Functional ATP-binding cassette (ABC) drug efflux transporters in isolated human and rat hepatocytes significantly affect assessment of drug disposition.

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Abbreviations: ABC-transporter, ATP-binding cassette transporter; ACN, acetonitrile; AU, arbitrary units; BCRP, breast cancer resistance protein; BSEP, Bile salt efflux pump; CL, clearance; CL_{int}, intrinsic clearance; CYP, cytochrome P450; FBS, fetal bovine serum; fu, fraction unbound, free fraction; DMSO, dimethylsulfoxide; FTC, Fumitremorgin C; HRP, horse-radish peroxidase; IVIVE, in vitro-in vivo extrapolation; KHL, Krebs-Henseleit buffer; LOQ, limit of quantitation; MRP, multidrug resistance associated protein; NTCP, Na⁺-taurocholate co-transporting protein; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PAGE; polyacrylamide gel electrophoresis; PBS, phosphate buffered saline solution; P-gp, P-glycoprotein; RT, room temperature; SDS, sodium dodecyl-sulfate; SLC, solute carrier; s-t-o, spinning through oil; TBS, Tris-buffered saline solution; TBST, Tris-buffered saline solution, 0.1% Tween-20; WE, William’s E medium
Abstract

Freshly isolated hepatocytes are considered the gold standard for in vitro studies of hepatic drug disposition. To ensure a reliable supply of cells, cryopreserved human hepatocytes are often used. ABC-super family drug efflux transporters are key elements in hepatic drug disposition. These transporters are often considered lost after isolation of hepatocytes. In the present study the expression and activity of ABC transporters BCRP, BSEP, P-gp, MRP2, MRP3, and MRP4, in human and rat cryopreserved hepatocytes were investigated. In commercially available human cryopreserved hepatocytes all drug efflux transporters except human BCRP (hBCRP) exhibited similar expression levels as in fresh liver biopsies. Expression levels of hBCRP were 60% lower in cryopreserved human hepatocytes than in liver tissue, which could lead to, at most, a 2.5-fold reduction in hBCRP-mediated efflux. Fresh rat hepatocytes showed significantly lower levels of rat BCRP (rBCRP) compared to liver expression levels, expression levels of other ABC transporters were unchanged. ABC-transporters in human cryopreserved cells were localized to the plasma membrane. Functional studies could demonstrate P-gp and BCRP activity in both human cryopreserved and fresh rat hepatocytes. Inhibiting P-gp mediated efflux by elacridar in in vitro experiments significantly decreased fexofenadine efflux from hepatocytes, resulting in an increase in apparent fexofenadine uptake. The results from the present study clearly indicates that ABC-transporter mediated efflux in freshly isolated as well as cryopreserved rat and human hepatocytes should be taken into account in in vitro experiments used for modeling of drug metabolism and disposition.
Introduction

Isolated hepatocytes are used to determine many properties of a candidate drug; its intrinsic clearance, $CL_{int}$, metabolic pathways, and increasingly also to assess drug-drug interactions mediated by drug metabolizing enzymes and/or drug transporters (Chiba et al., 2009; Soars et al., 2007a, 2009; McGinnity et al., 2009; Zhao, 2008; Brown et al., 2010; Giacomini et al., 2010).

Cryopreserved human hepatocytes are used as a convenient and reliable source of cells in drug discovery research while for preclinical species freshly isolated hepatocytes are commonly available (Chiba et al., 2009; Giacomini et al., 2010). Characterization of both freshly isolated and cryopreserved hepatocytes from a number of species has been reported, focusing on drug metabolizing enzymes (Chiba et al., 2009; Soars et al., 2009). The studies have concluded that the drug metabolizing enzymes are retained on the same level in cryopreserved hepatocytes as in freshly isolated cells (Floby et al., 2009; Stringer et al., 2008).

Several ABC efflux transporters such as P-glycoprotein (P-gp); multidrug-resistance protein 2 (MRP2); breast cancer resistance protein (BCRP); and the bile salt efflux pump (BSEP) are important for physiological functions of the liver but are also exporting organic compounds, including drugs, from the hepatocytes. These are all localized to the bile facing canalicular apical membrane of the hepatocytes and are important for biliary secretion of bile salts and drugs (Klaasen and Aleksunes, 2010). Additionally, some ABC transporters such as MRP3 and MRP4 are expressed on the basolateral membrane and export compounds into the blood stream (Klaasen and Aleksunes, 2010). MRP3 and MRP4 have been indicated to play a central role in basolateral efflux of sulfate- and glucoronide-conjugated drug metabolites (Zamek-
Gliszczynski et al., 2006) and have been shown to be involved in basolateral efflux of fexofenadine and rosvastatin (Pfeifer et al., 2013; Tian et al., 2008).

Fexofenadine, rosvastatin and digoxin are examples of drug compounds being eliminated, in whole or in part by bile secretion in man through efflux via ABC-transporters (Cvetkovic et al., 1999; Kitamura et al., 2008; Roberts et al., 2002, Yang et al., 2009). Erythromycin is an example of a drug that is cleared in the human liver by the concerted action of the ABC transporter P-gp (hP-gp) and cytochrome P450 3A4 (CYP3A4) (Kurnik et al., 2006). Fexofenadine and erythromycin are selective substrates of P-gp while rosvastatin is predominantly effluxed via BCRP (Morrissey et al., 2012). These compounds are thus valuable probes for the study of efflux transporter activity and of transporter-enzyme interplay and were extensively used in the present study together with selective transporter inhibitors.

The fate of ABC efflux transporters in isolated and cryopreserved hepatocytes has been debated. Some reports suggest that cryopreserved hepatocytes have lost most of their ABC transporters function as a result of a rapid and partial internalization after isolation of the hepatocytes (Bow et al., 2008, Oude Elferink et al., 1993). No quantification of the extent of transporter loss was attempted.

Other results have pointed to the presence and activity of several major efflux transporters in freshly isolated hepatocytes as well as cryopreserved hepatocytes from both rat and man (Lam et al., 2004, 2006; Li et al., 2008, 2009a, 2009b). In the study by Di et al., (2012) significant P-gp activity in cryopreserved hepatocytes was inferred from in vitro- in vivo extrapolation (IVIVE) of hepatic clearance (CL) for P-gp substrates from human liver microsomes and cryopreserved hepatocytes. However, in IVIVE of hepatic drug clearance using freshly isolated or cryopreserved
hepatocytes, the activity of ABC transporters is most often considered lost after hepatocyte isolation (Zamek-Gliszczynski et al., 2013).

The aim of the present study was to investigate the applicability of cryopreserved hepatocytes for drug disposition predictions. We specifically focused on efflux transporters and investigated the function and localization of these in human and rat cryopreserved hepatocytes. The abundance of efflux transporters was also investigated in isolated hepatocytes and compared with levels in liver tissue. The impact of ABC transporter-mediated efflux on in vitro uptake and metabolism measurements was assessed. Apart from BCRP no tested ABC efflux transporter (BSEP, P-gp, MRP2, MRP3, and MRP4) showed significant down regulation in human cryopreserved or rat freshly isolated hepatocytes. The efflux transporters exhibited activity that significantly affected in vitro measurements of drug disposition.
Material and methods

Nomenclature

As recommended by Hagenbuch and Stieger (2013) this article follows the gene and protein nomenclature guidelines of the HUGO Gene Nomenclature Committee (Gray et al., 2013) and the International Committee for Standardized Genetic Nomenclature in Mice (2009). Human genes are written in capital letters (e.g. ABCG2) while rodent genes are given in upper and lower case (e.g. Abcg2). Protein names for both human and rodent proteins are written in capital letters (e.g. BCRP). In the interest of clarity human protein names are prefixed with h while rat proteins are prefixed r (e.g. hBCRP, rBCRP). Protein names without prefix refer to the protein in general, not belonging to any particular species (e.g. a BCRP-inhibitor).

Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

Animals

Male Sprague-Dawley rats were acquired from Harlan Laboratories B.V., Venray, The Netherlands. Rats were housed up to 3 per cage in transparent plastic cages. All animals were acclimatized for 2 weeks prior to studies. The room was lit in a 12 h light-dark cycle. Bedding material consisted of aspen wood chips, plastic tunnels and aspen chewing blocks were provided. The rats had free access to food and water. All animal experiments were approved by the local animal ethics committee (Stockholms Södra djurförsöksetiska nämnd) and were conducted in compliance with national guidelines for the care and use of laboratory animals.
Preparation of hepatocytes

Rat hepatocytes were isolated according to a modified collagenase perfusion method previously described (Bizell and Guzelian, 1980) from 7-8 weeks old male Sprague-Dawley rats anesthetized with Isoflurane (Abbott Scandinavia AB, Kista, Sweden). Shortly, the liver was perfused in situ using a peristaltic pump, first with 37 °C calcium free buffer containing 0.5 M EGTA, followed by prewarmed William’s E medium, supplemented with 10 mM HEPES (Gibco; Life technologies, Paisley, UK), pH 7.4 (WE). To the buffer was added 0.16 mg/ml of Collagenase type XI. After completed perfusion the organ was cut from its ligaments and transferred to a prewarmed collagenase solution. Released from connective tissue the hepatocytes were washed to obtain a homogenized cell suspension without debris from extra cellular matrix. The suspension was filtered through gauze, repeatedly centrifuged at 50 x g for 2 minutes and resuspended in new Krebs-Henselleit buffer supplemented with 10 mM HEPES (Gibco), pH 7.4 (KHL) until a homogenous suspension of cells was obtained. Cell number and viability were determined using a Bürker counting chamber and the Trypan blue exclusion method. Viability typically exceeded 90%. Cell preparations with viability of less than 80% were discarded.

Handling of human and rat cryopreserved hepatocytes. Human: lots Hu4035, Hu4037, and Hu4152 (CellzDirect, Life technologies) and lots OZL, SQJ, and REL (Celsis In vitro technologies, Baltimore, MD, USA), donor information is listed in Supplemental Table 1. The lots represented a mix of ages and genders and displayed average CYP metabolism. Rat: derived from male Sprague-Dawley rats (CellzDirect), 7-9 weeks of age, only two lots were available. Cryopreserved hepatocytes were thawed in a 37°C water bath immediately after removal from the -150°C freezer and transferred to a 50 ml vial of WE at 37°C. Centrifugation was done according to the manufacturer’s instructions and the cell pellet was resuspended in a
small volume of KHL. Cell number and viability were determined as described for rat hepatocytes.

For direct efflux measurements hepatocytes were plated in collagen I coated 12-well plates (BD Biocoat, BD Biosciences, Franklin Lakes, NJ, USA). In each well was seeded 0.75 million cells, from a suspension of $10^6$ cells/ml in WE supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 0.01 % Insulin-transferrin-selenium A (Gibco). Prior to 10 min incubation at room temperature the plate was shaken carefully to let the cells distribute evenly. Cells were then left to attach to the plates for 90 min at 37°C in an incubator containing 5% CO₂. The plates were washed three times in 37°C KHL. The distribution of adhered cells was inspected using an inverted microscope after washing. Cells were then preincubated in KHL for 10 minutes before each experiment.

**Preparation of hepatocyte protein**

From liver tissue: rat liver was handled in WE in a petri dish, cut into smaller pieces and homogenized with a glass pestle homogenizer containing a cracking buffer, consisting of 5 mM Hepes, 0.5 mM EDTA, 1% Protease Inhibitor Cocktail (PIC, Sigma-Aldrich), pH 7.2. The resulting suspension was transferred to a tube and homogenized thoroughly with a small gauge syringe before centrifugation to remove unhomogenized tissue. Small samples of human liver tissue were taken from an in house tissue biobank and processed in the same manner (n= 3), donor information is given in Supplemental Table 1. The liver biobank derives from the Karolinska University Hospital, Stockholm, Sweden. Samples were collected from liver resections with approval from the Karolinska University Hospital ethical review board.

From hepatocytes: aliquots of 1 million fresh, snap-frozen after isolation, rat hepatocytes collected with the preparation technique described above, were thawed
and suspended in 200 μl cracking buffer. Homogenization was performed as described for liver tissue.

From cryopreserved hepatocytes: rat and human cryopreserved hepatocytes (CellzDirect; Celsis In vitro technologies), stored at -150°C, were thawed and pelleted by centrifugation at 90 rpm for 3 min and resuspended in cracking buffer. Homogenization was performed as described for liver tissue.

Determination of protein concentration was performed with a BCA protein assay kit (Pierce Biotechnology, Thermo Fischer Scientific Inc., Rockford, IL, USA) according to the manufacturer’s instructions. Samples were stored at -80°C until use.

**Determination of transporter expression by western blot**

10-40 μg of protein, prepared as described above, was diluted in dH₂O and loading buffer (0.5 ml Laemllli buffer, 50 μl β-mercaptoethanol and 2.5 μl 1M dithiothreitol, all from Bio-Rad (Hercules, CA, USA)) and separated by 9% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer of protein to a PVDF membrane (Perkin-Elmer, Waltham, MA, USA) at 200 mA for 2 h. A broad range unstained molecular weight ladder (Bio-Rad) was used as a reference and detected after Ponceau S staining. Ponceau S staining was also scanned and quantified to ensure that equal amounts of protein had been added to each lane.

The membranes were washed 2 x 5 minutes in Tris-buffered saline Tween-20 (0.1% Tween 20 in a buffer consisting of 150 mM NaCl and 25 mM Tris-HCl, pH 7.4 (TBST)) before continuing with blocking for 1 h in blocking buffer (consisting of 5% non-fat dry milk in TBST with pH adjusted to 7.2 with 1 M Tris base) at room temperature (rt).
Membranes were incubated with primary antibody (primary antibodies used are listed in Supplemental Table 2), diluted 1:500 or 1:1000, at 4°C over night followed by washing in TBST for 4 x 15 min. Blocking was again performed during 1 h at rt followed by incubation with a 1:1000 or polyclonal goat anti-mouse IgG conjugated to HRP (Dako, Glostrup, Denmark), depending on the species of the primary antibody. To detect GAPDH an HRP-conjugated primary antibody was used, omitting the need for a secondary antibody (Supplemental Table 2).

Membranes were washed again, first in TBST and thereafter for 2 x 5 min in dH₂O. Antibodies were chemiluminescently detected using Western Blotting ECL Plus reagents (GE Healthcare, Piscataway, NJ, USA), a substrate for HRP, according to the manufacturer’s instruction. The blot was photographed with Amersham hyperfilm ECL (GE Healthcare), developed and fixed. Protein bands were scanned and the background was subtracted. To normalize band intensities the ratio between the transporter band and the band from the housekeeping protein used as internal standard, GAPDH was calculated.

**Immunofluorescence**

Human cryopreserved hepatocytes were allowed to attach to slides (Super Frost, Thermo Fisher Scientific) for 1 h at RT. After washing, adherent cells were fixed in ice-cold 50% acetone for 2 min followed by a 5-minute fixation in 100% acetone. Cover slips were then dried and stored at -20°C until use.

Cover slips were washed 4 x 5 minutes in PBS and blocked with 10% FCS (Gibco) in PBS for 30 min at RT. They were then washed again and incubated with primary antibody in 1% FCS in PBS for 1 h at rt. Primary antibodies were used at a dilution of 1:100. Cells were washed again and blocked with 10% goat serum (Life
technologies) in PBS for 30 min at rt. Slides were then incubated with a 1:1000 dilution of goat anti-mouse Alexafluor conjugated fluorescent secondary antibodies (Life technologies) in PBS with 10% goat serum and double-stained with DAPI (Life technologies). Cover slips were washed one final time for 4 x 5 min in PBS and mounted with Prolong Gold anti-fade mounting media (Life technologies). Stained cells were photographed using a confocal microscope (DMIRE 2, Leica Microsystems, Wetzlar, Germany).

Liver tissue samples were obtained from donors undergoing surgical liver resection at Uppsala University Hospital (Uppsala, Sweden), as approved by Uppsala Regional Ethical Review Board. Small biopsies from liver resections were snap frozen in ethane cooled by liquid nitrogen in the operating theatre immediately after the surgical procedure (n=3), donor information is given in Supplemental Table 1. Frozen biopsies were stored at -80°C until use. Liver biopsies were cut into 8 μm thick sections on a cryotome and dried down on slides (Super Frost, Thermo Fischer Scientific). Liver sections were fixed, stained and visualised as described for hepatocyte samples above.

Uptake, efflux and metabolic stability measurements in hepatocytes

To directly measure efflux, plated hepatocytes in 12 well plates were used. To start uptake KHL was removed and 300 μl of 1 μM of rosuvastatin diluted in KHL, containing 0.1% DMSO, was added to each well. After 30 min of preloading the cells were rapidly washed 3 times with 37°C KHL after which 300 μl fresh KHL was added to each well. Efflux from the cells was followed by sampling 200 μl buffer from each well at different time points, 0.5, 1, 2 and 5 minutes. Efflux was quantified at the 1 minute time point, well within the linear range of efflux. Samples were mixed with two volumes of an ice cold stop solution of acetonitrile (ACN) with 200 nM Warfarin (internal standard). Plates containing samples were incubated on ice for at least 10
minutes to ensure complete precipitation of protein. The plate contents were shaken for a few minutes prior to centrifugation at 1200 x g for 10 minutes. An aliquot of 200 μl supernatant from each well were transferred to a 96 well analysis plate. Before analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) each fraction was diluted in KHL to 25 % ACN final concentration.

Uptake measurements in suspended cells were performed using the media loss technique as described (Soars et al., 2007). Briefly, hepatocytes (10^6 cells/ml) were incubated with test compound at a concentration of 1 μM in KHL with the addition of 0.1% DMSO. At intervals ranging from 0 and 15 seconds up to 90 minutes aliquots were transferred to a glass tube loaded into a bench top centrifuge and hepatocytes were pelleted by centrifugation (7000g, 15 s) after which a sample was taken from the supernatant. Samples were processed and analyzed as described above.

To assess metabolic elimination of compound an aliquot of cells and medium was removed from the incubation, lysed and processed as above omitting the centrifugation step.

Uptake of fexofenadine and uptake and metabolism of erythromycin was assessed using the spinning-through-oil (s-t-o) method (Petzinger and Fückel, 1992). An advantage with this method is that compound concentrations in cells and medium can be measured separately. The incubation of hepatocytes and test compound was layered on top of a layer of mineral oil (d = 1.02 g/ml) (Sigma-Aldrich). Below the oil meniscus a lower phase consisting of 50 μl 4 M CsCl was found. At the designated time points the uptake reaction was stopped by spinning the cells through the oil layer (1200 g, 30 s). A sample was taken of the supernatant after which it, the oil meniscus and the lower CsCl phase were removed by careful pipetting to not disturb
the cell pellet. The cells were then resuspended in 50 μl KHL, lysed and processed as described above. Compound concentrations were determined by LC-MS/MS.

The effects of efflux through ABC-transporters on drug efflux, drug metabolism, compound uptake and metabolite efflux were investigated using the P-gp and BCRP inhibitor elacridar (also known as GF120918) or the BCRP inhibitor fumitremorgin C (FTC) in rat fresh and human cryopreserved hepatocytes using terfenadine, fexofenadine, erythromycin or rosuvastatin as probe substrates (Supplemental Table 3). At the concentrations of 0.5-2 μM used in the experiments elacridar inhibition of BCRP is minor, less than 20% (Matsson et al., 2009). Fexofenadine and erythromycin were used in P-gp inhibition experiments using the inhibitor elacridar as these two compounds do not seem to interact with BCRP (Ozawa et al., 2004; Morrissey et al., 2012). Rosuvastatin and fexofenadine efflux from plated hepatocytes was measured in the presence or absence of inhibitors. After addition of terfenadine to hepatocytes P-gp activity was detected monitoring efflux of the terfenadine metabolite fexofenadine, in the presence or absence of elacridar. The effects on fexofenadine uptake by inhibiting P-gp mediated efflux with elacridar were then investigated using media-loss and spinning through oil experiments. Spinning through oil experiments with or without elacridar were performed to monitor uptake, metabolism and efflux of erythromycin and its metabolites. In each reaction the concentration of the inhibitor was optimized to give maximal effects on the reaction studied without affecting other parameters such as uptake or CYP metabolism of the test compound.

**Detection of test compounds**

Sample analysis was performed by LC-MS/MS using a Micromass Quattro Micro triple quadrupole (Micromass, Manchester, UK) coupled to a gradient pump composed of two Shimadzu LC-10AD VP isocratic pumps (Shimadzu Corporation,
Kyoto, Japan) and a CTC HTS PAL autosampler (CTC analytics, Zwingen, Switzerland). The software MassLynx (controlling the LC-system and mass spectrometer), which includes QuanLynx (quantification) and QuanOptimize (MS/MS optimization) was used (Waters Co., Milford, MS, USA). A High Purity C₁₈ 5 μm 30 x 2.1 mm (Thermo Electron Corporation, Waltham, USA) analytical column was used. Chromatography was performed using a generic gradient at a flow rate of 0.4 ml/min. The mobile phase consisted of solvent (A) 2 % acetonitrile in 0.1 % (v/v) acetic acid in water and (B) 80 % acetonitrile in 0.1% (v/v) acetic acid in water. The injection volume was 20 µl and warfarin was used as internal standard. Other source parameters (e.g., collision energy, cone voltage, ion mode, molecular weight of parent and daughter etc.) were individually optimized for each compound (see Supplemental Table 4). Responses were detected as the area under the curve, and the relative concentration was determined with the internal standard. Absolute concentrations were calculated from standards spanning from twice the initial experimental concentration to below the limit of quantification (LOQ). LOQ, signal to noise ratio for detection, was set to 5 times. Terfenadine metabolism was monitored by quantification of fexofenadine and erythromycin metabolism by the formation of N-desmethyl-erythromycin.

**Determination of intrinsic clearance**

The elimination rate constant was calculated from the first order elimination equation

\[ [S] = [S₀] \cdot e^{-kt} \]  

(1),

where [S] is concentration of substrate at a given time point, t, after start of the incubation at the initial concentration [S₀]. k is the elimination rate constant and was determined by non-linear regression within a time point interval considered to
represent the initial elimination rate either in metabolism or uptake experiments (Soars et al., 2007). Intrinsic clearance for metabolism or uptake (CL_{int,\text{met}} or \text{CL}_{int,\text{uptake}}) could then be calculated from:

\[
CL_{int} = k \cdot V \quad (2),
\]

where \(V\) represents the volume of incubated substrate.

In vitro to in vivo extrapolation (IVIVE) of in vitro \(CL_{int,\text{uptake}}\) values were performed for fexofenadine uptake experiments in the absence or presence of increasing concentrations of elacridar. IVIVE was performed using the well stirred model including fraction unbound in both in vitro and in vivo systems as described by Sohlenius-Sternbeck et al. (2012) omitting the regression line correction step employed in that study. The physiological scaling factors used are listed in Sohlenius-Sternbeck et al. (2012). In vivo \(CL_{\text{blood}}\) for fexofenadine was determined in house in Sprague-Dawley rats after intravenous administration of a dose of 3 µmol/kg (Lundquist et al., unpublished). Hepatic clearance (\(CL_{\text{hepatic}}\)) for fexofenadine was calculated from the \(CL_{\text{blood}}\) using data on the hepatic fraction of clearance from the literature (Swift et al., 2009). Human pharmacokinetic parameters for erythromycin are from Sun et al., 2010.

**Statistical analysis**

Data were analyzed using Graphpad Prism 5 software (Graphpad Software, Inc., La Jolla, CA, USA). Statistical differences were tested using the two-tailed Student’s \(t\)-test. Probability values (P-values) are symbolized by: \(* = p < 0.05\), \(** = p < 0.01\), \(*** = p < 0.001\), p-values of \(p < 0.05\) were considered statistically significant.
Results

ABC efflux transporter expression in human and rat hepatocytes

Western blotting was used to determine expression levels of drug efflux transporters from the ABC superfamily, hBCRP, hBSEP, hMRP2, hMRP3, hMRP4 and hP-gp in human cryopreserved hepatocytes and human liver samples. In total 6 different batches of cryopreserved hepatocytes (3 obtained from CellzDirect and 3 from Celsis In vitro technologies) and samples from 3 liver donors were tested. Both cryopreserved hepatocytes and liver samples derived from donors of mixed gender (Supplemental Table 1). No differences could be seen between cryopreserved cells obtained from the different providers. No statistically significant differences of efflux transporter protein levels in cryopreserved human hepatocytes and liver tissue were seen, with the exception of hBCRP which was approximately 60% lower in cryopreserved hepatocytes compared to levels in fresh liver (Fig. 1A, B).

Three batches of fresh rat hepatocytes were prepared from Sprague-Dawley rats. Two batches of cryopreserved rat hepatocytes from the same strain were purchased from CellzDirect. In freshly isolated rat hepatocytes rBCRP protein levels were significantly lower (75%) than in liver tissue (Fig. 2A, B). In cryopreserved rat hepatocytes the transporters rBCRP, rMRP2, and rP-gp were 90, 40, and 60% lower, respectively, compared to liver tissue.

Immunofluorescent microscopy of cryopreserved human hepatocytes indicated that hBCRP, hMRP2 and hP-gp transporters were localized to the plasma membrane (Fig. 3A-C). Some intracellular staining was also evident but was much weaker than the plasma membrane associated signal. hMRP2 and hP-gp were found in distinct areas of the membrane possibly constituting remaining apical domains. BCRP on the
other hand showed no clear localization but seemed distributed over the surface of the cryopreserved hepatocytes.

Immunofluorescent staining of human liver tissue sections by the same three primary antibodies towards hBCRP, hMRP2 and hP-gp (Fig. 4 A-D) showed clear and specific staining of the apical domains of hepatocytes. Localization of the transporters along the bile canalicular ducts was clearly visible.

**Efflux transporter activity in human cryopreserved and rat fresh hepatocytes**

To investigate transporter functions in fresh and cryopreserved hepatocytes the transport of rosuvastatin, a known BCRP substrate, and the mother compound-metabolite pair terfenadine-fexofenadine, both P-gp substrates, were used as probe compounds (Kitamura et al., 2008; Cvetkovic et al., 2006) in combination with the P-gp and BCRP inhibitor elacridar or the BCRP inhibitor FTC.

Figure 5 shows efflux of rosuvastatin from plated human cryopreserved hepatocytes (Fig. 5A) and rat fresh hepatocytes (Fig. 5B). Rosuvastatin efflux was in both cases inhibited by FTC. Efflux from rat cells was 20-fold higher than efflux from human cryopreserved hepatocytes, due to the higher uptake of rosuvastatin and resulting increase in intracellular concentration seen in fresh rat hepatocytes (data not shown).

In Fig 6A the uptake and metabolism of terfenadine in human cryopreserved hepatocytes is shown, uptake was measured using the media loss method and metabolism by metabolic stability measurements (total metabolism, cells + medium) in suspended cells. The compound showed rapid uptake into hepatocytes and moderate metabolism. Adding 1 μM elacridar did not influence the rate of terfenadine metabolism in metabolic stability experiments (Fig. 6B) and the formation of the terfenadine metabolite fexofenadine was not affected (Fig. 6C).
Elacridar (1 µM) added to loss from media experiments with terfenadine reduced the efflux of intracellularly formed fexofenadine into the medium by approximately 40% indicating inhibition of hP-gp mediated efflux (Fig. 6D). In fresh rat hepatocytes elacridar did not inhibit the formation of fexofenadine but inhibited the efflux in a similar way as shown in cryopreserved human hepatocytes (Fig. 6E, F).

**Uptake-efflux interactions**

To test whether efflux affects the results from loss from media uptake experiments, experiments with freshly isolated rat hepatocytes using the OATP and P-gp substrate fexofenadine were performed. The uptake of fexofenadine in human cryopreserved hepatocytes was studied using the spinning through oil technique since the uptake was too slow to be reliably measured in loss from media experiments.

In freshly isolated rat hepatocytes the measured loss of fexofenadine from the medium increased with increasing concentrations of elacridar up to 2 µM indicating an active efflux of fexofenadine back to the medium by rP-gp (Fig. 7A). Solid lines represent initial fexofenadine uptake rates in the presence of 0, 1, and 2 µM elacridar (Fig. 7A). The apparent uptake rate increased four-fold when comparing CL<sub>int, uptake</sub> in incubations with 2 µM elacridar and control experiments without elacridar (Fig. 7B).

Fig. 7A also indicates that the medium concentration of fexofenadine reached a steady state after 60 minutes. At this time point the concentration of fexofenadine left in the medium was reduced with increasing elacridar concentration. Without elacridar 20% of fexofenadine had been taken up by the cells after uptake completion. After addition of 2 µM elacridar 55% of fexofenadine had been taken up by the hepatocytes after 60 min. We can thus infer that the total intracellular concentration of fexofenadine had more than doubled in the presence of 2 µM elacridar.
Measuring fexofenadine uptake in cryopreserved human hepatocytes with the spinning through oil method showed a significant increase in fexofenadine uptake after addition of 1 μM elacridar (Fig. 7D).

$CL_{\text{int, uptake}}$ measurements shown in Fig. 7B were used for IVIVE of rat hepatic fexofenadine CL (Table 4). The higher $CL_{\text{int, uptake}}$ values measured in the presence of elacridar led to predicted in vivo $CL_{\text{hepatic}}$ closer to observed in vivo values.

**Metabolism-efflux interactions**

Effects of efflux inhibition on drug metabolism in human cryopreserved hepatocytes were investigated using erythromycin as a probe substrate together with 0.5 μM of the P-gp inhibitor elacridar.

In Fig 8A-B the results from a metabolic stability experiment with erythromycin is shown using human cryopreserved hepatocytes in suspension. The addition of 0.5 μM elacridar to the incubation did not change the total (cells + medium) metabolism of erythromycin significantly, neither was the total (cells + medium) formation rate of the main metabolite $N$-desmethyl-erythromycin affected.

The extracellular concentration of erythromycin in spinning through oil uptake experiments with human cryopreserved hepatocytes was not affected by elacridar (Fig. 8C) whereas extracellular levels of $N$-desmethyl-erythromycin were reduced (Fig. 8D).

Measurements of intracellular levels showed that the net uptake of erythromycin was increased by approximately two-fold after addition of elacridar (Fig. 8E). In parallel with this intracellular concentrations of $N$-desmethyl-erythromycin more than doubled in the presence of the inhibitor (Fig. 8F).
Uptake of erythromycin was used for IVIVE of human hepatic CL (Table 4). The higher uptake rates measured in the presence of elacridar significantly improved the IVIVE.

The experiment on human cryopreserved hepatocytes shown in Fig. 8 could be confirmed with fresh rat hepatocytes reproducing the above findings of intracellular retention of erythromycin and its metabolite after P-gp inhibition by elacridar (data not shown).
Discussion

In the present study protein levels of most tested efflux transporters with the exception of hBCRP were present at similar levels in human liver and cryopreserved hepatocytes. Our data corresponds well with LC-MS/MS quantification of hepatocyte transporters by Li (et al., 2009a, b) showing retention of hMRP2, hBCRP and hBSEP in human liver, fresh and cryopreserved hepatocytes. Li et al. (2009a, b) detected lower levels of rMRP2 in isolated and cryopreserved rat hepatocytes and a loss of rBCRP in cryopreserved cells whereas in the present study rBCRP showed the largest reduction of the transporters studied in isolated as well as in cryopreserved hepatocytes as compared to fresh liver tissue levels.

The levels of the basolateral efflux transporters hMRP3 and hMRP4 were unchanged in cryopreserved human hepatocytes, compared to liver tissue. Basolateral efflux of drugs and conjugated drug metabolites is thus likely to be present at physiological levels in in vitro experiments with isolated hepatocytes.

Retention of hepatocyte canalicular membrane domains after cell isolation has been demonstrated previously (Rippin et al., 2001; Tietz et al., 2005) and is likely representing the localization of hP-gp and hMRP2 observed in the present study. In contrast hBCRP was found to be spread over large areas of the cell surface in isolated cells. This is reminiscent of the redistribution of some canalicular membrane markers such as alkaline phosphatase over the cell surface demonstrated by Groothius et al. (1981). It seems that BCRP is both the least stable and more prone to lateral diffusion compared to the other tested ABC-transporters. BCRP exists as a multimer of several “half-transporters”, while the other tested ABC-transporters consist of single polypeptide chains, possibly making BCRP more prone to disassociation and break-down (Klaasen and Aleksunes, 2010). Another explanation
could be that P-gp, BSEP and MRP2 are anchored to the cytoskeleton and are localized to stable caveolin-1 containing membrane micro-domains (Hegedüs et al., 2003; Ortiz et al., 2003; Ismair et al., 2009). Such stabilizing interactions have not been demonstrated for BCRP and their absence might make the transporter more sensitive to degradation. Some intracellular staining for hBCRP, hMRP2, and hP-gp was detected that may be internalized transporters or new transporter proteins that have yet to be exported to the membrane.

Bow et al. (2008) showed that rP-gp and rMRP2 in hepatocytes immediately after isolation were localized to remaining canalicular structures of the hepatocyte membrane. When culturing hepatocytes for one hour a rapid but partial internalization of the transporters were observed which was taken as evidence that transporter mediated drug efflux is inactive in primary hepatocytes. However, little effort has been made to quantify the impact of this possible internalization on in vitro efflux activity. The fold-reduction of efflux activity due to a loss of transporter proteins can be calculated as the inverse of the remaining transporter activity (Zamek-Gliszczynski et al., 2008). A 60% loss of BCRP activity in human cryopreserved or rat fresh hepatocytes would thus give a 2.5-fold (1/0.4) decrease in in vitro efflux and increase in intracellular exposure of a BCRP selective substrate, less if other efflux transporters are also involved. This is within the error margins deemed acceptable in IVIVE. BCRP expression in the human population is variable, showing a span of 2-fold around the mean, only slightly smaller than the reduction seen in cryopreserved samples (Prasad et al., 2013). This indicates that the remaining BCRP level is close to the levels in the lower range of the human population. In rat cryopreserved hepatocytes effects will be larger; 10-, 1.7-, and 2.5-fold reductions in in vitro efflux for selective rBCRP, rMRP2, and rP-gp substrates, respectively.
Several other studies have suggested that efflux transporter protein and activity is retained to some extent in isolated or cryopreserved hepatocytes. Protein levels similar to liver expression levels of rBCRP, rBSEP, rP-gp, and rMRP2, have been detected in short term cultures of isolated rat hepatocytes (Rippin et al., 2001, Tchaparian et al., 2011). Efflux activity in rat isolated hepatocytes has been shown for rP-gp using the inhibitor elacridar and the substrate erythromycin (Lam et al., 2004, 2006). Li and coworkers could demonstrate BCRP activity in human fresh and cryopreserved hepatocytes, but not rat cells, using the selective inhibitor FTC, and P-gp activity using efflux of 3,3-diethyloxacarbocyanine iodide (Li et al., 2008, Matsson et al., 2009). Full or partial retention of BCRP, BSEP, MRP2, and P-gp protein in isolated rat and human hepatocytes has been demonstrated by proteomics but these measurements might include internalized transporters (Li et al., 2008, 2009a, 2009b). Retention of efflux transporter activity in human cryopreserved hepatocytes has recently been inferred from comparisons of CL_int determinations from hepatocytes and microsomes for efflux transporter substrates (Di et al., 2012). These findings correspond well with the present study were P-gp activity in rat fresh and human cryopreserved hepatocytes could be detected by inhibiting efflux of fexofenadine and rosvastatin with the inhibitor elacridar and FTC, respectively. In contrast to Bow et al. (2008) only limited internalization of efflux transporters were seen in our experiments on human cryopreserved hepatocytes. Data in the present study is in agreement with studies suggesting that only a fraction of ABC transporters are internalized leaving substantial active efflux in freshly isolated as well as cryopreserved hepatocytes (Li et al., 2009a; 2009b; Di et al., 2012).

Efflux transporter activity may reduce intracellular concentrations of certain drugs and thus have an impact on both uptake rate estimations and drug metabolism in the hepatocytes; often called transporter-enzyme interplay. In vivo, in the liver, uptake and efflux transporters work in unison which results in a vectorial flux of compounds
from blood to bile. When performing in vitro hepatocyte uptake studies with suspended hepatocytes the cells polarization is lost. Compound that is taken up by uptake transporters is effluxed back into the same medium compartment by active ABC-transporters that in this manner diminishes measurements of apparent uptake (Benet, 2009). In the present study measured $CL_{\text{int, uptake}}$ for fexofenadine in rat hepatocytes was increased after addition of the P-gp inhibitor elacridar. Fexofenadine is also effluxed by MRP2, so efflux inhibition by elacridar was likely not complete (Matsushima et al., 2008a; Tian et al., 2008; Matsson et al., 2009). Fexofenadine shows low permeability, low metabolism and high solubility and its hepatic CL is likely rate limited by uptake transporters (Benet, 2009). Scaling rat in vivo hepatic CL for fexofenadine from hepatocyte $CL_{\text{int, uptake}}$ data resulted in a large under prediction. Predictions of fexofenadine hepatic CL was considerably improved using $CL_{\text{int, uptake}}$ generated in the presence of elacridar demonstrating that in vitro uptake measurements can be perturbed by efflux transporter activity retained in the hepatocytes used. Scaling of human hepatic clearance from erythromycin uptake in human cryopreserved hepatocytes was improved in a similar manner using the higher uptake rates measured in the presence of elacridar. Human hepatic CL was under-predicted 4-5 fold, presumably due to reduced uptake of erythromycin in human cryopreserved hepatocytes as a consequence of the large down regulation of OATPs seen in human cryopreserved hepatocytes (Kimoto et al., 2012).

Uptake transporters are rate determining for the clearance of many drugs, but IVIVE of hepatic clearance from hepatocyte uptake data generally under-predicts the in vivo CL (Giacomini et al., 2010; Chiba et al., 2009; Watanabe et al., 2009). Modeling of in vivo CL from rat and human hepatocyte uptake data in recent studies needed inclusion of empirical scaling factors ranging from 2 to 67 to predict in vivo hepatic CL within 2-fold (Gardiner et al., 2011; Menochet et al., 2012). One reason for this under-prediction may be that the studies did not take any active efflux transporters in
the cell models into account when predicting hepatic clearance. Additionally, loss of OATPs and other uptake transporters when isolating and cryopreserving rat and human hepatocytes may further add to the underprediction of hepatic CL (Kimoto et al., 2012; Lundquist et al., unpublished).

In the present study, no increase in erythromycin metabolism could be detected in experiments using cryopreserved human hepatocytes after addition of elacridar. The addition of elacridar slightly diminished the total amount of N-desmethyl-erythromycin metabolite formed. However, the intracellular levels of both erythromycin and the N-desmethyl-erythromycin metabolite increased after P-gp inhibition, which is in accordance with the results by Lam et al. (2006). Measurements of metabolite concentrations in the extracellular medium showed that P-gp inhibition had reduced the efflux of the metabolite that previously was identified as a P-gp substrate (Lam et al., 2006). The increase in intracellular metabolite levels is thus probably to a large extent a result of inhibited efflux of the metabolite and not a result of increased metabolism.

In conclusion, the present study shows that the four ABC efflux transporters P-gp, BCRP, MRP2, and BSEP, important for biliary drug clearance are retained in human cryopreserved and freshly isolated rat hepatocytes. The transporters exhibit activity that clearly affects the drug disposition in the experimental system and should thus be considered when hepatocyte drug metabolism and transport kinetics are studied.
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Contributed new reagents or analytical tools: Johansson

Performed data analysis: Lundquist, Englund, Skogastierna, Johansson, Lööf

Wrote or contributed to the writing of the manuscript: Lundquist, Hoogstraate, Afzelius, Andersson


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Footnotes

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Figure legends.

Fig. 1A
Quantification of ABC transporter expression in human liver and cryopreserved hepatocytes. Transporter protein levels were normalized against GAPDH, expression levels in liver tissue were set to 1. Of the tested transporters only hBCRP was downregulated (by 60%) after cryopreservation of hepatocytes. (Mean ± SD, n = 3; * = p < 0.05.)

B: Representative Western blots of hP-gp, hMRP2, and hBCRP derived from human liver and cryopreserved hepatocytes are shown. Lower panels show the internal standard hGAPDH. Approximate molecular weights were: hBCRP, 140 kDa (dimer); hMRP2 190 kDa; hP-gp, 170 kDa; and hGAPDH, 35 kDa. Black bars denote different sample groups; L, liver; CH, cryopreserved hepatocytes.

Fig. 2A:
Quantification by western blot of ABC efflux transporter expression in rat liver, freshly isolated hepatocytes and cryopreserved cells. Transporter protein levels were normalized against GAPDH, expression levels in liver tissue were set to 1. rBCRP showed significant down regulation after isolation of cells of hepatocytes. All three transporters were down regulated after cryopreservation. (Mean ± SD, n=3; * = p < 0.05; for liver and fresh hepatocytes, for cryopreserved hepatocytes two individual measurements were performed.)

B: Western blots of rP-gp, rMRP2, and rBCRP derived from rat liver, fresh hepatocytes and cryopreserved hepatocytes are shown. Lower panels show the internal standard rGAPDH. Approximate molecular weights were: rBCRP, 140 kDa (dimer); rMRP2 190 kDa; rP-gp, 170 kDa; and rGAPDH, 35 kDa. Black bars denote different sample groups; L, liver; FH, fresh hepatocytes; CH, cryopreserved hepatocytes.
Fig. 3
Immunofluorescent detection of hBCRP (A), hMRP2 (B), and hP-gp (C) in human cryopreserved hepatocytes. Right hand panels show a higher magnification of individual cells. The large majority of transporters seemed to be localized to the plasma membrane while intracellular staining was comparatively weak. hMRP2 and hP-gp were localized to distinct membrane domains reminiscent of intact canalicular membrane domains. hBCRP on the other hand was found spread over a large membrane area showing no specific localization.

Fig. 4
Immunofluorescent detection of hBCRP (A), hMRP2 (B), and hP-gp (C) in human liver tissue sections. In figure 4D a negative control section stained, omitting the primary antibody is shown. Transporters are localized to apical membranes of hepatocytes tracing an outline of the bile canalicular network.

Fig. 5
Efflux of rosuvastatin from plated hepatocytes can be inhibited with FTC. A: Efflux from plated human cryopreserved hepatocytes. B: Efflux from plated rat fresh hepatocytes (Mean ± SD, n = 3; * = p < 0.05).

Fig. 6
Terfenadine uptake and metabolism and fexofenadine efflux in human cryopreserved hepatocytes. A: Terfenadine shows rapid uptake (loss from media) and moderate metabolism (metabolic stability assay, cells + medium). B: Metabolic break down of terfenadine is unaffected by 1 µM elacridar (metabolic stability assay, cells + medium). C: Total formation of fexofenadine (cells + medium) is unaffected by 1 µM elacridar. D: Efflux of fexofenadine is reduced by 40% upon addition of 1 µM elacridar. E: Total formation of fexofenadine (cells + medium) in fresh rat
hepatocytes, with and without 1 μM elacridar. **F**: Efflux of fexofenadine from fresh rat hepatocytes, with and without 1 μM elacridar (Mean ± SD, n = 3; ** = p < 0.01, *** = p < 0.001).

**Fig. 7**

Uptake-efflux interactions of fexofenadine in fresh rat hepatocytes. **A**: In loss from media experiments apparent uptake rates for fexofenadine increased with increasing concentrations of the P-gp inhibitor elacridar. Solid lines represent best fit curves of uptake rates. Steady state medium concentrations of fexofenadine seen after 60 minutes were decreased in a dose dependent manner by elacridar. **B**: Fexofenadine apparent uptake CLint increased 4-fold with increasing concentrations of elacridar from 0 to 2 μM. **C**: In human cryopreserved hepatocytes fexofenadine uptake was measured with the spinning through oil method. Fexofenadine uptake was significantly increased by 1 μM elacridar (Mean ± SD; n = 3, ** = p < 0.01, *** = p < 0.001).

**Fig. 8**

Metabolism-efflux interactions of erythromycin in human cryopreserved hepatocytes. **A**: Total metabolic break down (cells + medium) of erythromycin was unaffected by addition of 0.5 μM elacridar (metabolic stability assay). **B**: Addition of 0.5 μM elacridar did not change the formation rate of the major metabolite N-desmethylerythromycin significantly (cells + medium, metabolic stability assay). **C**: No change in extracellular erythromycin concentrations could be seen after addition of elacridar to spinning through oil experiments (s-t-o). **D**: Efflux of the metabolite N-desmethylerythromycin was diminished by addition of elacridar (s-t-o). **E**: Elacridar (0.5 μM) increased uptake and intracellular concentrations of erythromycin in human cryopreserved hepatocytes (s-t-o). **F**: Intracellular levels of the metabolite N-
desmethyl-erythromycin increased two-fold in the presence of elacridar (s-t-o) (AU =

arbitrary units; Mean ± SD, n = 3; * = p < 0.05, ** = p < 0.01, *** = p < 0.001,).
Tables

**Table 1.** IVIVE of fexofenadine and erythromycin $\text{CL}_{\text{hepatic}}$ utilizing in vitro $\text{CL}_{\text{int, uptake}}$ measurements demonstrating the impact of elacridar.$^a$

<table>
<thead>
<tr>
<th></th>
<th>$\text{CL}_{\text{int, uptake}}$ $^b$ (µl/min/10^6 cells)</th>
<th>Predicted $\text{CL}_{\text{blood}}$ $^c$ (ml/min/kg)</th>
<th>Predicted $\text{CL}_{\text{hepatic}}$ $^d$ (ml/min/kg)</th>
<th>In vivo $\text{CL}_{\text{blood}}$ $^e$ (ml/min/kg)</th>
<th>In vivo $\text{CL}_{\text{hepatic}}$ $^d$ (ml/min/kg)</th>
<th>Fold error $\text{CL}_{\text{hepatic}}$ (observed/predicted)</th>
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</thead>
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<tr>
<td><strong>Rat, Fexofenadine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.92 ± 0.8</td>
<td>1.7 ± 0.7</td>
<td>1.1 ± 0.5</td>
<td>10.9 ± 3.4</td>
<td>7.2 ± 2.7</td>
<td>6.5</td>
</tr>
<tr>
<td>Elacridar, 1 µM</td>
<td>4.6 ± 1.6**</td>
<td>3.8 ± 1.3*$^*$</td>
<td>2.5 ± 0.9*$^*$</td>
<td></td>
<td></td>
<td>2.9</td>
</tr>
<tr>
<td>Elacridar, 2 µM</td>
<td>7.6 ± 1.1**</td>
<td>6.0 ± 0.8**</td>
<td>4.1 ± 0.6*$^*$</td>
<td></td>
<td></td>
<td>1.8</td>
</tr>
<tr>
<td><strong>Human, Erythromycin</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>4.4 ± 0.3</td>
<td>1.9± 0.3</td>
<td>1.9± 0.3</td>
<td>8.8 ± 2.2</td>
<td>8.5 ± 2.2</td>
<td>4.5</td>
</tr>
<tr>
<td>Elacridar, 0.5 µM</td>
<td>5.7 ± 0.8**</td>
<td>2.4± 0.4**</td>
<td>2.4± 0.4**</td>
<td></td>
<td></td>
<td>3.6</td>
</tr>
</tbody>
</table>

$^a$Mean ± SD; n = 3; *** = p < 0.001, ** = p < 0.01, * = p < 0.05, significantly different from control experiments without elacridar.

$^b$CL$_{\text{int, uptake}}$ was measured using suspended hepatocytes.

$^c$CL$_{\text{blood}}$ was predicted from CL$_{\text{int, uptake}}$ using the well stirred model as described (Sohlenius-Sternbeck et al., 2012).

$^d$CL$_{\text{hepatic}}$ was calculated from CL$_{\text{blood}}$ using data from Swift et al. (2013).

$^e$In vivo CL$_{\text{blood}}$ was measured in house in Sprague-Dawley rats (Lundquist et al., unpublished). Human pharmacokinetic parameters are from Sun et al., 2010.
Figure 1

A

Human
Liver
Cryopreserved hepatocytes

Normalized transporter expression

P-gp  MRP2  BCRP  BSEP  MRP3  MRP4

B

hP-gp

L  CH

hMRP2

L  CH

hBCRP

L  CH
**Figure 2**

(A) Normalized transporter expression for rat hepatocytes. The chart illustrates the expression levels of rP-gp, rMRP2, and rBCRP in liver, fresh hepatocytes, and cryopreserved hepatocytes. Significant differences are indicated by an asterisk.

(B) Western blot analysis of rP-gp, rMRP2, and rBCRP in liver (L), fresh hepatocytes (FH), and cryopreserved hepatocytes (CH). The blots show the protein expression levels for each condition.
Figure 3

A hBCRP

B hMRP2

C hP-gp
Figure 4

A hBCRP

B hMRP2

C hP-gp

D Negative control
Figure 7

A

Rat fresh hepatocytes
- Control
- Elacridar (1 μM)
- Elacridar (2 μM)

[Fexofenadine] nM vs t (min)

B

Rat fresh hepatocytes

Clint uptake (μl/min/10^6 hepatocytes)

Control
Elacridar (1 μM)
Elacridar (2 μM)

**

C

Human cryopreserved hepatocytes

Fexofenadine uptake (pmol/15 min/mg protein)

Control
Elacridar (1 μM)

**