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The impact of solute carrier (SLC) drug uptake transporter loss in human and rat cryopreserved hepatocytes on clearance predictions.

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Running title: Drug uptake transporters in cryopreserved hepatocytes.

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Abbreviations: ABC transporter, ATP binding cassette transporter; ACN, acetonitrile; AZ, AstraZeneca; C_{blood}/C_{plasma}, blood-plasma concentration ratio; CL, clearance; CL_{int}, intrinsic clearance; CYP, Cytochrome P450; DMSO, Dimethyl-sulfoxide; fu, fraction unbound, free fraction; HRP, Horse radish peroxidase; IVIVE, in vitro – in vivo extrapolation; KHL, Krebs-Henseleit buffer; LOQ, limit of quantification; LC-MS/MS, Liquid chromatography tandem mass spectrometry; MCT1, Monocarboxylate transporter 1; mPGES-1, microsomal Prostaglandin E synthase-1; NTCP, Na^+-taurocholate cotransporting polypeptide; OAT, Organic anion transporter; OATP, Organic anion transporting polypeptide; OCT, Organic cation transporter; PBS, Phosphate buffered saline solution; PEPT1, Peptide transporter 1; SDS, Sodium dodecyl-sulfate; PAGE, Polyacrylamide gel electrophoresis; SLC, Solute carrier; TBS, Tris-buffered saline solution; TBST, Tris-buffered saline solution with 0.1% Tween-20; UGT, UDP-glucoronosyltransferase; WE, William's E medium

Abstract

Cryopreserved hepatocytes are often used as a convenient tool in studies of hepatic drug metabolism and disposition. In this study the expression and activity of drug transporters in human and rat fresh and cryopreserved hepatocytes was investigated. In human cryopreserved hepatocytes Western blot analysis indicated that protein expression of the drug uptake transporters (hNTCP, hOATPs, hOATs and hOCTs) was considerably reduced compared to liver tissue. In rat cryopreserved cells the same trend was observed but to a lesser extent. Several rat transporters were reduced as a result of both isolation and cryopreservation procedures. Immunofluorescence showed that a large portion of remaining hOATP1B1 and hOATP1B3 transporters were internalized in human cryopreserved hepatocytes. Measuring uptake activity using known substrates of OATPs, OCTs and NTCP showed decreased activity in cryopreserved as compared to fresh hepatocytes in both species. The reduced uptake in cryopreserved hepatocytes limited the in vitro metabolism of several AstraZeneca compounds. A retrospective analysis of clearance predictions of AstraZeneca compounds suggested systematic lower clearance predicted using metabolic stability data from human cryopreserved hepatocytes compared to human liver microsomes. This observation is consistent with a loss of drug uptake transporters in cryopreserved hepatocytes. In contrast the predicted metabolic clearance from fresh rat hepatocytes was consistently higher than those predicted from liver microsomes consistent with retention of uptake transporters. The uptake transporters, which are decreased in cryopreserved hepatocytes, may be rate limiting for the metabolism of the compounds and thus be one explanation for under-predictions of in vivo metabolic clearance from cryopreserved hepatocytes.

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Introduction

Isolated hepatocytes are often used to determine in vitro pharmacokinetic characteristics of drug candidate compounds (Chiba et al., 2009; Soars et al., 2007b, 2009). To provide useful information for in vivo pharmacokinetic predictions the isolated hepatocyte should accurately reflect the cells functions in liver tissue. This includes drug transporter expression and localization, activity of drug metabolizing enzymes, gene regulatory mechanisms, and cofactor concentrations (Chiba et al., 2009; Soars et al., 2009; Sohlenius-Sternbeck et al., 2005).

In the intact liver tissue hepatocytes express a number of drug uptake transporters of the SLC-super family on their basolateral, blood facing, sinusoidal membranes. The major families of hepatic drug uptake transporters are organic anion transporters (OATs), organic anion transporting polypeptides (OATPS), organic cation transporters (OCTs), and the Na^+-taurocholate cotransporting polypeptide (NTCP). These transporters facilitate uptake of drugs and other xenobiotics from the blood into the hepatocyte (Klaasen and Aleksunes 2010). In the hepatocytes drug molecules can be metabolized by a number of enzymes (such as Cytochrome P450s, CYPs, and UDP-glucuronosyltransferases, UGTs) (Soars et al., 2007b; Chiba et al., 2009). Drugs and drug metabolites can be eliminated by biliary secretion mediated by drug efflux transporters belonging to the ATP binding cassette transporter (ABC transporter) super-family located on the bile-facing, canalicular hepatocyte membrane.

Uptake transporters may be rate determining for drug clearance (CL), and it has been shown that intra-hepatocyte accumulation by active uptake of a compound can influence both its metabolism and its CYP inhibitory effect (Kusuhara and Sugiyama, 2009; Watanabe et al., 2009; Grime et al., 2008; Brown et al., 2010). The

International Transporter Consortium has recently published guide lines for the application of drug uptake transporter kinetics in pharmacokinetics and strongly encourages screening for interactions with the major drug uptake transporters during drug development (in the liver: OAT2, OATP1B1, OATP1B3, OCT1, and OCT3) (Giacomini et al., 2010; Zamek-Gliszczynski et al., 2013).

Freshly isolated hepatocytes are easily available from preclinical animal model species. However, since the access to freshly isolated human hepatocytes is erratic many drug development laboratories are using cryopreserved hepatocytes (Chiba et al., 2009; Soars et al., 2009). Therefore human cryopreserved hepatocytes have been extensively characterized focusing mainly on drug metabolic capacity (Floby et al., 2009; Stringer et al., 2008).

Most studies on uptake transporter activity in cryopreserved human hepatocytes have used the uptake of selective marker substrates to investigate a certain class of uptake transporters in the cells (Shitara et al., 2003; Lu et al., 2006; Houle et al., 2003; Soars et al., 2009; Badolo et al., 2011). Previous studies have reported partly conflicting results, ranging from no change to significant losses of either OATP or OCT uptake activity in cryopreserved hepatocytes as compared with freshly isolated cells. Recently, absolute quantification of transporter expression have indicated that OATP1B1, OATP1B3 and OATP2B1 protein levels were lower in human cryopreserved hepatocytes compared to liver tissue (Kimoto et al., 2013). The amount of NTCP seemed unaffected by cryopreservation in human hepatocytes while displaying some decline in rat cryopreserved hepatocytes (Qiu et al., 2013). As hepatocyte measurements were performed on a single, unmatched, batch of cryopreserved cells from each species, any conclusions on NTCP expression in these cells are equivocal. A loss of drug transporters in cryopreserved hepatocytes, resulting in reduced membrane permeability for some drugs, may explain why in vitro

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CL_{int} obtained from cryopreserved hepatocytes often lead to an under-prediction of in vivo clearance, progressively larger for higher clearances (Foster et al., 2010; Halifax et al., 2012).

The aim of this study was to investigate whether under-predictions of in vivo CL based on in vitro CL_{int} measurements from cryopreserved hepatocytes can be due to loss of drug uptake transporters in cryopreserved hepatocytes. We studied the protein expression, protein localization and activity of several of the most important hepatic drug uptake transporters, including OATs, OATPs, OCTs and NTCP. We compared activity and expression of the transporters in freshly isolated and cryopreserved hepatocytes from human and rat. The expression levels in hepatocytes were also compared with liver tissue levels from both species. Our study showed that cryopreserved hepatocytes from both human and rat exhibited large reductions in drug uptake transporter expression (including OATPs, OATs, and OCTs in both species, as well as NTCP in human cells) and activity compared to both fresh hepatocytes and intact liver tissue. This loss of uptake transporters may be one important factor explaining the under-predictions when scaling hepatic clearance from cryopreserved hepatocytes.

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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 gene names are written in capital letters (e.g. SLCO2B1) while rodent genes are given in upper and lower case (e.g. Slco2b1). Protein names for both human and rodent proteins are written in capital letters (e.g. OATP2B1). In the interest of clarity human protein names are prefixed with h while rat proteins are prefixed r (e.g. hOATP2B1, rOATP2B1). Protein names without prefix refer to the protein in general, not belonging to any particular species (e.g. an OATP2B!-inhibitor).

Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. A series of AstraZeneca (AZ) project compounds, microsomal Prostaglandin E synthase-1 inhibitors (mPGES-1, chemical structures are presented in Bylund et al., 2013), was obtained from AstraZeneca's compound collection.

Animals

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 fresh and cryopreserved 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-Henseleit buffer supplemented with 10 mM Hepes (Gibco), pH 7.4, (KHL). 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 <80% were discarded.

Liver tissue samples were obtained from donors undergoing surgical liver resection in Uppsala University Hospital (Uppsala, Sweden), as approved by Uppsala Regional Ethical Review Board. Small biopsies from histologically normal areas of liver resections were snap frozen in ethane cooled by liquid nitrogen in the operating theatre immediately after the surgical procedure (n=3). Frozen biopsies were stored at -80°C until use. Fresh human hepatocytes were prepared from liver resections as described elsewhere (LeCluyse and Alexandre, 2010). Fresh human hepatocytes

were not available for Western blotting experiments or for studies on AZ project compounds.

Human and rat cryopreserved hepatocytes. Human: lots Hu4035, Hu4037, Hu4152 (CellzDirect, Life technologies), all of plateable quality, and lots OZL, SQJ, and REL (Celsis In vitro technologies, Baltimore, MD, USA). The lots represented a mix of genders and displayed average CYP metabolism. Rat: derived from male Sprague-Dawley rats, 7-8 weeks of age, (CellzDirect), 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 counting and viability were determined as described for rat hepatocytes.

Donor information for liver tissue, fresh and cryopreserved hepatocytes are given in Supplemental Table 1.

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 cracking buffer, consisting of 5 mM Hepes, 0.5 mM EDTA, Protease Inhibitor Cocktail (Sigma) according to the manufacturer's instruction, 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 frozen samples from human liver biopsies were handled in the same manner.

From fresh hepatocytes: Aliquots of 1 million fresh rat hepatocytes collected from the preparation technique described above were snap frozen after isolation. These were

later 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. Homogenization was performed as described for liver tissue.

The preparations were supplemented with 50 mM mannitol and stored at -20ºC until use. Determination of protein concentration was done according to BCA protein assay kit (Pierce Biotechnology, Thermo Fischer Scientific Inc., Rockford, IL, USA) according to the manufacturer's instructions.

Determination of transporter expression by western blot

10-40 µg of hepatocyte or liver protein was diluted in dH_2O and loading buffer (0.5 ml Laemlli buffer, 50 µl β-mercaptoethanol, and 2.5 µl 1M dithiothreitol, all from Bio-Rad (Hercules, CA, USA)) and separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 9% gels followed by transfer of protein to a PVDF membrane (Perkin-Elmer, Waltham, MA, USA) at 200 mA for 2 h. Membranes were washed in 3 x 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 pH 7.2). Membranes were incubated with primary antibody (Supplemental Table 2), diluted 1:500 or 1:1000, at 4°C over night followed by washing in TBST. Reblocking was performed during 1 h followed by incubation with 1:1000 diluted polyclonal goat anti-rabbit IgG conjugated to Horse radish peroxidase (HRP) (Abcam, Cambridge, UK). To detect GAPDH (internal standard) a HRP-conjugated primary antibody was

used (Santa Cruz Biotechnology, Houston, TX, USA, Table 1). Incubations were followed by a new washing step.

Antibodies were detected by chemiluminescence (Western blotting ECL Plus reagents, GE Healthcare, Piscataway, NJ, USA), a substrate for HRP, according to manufacturer's instruction. Molecular weights were determined using the Broad Range Molecular Weight Marker (Bio-Rad). The blot was photographed with Amersham Hyperfilm ECL (GE Healthcare), developed and fixed. Several exposures were made to ensure that quantification was performed on film that had not been overexposed. 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 GAPDH was used.

Immunofluorescence

Human cryopreserved hepatocytes were seeded on Collagen type I covered cover slips (BD Biosciences, Franklin Lakes, NJ, USA) for 1 h in WE in a cell incubator at 37°C and 5% CO_2. After washing in KHL, 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 -20ºC until use.

Cover slips were washed 4 x 5 minutes in PBS and blocked with 10% Fetal Calf Serum (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 against human OATP1B1 and OATP1B3 (both rabbit polyclonals kindly provided by Dr Bruno Stieger, Department of Clinical Pharmacology, University of Zürich, Switzerland) 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 goat anti-rabbit Alexafluor conjugated fluorescent secondary antibodies (Life

technologies). Secondary antibodies were used at a dilution of 1:1000 in PBS with 10% goat serum. 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 inspected and photographed using a confocal microscope (DMIRE 2, Leica Microsystems, Wetzlar, Germany).

Human liver biopsies were cut into 8 µm thick sections on a cryotome and dried down on slides. Liver sections were fixed, stained and visualised as described for hepatocyte samples above.

Uptake and metabolic stability measurements in hepatocytes

Uptake measurements in suspended cells were performed using the media loss technique described by Soars et al., (2007a). Briefly, hepatocytes (10^6 / ml) were incubated with test compound in KHL at a concentration of 1 µM and a final dimethyl-sulfoxide (DMSO) concentration of 0.1%. Experiments were completed within 2 h of initial thawing or isolation of the hepatocytes. To distinguish media loss due to uptake or binding, test compound was incubated under the same conditions with cells that had been subjected to repeated freeze-thawing. Trypan blue staining revealed these cells to be intact but totally permeabilized. Uptake was followed for 30-45 minutes, the initial uptake phase used to determine $CL_{int,\ uptake}$ were 5-15 minutes for different compounds, the longest time used to quantify the slow uptake of fexofenadine. Aliquots were removed to a glass tube loaded into a bench top centrifuge and hepatocytes were pelleted by centrifugation. A sample of the buffer supernatant was removed and mixed with two volumes of an ice cold stop solution of acetonitrile (ACN) containing 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. An aliquot of 200 µl supernatant from each well were transferred to a 96 well analysis

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plate. Before analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS), each fraction was diluted in KHL to contain 25 % ACN. Compound binding was measured in glass vials and were found to amount to less than 1% of initial compound concentrations.

Hepatocytes in suspension (10^6/ml) were used to determine the metabolic stability of test compounds. Hepatocytes were incubated with test compound in KHL with 0.1% DMSO for 0 to 90 minutes at 37ºC. Samples were taken at intervals, stopped and precipitated with 2 volumes of stop solution, and processed as described above. The activity of phase I and II drug metabolising enzymes in rat fresh and cryopreserved hepatocytes in suspension were compared using a cocktail containing highly permeable substrates for CYP and phase II enzymes (Floby et al., 2009); 7-hydroxycoumarin (phase II, UGT), bufuralol (CYP2D2), diazepam (CYP2C11), diclofenac (CYP2C6), midazolam (CYP3A) and phenacetin (CYP1A2), each at 2 uM final concentration. CYP isoform specificites were from (Kobayashi et al., 2002; Sakai et al., 2009).

Uptake experiments with plated fresh or cryopreserved hepatocytes were performed essentially as described (Menochet et al., 2012). Briefly hepatocytes were seeded in 24-well collagen type I covered plates (BD Biocoat, BD Biosciences, Franklin Lakes, NJ, USA) in WE supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01% Insulin-transferrin-selenium A solution (Gibco) according to the provider's instructions. Cells were left to attach for 2 h at 37ºC in 5% CO_2. After washing and removal of unattached cells, leaving a semi-confluent cell monolayer (> 90% confluent), uptakes were performed at 1 µM substrate concentration in KHL. The activity of uptake transporters were tested with several well characterized substrates; rosuvastatin (AstraZeneca), OATPs (Ho et al., 2006); fexofenadine, OATPs (Cvetkovic et al., 1999); ipratropium, OCTs (Nakanishi et al., 2010); taurocholate,

NTCP (Ozawa et al., 2004). Rosuvastatin is also an NTCP substrate in human but not rat cells (Ho et al., 2006). After uptake completion cells were washed three times with ice-cold KHL, lysed with ACN stop solution, processed and analyzed as described above. Uptake rates were quantified after 2 minutes of uptake, well within the linear phase of uptake. All experiments with plated hepatocytes were completed within 4 h of initial thawing or isolation of the hepatocytes including preparation and plating. Compound binding was measured in empty plates and subtracted from uptake measurements.

Metabolic stability measurements with human and rat microsomes

A pool of microsomes from 33 donors (28 male and 5 female) was purchased from BD Gentest (Woburn, MA, USA). Rat microsomes derived from an in house preparation. Microsomes were incubated at a concentration of 0.5 mg/ml in 96-well microplates in the presence of 1 µM of the drug compound at a final DMSO concentration of 0.1%. The reaction was initiated by the addition of NADPH (final concentration 1.5 mM). The final volume was of 100 µl and the incubations were performed in duplicate for 5, 15, 30 and 60 minutes. The reactions were stopped by the addition of 100 µl ACN, after which the samples were mixed and centrifuged and the supernatant thus obtained, analyzed. Control incubations (without microsomes or with microsomes but without NADPH) were performed for all compounds. Samples were processed and analyzed by LC-MS/MS as described above.

Determination of plasma protein binding

Plasma protein binding was determined by equilibrium dialysis. Human plasma was obtained from AstraZeneca´s internal blood tapping at Clinical Pharmacology & DMPK. Plasma from three individuals was pooled and mixed with the compound at a concentration of 10 µM (in 0.1% DMSO). A dialysis membrane (Spectra/Por MWCO 6-8000, Spectrum Laboratories, Inc, Rancho Dominguez, CA, USA) was soaked in

distilled water and thereafter placed between two dialysis plate halves with 48 wells. A volume of 180 µl 0.122 M phosphate buffer, pH 7.4 with 75 mM NaCl was added to each well on one side of the dialysis plate side, and 180 µl of the plasma/compound mix to the opposite side. After incubation on an orbital shaker (4 mm in diameter and 100 rpm) at 37^{o}C for 18 hrs the samples on both sides of the membrane were analyzed as described below. The fraction unbound (fu) in plasma was calculated from the ratio of the MS-area of the compound in the buffer to the MS-area of the compound in the plasma. Recovery was measured from the ratio of the sum of the mass spectrometry peak areas (MS-areas) in buffer and sample to the MS-area in the plasma/compound mix at zero time. All compounds were found to be stable during the 18 h incubation. The volume change over time was negligible (<10%).

Plasma clearance of test compound

Blood samples were drawn from male Sprague-Dawley rats, at an age of approximately 8 weeks, just before and at 1 min; 5 min; 20 min; 40 min; 1 h; 1.5 h; 3 h; 6 h and at 24 h after intravenous bolus administration of test compound (in 0.3 M N-methyl-D-glucamine). A dose of 3 µmol/kg was given for all compounds. Standards, diluted in 50% ACN with dH_2O, were subsequently diluted 1:10 with blank plasma. 50 µl of each standard or analytic sample together with 150 µl cold stop solution was mixed in a 96 deep well working plate and centrifuged at 4ºC and 4000 rpm for 20 min. An aliquot of 120 µl supernatant was transferred to a 96 deep well analysis plate and diluted with 300 µl of the liquid chromatography mobile phase A and analyzed as described below.

Analysis of study compounds

Analysis of parent compounds and metabolites were 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 Corporation, Milford, MA, USA). A High Purity C_{18} 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 (Supplemental Table 3). Quantification of each compound was achieved by comparison of the analyte/internal standard peak area ratios. A standard curve was included for each compound analysed spanning a concentration range 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.

Pharmacokinetic calculations

IVIVE was performed for a number of selective uptake transporter substrates to compare predictions deriving from uptake clearance measurements ($CL_{int,\ uptake}$) in freshly isolated and cryopreserved hepatocytes.

In a retrospective study scaled in vivo CL_{int} (scaled $CL_{int,\ in\ vivo}$, see Eq. 6) was calculated for 83 chemically diverse AstraZeneca project compounds. In vitro data on rat and human metabolic CL_{int} ($CL_{int,\ met}$) using fresh and cryopreserved hepatocytes, respectively, $CL_{int,\ met}$ using liver microsomes, fraction unbound in plasma (fu_{plasma}), and blood-plasma concentration ratio (C_{blood}/C_{plasma}) were derived from AstraZeneca in house data bases. All compounds derived from the CNS and Pain therapeutic area

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and had been synthesized at the AstraZeneca R&D facility in Södertälje, Sweden. The study included all compounds that had the in vitro data listed above available for both rat and human determined using the same protocols as presented in this study.

Determination of intrinsic clearance

The elimination rate constant for metabolic stability or uptake experiments was calculated from the first order elimination equation

$$[S] = [S_0] \cdot e^{-k \cdot t} \quad (1),$$

where [S] is concentration of substrate at a given time point, t, after the incubation concentration of the substrate, $[S_0]$. 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 (Soars et al., 2007a). In vitro intrinsic clearance ($CL_{int\ in\ vitro}$) for the elimination phase could then be calculated from:

$$CL_{\text{int } in\ vitro} = k \cdot V \quad (2),$$

where V represents the volume of the incubation.

Fraction unbound in the incubations

The fraction unbound in the hepatocyte incubation was predicted according to Kilford et al. (2008):

$$fu_{hep} = \frac{1}{1+125\times V_R \times 10^{0.072\times \log P/D^2 + 0.067\times \log P/D - 1.126}} \quad (3)$$

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where V_R is the ratio between the cell volume and the incubation volume and it has a value of 0.005 at the cell concentration of 10^6 cells/ml.

The fraction unbound in the microsome incubation was predicted according to Hallifax and Houston (2006):

$$fu_{mic} = \frac{1}{1 + P \times 10^{0.072 \times \log P/D^2 + 0.067 \times \log P/D - 1.126}} \quad (4)$$

where P is the microsomal protein concentration (0.5 mg/ml). In both equation 3 and 4 log P is used for bases and log D for other ion classes. Predicted log P and log D values were generated using a commercial package from Advanced Chemistry Development (Toronto, Canada).

Calculations of fraction unbound in the blood

The fraction unbound in the blood (fu_{blood}) was calculated using the following equation

$$fu_{blood} = \frac{fu_{plasma}}{\left(C_{blood}/C_{plasma}\right)} \quad (5)$$

When C_{blood}/C_{plasma} was unavailable, a value of 1 was assumed for neutral and basic compounds and 0.55 for acidic compounds.

Scaled in vivo CL_{int}

$CL_{int\ in\ vitro}$ from microsomes or hepatocytes was scaled to an in vivo CL_{int}

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$$Scaled\ CL_{\text{int } in\ vivo} = \frac{CL_{\text{int } in\ vitro} \times scaling\ \ factors \times fu_{blood}}{fu_{inc}} \quad (6)$$

The scaling factors were: For human hepatocytes: (120×10^6 cells / g liver) $\times$ (1500 g liver / 70 kg body weight). For human microsomes: (45 mg protein / g liver) $\times$ (1500 g liver / 70 kg body weight) (Bayliss et al., 1990; Obach et al., 1997; Sohlenius-Sternbeck 2006) In the rat liver and body weight was 10 g and 250 g, respectively, and the hepatocellularity and microsome protein content per gram of liver the same as for human.

Prediction of in vivo hepatic blood CL

Predictions were based on the well stirred model (Yang et al., 2007).

$$Predicted\ CL_{hepatic} = \frac{Q_{h,b} \times CL_{\text{int},in\ vivo}}{\left(Q_{h,b} + CL_{\text{int},in\ vivo}\right)} \quad (7)$$

Hepatic blood flow, $Q_{H,B}$, was set to 72 and 20 ml/min/kg for rat and human, respectively (Brown, et al., 1997; Delp et al., 1998).

Determination of in vivo clearance

In vivo blood clearance was calculated according to

$$CL_{blood} = \frac{Intravenous\ Dose}{AUC_{blood}} \quad (9)$$

where $AUC_{blood} = \frac{AUC_{plasma} \times C_{blood}}{C_{plasma}}$ (10)

The blood $CL_{hepatic}$ was calculated from the CL_{blood} and the hepatic fraction, $f_{hepatic}$ given in the literature (listed in Table 1).

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Statistical analysis

Data were analyzed using Graphpad Prism 5 software (Graphpad Software, Inc, La Jolla, CA, USA). Data are given as mean ± SD. Statistical significances were tested by 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.

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Results

Uptake transporter expression in human and rat hepatocytes

Western blotting was used to determine total protein expression levels in liver cells and liver tissue of several major drug uptake transporters, hOATP1B1, hOATP1B3, hOATP2B1, hOAT2, hOCT1, hOCT3, and hNTCP as well as transporters such as hPEPT1 and hMCT1 involved in uptake of endogenous substrates. The expression levels of transporter in human liver tissue and freshly thawed cryopreserved hepatocytes measured by densitometry of Western blots indicated that the drug uptake transporters were significantly lower in the cryopreserved hepatocytes (Fig. 1A). Liver tissue and cryopreserved hepatocytes derived from different donors. The hOATP family of transporters was the most affected showing of 80% - 95% lower levels in human cryopreserved hepatocytes as compared to liver tissue. hOAT2 as well as hOCT1 and hOCT3 were 70% to 80% lower in cryopreserved hepatocytes as compared with liver tissue whereas hNTCP showed 50% lower levels in the cryopreserved hepatocytes. The transporters hMCT1 and hPEPT1 displayed no reductions in protein levels after cryopreservation as compared to the levels in liver tissue (Fig. 1A). No significant differences in transporter expression were found between cryopreserved hepatocytes from different vendors (data not shown). Fig. 1B depicts Western blotting of hOATP1B1 and hOATP1B3 clearly showing the loss of the transporters proteins in human cryopreserved hepatocytes as compared to liver tissue expression levels (Fig. 1B).

Western blot analysis of uptake transporters in rat liver, freshly isolated hepatocytes, and cryopreserved hepatocytes, indicated that rOATP2B1, rOCT3, and rOAT2 levels were significantly lower already after cell isolation (Fig. 2). The loss of these transporters then increased further after cryopreservation. rMCT1 showed little loss

after hepatocyte isolation but expression levels decreased more than 50% after cryopreservation. rOCT1, rNTCP, and rPEPT1 expression levels were unaffected by isolation and cryopreservation of rat hepatocytes. rOATP2B1 was the uptake transporter most affected in isolated and cryopreserved rat hepatocytes, which is similar to the results in human cells.

Immunolocalization of hOATP1B1 and hOATP1B3 in human cryopreserved hepatocytes and liver

Despite the large loss of transporters after cryopreservation, OATP1B1 and OATP1B3 could readily be detected by immunohistochemistry in human cryopreserved hepatocytes attached to collagen-covered cover slips (Fig. 3A and 3B). Transporter proteins were detected on the plasma membranes but also seemed to have been internalized to a large extent. This internalization was particularily evident for hOATP1B3. In liver tissue hOATP1B1 and hOATP1B3 was mainly localized to plasma membranes of the hepatocytes (Fig. 4A and 4B). Negative control stainings of liver sections, omitting primary antibody, were devoid of signal (Fig. 4C).

Uptake transporter activity in fresh and cryopreserved human and rat hepatocytes

The uptake of several well characterized substrates was tested in plated human fresh and cryopreserved hepatocytes. Rosuvastatin, an hNTCP and hOATP substrate; fexofenadine, a hOATP substrate; and ipratropium, a hOCT substrate, all showed significantly lower uptake rates by 75%, 90%, and 50%, respectively in cryopreserved hepatocytes as compared with fresh cells (Fig. 5A). In agreement with Western blotting data, the hNTCP substrate taurocholate showed similar uptake rates in both fresh and cryopreserved hepatocytes.

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In rat cryopreserved hepatocytes the uptake of the rOATP substrate fexofenadine was reduced by more than 90% as compared with fresh hepatocytes (Fig. 5B). The OATP-mediated uptake of rosuvastatin was reduced by 80%. rOCT-mediated uptake of ipratropium was reduced by 40% in cryopreserved cells compared to fresh hepatocytes. As for human NTCP, taurocholate uptake by rNTCP was unaffected by cryopreservation of rat hepatocytes.

Activity of phase I and phase II drug metabolizing enzymes in fresh and cryopreserved rat hepatocytes

No significant differences in drug metabolism between fresh and cryopreserved cells were indicated when measuring the metabolism of a cocktail of highly permeable model substrates for CYP and UGT enzymes, bufuralol (CYP2D2), diazepam (CYP2C11), diclofenac (CYP2C6), midazolam (CYP3A) , phenacetin (CYP1A2), and 7-hydroxycoumarin (phase II, UGT), (Fig. 6). Since these substances are highly permeable transporter mediated uptake is unlikely to be rate determining for their metabolism (Benet et al., 2011).

IVIVE of clearance for uptake transporter substrates in human and rat

In Table 1 the IVIVE of in vivo $CL_{hepatic}$ using the well stirred model (Eqs. 6 and 7) and vitro uptake data for fexofenadine, rosuvastatin, and ipratropium from human and rat fresh as well as cryopreserved hepatocytes is shown. Uptake rates were measured both in hepatocyte suspensions and with plated cells. In vivo CL_{blood} was calculated according to Eqs. 8 and 9 for rat or given in the literature for human CL_{blood} (Supplemental references). $CL_{hepatic}$ was consistently under-predicted when using $CL_{int,\ uptake}$ from cryopreserved hepatocytes, regardless of species or experimental method used. . Using $CL_{int,\ uptake}$ from fresh human or rat hepatocytes resulted in significantly higher predictions of $CL_{hepatic,,}$.

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Effects of transporter loss on metabolic and uptake $CL_{int\ in\ vitro}$ of a chemical series of AZ compounds

Measuring in vitro metabolic stability for four close chemical analogs from an AstraZeneca series of mPGES-1 inhibitors showed that they displayed similar in vitro metabolism patterns; rapid metabolism in metabolic stability assays by human microsomes but stable when incubated with human cryopreserved hepatocytes (a representative example is shown in Table 2 and Fig. 7, structures are published by Bylund et al., 2013). No active uptake was indicated in human cryopreserved hepatocytes using the media loss method as the decrease in extracellular concentration seen when incubating cryopreserved cells and compound closely corresponded to the binding seen when incubating compound with dead hepatocytes (Fig. 7A). In contrast rat liver microsomes metabolized the compounds rapidly as did freshly isolated rat hepatocytes (Table 2). In fresh rat hepatocytes the compounds showed a pronounced uptake, well above the binding in dead cells (Table 2, Fig. 7B). Rat cryopreserved hepatocytes showed both slower uptake and metabolism than fresh hepatocytes (Table 2, Fig. 7C). Metabolic $CL_{int\ in\ vitro}$ (Eq. 2) was 75% lower ($p < 0.001$) and the uptake $CL_{int\ in\ vitro}$ was 50% lower ($p < 0.01$) in cryopreserved hepatocytes as compared to fresh cells (Table 2). This suggests that metabolism of the compound is rate limited by active uptake since metabolic capacity in rat freshly isolated and cryopreserved isolated hepatocytes is similar as presented above (Fig. 6).

Scaling $CL_{int\ in\ vivo}$ from rat and human microsomes and hepatocytes

A retrospective investigation was performed to scale the in vivo CL_{int} (Eq. 6) for a set of 83 chemically diverse, AstraZeneca new chemical entities synthesized at AstraZeneca R&D, Södertälje, Sweden, including the four mPGES-1 inhibitors mentioned above. Figure 8A shows the scaled in vivo CL_{int} (calculated using Eq. 6)

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from human microsomes versus human cryopreserved hepatocytes for the 83 compounds. Data from fresh human hepatocytes were not available. Scaling from cryopreserved human hepatocytes indicated higher values for 23% of the compounds than the values from liver microsomes. However in rat fresh hepatocytes 81% of the compounds showed higher scaled in vivo CL_{int} values (Eq. 6) than in microsomes (Fig. 8B). Acids, neutrals and bases in the data set showed similar behaviors (data not shown).

Discussion

In the present study we could clearly show that both human and rat cryopreserved hepatocytes have considerably lower levels of uptake transporter expression of especially OATPs, OAT2 and to a somewhat lesser extent OCTs and NTCP than in freshly isolated cells and in liver tissue. PEPT1 and MCT1 showed smaller reductions in protein levels. In addition to lower levels in cryopreserved hepatocytes hOATP1B1 and hOATP1B3 also showed some internalization. This internalization may be a step in the breakdown of the transporters translocating the proteins from the cell surface to the lysosome, a process that might be induced by the isolation and cryopreservation procedure. Such internalization has earlier been described in the down regulation of ABC transporters in plated rat hepatocytes and hOATP1B1 and hOATP1B3 in plated human hepatocytes (Bow et al., 2008, Ulvestad et al., 2011).

In rat cells rOAT2, rOATP2B1, and rOCT3 levels were decreased already in freshly isolated cells, suggesting that the isolation procedure itself initiates the loss of uptake transporters, which are then further decreased by cryopreservation. Houle et al. (2003) reported that cryopreservation of rat hepatocytes did not affect NTCP activities while OATP activity was only slightly reduced after cryopreservation. In the study by Houle et al. (2003), cells had been cryopreserved quickly after isolation using an optimized protocol, suggesting that the cryopreservation method may be important for the retention of functional uptake transporters. Loss of uptake transporters compared to liver levels was however not investigated.

There is now mounting evidence that cryopreserved human hepatocytes lose some or most of their drug uptake transporters. Recently it was demonstrated by LC-MS/MS protein quantification that hOATP1B1, hOATP1B3 and hOATP2B1 were significantly lower in human cryopreserved hepatocytes compared to expression

levels in human liver, supporting the results presented in this study (Kimoto et al., 2013). In contrast hNTCP expression was found to be retained in human cryopreserved hepatocytes and slightly down regulated in rat cryopreserved cells compared to liver levels (Qiu et al., 2013). Expression levels in cryopreserved cells were measured in one single batch, unrelated to the liver tissue samples, from each species. A small decrease in hNTCP expression as seen in the present study may thus have been masked by individual variations in the study by Qui et al. (2013). In a study by Soars et al. (2009), freshly isolated human hepatocytes and cryopreserved cells were shown to exhibit similar levels of OATP activity, while OCT activity was reduced in cells after cryopreservation. Badolo et al. (2011) have reported a loss of OCT activity in human cryopreserved hepatocytes, while OATP activity was similar in fresh and cryopreserved human hepatocytes in their study. The present study is to our knowledge the first study on uptake transporters in human cryopreserved hepatocytes combining measurements of protein expression levels of OATPs, OCTs, and NTCP with activity measurements.

Commercially available human cryopreserved cells often derive from liver resections or transplantation organs that have not found a recipient. The tissue may have been handled and shipped to laboratory for a significant amount of time before isolation of hepatocytes can be initiated (Richert et al., 2004). Ischemia and resulting hypoxia is always a consequence of hepatic surgery needed to isolate hepatocytes, regardless of species, and has been described to be critical for the quality of the isolated cells (Richert et al., 2004**,** Berendsen et al., 2011). Down-regulation of drug uptake transporters in hepatocytes has been described to be a result of the time in ischemia and hypoxia as well as liver injury and cholestasis, starting within 30 minutes of liver injury in rat (Gerloff et al., 1999; Gartung et al., 1996; Vos et al., 1999; Donner et al., 2012). The down regulation of drug uptake transporters is likely a physiological stress response protecting the hepatocyte from toxic levels of bile salts, bilirubin or other

endotoxic compounds upon loss of circulation (Vos et al., 1999; Donner et al., 2012). The down-regulation of hepatic uptake transporters is initiated by proinflammatory cytokines released upon tissue damage and the signaling pathways involved are being elucidated (Donner et al., 2012). There are also indications that this down-regulation of uptake transporters can be minimized, for example by limiting the time of tissue ischemia during cell isolation, or by induction of heme oxygenase-1 (Donner et al., 2012). In earlier studies where uptake transporter activity in human cryopreserved hepatocytes seemed retained, livers had been stored for extended periods before cells were isolated. In a study by Shitara et al., the livers had been stored in the cold for up to 24 hours before cell isolation and cryopreservation (Shitara et al., 2003). It is possible that loss of uptake transporters had already taken place during the storage of livers, before initial cell isolation. In their study most batches of cryopreserved hepatocytes showed some loss of NTCP and OATP activities compared to freshly isolated hepatocytes, while some batches exhibited no change. No comparison was made to liver tissue expression levels. Freshly isolated human hepatocytes used in the present study were isolated within 3-4 hours of hepatic surgery, which is close to the minimum elapsed time possible and only achievable if the lab isolating the hepatocytes is located in close proximity to the hospital performing the surgery. Information on the donors of liver tissue, fresh and cryopreserved hepatocytes in the present study is listed in Supplemental Table 1. They display mixed ages and sexes with varied medical history and use of medications. Information on medical history and medications used are cursory but it is known that two donors of cryopreserved hepatocytes have been taking anti-hypertensives while two donors of liver tissue and fresh hepatocytes have received cytostatica. These drugs are not known to affect expression levels of SLC-transporters and are unlikely to explain the differences seen in the present study.

Uptake transporters might be rate determining for drug clearance and data from isolated or cryopreserved hepatocytes often under-predict in vivo CL. The 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 achieve accurate CL predictions (Gardiner et al., 2011; Menochet et al., 2012). Some researchers have taken a pragmatic approach recommending regression line-based methods to compensate for under-predictions of CL from in vitro systems until the underlying causes can be identified (Sohlenius-Sternbeck et al., 2012). It has been noted that one possible reason for under predictions could be a change in absorptive capacity of the cryopreserved hepatocytes consistent with a loss of uptake transporters (Halifax et al., 2012, Foster et al., 2010). In the present study we showed that IVIVE of in vivo hepatic CL was improved using uptake data from freshly isolated hepatocytes over data from cryopreserved cells for several uptake transporter substrates suggesting that under-predictions of CL is partly due to reduced uptake transporter activity in the cryopreserved hepatocytes.

In our retrospective analysis of in vivo CL predictions, the down regulation of drug uptake transporters in human cryopreserved hepatocytes seemed to limit measurements of drug metabolism for a collection of AstraZeneca compounds. Scaling of in vivo CL_{int} from human cryopreserved hepatocyte in vitro CL_{int} for a large majority of compounds gave lower values than scaling from human microsomal CL_{int}, while scaling from fresh rat hepatocytes gave higher in vivo CL_{int} values than did rat liver microsomes. A series of four AstraZeneca mPGES-1 inhibitors seemed to display uptake rate limited metabolic clearance that would be consistent with such an uptake limitation present in human cryopreserved hepatocytes that is not present in fresh rat hepatocytes. It is likely that the compounds showing large discrepancies when scaling from microsomes or hepatocytes are substrates of drug uptake transporters. Our data illustrates the need for compensatory scaling methods such as

regression line methods that have been proposed by Sohlenius-Sternbeck et al. (2012).

In summary this study clearly indicates that drug uptake transporters in cryopreserved hepatocytes from both human and rat to a large extent are lost during preparation and may significantly affect the prediction of in vivo clearance when uptake is limiting the metabolism of the studied compounds. Cryopreserved hepatocytes should be used with awareness of their limitations and it would be prudent to primarily use lots exhibiting high remaining uptake activity. Improved cell isolation and tissue-handling protocols are needed to maintain transporter phenotype in both fresh and cryopreserved hepatocytes.

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Footnotes

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Figure legends.

Fig. 1A

Comparison of expression levels of SLC uptake transporters in human cryopreserved hepatocytes and human liver tissue. Human liver tissue was acquired from patients undergoing hepatectomy due to different forms of liver cancer while cryopreserved hepatocytes were acquired from commercial sources. Transporter protein levels were normalized against GAPDH, expression levels in liver tissue were set to 1. (Mean ± SD, n=3**,** * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.)

B: Representative Western blots depicting the levels of hOATP1B1, hOATP1B3, and hOAT2 transporters in three human cryopreserved hepatocyte preparations and three human liver samples. hOATP1B1 and hOATP1B3 were seen at approximately 75 kDa and hOAT2 at 70 kDa. All proteins could be detected in all human samples. Lower panels depict the internal standard GAPDH, detected at 35 kDa. Black bars denote different sample groups.

Fig. 2

Comparison of expression levels of SLC uptake transporters in rat cryopreserved hepatocytes, freshly isolated rat hepatocytes and rat liver tissue. Transporter protein levels were normalized against GAPDH, expression levels in liver tissue were set to 1. (Mean ± SD, n=3, * = $p < 0.05$, ** = $p < 0.01$ for liver and fresh hepatocytes. For cryopreserved hepatocytes two individual measurements were performed, statistical significance of differences can therefore not be calculated for these samples.)

B: Representative Western blot depicting the levels of rOAT2 transporters in rat liver, fresh hepatocytes and cryopreserved hepatocytes. rOAT2 was detected at approximately 60 kDa. One animal seemed devoid of rOAT2 staining. Lower panel

depict the internal standard GAPDH, detected at 35 kDa. Black bars denote different sample groups; L, liver; FH, fresh hepatocytes; CH, cryopreserved hepatocytes.

Fig. 3

Immunofluorescent detection of OATP1B1 (**3A**) and OATP1B3 (**3B**) in human cryopreserved cells attached to collagen type I covered cover slips.

Fig. 4

Immunofluorescent detection of OATP1B1 (**4A**) and OATP1B3 (**4B**) in human liver sections. The OATP transporters were seen almost exclusively on hepatocyte membranes. In **Fig 4C** a negative control section stained omitting the primary antibody is shown, neither unspecific staining nor auto-fluorescence is evident.

Fig. 5

Activity of drug uptake transporters in plated fresh and cryopreserved hepatocytes. **A:** Uptake rates were determined for uptake substrates in plated fresh or cryopreserved human hepatocytes (fexofenadine is a substrate of hOATP transporters; rosuvastatin, hOATP and hNTCP transporters; Ipratropium, hOCT transporters; taurocholate, hNTCP transporter). **B:** The same experiment was performed using plated rat fresh and cryopreserved hepatocytes (fexofenadine is a substrate of rOATP transporters; rosuvastatin, rOATP but not rNTCP transporters; Ipratropium, rOCT transporters; taurocholate, rNTCP transporter). (Mean ± SD, n=3, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n = 3).

Fig. 6

Drug metabolism in rat fresh and cryopreserved hepatocytes. Drug metabolism of CYP and phase II enzyme substrates was measured in rat fresh and cryopreserved hepatocytes in suspension. Enzyme specificities: 7-hydroxycoumarin, UGT; bufuralol,

CYP2D2; diazepam, CYP2C11; diclofenac, CYP2C6; midazolam, CYP3A; and phenacetin, CYP1A2. (Mean ± SD, n=3).

Fig. 7

The uptake and metabolism of an AZ project compound is shown in human cryopreserved (**7A**), rat fresh hepatocytes (**7B**), and rat cryopreserved hepatocytes (**7C**). Graphs show the total loss of compound from the incubation (cells and media) by metabolism, or loss from the extracellular medium by uptake or binding (to dead cells) measured after removal of cells by centrifugation. In human cryopreserved hepatocytes metabolism was slow and uptake was limited to binding to cells. In fresh rat hepatocytes, and to a lesser extent in rat cryopreserved hepatocytes, the compound was rapidly taken up and metabolized. (Mean ± SD, n=3).

Fig. 8

Comparison between the logarithms of scaled in vivo CL_{int} (calculated using Eq. 6) for human liver microsomes and human cryopreserved hepatocytes (**8A**) and rat microsomes and rat fresh hepatocytes (**8B**) for 83 AZ new chemical entities. Human liver microsomes tended to give higher scaled in vivo CL_{int} than human cryopreserved hepatocytes. The reverse was true for rat experiments were fresh rat hepatocytes tended to give higher scaled in vivo CL_{int} than rat liver microsomes. The dashed line represents the line of unity.

Tables

Table 1. IVIVE of fexofenadine, ipratropium and rosuvastatin in vivo $CL_{hepatic}$.[a]

	Hepatocyte $CL_{int,\ uptake}$ (µl/min/10^6 cells)		Predicted $CL_{hepatic}$[b] (ml/min/kg)		In vivo $CL_{,hepatic}$[c] (ml/min/kg)	In vivo CL_{blood}[d] (ml/min/kg)
	Fresh	Cryopreserved	Fresh	Cryopreserved		
Human (hepatocyte suspension)						
Fexofenadine	11.4 ± 2.4	1.9[e]	4.2 ± 1.6	1.5	2.2	3.3
Rosuvastatin	21.2 ± 3.7	5.0 ± 1.3***	3.5 ± 0.7	1.2 ± 0.4	9.8	13.9
Ipratropium	25 ± 6	14.5 ± 3 NS	12.4 ± 2.8	9.6 ± 2.1	19.5	31.4
Human (plated hepatocytes)						
Fexofenadine	8.9 ± 3.4	0.8 ± 0.5***	3.4 ± 1.4	0.4 ± 0.2	2.2	3.3
Rosuvastatin	16.5 ± 2.1	3.9 ± 1.1***	2.3 ± 0.8	0.6 ± 0.2	9.8	13.9
Ipratropium	18.3 ± 2.7	11.2 ± 2.3*	11.1 ± 2	8.3 ± 2.6	19.5	31.4
Rat (hepatocyte suspension)						
Fexofenadine	6 ± 1	2 ± 0.5**	4.8 ± 1.2	1.5 ± 0.6	7.2	10.9 ± 3.4
Rosuvastatin	36.2 ± 5.7	11 ± 2.5***	14.3 ± 4.4	4.9 ± 1.6	50	62.8 ± 8.2
Ipratropium	53 ± 8.5	34 ± 6*	36.2 ± 5.3	30.3 ± 4.1	36.1	58.3
Rat (plated hepatocytes)						
Fexofenadine	11.2 ± 3.1	3 ± 0.9***	12.9	4.2	7.2	10.9 ± 3.4
Rosuvastatin	40 ± 12	9 ± 1.5***	15.3	4.4	50	62.8 ± 8.2
Ipratropium	51 ± 11.1	28 ± 7.8*	37.1	29	36.1	58.3

[a]Mean ± SD; n = 3; *** = p< 0.001, ** = p< 0.01, * = p< 0.05, significantly different from fresh hepatocytes.

[b]Predicted in vivo $CL_{hepatic}$ was derived from $CL_{int,\ uptake}$ and Eqs. 6, 7, and 8.

[c]In vivo unbound $CL_{hepatic}$ was calculated from CL_{blood} and the hepatically cleared fraction, $f_{hepatic.}$ Values for $f_{hepatic}$ are from Kamath et al., 2005; Swift et al., 2009; Kitamura et al., 2008;Varma et al.,

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2009; and Ensing et al., 1989. For ipratropium rat $f_{hepatic}$ is unknown and the human value was used as a substitute (references are listed in Supplemental references).

[d]In vivo unbound CL_{blood} was calculated from rat plasma CL using Eqs. 8 and 9. Human CL_{blood} values are from Ensing et al., 1989; Swift et al., 2009; and Varma et al., 2009. Human and rat CL_{blood} for ipratropium were from Ensing et al., (1989) and Urso et al., (1991), respectively (references are listed in Supplemental references).

[e]Fexofenadine uptake in human cryopreserved hepatocytes in suspension was below 2 µl/min/10^6 cells, the LOQ of the assay. The $CL_{int,\ uptake}$ value of 1.9 represents a hypothetical maximum uptake value used for comparative purposes.

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Table 2. Metabolism and uptake $CL_{int,\ in\ vitro}$ determinations for an AZ project compound.[a]

	Metabolic $CL_{int,\ in\ vitro}$[b]			Uptake $CL_{int,\ in\ vitro}$[b]	
	µl/min/mg	µl/min/10^6 cells	µl/min/10^6 cells	µl/min/10^6 cells	
Species	Microsomes	Fresh Hepatocytes	Cryopreserved Hepatocytes	Fresh Hepatocytes	Cryopreserved Hepatocytes
Rat	10 ± 2	44 ± 5	11 ± 3***	432 ± 53	204 ± 31**
Human	12 ± 3	N.D.[c]	<5[d]	N.D.[c]	96 ± 22

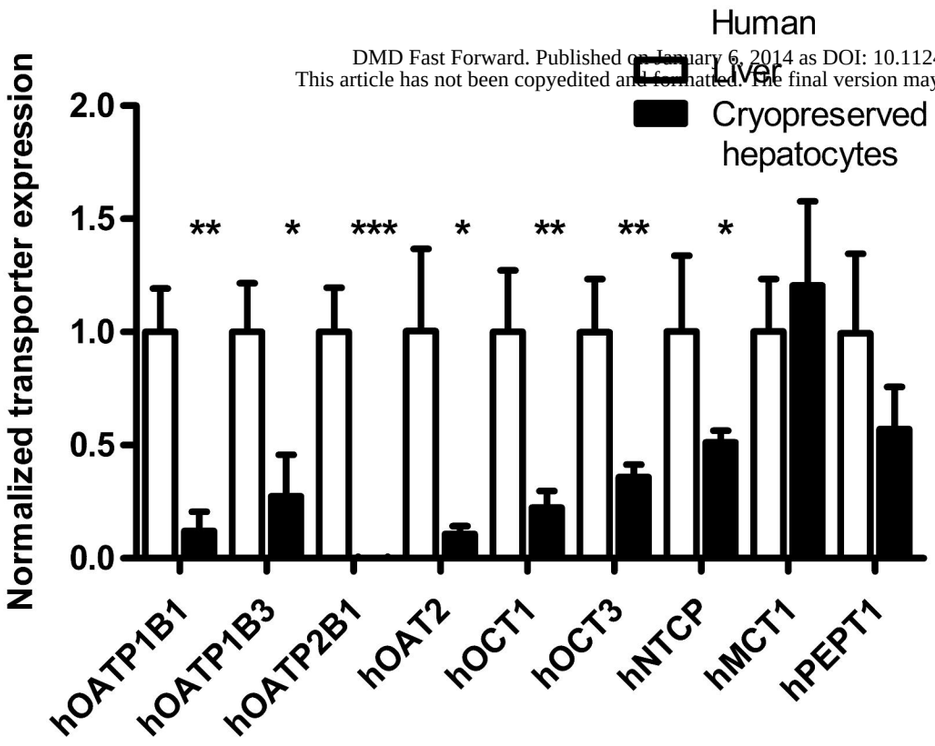
[a]Metabolic stability and uptake (loss from media method) curves are shown in Fig. 7.

[b]Mean ± SD; n = 3; *** = $p< 0.001$, ** = $p< 0.01$, significantly different from fresh hepatocytes;

[c]N.D. = not determined

[d]<5 µl/min/10^6 hepatocytes, below the detection limit of the assay.

Figure 1



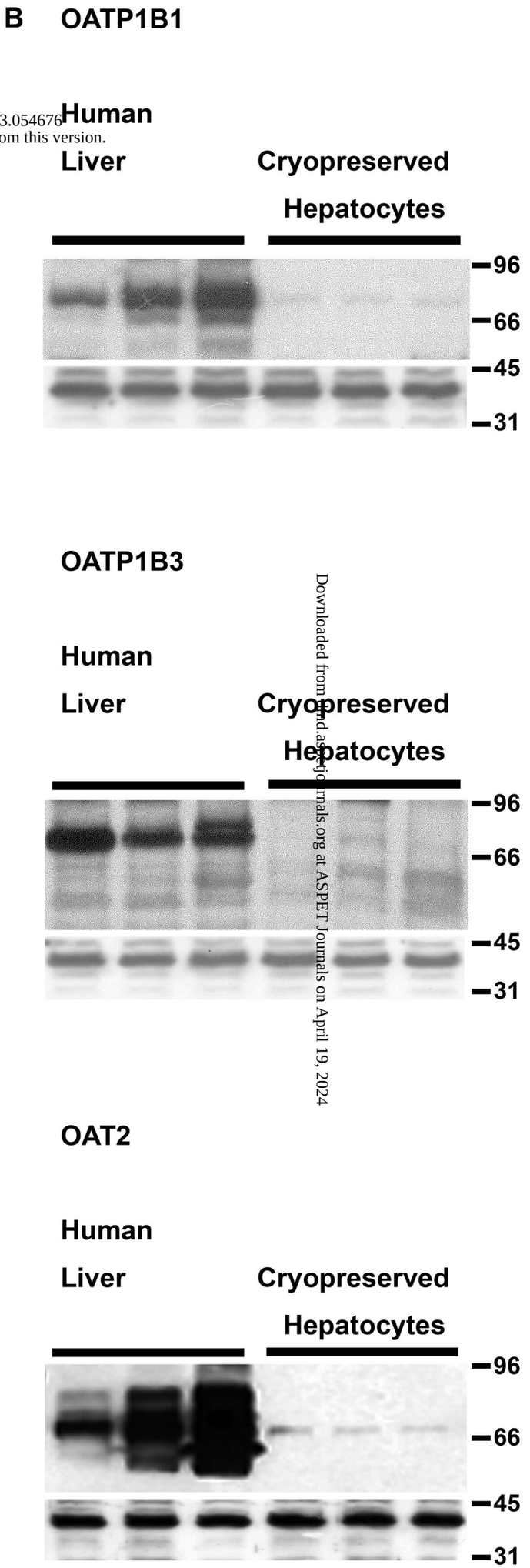
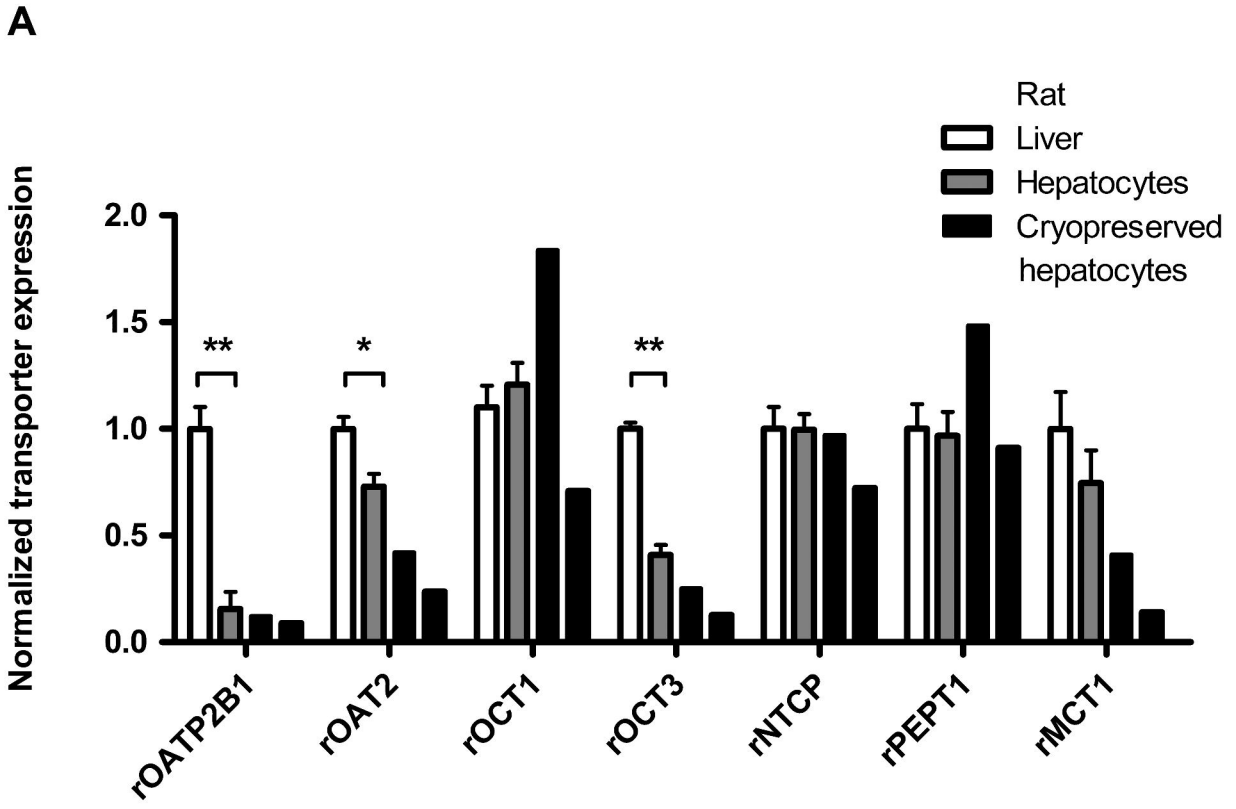


Figure 2



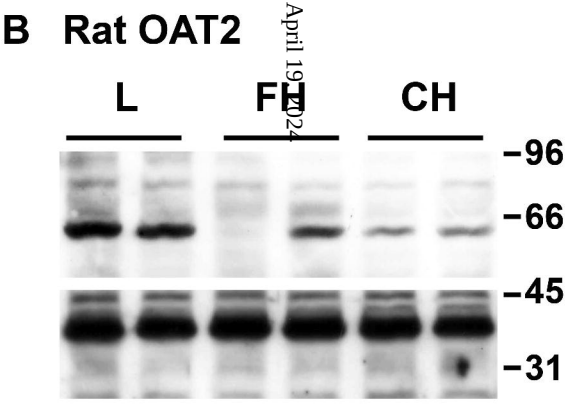
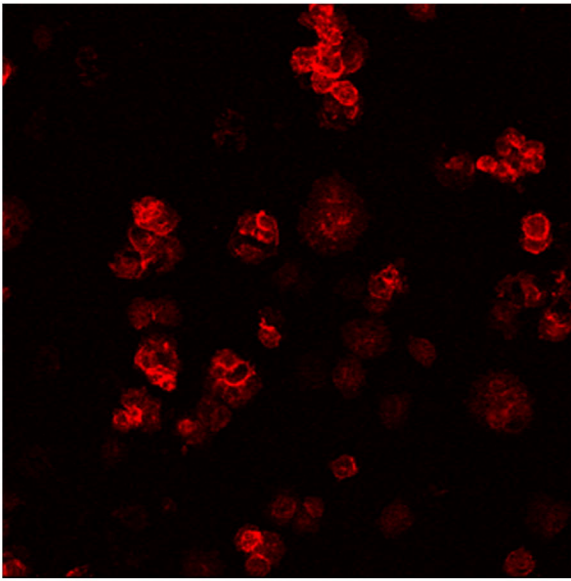


Figure 3

A hOATP1B1



B hOATP1B3

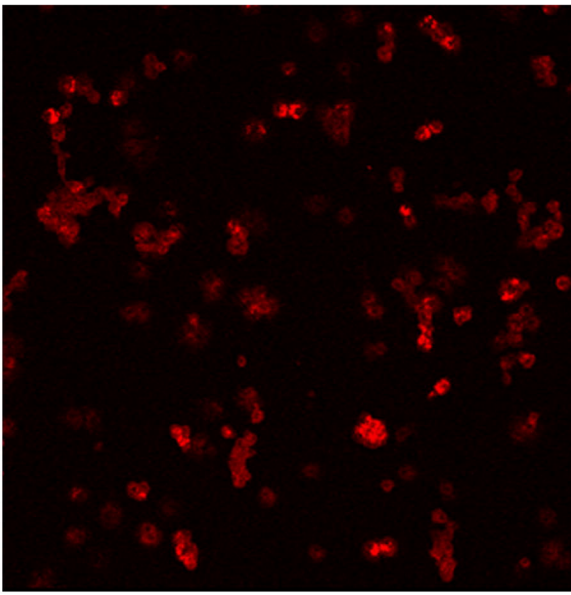
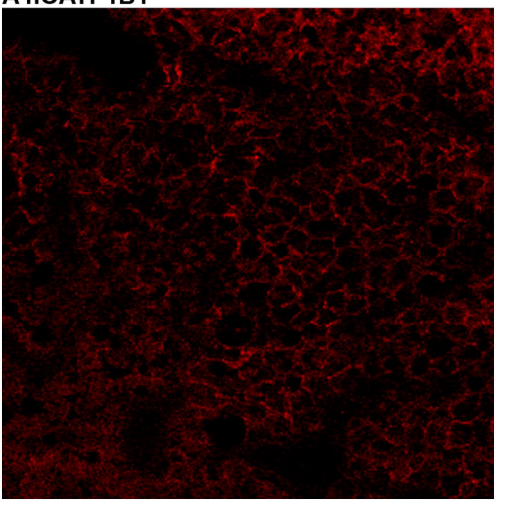
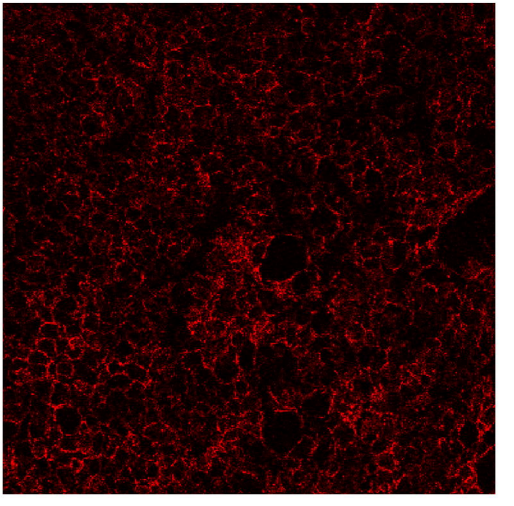


Figure 4

A hOATP1B1

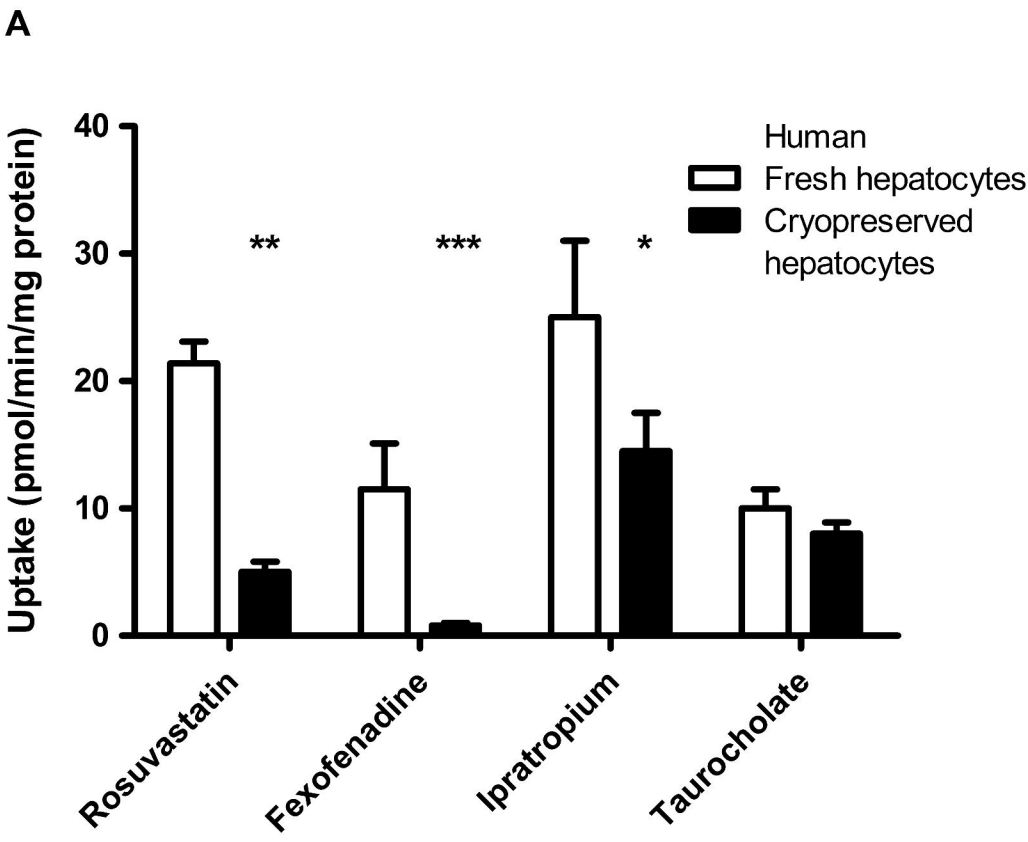


B hOATP1B3



C Negative control

Figure 5


A
Uptake (pmol/min/mg protein)
40
30
20
10
0
**
***
*
Human
Fresh hepatocytes
Cryopreserved hepatocytes
Rosuvastatin
Fexofenadine
Ipratropium
Taurocholate

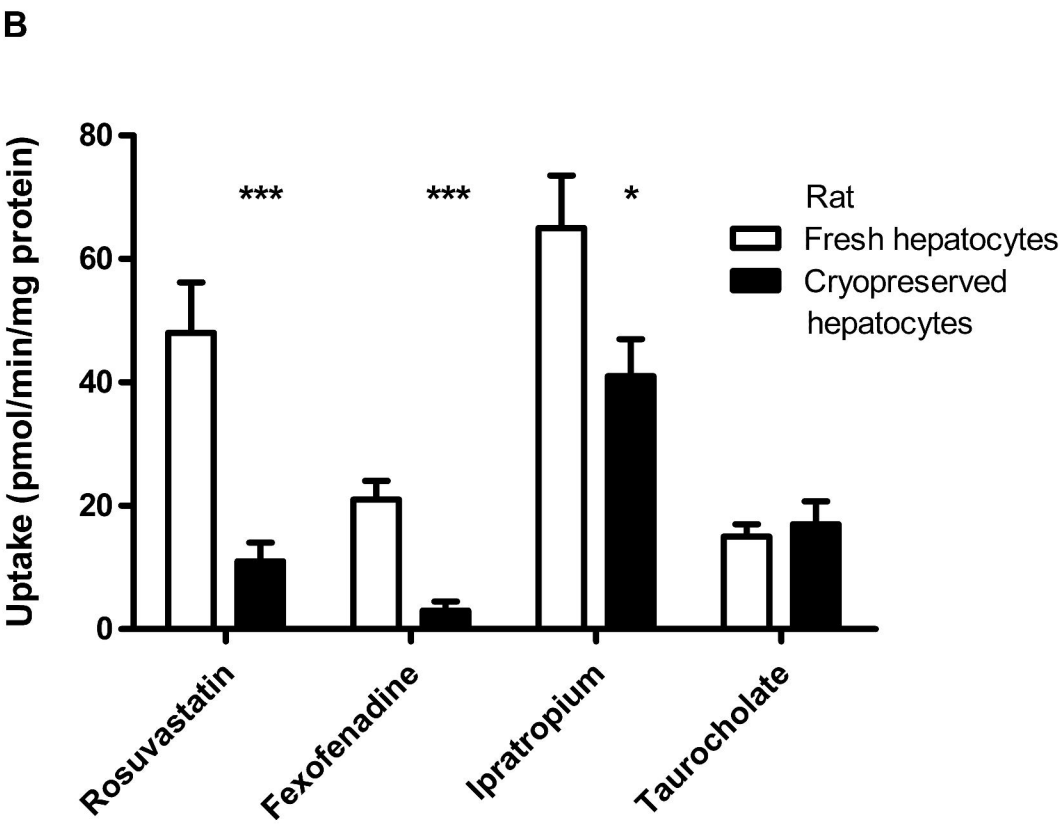

B
Uptake (pmol/min/mg protein)
80
60
40
20
0
***
***
*
Rat
Fresh hepatocytes
Cryopreserved hepatocytes
Rosuvastatin
Fexofenadine
Ipratropium
Taurocholate

Figure 6

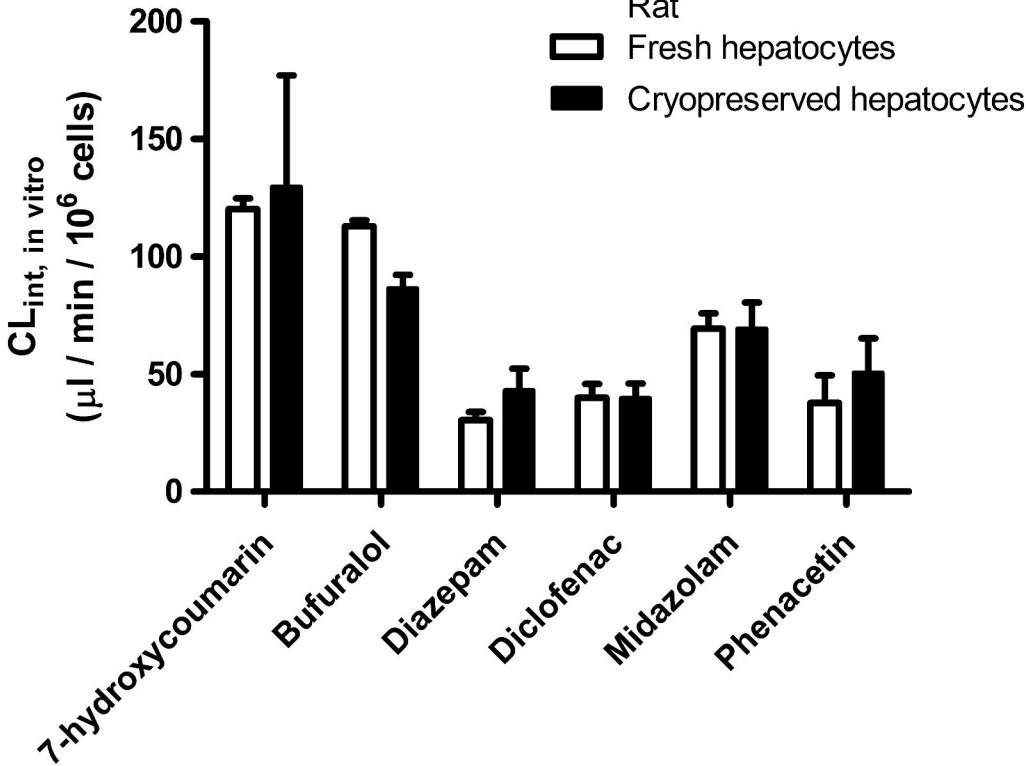

Rat
Fresh hepatocytes
Cryopreserved hepatocytes
$CL_{int, in\ vitro}$
(µl / min / 10^6 cells)
200
150
100
50
0
7-hydroxycoumarin
Bufuralol
Diazepam
Diclofenac
Midazolam
Phenacetin

Figure 7

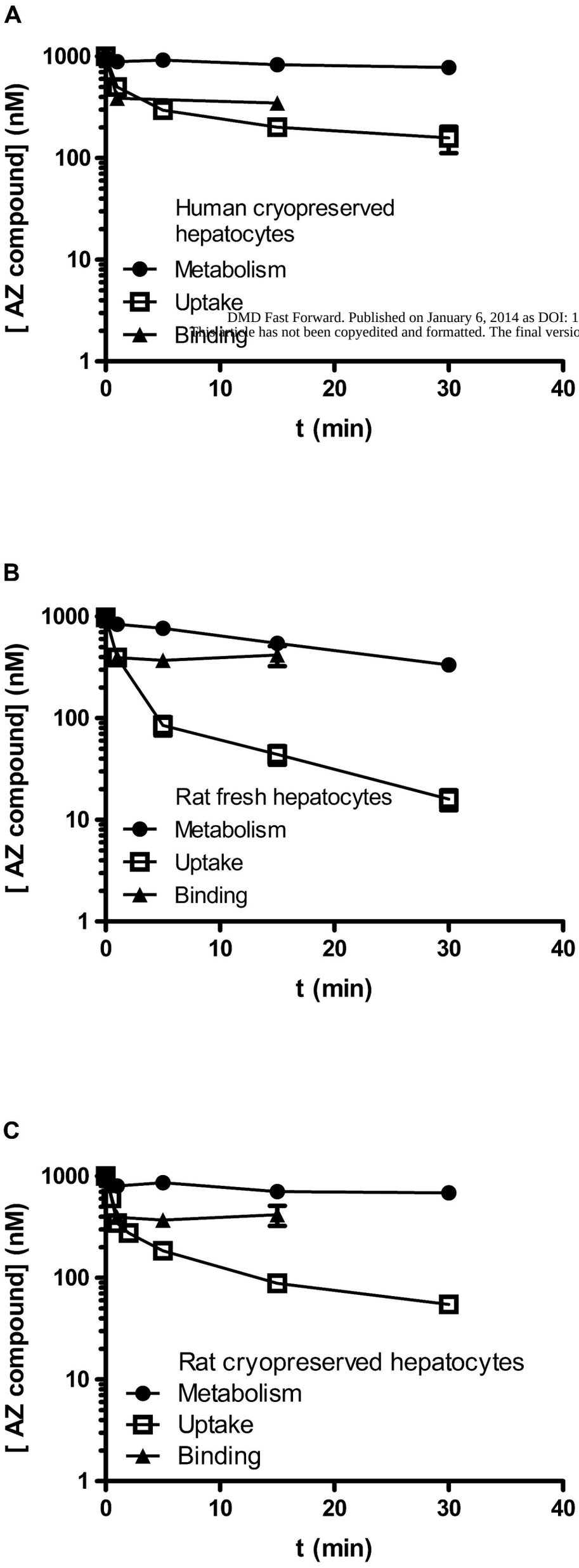
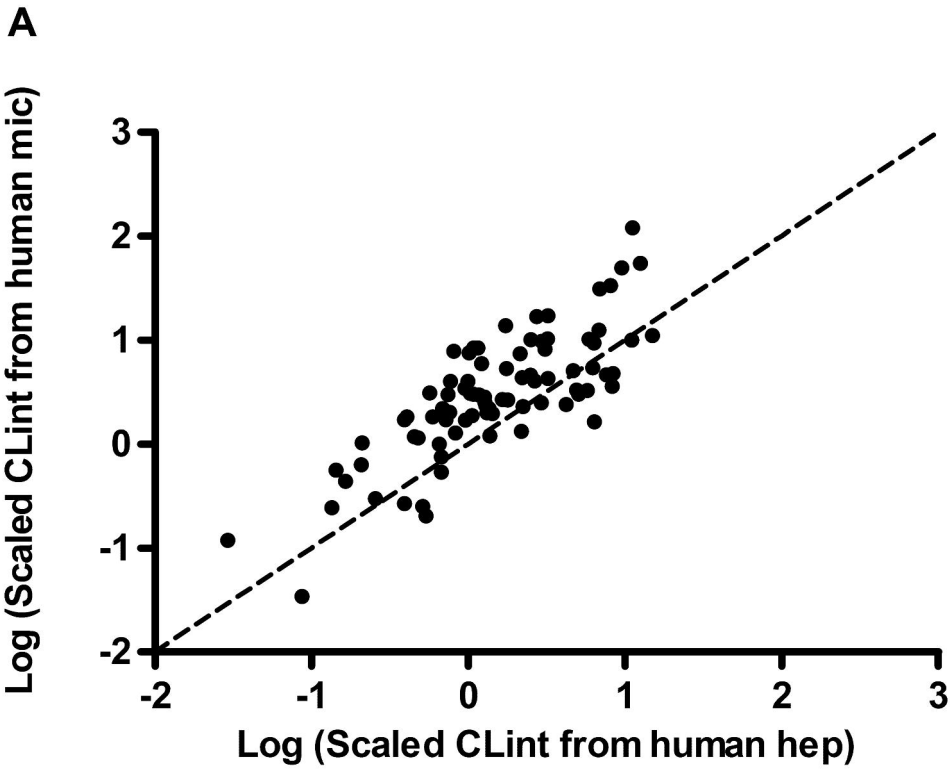
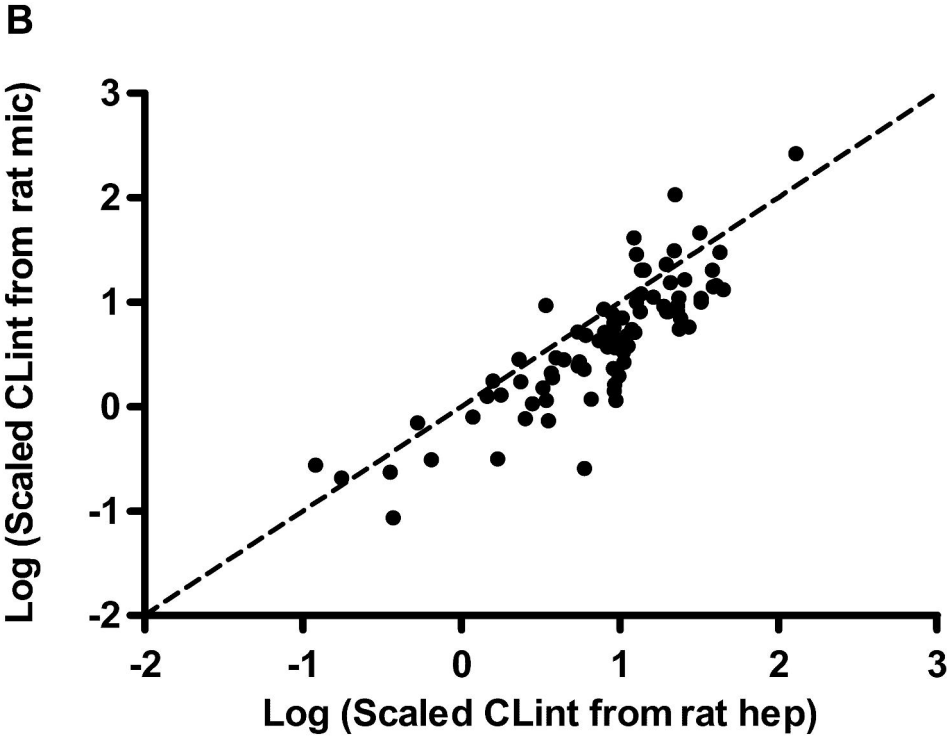


Figure 8


A
Log (Scaled CLint from human mic)
3
2
1
0
-1
-2
-2
-1
0
1
2
3
Log (Scaled CLint from human hep)


B
Log (Scaled CLint from rat mic)
3
2
1
0
-1
-2
-2
-1
0
1
2
3
Log (Scaled CLint from rat hep)

Supplemental Material

Major loss of solute carrier (SLC) – super family drug uptake transporters in human and rat cryopreserved hepatocytes.

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Drug Metabolism and Disposition

Supplemental Tables

Supplemental Table 1. Liver tissue and hepatocyte donor information

Cryopreserved hepatocytes donors						
Provider	Donor	Sex	Age	Cause of death[a]	Medical History	Medications
Celsis	OZL	M	53	CVA	Hypertension Diabetes	Anti-hypertensives
Celsis	REL	F	60	ICH	None reported	None reported
Celsis	SQJ	F	67	ICH	Hypertension	Anti-hypertensives
CellzDirect	4035	M	34	Head Injury	Colitis	None reported
CellzDirect	4037	F	64	CVA	Hypertension Multiple Sclerosis	None reported
CellzDirect	4152	F	50	Anoxia	None reported	None reported
Liver tissue and fresh hepatocyte donors						
	Donor	Sex	Age	Reason for hepatectomy[b]	Medical History	Medications
	A	F	23	FNH	None reported	None reported
	B	M	59	RC	None reported	Folinic acid[c, d] 5-Fluorouracil Oxaliplatin
	C	F	59	CC	None reported	Folinic acid[c, d] 5-Fluorouracil Oxaliplatin

[a]CVA, cerebrovascular accident/stroke ; ICH, intracerebral hemorrhage
[b]FNH, focal nodular hypoplasia; RC, rectal carcinoma; CC, carcinoid coli
[c]This combination of cytostatica is known as Folfox
[d]Livers from donors receiving oxaliplatin were devoid of obvious histological changes.

Supplemental Table 2. Primary antibodies: sources and species reactivity.

Primary Antibody (catalogue #)	Observed species reactivity
Rabbit polyclonal to MCT1[c], Human Protein Atlas[a]	Hu, Rat
Rabbit polyclonal to NTCP, University of Zürich[b]	Hu, Rat
Rabbit polyclonal to OAT2, Abcam (ab58683)	Hu, Rat
Rabbit polyclonal to OATP1B1, University of Zürich[b]	Hu
Rabbit polyclonal to OATP1B3, University of Zürich[b]	Hu
Rabbit polyclonal to OATP2B1, University of Zürich[b]	Hu, Rat
Rabbit polyclonal to OCT1, Abcam (ab55916)	Hu, Rat
Rabbit polyclonal to OCT3, Abcam (ab74152)	Hu, Rat
Rabbit polyclonal to PEPT1[d], Human Protein Atlas[a]	Hu, Rat
Goat polyclonal to GAPDH-HRP, Santa Cruz Biotechnology (sc20357)	Most mammals

[a]Antibodies from Human Protein Atlas (www.proteinatlas.org) have been described previously (Nilsson et al., 2005, supplemental references).

[b]Antibodies from University of Zürich were kindly provided by Dr Bruno Stieger (Rippin et al., 2001, supplemental references).

[c]Monocarboxylate transporter 1, MCT1

[d]Peptide transporter 1, PEPT1

Supplemental Table 3. Test compound optimized settings for LC-MS/MS analysis.

Compound	Transition m/z	Ion source mode	Cone voltage [V]	Collision energy [eV]
7-Hydroxycoumarin	160.90 → 133.10	Negative	37.00	19.00
Diazepam	285.10 → 154.10	Positive	37.00	26.00
Diclofenac	293.80 → 250.10	Negative	19.00	12.00
Fexofenadine	502.00 → 171.12	Positive	30.00	40.00
Ipratropium	332.86 → 124.15	Positive	37.00	28.00
Midazolam	260.00 → 291.10	Positive	46.00	26.00
Phenacetin	180.10 → 110.10	Positive	37.00	19.00
Rosuvastatin	482.14 → 258.46	Positive	55.00	34.00
Taurocholate	514.09 → 123.85	Negative	64.00	50.00
Warfarin	308.90 → 163.01	Positive	18.00	16.00

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