3-mediated transport.

The decline in OATP1B1/1B3 activity over time in culture exhibited a large variability between donors. The observed extensive increase and variability in the passive uptake of E17βG into plated human hepatocytes over time contributed to the large variability in OATP1B1/1B3 activity after longer culturing time, and profoundly limits the time possible to study OATP1B1/1B3-mediated uptake in this system. Furthermore, the increase and variability in passive uptake over time led to increased uncertainty in the K_m and V_{max} estimations.

In the human liver, OATP1B1 and OATP1B3 are localized to the basolateral membrane of hepatocytes [2,3,7]. However, it has been shown that during isolation, the polarity of the hepatocytes is rapidly lost [41]. Our study supports this observation as the examination of the protein expression of OATP1B1 studied by confocal microscopy showed that OATP1B1 was evenly distributed over the whole cell surface at 2 h. The decline in protein expression of OATP1B1 on the cell surface also supported the present parallel decrease in activity over time in culture. In addition, the significant reduction in gene expression of both *SLCO1B1* and *SLCO1B3* in plated human hepatocytes also supported the loss of OATP1B1/1B3 activity over time in culture. These results are consistent with previous results by Richert et al., who reported a decrease in *SLCO1B1* and *SLCO1B3* expression in plated human hepatocytes after 24 h in culture [36]. No functional data was presented in the study by Richert et al.

A previous study by Jigorel et al. investigated the functional expression of the major sinusoidal transporters (OATPs, OCT1, OAT2 and NTCP) in primary hepatocyte cultures [42]. They found that the functional expression of sinusoidal transporters was markedly decreased with time in plated primary rat hepatocytes, but not in plated primary human hepatocytes. In their study, OATP activity in human hepatocytes cultured for 3 and 5 days was compared with 1 day old hepatocytes. However, as our results show that the OATP1B1/1B3 activity is extensively decreased already hours after isolation, the findings by Jigorel et al. may indicate that only a limited further loss in OATP1B1/1B3 expression and activity after 24 h in culture takes place.

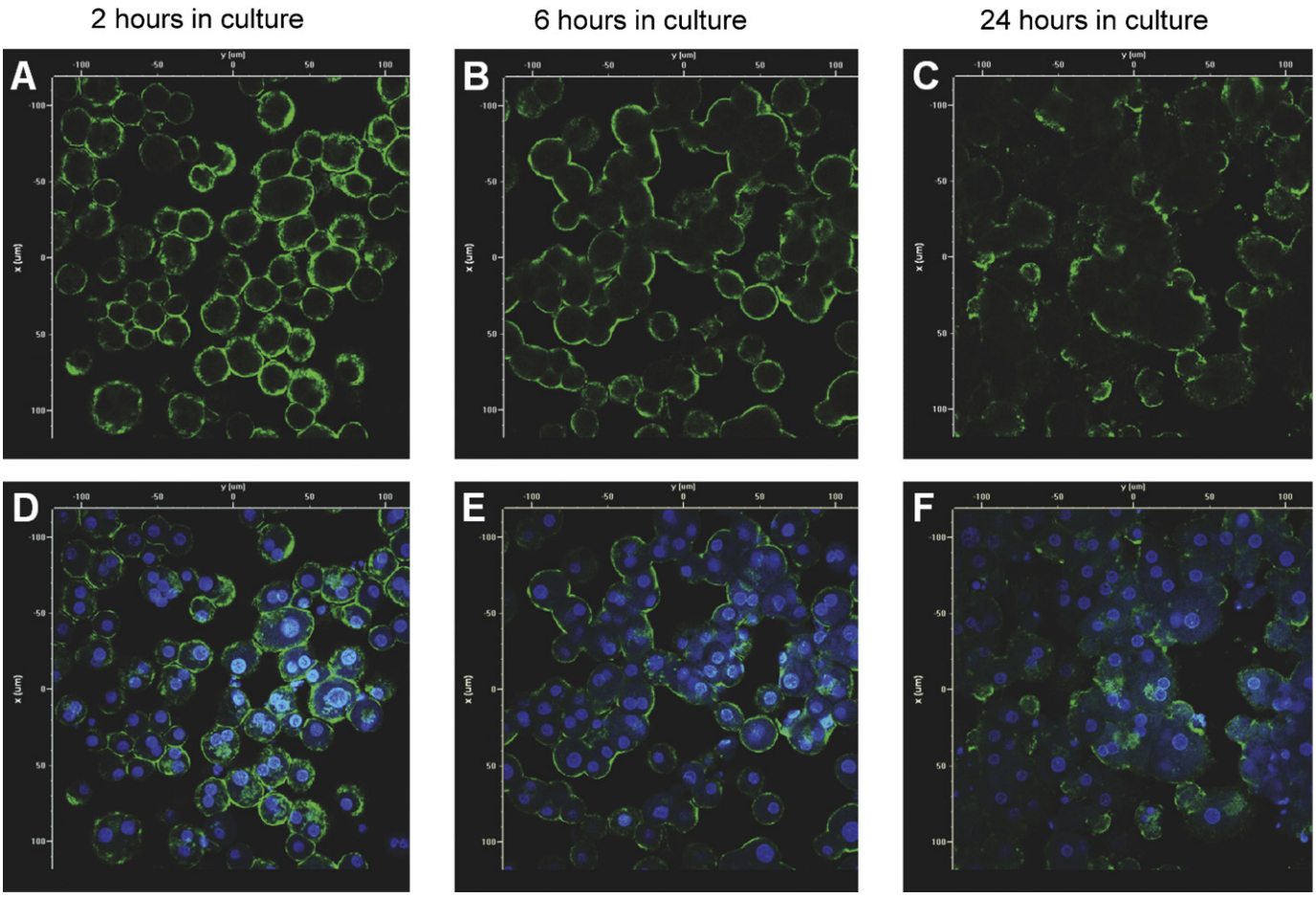

Fig. 3. Confocal images of OATP1B1 (green) (A–C) and co-staining of OATP1B1 (green) and cell nuclei (blue) (D–F) in plated primary human hepatocytes from donor # 3 after 2, 6 and 24 h in culture.

Polymorphism in genes encoding transport proteins could be a determinant of interindividual variability of pharmacokinetics and drug response [29]. The *521T>C* (174Val > Ala) polymorphism, which is present in the *SLCO1B1*5*, **15 and *17* haplotypes, has been associated with reduced clearance of OATP1B1 substrates [12,23,43–45]. In our *in vitro* study, the functional data after 2 h in culture did not reveal any obvious affects of the genotype on the V_{max} or K_m values. The number of individuals in this study is of course too low to study a possible functional affect of certain genotypes. However, a possible explanation for the apparent lack of impact of the *521T>C* polymorphism on the uptake activity of E17βG might be that factors associated to plating provide greater variability *per se* than the genotype, e.g. collagenase treatment, differences in attachment status [46] and/or the previously discussed inter-lot differences in passive diffusion of E17βG.

In hepatic uptake studies, the passive diffusion is typically assessed in a parallel experiment at 4 °C [30,47–49]. At this temperature, the transport proteins are disabled. Alternatively, the passive diffusion can be assessed by specific inhibition of the active uptake [10,42,50], but such inhibitors are often not available or not sufficiently evaluated with respect to potential influence of other transporters or cell processes. In our study, the passive diffusion of E17βG was determined by uptake experiments at 4 °C. Decreasing temperature can potentially alter the passive diffusion of a compound as membrane fluidity decreases [51,52], and thus cause misprediction of kinetic parameters [53]. However, control experiments in HepG2 cells, a human liver carcinoma cell line expressing no OATP1B1 or OATP1B3 [54], showed no significant difference in the uptake of E17βG at 37 °C and 4 °C, suggesting that the passive uptake of E17βG into hepatocytes is not affected by decreasing temperature.

E17βG is regarded as a probe substrate for OATP1B1 and OATP1B3-mediated uptake [4,13,55,56]. In this study, the K_m values for uptake of E17βG into plated primary human hepatocytes after 2 h in culture (7–22 μM) were in agreement with K_m values earlier reported in uptake studies with E17βG using recombinant OATP1B1 and OATP1B3 models, as well as human hepatocytes, in four out of our five donors [4,13,47,57]. The relative gene expression level of *SLCO1B3* was 0.52 compared to the *SLCO1B1* expression which is consistent with previous results by Hilgendorf et al. [54].

Primary human hepatocytes are frequently used as an *in vitro* tool to predict hepatic clearance of drugs. For drugs being OATP1B1 and/or OATP1B3 substrates, uptake transport activity may have important implications on hepatic clearance. Our findings indicate that the time in culture profoundly affect the uptake transporter function in plated primary human hepatocytes. This may confound the *in vitro* estimations of total hepatic clearance when a compound is subjected to significant OATP1B1/1B3-mediated uptake into the cell. Although, primary human hepatocytes cultured for longer periods could still be used to predict hepatic clearance, it would probably be more reliable to use freshly isolated human hepatocytes in suspension, which possess both metabolism and transport activity, for this purpose [32,33,47]. Recently, Badolo et al. showed that cryopreserved human hepatocytes in suspension exhibited carrier-mediated uptake of E17βG [47]. In their study, The V_{max} and CL_{int} values decreased in average by approximately 50% after cryopreserva-

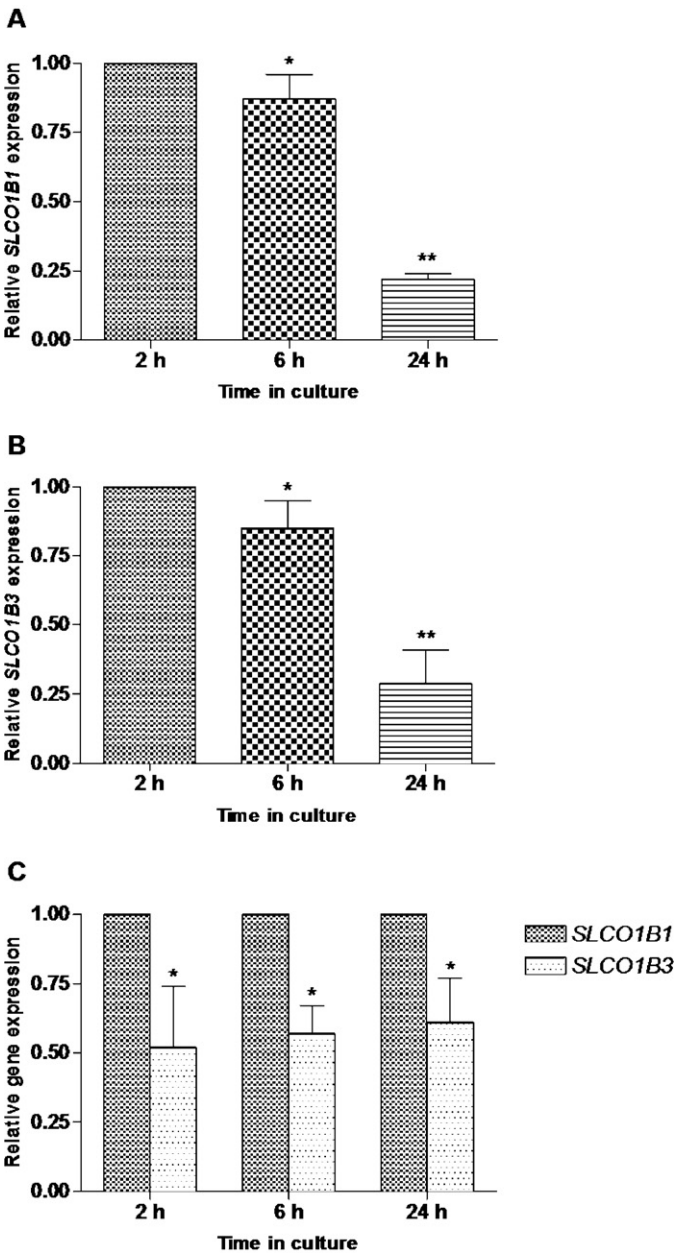

Fig. 4. Relative mRNA expression of *SLCO1B1* (A) and *SLCO1B3* (B) in plated primary human hepatocytes after 6 and 24 h in culture compared to after 2 h, and relative gene expression of *SLCO1B3* compared to *SLCO1B1* after 2, 6 and 24 h in culture (C). Each bar represents the mean ± SD, $n = 4–5$. $^{*}p < 0.05$; $^{**}p < 0.001$.

tion. Although cryopreserved human hepatocytes still could be a useful tool for qualitative evaluations of OATP1B1 and OATP1B3-mediated transport, transfected cell systems over-expressing OATP1B1 or OATP1B3 are probably better alternatives.

The present study did not explore the mechanisms behind the decline in OATP1B1/1B3 activity with time. It is however possible that the OATP1B1/1B3 phenotype will change when cells lose their natural cell contacts and are not organized in a three dimensional system. Other culture conditions, e.g. sandwich-cultures or three-dimensional systems such as a bioreactor, may better retain and stabilize the transporter phenotype. Hoffmaster et al. reported that the OATP1B1 and OATP1B3 protein expression is retained in sandwich-cultured human hepatocytes up to six days, but the metabolic capacity of this model has been questioned [34]. Moreover, other human *in vitro* models, such as HepaRG cells or human pluripotent stem cells-derived hepatocytes may have the potential to provide a model which combines appreciable drug metabolism and transport activities in order to predict *in vivo* hepatic clearance of drugs.

In conclusion, the present findings indicate that plated primary human hepatocytes is a useful *in vitro* model for OATP1B1/1B3-mediated uptake studies, but the time in culture dramatically changes the uptake kinetics. Longer culturing times induce an extensive variability in OATP1B1/1B3 activity and passive permeability which may confound the results. Together with restricted tissue availability, this limits the potential of plated primary human hepatocytes as an *in vitro* tool for evaluation of hepatic uptake and clearance, and alternative models should be explored for use in the future.

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