Evaluation of Organic Anion-Transporting Polypeptide 1B1 and CYP3A4 Activities in Primary Human Hepatocytes and HepaRG Cells Cultured in a Dynamic Three-Dimensional Bioreactor System

Maria Ulvestad, Malin Darnell, Espen Molden, Ewa Ellis, Anders Åsberg, and Tommy B. Andersson

DMPK Innovative Medicines, AstraZeneca R&D Mölndal, Mölndal, Sweden (M.U., M.D., T.B.A.); Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, Oslo, Norway (M.U., E.M., A.A.); Cellectis Stem Cells, Cellartis AB, Gothenburg, Sweden (M.U.); and Departments of Physiology and Pharmacology (M.D., T.B.A.) and Clinical Science, Intervention, and Technology (E.E.), Karolinska Institutet, Stockholm, Sweden

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ABSTRACT

The long-term stability of liver cell functions is a major challenge when studying hepatic drug transport, metabolism, and toxicity in vitro. The aim of the present study was to investigate organic anion-transporting polypeptide (OATP) 1B1 and CYP3A4 activities in fresh primary human hepatocytes and differentiated cryopreserved HepaRG cells when cultured in a three-dimensional (3D) bioreactor system. OATP1B1 activity was determined by loss from media experiments of [3H]estradiol-17β-D-glucuronide and atorvastatin acid (ATA) for up to 7 days in culture. ATA metabolite formation was determined at days 3 to 4 to evaluate CYP3A4 activity. Overall, the results showed that freshly isolated human hepatocytes inoculated in the bioreactor retained OATP1B1 activity for at least 7 days, whereas in HepaRG cells no OATP1B1 activity was observed beyond day 2. The activity data were in agreement with immunohistochemical stainings, which showed that OATP1B1 protein expression was preserved for at least 9 days in fresh human hepatocytes, whereas OATP1B1 was expressed markedly lower in HepaRG cells after 9 days in culture. Fresh human hepatocytes and HepaRG cells exhibited similar CYP3A4 activity in bioreactor culture, and immunohistochemical stainings supported these findings. Activity and mRNA expression of OATP1B1 and CYP3A4 in primary human hepatocytes compared with HepaRG cells in fresh suspensions were in agreement with data obtained in bioreactor culture. In conclusion, freshly isolated human hepatocytes cultured in a 3D bioreactor system preserve both OATP1B1 and CYP3A4 activities, allowing long-term in vitro studies on drug disposition and toxicity.

Introduction

Drug-drug interactions, large interpatient variability in pharmacokinetics, and the propensity of drugs to induce liver injury are major concerns when developing new drugs. Today, hepatic membrane transporters are recognized as important determinants of disposition and safety of many drugs and work in cooperation with metabolizing enzymes to restrict the systemic exposure of exogenous substances (International Transporter Consortium et al., 2010). Several transporter-mediated drug-drug interactions have been reported (Zhang et al., 2009), and inhibition of hepatic uptake and/or efflux has been indicated as a possible mechanism of hepatotoxicity (Kostrubsky et al., 2003; Marion et al., 2007; Morgan et al., 2010; Ye et al., 2010). Moreover, hepatic membrane transporters are major determinants of pharmacokinetic variability of many drugs (International Transporter Consortium et al., 2010). In drug development, it is therefore crucial to develop in vitro
models for drug disposition that can be run long enough to detect the above-mentioned effects at clinically relevant concentrations. These models should also express the activities of membrane transporters and drug-metabolizing enzymes reflecting the in vivo situation.

Although CYP3A4 has been identified as the most frequently involved enzyme in drug metabolism (Guengerich, 1999), the organic anion-transporting polypeptide 1B1 (OATP1B1) is crucial for the uptake of many acidic drugs into the liver, e.g., HMG-CoA reductase inhibitors (statins) (Nakai et al., 2001; Hirano et al., 2004). Several OATP1B1-mediated drug-drug interactions involving these agents have been reported (Shitara and Sugiyama, 2006; Lau et al., 2007; Nakagomi-Hagihara et al., 2007; Noé et al., 2007; Treiber et al., 2007). An important example is the substantially increased statin exposure and risk of toxicity observed during coadministration with the potent OATP1B1 inhibitor cyclosporine A (Asberg et al., 2001; Amundsen et al., 2010).

Primary hepatocytes, plated or in suspension, represent the most common in vitro model for the evaluation of human hepatic drug transport, metabolism, clearance, and toxicity in the pharmaceutical industry (Hewitt et al., 2007). These cells express a complete set of enzymes and transporters involved in hepatic drug clearance, but their spontaneous dedifferentiation and rapid loss of enzyme and transporter activity in two-dimensional (2D) culture prevents long-term studies (Rodríguez-Antona et al., 2002; Richert et al., 2006; Ulvestad et al., 2011). Furthermore, restricted tissue availability and interdonor variability caused by genetic differences and variability in the cell preparation steps limits the use of primary human hepatocytes (Li, 2008). This has led to the development of protocols for the cryopreservation of human hepatocytes. Cryopreserved human hepatocytes exhibit both cytochrome P450 (P450), UDP-glucuronosyltransferase, and transporter activities (Li et al., 1999; Steinberg et al., 1999; Jørgensen et al., 2007; Fournel-Gigleux et al., 2010; Badolo et al., 2011; De Bruyn et al., 2011), but as in primary human hepatocytes, a rapid loss of enzyme and transporter expression is observed when these cells are cultured in 2D models (Richert et al., 2006). Thus, novel cell culture systems with improved conservation of hepatocyte functions allowing predictive long-term in vitro pharmacokinetic or toxicological studies are warranted.

Gerlach et al. (1994) introduced the multicompartment bioreactor technology for dynamic three-dimensional (3D) perfusion culture of human liver cells. This technique uses interwoven hollow-fiber capillary membranes that provide independent, decentralized medium and gas exchange to the cells located between the capillaries (Fig. 1B). When cultured in a perfused 3D bioreactor, human liver cells retain in vivo-like properties and are arranged in tissue-like structures (Zeilinger et al., 2004; Schmelzer et al., 2009). Zeilinger et al. (2002) showed that liver-specific functions such as urea and albumin synthesis, glucose metabolism, and P450 activities all were maintained for at least 14 days in bioreactor culture.

HepaRG cells, a human hepatoma cell line, represents an alternative to primary human hepatocytes. Differentiated HepaRG cells maintain important functions for drug metabolism, such as P450 and UDP-glucuronosyltransferase activities, in both 2D and 3D models over several weeks (Josse et al., 2008; Anthérieu et al., 2010; Darnell et al., 2011). Furthermore, Le Vee et al. (2006) reported activity of certain canalicular and biliary transporters in HepaRG cells in 2D culture.

Preservation of drug transport and metabolism activities over longer time periods is essential in vitro liver models for drug discovery research. The aim of the present study was to investigate OATP1B1 and CYP3A4 activities in fresh primary human hepatocytes and differentiated cryopreserved HepaRG cells cultured in a 3D bioreactor system over several days.

**Materials and Methods**

**Chemicals and Reagents.** [3H]E17βG was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Atorvastatin acid (ATA), atorvastatin lactone (ATL), 2-OH-ATA, 2-OH-ATL, 4-OH-ATA, and 4-OH-ATL were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Unlabeled estradiol-17β-d-glucuronide, estrone-3-sulfate (E3S), and L-glutamine (200 mM) were obtained from Sigma-Aldrich (Steinheim, Germany). Cryopreserved human hepatocytes were obtained from Celsis In Vitro Technologies (Brussels, Belgium). Differentiated HepaRG cells cultured in a 3D bioreactor system over several days.

**Fig. 1.** A, bioreactor perfusion system with tubing for medium recirculation, feeding, and outflow. The recirculation pump controls the speed of medium perfusion through the bioreactor, and the feed pump supplies the system with fresh medium from the medium bottle. The sampling port enables substance injection and sampling. B, multicompartment perfusion bioreactor with two capillary layers used for 3D culture of fresh human hepatocytes and HepaRG cells. Each capillary layer consisted of hydrophobic oxygenation capillaries (yellow) and hydrophilic medium perfusion capillaries (blue and red) supplying the cells located in the intercapillary space with oxygen/CO2 and nutrients.
cryopreserved HepaRG cells were obtained from Biopredic International (Rennes, France). HEPES, phosphate-buffered saline, and Williams medium E without phenol red were obtained from Invitrogen (Carlsbad, CA). HCl and NaOH were obtained from Merck (Darmstadt, Germany). High-purity water was obtained from an ELGA purification system (ELGA, High Wycombe, UK). All other chemicals and reagents were of analytical grade and available from commercial sources. Bioreactors, tubing systems, and a perfusion device were purchased from Stem Cell Systems (Berlin, Germany).

**Isolation of Human Hepatocytes.** All tissues were obtained from Karolinska University Hospital (Huddinge, Sweden) by qualified medical staff, with donor informed consent following ethical and institutional guidelines. Liver tissues from three patients (donors 1, 2, and 3) undergoing liver resection because of metastatic colon or liver cancer were used in this study. Donor information regarding age, sex, disease, and cell viability is summarized in Table 1. Residual tissue not needed for diagnostic purposes was transported to the laboratory from the operating rooms in cold Eagle’s minimum essential medium (Lonza Walkersville, Inc., Walkerville, MD) within 30 min after removal. Tissue dissociation and hepatocyte isolation were performed by using a two-step collagenase perfusion protocol originally developed by Seglen (1976) and modified as described previously (Gramignoli et al., 2011). The isolated fresh human hepatocytes were transported from Karolinska University Hospital to AstraZeneca R&D Mödlal in a cold package within the same day. Immediately upon arrival, the cells were centrifuged for 3 min at 100g, and the supernatant was removed. The cells were washed with 45 ml of Williams medium E without phenol red supplemented with 25 mM HEPES and 2 mM l-glutamine, pH adjusted to 7.4 (modified Williams medium E) and centrifuged for 3 min at 100g. The cells were counted and trypan blue exclusion test was used to calculate the cell viability. The cell suspension was diluted in medium to required concentration. The cells were kept on ice and used within 3 h after isolation.

**Thawing of Cryopreserved Human Hepatocytes and HepaRG Cells.** Cryopreservation of human hepatocytes was conducted at Celsis In Vitro Technologies using a controlled freezing protocol according to in-house procedures. Pooled cryopreserved human hepatocytes from three different batches (IRK, UMJ, and PHL), each containing hepatocytes from 10 donors, were used in the experiments. Immediately before experiments in suspension, cryopreserved human hepatocytes were rapidly thawed by gently shaking the vial in a 37°C water bath. The content was transferred to a 50-ml tube containing prewarmed (37°C) modified Williams medium E. The cryogenic tubes were rinsed twice. The 50-ml tube was centrifuged at 600g for 3 min at 100g, and the supernatant was removed. The cells were washed with 45 ml of Williams medium E without phenol red supplemented with 25 mM HEPES and 2 mM l-glutamine, pH adjusted to 7.4 (modified Williams medium E) and centrifuged for 3 min at 100g. The cells were counted and trypan blue exclusion test was used to calculate the cell viability. The cell suspension was diluted in medium to required concentration. The cells were kept on ice and used within 3 h after isolation.

<table>
<thead>
<tr>
<th>Donor ID</th>
<th>Age</th>
<th>Sex</th>
<th>Disease</th>
<th>Cell Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>81</td>
<td>Female</td>
<td>Hepatocellular carcinoma</td>
<td>76</td>
</tr>
<tr>
<td>Donor 2</td>
<td>83</td>
<td>Female</td>
<td>Metastasis (colon cancer)</td>
<td>75</td>
</tr>
<tr>
<td>Donor 3</td>
<td>41</td>
<td>Female</td>
<td>Metastasis (rectal cancer)</td>
<td>70</td>
</tr>
</tbody>
</table>

**Bioreactor and Perfusion System.** The bioreactor consists of two hollow fiber capillary layers integrated into a polyurethane housing. Each capillary layer includes hydrophobic oxygenation capillaries and hydrophilic medium perfusion capillaries (Fig. 1B). The medium capillaries were arranged as two layers with countercurrent perfusion to increase mass exchange between the inside of capillaries and the cells located within the intercapillary space. A port enabled substance injection, mimicking bolus administration, and sampling from the recirculating medium (Fig. 1A). A detailed description of the bioreactor structure is given elsewhere (Hirano et al., 2006). In this study, a downscaled bioreactor with a cell compartment volume of 0.5 ml was used for comparison with the 2-ml cell compartment bioreactor used in previous studies (Darnell et al., 2011; Zeilinger et al., 2011). The bioreactors were operated by means of an electronically controlled perfusion device (Zeilinger et al., 2011), allowing two bioreactors to be run in parallel. Temperature, medium feed, and medium recirculation rates were monitored and regulated via a connected computer. A valve-controlled gas mix unit was used for air/oxygen/CO₂ supply.

**Bioreactor Cell Inoculation and Culture.** Bioreactors were connected with preassembled sterile tubing systems and rinsed with phosphate-buffered saline and medium before cell inoculation. Cell suspension (2.5 ml; 10 x 10⁶ viable cells/ml) was inoculated in the cell compartment of each bioreactor to a final amount of 25 x 10⁶ cells per bioreactor. The bioreactors were waggled carefully during the filling to allow even distribution.

Fresh human hepatocytes from donor preparations 1, 2, and 3 were inoculated into three bioreactors at separate occasions. The bioreactors were perfused with Heparmed medium Vito 143 (Biochrom, Berlin, Germany), a modification of Williams medium E specifically adapted for serum-free perfusion culture of liver cells by enrichment with amino acids, free fatty acids, and trace elements. Before use, the medium was supplemented with 20 IU/liter insulin, 5 mg/liter transferrin, 3 µg/liter glucagon (Biochrom), 100,000 U/liter penicillin, 100 mg/liter streptomycin (Invitrogen), and 20 µg/ml gentamycin (PAA Laboratories GmbH, Pasching, Austria). Three different batches of cryopreserved differentiated HepaRG cells were inoculated into three bioreactors, and hepatic cell medium MIL600 supplemented with ADD670 was used during the whole culture period.

Bioreactor cultures were perfused with a total medium volume of 12 ml recirculating at a flow rate of 3 ml/min. The temperature in the bioreactor chamber was maintained at 37°C, and bioreactors were oxygenated through the integrated gas capillaries with a gas mix of 95% air and 5% CO₂ at a gas flow velocity of 5 ml/min. Before and between experiments, medium flow rate was set to 0.6 ml/h, and the standard operation mode with continuous medium feed (open-system perfusion) was used.

**Transport Studies with E17/βG in Cell Suspension.** Loss from media and intracellular accumulation of E17/βG with and without E3S (OATP1B1 inhibitor) was determined in cell suspension from three different batches of pooled cryopreserved human hepatocytes, three different batches of HepaRG cells, and fresh human hepatocytes from donor preparations 1, 2, and 3 on different occasions. Cells were suspended in medium to a final density of 3 x 10⁶ viable cells/ml. The experiments were performed in triplicate in 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark) with 50 µl of supplemented with serum-free ADD650 according to the manufacturer’s instructions (Biopredic International). These reagents are part of a commercially available assay that anyone can use to replicate the work.

Cryopreserved human hepatocytes and HepaRG cells were counted by using a Bürker chamber, and trypan blue exclusion test was used to calculate the cell viability. The cells were kept on ice and used within 3 h after thawing.

**TABLE 1**

<table>
<thead>
<tr>
<th>Donor ID</th>
<th>Age</th>
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<th>Disease</th>
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<tr>
<td>Donor 1</td>
<td>81</td>
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<td>41</td>
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<td>70</td>
</tr>
</tbody>
</table>
cell suspension/well. The cells were preincubated with either 25 μl of estrone-3-sulfate (30 μM) or medium (control samples) for 10 min at 37°C. The transport studies were initiated by adding 75 μl of substrate solution, giving a final [3H]E17βG concentration of 1 μM (0.5 μCi/ml) and a final cell concentration of 1 × 10^6 cells/ml. The cells were incubated for 1, 4, 8, 15, 30, and 60 min. The whole sample was transferred to an Eppendorf tube and centrifuged for 30 s at 10,000g. Fifty microliters of the supernatant was immediately transferred to a 96-well plate (Optiplate; PerkinElmer Life and Analytical Sciences), and 200 μl of scintillation liquid (MicroScint 20, PerkinElmer Life and Analytical Sciences) was added. Immediately after removal of the supernatant, 350 μl of ice-cold medium was added to the pellet to stop the transporter-mediated uptake. The tubes were centrifuged for 30 s at 10,000g, and the pellets were washed twice with 350 μl of ice-cold buffer. After the third centrifugation, 100 μl of 1 M NaOH was added to the pellet to lyse the cells, and the samples were placed in the refrigerator (4°C) over night. A 50-μl sample was transferred to a 96-well plate (Optiplate; PerkinElmer Life and Analytical Sciences), and 200 μl of scintillation liquid (MicroScint 20; PerkinElmer Life and Analytical Sciences) was added. The radioactivity in the samples was determined by a microplate scintillation counter (Top-Count; PerkinElmer Life and Analytical Sciences).

**Transport Studies with E17βG in the Bioreactor.** Loss from media of [3H]E17βG with and without E3S was assessed in fresh human hepatocytes from donor preparations 1, 2, and 3 and two different batches of HepaRG cells at days 2 and 7 in bioreactor culture (Fig. 2). In experiment 1, 1 ml of substrate solution was injected through the sampling port (Fig. 1A), giving a final concentration of 1 μM (0.5 μCi/ml). The transport studies were performed at continuous medium perfusion, but without feeding medium into the perfusion circuit (closed-system perfusion) (Fig. 1A). Samples (150 μl) were collected at 8, 20, 40, 60, 90, 120, and 180 min. The bioreactor circuit was rinsed with 50 ml of culture medium (single-pass perfusion) before experiment 2. In experiment 2, the cells were preincubated with E3S for 30 min by injecting 1 ml of inhibitor solution. After 30 min, 1 ml of medium was removed from the recirculation and replaced by 1 ml of substrate solution giving a final concentration of 1 μM (0.5 μCi/ml) [3H]E17βG and 30 μM E3S. Except for the preincubation with inhibitor, experiment 2 was executed exactly as experiment 1. After the experiments, the bioreactor circuit was rinsed with 50 ml of culture medium (single-pass perfusion) and reset to the standard operation mode with continuous medium feed (open-system perfusion). Duplicate samples of 50 μl were mixed with 5 ml of scintillation liquid (OptiPhase HiSafe 2; PerkinElmer Life and Analytical Sciences). The radioactivity in the samples was determined by liquid scintillation spectroscopy (Wallac Win Spectral, 1414 Liquid Scintillation Counter; PerkinElmer Life and Analytical Sciences).

In addition to the transport studies in the bioreactor, [3H]E17βG was injected in a bioreactor system without cells to assess potential nonspecific binding to the bioreactor system. Samples were analyzed at 0, 8, 20, 40, 60, 90, 120, and 180 min, and the results did not indicate any such nonspecific binding.

**Transport and Metabolism Studies with ATA in Cell Suspension.** Total loss of ATA and ATA metabolite formation with and without E3S was determined in suspension from three different batches of pooled cryopreserved human hepatocytes, three different batches of HepaRG cells, and fresh human hepatocytes from donor preparations 1, 2, and 3. Cells were suspended in medium to a final density of 4 × 10^6 viable cells/ml. The experiments were performed in triplicate in 96-well tissue culture plates with flat bottoms (TPP, Trasadingen, Switzerland). Fifty microliters of cell suspension/well were preincubated for 10 min at 37°C. Fifty microliters of prewarmed (37°C) substrate solution with or without E3S (final concentration 30 μM) were added to a final concentration of 10 μM ATA and a final cell concentration of 2 × 10^6 cells/ml. For cryopreserved human hepatocytes and HepaRG cells, the plates were incubated at 37°C for 2, 6, and 24 h. For fresh human hepatocytes, the plates were incubated at 37°C for 2, 14, and 24 h. Longer incubations were performed with ATA compared with E17βG to ensure formation of quantifiable levels of metabolites. The reactions were stopped with 100 μl of ice-cold ACN. The 0-h plate was stopped with 100 μl of ACN before adding the substrate. The plates were placed in the freezer (−20°C) for 20 min and centrifuged for 20 min at 4000g and 4°C. The supernatant was mixed with an equal amount of ELGA water. The triplicate samples were pooled and stored at −80°C until analysis. Before analysis, the samples were thawed and mixed with equal amount of ACN. The samples were placed in the freezer (−20°C) for 20 min and centrifuged for 20 min at 4000g and 4°C. The supernatant was diluted with ELGA water and analyzed by liquid chromatography/mass spectrometry (LC/MS).

**Transporter and Metabolism Studies with ATA in the Bioreactor.** Loss from media of ATA and ATA metabolite formation with and without E3S was assessed in fresh human hepatocytes from donor preparations 1, 2, and 3 and three different batches of HepaRG cells at days 3 and 4 in bioreactor culture (Fig. 2). In experiment 1, 1 ml of substrate solution was injected through the sampling port, giving a final concentration of 5 μM ATA. The transport and metabolism studies were performed in closed-system perfusion mode as described above. Samples (150 μl) were collected at 15 and 30 min and 1, 2, 4, 6, and 24 h. Longer incubations were performed with ATA compared with E17βG to ensure formation of quantifiable levels of metabolites. The bioreactor circuit was rinsed with 50 ml of culture medium (single-pass perfusion) before experiment 2. In experiment 2, the cells were preincubated with E3S for 30 min. After 30 min, 1 ml of medium was removed from the recirculation and replaced by 1 ml of substrate solution, giving a final concentration of 5 μM ATA and 30 μM E3S. Except for the preincubation with inhibitor, experiment 2 was executed exactly as experiment 1. After the experiments, the bioreactor circuit was rinsed and reset to the standard operation mode. Samples were frozen directly after sample taking. Before analysis, the samples were thawed and mixed with ice-cold ACN.
(1:2). The samples were centrifuged for 20 min at 4000g and 4°C. The supernatant was diluted with ELGA water and analyzed by LC/MS.

In addition to the transport and metabolism studies in the bioreactor, ATA was injected in a bioreactor system without cells to assess potential nonspecific binding to the bioreactor system. Samples were analyzed at 0, 15, and 30 min and 1, 2, 4, 6, and 24 h, and the results did not indicate any such nonspecific binding.

**Analysis of ATA and ATA Metabolites.** ATA, ATL, 2-OH-ATA, 2-OH-ATL, 4-OH-ATA, and 4-OH-ATL were analyzed by LC/MS. The liquid chromatography system consisted of an HPLC PAL injector, a CTC Analytics, Zwingen, Switzerland) combined with an HP 1100 LC binary pump and column oven (Agilent Technologies, Waldbronn, Germany). The separation was performed on a reversed-phase HyPurity C18 analytical column (50 × 2.1 mm, 5 μm; Thermo Fisher Scientific, Waltham, MA) at 40°C and a flow rate of 0.75 ml/min. The mobile phases consisted of 0.1% (v/v) formic acid in 5% ACN (A) and 0.1% (v/v) formic acid in 95% ACN (B). Mobile phase B was maintained at 2% for 0.8 min, and then increased linearly from 2 to 98% for 3.7 min. After maintenance at 98% for 0.5 min, mobile phase B was decreased linearly to 2% in 0.1 min and maintained at 2% for 0.9 min. The retention times of 4-OH-ATA, 4-OH-ATL, 2-OH-ATA, 2-OH-ATL, and ATL were 3.05, 3.20, 3.43, 3.51, 3.57, and 3.66 min, respectively. Detection was performed with a triple quadrupole mass spectrometer (API4000) equipped with an electrospray interface (Applied Biosystems/MDS Sciex, Concord, ON, Canada). The mass spectrometry parameters were optimized by using each analyte. The compound-dependent parameters were as follows: the collision energy was set to 31, 28, 30, 31, 25, and 25 V for 4-OH-ATA, 4-OH-ATL, 2-OH-ATA, 2-OH-ATL, and ATL, respectively. The collision-activated dissociation gas was set to 6 for all analytes. The analytes were separated into two periods. In period 1, the multiple reaction monitoring transitions chosen were 575.2 > 440.3 for 4-OH-ATA and 557.1 > 448.2 for 4-OH-ATL. In period 2, the multiple reaction monitoring transitions chosen were 575.2 > 440.2 for 2-OH-ATA, 559.3 > 440.2 for ATA, 557.1 > 448.1 for 2-OH-ATL, and 541.1 > 448.2 for ATL. Instrument control, data acquisition, and data evaluation were performed with Analyst 1.4 software (Applied Biosystems/MDS Sciex).

**Immunohistochemical Staining.** Samples from cell culture material were collected from different locations within the bioreactor capillary network upon culture termination. After fixation in 5% paraformaldehyde the material was dehydrated and embedded in paraffin and cut into approximately 4-μm sections. The immunohistochemical staining was performed on the staining module Discovery XT (Ventana Medical Systems, Tucson, AZ), using antibodies against OATP1B1 (kind gift from Dr. Bruno Stieger, University Hospital Zurich, Zurich, Switzerland) and CYP3A4 (CYP4X PAP01) (Cyper Ltd, Dundee, Scotland UK). All solutions for deparaffinization, pretreatment, detection, counterstaining, and rinsing steps were supplied by Ventana Medical Systems. Heat (40 min in a Trisborate/EDTA buffer, pH 8, at 98°C) was used as an antigen retrieval pretreatment. Primary antibodies were diluted in Discovery Ab Diluent. As a second step the Ventana OmniMap antiretinoblastoma horseradish peroxidase was used. The immunohistochemical staining was visualized with diaminobenzidine chromogen, and the counterstaining was performed with hematoxylin.

**RNA Isolation and cDNA Synthesis.** Total RNA was isolated from fresh human hepatocytes, cryopreserved human hepatocytes, and HepaRG cells by using TRIzol reagent according to the manufacturer’s instructions. cDNA was prepared from 0.5 μg of total RNA by using the SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (PCR) with random hexamer primers according to the manufacturer’s protocol (Invitrogen).

**Quantitative Real-Time PCR.** Quantitative real-time PCR amplification reactions were carried out in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene expression was estimated by using TaqMan Gene Expression Assays with specific primers and probes (Applied Biosystems) according to the manufacturer’s recommendations. In brief, the reaction mixtures (25 μl/well) contained 30 ng of cDNA, 1× TaqMan Universal Master Mix (Applied Biosystems), and 1.25 μl of Gene Expression Assays Mix and 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 duplicates, and data were captured by using 7500 Real-Time PCR System Sequence Detector Software version 1.3.1 (Applied Biosystems). Gene products measured by quantitative real-time PCR were SLC01B1 (Hs00273734_m1) and CYP3A4 (Hs00064506_m1). Expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase. The relative gene expression (ΔΔCt) and the fold change of gene expression (2−ΔΔCt) were calculated by using the cryopreserved human hepatocytes as a calibrator.

**Metabolic Parameters and Cell Damage Markers.** Metabolic parameters and cell damage markers in fresh human hepatocytes and HepaRG cells cultured in the 3D bioreactor were measured daily in samples from the culture perfusate and/or the medium outflow. The samples were stored at −20°C and analyzed at the Laboratory of Clinical Chemistry at Sahlgrenska University Hospital (Gothenburg, Sweden). Urea and glucose production/consumption and release of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transferase (GGT) were determined in all samples.

The daily production, consumption, and release of biochemical parameters in the bioreactors were calculated on the basis of concentrations measured in the outflow bottle and the perfusion circuit (Fig. 1A), according to the following equation:

\[
P = \frac{C_0 - C_{d}}{t}V_d + \frac{C_{d} - C_{d-1}}{t}V_d\Delta t
\]

where \(P\) is the total production rate, \(\Delta t\) is the time interval between two sampling time points, \(C_0\) is concentration in the outlet vessel, \(C_{d}\) is medium blank concentration, \(V_d\) is volume of medium in the outlet vessel, \(C_{d-1}\) is concentration in perfusate at the time point of sample taking, \(C_{d-2}\) is concentration in the previously collected sample from the perfusate, and \(V_p\) is recirculating volume. The mean and S.D. values were calculated for the three bioreactors. The parameters are presented as milligrams per day per bioreactor or unit per day per bioreactor.

**Calculations and Statistical Analysis.** Nonlinear regression was used to describe loss from media data of E17βG (one-phase exponential decay) and ATA (two-phase exponential decay) in bioreactor culture (Figs. 4 and 6, respectively) using GraphPad Prism 4.03 (GraphPad Software Inc., San Diego, CA). Area under the curve (AUC) of substrate concentration and/or metabolite concentration was determined without and with coinoculation with E3S in each experiment by using the trapezoidal method.

Triplicate experiments were performed in all studies except for loss from media of E17βG in HepaRG cells cultured in the bioreactor where duplicate experiments were performed. The mean value ± S.D. was calculated. The significance of difference of AUC of substrate concentration or metabolite concentration without and with coinoculation with E3S was estimated by using the one-tailed, paired Student’s t test. The significance of difference in gene expression of SLC01B1 and CYP3A4 between fresh human hepatocytes, cryopreserved human hepatocytes, and HepaRG cells was estimated by using the two-tailed, unpaired Student’s t test. P values <0.05 were considered statistically significant.

**Results**

Intracellular Accumulation and Loss from Media of E17βG in Cell Suspension. OATP1B1-mediated uptake of E17βG was determined by combined time-dependent uptake studies and loss from media experiments of E17βG in cell
TABLE 2

AUC of medium concentration and intracellular concentration of E17βG without and with coincubation of E3S in fresh human hepatocytes, cryopreserved human hepatocytes, and HepaRG cells in suspension at day 0

The significance levels of difference in the average loss from media or intracellular concentration of E17βG with and without E3S are shown. Results shown are average ± S.D.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Experiment</th>
<th>AUC Medium Concentration</th>
<th>AUC Intracellular Concentration</th>
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<tr>
<td></td>
<td></td>
<td>µmol·h/l</td>
<td>pmol·h/mg protein</td>
</tr>
<tr>
<td>Fresh hepatocytes</td>
<td>E17βG</td>
<td>893 ± 8</td>
<td>103 ± 7</td>
</tr>
<tr>
<td>Fresh hepatocytes</td>
<td>E17βG + E3S (30 μM)</td>
<td>918 ± 3**</td>
<td>43 ± 13**</td>
</tr>
<tr>
<td>Cryo hepatocytes</td>
<td>E17βG</td>
<td>963 ± 29</td>
<td>127 ± 11</td>
</tr>
<tr>
<td>Cryo hepatocytes</td>
<td>E17βG + E3S (30 μM)</td>
<td>987 ± 33**</td>
<td>36 ± 3**</td>
</tr>
<tr>
<td>HepaRG</td>
<td>E17βG</td>
<td>1020 ± 27</td>
<td>46 ± 7</td>
</tr>
<tr>
<td>HepaRG</td>
<td>E17βG + E3S (30 μM)</td>
<td>1025 ± 40</td>
<td>31 ± 6**</td>
</tr>
</tbody>
</table>

*, P < 0.05, **, P < 0.01.

suspensions. AUCs of the E17βG medium concentration and the intracellular E17βG concentration without and with E3S (30 μM) are shown in Table 2. Results from the time-dependent uptake studies showed an OATP1B1-dependent active uptake of E17βG in both fresh human hepatocytes (P < 0.01), cryopreserved human hepatocytes (P < 0.01), and HepaRG cells (P < 0.01) (Fig. 3; Table 2). In the loss from media experiments, a significant OATP1B1-mediated loss from media of E17βG was observed in fresh (P < 0.01) and cryopreserved (P < 0.05) human hepatocytes, whereas no significant difference in loss from media of E17βG with and without E3S was observed in the cryopreserved HepaRG cells (P > 0.05) (Table 2).

Loss from Media of E17βG in Cells Cultured in the Bioreactor System. OATP1B1-mediated loss from media of E17βG was determined at days 2 and 7 in the bioreactor. Figure 4 shows the E17βG medium concentration as a percentage of start concentration without and with E3S in fresh human hepatocytes (A and B) and HepaRG cells (C and D) at days 2 and 7, respectively. AUCs of the E17βG medium concentration without and with E3S over time are shown in Table 3. A significant OATP1B1-mediated loss from media was observed in fresh human hepatocytes at day 2 in culture (P < 0.05). At day 7, two of three donors showed an OATP1B1-mediated loss from media of E17βG (P > 0.05). In HepaRG cells, a significant OATP1B1-mediated loss from media was observed at day 2 in culture (P < 0.05), but the results showed no OATP1B1 activity at day 7 (P > 0.05).

Loss of ATA and ATA Metabolite Formation in Cell Suspension. Total loss of ATA and ATA metabolite formation with and without E3S was determined in cell suspension at day 0. The results are shown in Fig. 5. AUCs of the ATA concentration and the total hydroxy metabolite concentration in the experiments with and without E3S are shown in Table 4. Significantly more loss of ATA and more metabolite formation were observed in the experiments without E3S in both fresh (P < 0.05) and cryopreserved (P < 0.05) human hepatocytes, indicating an active OATP1B1-mediated uptake of ATA. In HepaRG cells, no significant difference was observed in the loss of ATA or metabolite formation without and with E3S (P > 0.05). The CYP3A4-dependent formation of 2-OH-ATA acid and 4-OH-ATA were similar in all three cell systems. Concentrations of ATL, 2-OH-ATL, and 4-OH-ATL were below the limit of quantification.

Loss from Media of ATA in Cells Cultured in the Bioreactor System. OATP1B1-mediated loss from media of ATA was determined after 3 to 4 days in the bioreactor. 

Fig. 3. Time-dependent uptake of [3H]E17βG in suspension of fresh human hepatocytes (A), cryopreserved human hepatocytes (B), and HepaRG cells (C) without (■; solid lines) and with (□; dotted lines) coincubation of E3S. Each point represents the mean ± S.D. (n = 3). Where the vertical error bars are not shown, the S.D. values are within the limits of the symbols.
Figure 6 shows the ATA medium concentration as the percentage of start concentration with and without E3S in fresh human hepatocytes and HepaRG cells. AUCs of the ATA medium concentration with and without E3S over time are shown in Table 5. Comparing the AUC values with and without coincubation with E3S, no statistical significant difference was observed in either fresh human hepatocytes or HepaRG cells at days 3 and 4 in culture. However, visual examination of Fig. 6A presenting the loss from media of ATA/E17βG in fresh human hepatocytes, and the relatively low P value of 0.080 obtained comparing AUC of ATA without and with E3S, suggests an OATP1B1-dependent loss from media in fresh human hepatocytes.

Compared with the amount of ATA metabolites formed in the first experiment, the formation of 2-OH-ATA and 4-OH-ATA was increased in the subsequent experiment, when E3S was coincubated with ATA (Fig. 2), in both fresh human hepatocytes and HepaRG cells (Fig. 7), as a result of CYP3A4 induction by atorvastatin and increased metabolite formation over time. Concentrations of ATL, 2-OH-ATL, and 4-OH-ATL were below the limit of quantification.

**Immunohistochemical Staining of OATP1B1 and CYP3A4.** Protein expression and cellular localization of OATP1B1 and CYP3A4 were determined in bioreactors inoculated with freshly isolated human hepatocytes or HepaRG cells by immunohistochemical staining after 9-day culture. Examination of primary human hepatocytes in the bioreactor showed that OATP1B1 was expressed and localized to the hepatocyte plasma membrane (Fig. 8A). OATP1B1 was evenly distributed on the cell surface. In HepaRG cells, the OATP1B1 protein expression was markedly lower than in hepatocytes in bioreactor culture at day 9 (Fig. 8B). Similar protein levels of CYP3A4 were expressed in fresh human hepatocytes and HepaRG cells (Fig. 8, C and D). Unfortunately, the bioreactor tissue structure is easily ruptured because of technical difficulties in removing the fixated cell material from the bioreactor. This phenomenon can be observed in the histological pictures of fresh human hepatocytes in bioreactor culture, especially in Fig. 8C, but should not affect the immunostaining of the cells.

**mRNA Expression of SLCO1B1 and CYP3A4.** The gene expression of SLCO1B1 and CYP3A4 in freshly isolated human hepatocytes and HepaRG cells is shown in Fig. 9. No significant difference in the gene expression level of SLCO1B1 was observed between fresh and cryopreserved human hepatocytes.
cytes. The SLC01B1 gene expression level was 95% (P < 0.01) and 90% (P < 0.05) lower in HepaRG cells than in fresh human hepatocytes and cryopreserved human hepatocytes, respectively. No significant differences in the gene expression levels of CYP3A4 were observed.

Metabolic Parameters. Daily urea and glucose production/consumption and release of ALAT, ASAT, ALP, and GGT were determined in fresh human hepatocytes and cryopreserved HepaRG cells cultured in the bioreactor. Daily release of ALAT, ASAT, ALP, and GGT and production/consumption of urea and glucose in fresh human hepatocytes from three different donors cultured in separate bioreactors were similar with the exception that donor preparation 2 showed higher ASAT and ALAT during the first 2 days in culture.

![Fig. 5. Time-dependent loss of ATA (squares) and formation of ATA hydroxy metabolites (triangles) in suspension of fresh human hepatocytes (A), cryopreserved human hepatocytes (B), and HepaRG cells (C) without (solid lines) and with (dotted lines) coincubation of E3S. Each point represents the mean ± S.D. (n = 3). Where the vertical error bars are not shown, the S.D. values are within the limits of the symbols.](image)

![Fig. 6. Time-dependent loss from media of ATA in fresh human hepatocytes (A) and HepaRG cells (B) without (■; solid lines) and with (○; dotted lines) coincubation of E3S at days 3 and 4 in bioreactor culture. The medium concentration is expressed as the percentage of start concentration ATA. Each point represents the mean ± S.D. (n = 3). Where the vertical error bars are not shown, the S.D. values are within the limits of the symbols.](image)

**TABLE 4**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Experiment</th>
<th>AUC (μmol · h⁻¹)</th>
<th>AUC OH-ATA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh hepatocytes</td>
<td>ATA</td>
<td>152 ± 3</td>
<td>64 ± 20</td>
</tr>
<tr>
<td>Fresh hepatocytes</td>
<td>ATA + E3S (30 μM)</td>
<td>191 ± 13*</td>
<td>51 ± 23*</td>
</tr>
<tr>
<td>Cryo hepatocytes</td>
<td>ATA</td>
<td>164 ± 7</td>
<td>67 ± 17</td>
</tr>
<tr>
<td>Cryo hepatocytes</td>
<td>ATA + E3S (30 μM)</td>
<td>196 ± 23*</td>
<td>47 ± 11*</td>
</tr>
<tr>
<td>HepaRG</td>
<td>ATA</td>
<td>208 ± 20</td>
<td>52 ± 7</td>
</tr>
<tr>
<td>HepaRG</td>
<td>ATA + E3S (30 μM)</td>
<td>207 ± 21</td>
<td>50 ± 6</td>
</tr>
</tbody>
</table>

*, P < 0.05.

**TABLE 5**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Experiment</th>
<th>AUC (μmol · h⁻¹)</th>
<th>AUC ATA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh hepatocytes</td>
<td>ATA</td>
<td>21.1 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>Fresh hepatocytes</td>
<td>ATA + E3S (30 μM)</td>
<td>24.6 ± 4.9*</td>
<td></td>
</tr>
<tr>
<td>HepaRG</td>
<td>ATA</td>
<td>31.5 ± 5.6</td>
<td></td>
</tr>
<tr>
<td>HepaRG</td>
<td>ATA + E3S (30 μM)</td>
<td>32.5 ± 7.6</td>
<td></td>
</tr>
</tbody>
</table>

*, P = 0.08.
The major finding of the present study is that fresh primary human hepatocytes cultured in a 3D bioreactor system retain both OATP1B1 and CYP3A4 activities longer than in currently available in vitro models. Activity measures were in line with conserved protein expression during the same time period. Thus, primary human hepatocytes cultured in a 3D bioreactor system seem to be a promising model for drug discovery research.

The rapid loss of transporter and enzyme activity in 2D cultures of human hepatocytes is a major concern when studying drug uptake, metabolism, and extrusion from cells in vitro and prevents reliable long-term studies to be performed (Rodríguez-Antona et al., 2002; Richert et al., 2006; Ulvestad et al., 2011). Ulvestad et al. (2011) showed an extensive decrease in OATP1B1/1B3 activity in plated primary human hepatocytes, along with increased passive diffusion, when cultured for more than 2 h. In the bioreactor inoculated with freshly isolated human hepatocytes, OATP1B1 activity was retained for at least 7 days, whereas in HepaRG cells no OATP1B1 activity was observed later than day 2. The activity data were in agreement with immunohistochemical stainings that showed that OATP1B1 protein expression was preserved for at least 9 days in fresh human hepatocytes, whereas in HepaRG cells OATP1B1 was markedly lower than in hepatocytes at day 9 in culture, indicating that freshly isolated human hepatocytes are preferred compared with HepaRG cells. Hoffmaster et al. (2004) have reported previously that OATP1B1 and OATP1B3 protein expression is retained in sandwich-cultured human hepatocytes for up to 6 days, but the metabolic capacity of this model has been questioned.

A significant OATP1B1-mediated uptake of E17βG was observed in all three cell types in suspension. However, the OATP1B1-mediated transport was significantly higher in human hepatocytes than in HepaRG cells, both in suspension and bioreactor culture at day 2. The OATP1B1 activity data are consistent with the mRNA expression of SLCO1B1 in cell suspensions showing a significantly lower gene expression in HepaRG cells than in both fresh and cryopreserved human

![Fig. 7. Time-dependent ATA hydroxy metabolite formation in fresh human hepatocytes (A) and HepaRG cells (B) without (●, solid lines) and with (□, dotted lines) coincubation of E7S at days 3 and 4 in bioreactor culture. The medium concentration is expressed as the percentage of start concentration ATA. Each point represents the mean ± S.D. (n = 3). Where the vertical error bars are not shown, the S.D. values are within the limits of the symbols.](image)

![Fig. 8. Immunohistochemical staining (brown) of the basolateral uptake transporter OATP1B1 and the metabolic enzyme CYP3A4 in bioreactor tissue of fresh human hepatocytes from donor preparation 3 (A and C) and HepaRG cells (B and D) cultured for 9 days. OATP1B1 is evenly distributed throughout the whole cell membrane in the bioreactor tissue. Magnification, 40-fold.](image)
hepatocytes. The mRNA expression data are also in agreement with previous results on SLCO1B1 expression in HepaRG cells compared with human hepatocytes (Le Vee et al., 2006; Kanebratt and Andersson, 2008). Furthermore, in freshly thawed HepaRG cells in suspension, no significant OATP1B1-mediated uptake of ATA or loss from media of E17βG was observed. The apparent inconsistent results from the studies on ATA and E17βG regarding active uptake in HepaRG cell suspension could possibly be explained by a different experimental setup or the variation in passive diffusion of the two substances. When the active OATP1B1-mediated uptake of a substance is low compared with the passive diffusion, which may be the case with the more lipophilic ATA, HepaRG cells in suspension is not sufficiently sensitive to separate these processes because of the low OATP1B1 expression.

In previous studies, immunohistochemical staining of the apical efflux transporters multidrug resistance-associated protein (MRP2) and multidrug resistance protein (MDR1) showed expression of both transporters in 3D bioreactor cultures of fresh human hepatocytes and HepaRG cells for at least 2 weeks (Darnell et al., 2011; Zeilinger et al., 2011). The localization and distribution of the transporters were similar to that found in liver tissue. To our knowledge, our study is the first to investigate the protein expression and activity of basolateral transporters in hepatocyte-like cells cultured in the bioreactor. Apparently, this is also the first study to show that loss from media experiments can be performed to evaluate the involvement of hepatic uptake transporters in the bioreactor system using specific substrates and inhibitors.

E17βG is described as a probe substrate for both OATP1B1 and OATP1B3 (König et al., 2000; Nakai et al., 2001; Iwai et al., 2004; Sasaki et al., 2004), whereas ATA is a clinically relevant substrate that is subject to hepatic uptake by OATP1B1 (Kameyama et al., 2005). E3S at a concentration of 30 μM is a selective inhibitor of OATP1B1-mediated uptake with little effect on OATP1B3 (Ishiguro et al., 2006).

In the liver, hepatic uptake of E17βG and ATA is mediated by OATP1B1 and/or OATP1B3 as discussed above. MRP2, and to less extent MDR1, is responsible for the biliary efflux of E17βG, whereas ATA is excreted via MDR1, MRPI, and breast cancer resistance protein (Wu et al., 2000; Chen et al., 2005; Lau et al., 2006; Keskitalo et al., 2009). Formation of bile structures in hepatocytes cultured in a 3D bioreactor have been reported previously (Zeilinger et al., 2004; Schmelzer et al., 2009), but the organization of these bile structures is not known and the bioreactor technique does not allow collection of bile at this point. In the bioreactor experiments performed in this study, intracellular E17βG probably is partly transported back into the medium via MRPI (and MDR1), whereas ATA is excreted by MDR1, MRPI, and breast cancer resistance protein. The curve plateau that is observed in the loss from media experiments over time (Figs. 4 and 6) could possibly be explained by an equilibrium between uptake and efflux of E17βG and ATA.

Lau et al. (2007) reported a 2.7-fold increase in the elimination half-life of ATA in vivo after oral, single-dose coadministration of rifampin, a potent OATP1B1 inhibitor (and a potent CYP3A4 inducer over days). This was in line with our observations in the bioreactor experiments with fresh human hepatocytes, where the AUC of ATA increased 1.2-fold during coadministration of E3S, another OATP1B1 inhibitor. Possible explanations for the lower interaction effect in vitro include efflux of ATA back into the media and less potent OATP1B1 inhibition of E3S compared with rifampin. Nevertheless, the in vitro findings demonstrate the applicability of the bioreactor as a model for preclinical interaction studies.

Atorvastatin is a HMG-CoA reductase inhibitor administered clinically as the active acid form (Cilla et al., 1996). In the liver, ATA and its corresponding inactive lactone form are metabolized by CYP3A4 to 2- and 4-hydroxylated metabolites, of which the acidic forms are also pharmacologically active (Kearney et al., 1993; Ishigami et al., 2001). In this study, ATA was metabolized to 2-OH-ATA and 4-OH-ATA in all cell systems, demonstrating an active CYP3A4 metabolism. These data are in agreement with studies on fresh human hepatocytes and HepaRG cells in bioreactor culture where CYP3A4 activity was observed for at least 2 weeks (Zeilinger et al., 2002; Darnell et al., 2011), as well as studies on CYP3A4 activity in fresh and cryopreserved human hepatocytes and HepaRG cells in suspension (Steinberg et al., 1999; Gómez-Lechon et al., 2004; Aninat et al., 2006). Similar metabolite levels were observed in human hepatocytes and HepaRG cells in both the bioreactor experiments at days 3 and 4 and in experiments in cell suspensions. These data are in agreement with the comparable CYP3A4 protein expression levels observed in fresh human hepatocytes and HepaRG cells in bioreactor culture at day 9 and CYP3A4 mRNA expression data that showed no significant difference in gene expression level between freshly isolated human hepatocytes and freshly thawed cryopreserved human hepatocytes and HepaRG cells in suspension.

In the present study, the inhibition of ATA uptake by E3S in both fresh and cryopreserved human hepatocyte suspension resulted in a significant decreased formation of ATA metabolites because of inhibition of the OATP1B1-mediated uptake. On the contrary, in the bioreactor, an increased metabolite formation was observed in the experiments with the inhibitor. Atorvastatin has been shown previously to induce CYP3A4 expression in vitro (Kocarek et al., 2002; Monostory et al., 2009). In the bioreactor, ATA actually increased CYP3A4 expression over time in the first experiment, which resulted in an autoinduction in metabolism and increased metabolite formation in the subsequent ATA plus E3S experiment in both fresh human hepatocytes and HepaRG cells. Induction of CYP3A4 in HepaRG cells in bioreactor culture have been demonstrated previously (Darnell et al., 2011). This study demonstrates an induction response.

![Fig. 9. mRNA expression of SLCO1B1 and CYP3A4 in fresh human hepatocytes, cryopreserved human hepatocytes, and HepaRG cells in suspension. The relative gene expression (ΔΔCt) and the fold change of gene expression (2^ΔΔCt) were calculated by using cryopreserved human hepatocytes as a calibrator. The bars represent the mean values ± S.D. (n = 3). * P < 0.05; **, 0.01.](image-url)
by ATA in both primary human hepatocytes and HepaRG cells cultured in the bioreactor.

Variability in the quality of freshly isolated human hepatocytes is a well known limitation of this model. Release of ASAT and ALAT values are parameters used to evaluate the quality of hepatocytes. In this study, donor preparation 2 showed higher ASAT and ALAT values compared with donor preparations 1 and 3, indicating an inferior quality of the hepatocytes from donor preparation 2. This could explain the less stable OATP1B1 activity observed in donor preparation 2. A considerable improvement of hepatocyte cryopreservation protocols, allowing storage, transport, scheduling of experiments, and repeated experiments with hepatocytes isolated from the same donors has been achieved in recent years. In this study, similar data were obtained on activity and mRNA expression of both OATP1B1 and CYP3A4 in fresh and cryopreserved human hepatocytes (Hengstler et al., 2000; Badolo et al., 2011) and show the improved functions and properties of cryopreserved cells (Li, 2008). In this study, cryopreserved human hepatocytes were, however, not applied in the bioreactor system based on prior experience showing that nonplatable cryopreserved human hepatocytes are not functioning in the bioreactor (data not shown). The bioreactor technique requires cell attachment to the hollow-fiber capillary membranes, and the incapability of nonplatable cryopreserved hepatocyte to attach to surfaces probably explains the negative results. In future experiments, platable cryopreserved human hepatocytes should be applied in the bioreactor to test this hypothesis.

The rapid loss of enzyme and transporter activity in 2D culture of human hepatocytes is a restriction of these systems, which prevents reliable long-term studies. For OATP1B1 substrates, uptake transport activity may have important implications for cellular drug concentrations, which also affect drug metabolism and possible toxic effects. This study shows that freshly isolated human hepatocytes cultured in a 3D bioreactor system preserve functionality of both OATP1B1 and CYP3A4. This allows long-term preclinical studies on drug uptake and metabolism, which is especially important for slowly metabolized drugs.

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Authorship Contributions
Participated in research design: Ulvestad, Darnell, Molden, ¨Asberg, and Andersson.

Conducted experiments: Ulvestad, Darnell, and Ellis.

Performed data analysis: Ulvestad.

Wrote or contributed to the writing of the manuscript: Ulvestad, Darnell, Molden, ¨Asberg, and Andersson.

References


Kearney AS, Crawford LF, Mehta SC, and Radebaugh GW (1993) The interconversion of Clinical Pharmacology and Toxicology, University Hospital Zurich, Zurich, Switzerland) for the human OATP1B1 antibody.

**Address correspondence to**: Dr. Tommy B. Andersson, DMPK Innovative Medicines, AstraZeneca R&D Mömlund, Pepparedsleden 1, SE-431 83 Mömlund, Sweden. E-mail: tommy.b.andersson@astrazeneca.com