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Polarized expression of drug transporters in differentiated human hepatoma HepaRG cells

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Abstract

The HepaRG cell line is a well-differentiated human hepatoma cell line proposed as a surrogate for human hepatocytes, especially for hepatic detoxification studies. Polarized status of drug transporters, i.e., their coordinated location at sinusoidal or canalicular membranes, which represents a key hallmark of hepato-biliary drug transport, remains however incompletely documented in HepaRG cells. The present study was therefore designed to analyze transporter location in HepaRG cells, which exhibit mRNA expressions of most of hepatic transporters. HepaRG cells were demonstrated, through immunofluorescence staining, to express several drug transporters at their sinusoidal pole, especially the influx transporters organic anion transporting polypeptide (OATP) 1B1, OATP2B1 and organic cation transporter (OCT) 1 and the efflux transporter multidrug resistance-associated protein (MRP) 3. In addition, the efflux transporters P-glycoprotein and MRP2 were detected at the canalicular pole of HepaRG cells. Moreover, saturable uptake of reference substrates for the sinusoidal transporters sodium-taurocholate cotransporting polypeptide, OATPs and OCT1 and canalicular secretion of reference substrates for the efflux transporters bile salt export pump and MRP2 were observed. This polarized and functional expression of various sinusoidal and canalicular transporters in HepaRG cells highlights the interest of using these hepatoma cells in xenobiotic transport studies.

Key-words: Drug transporter, Hepatocytes, HepaRG cells, Polarization, Hepato-biliary secretion.

1. Introduction

The HepaRG cell line is a recently-characterized hepatic cell line, established from a hepatocholangiocarcinoma of a female patient (Gripon et al., 2002). In contrast to most, if not all, other available hepatic cell lines, the HepaRG cell line exhibits various liver-specific functions and is therefore proposed as a surrogate for the use of primary human hepatocytes, especially for xenobiotic metabolism and toxicity studies (Andersson et al., 2012; Guillouzo et al., 2007). Indeed, when cultured in appropriate conditions, i.e., in the presence of 2 % (vol/vol) dimethylsulfoxide (DMSO), HepaRG cells express high levels of hepatic drug detoxifying pathways, including phase 1 enzymes, such as cytochromes P-450 1A2, 2B6 and 3A4, and phase 2 enzymes such as glutathione S-transferases A1/A2, A4 and M1 and UDP-glucuronosyl transferase 1A1 (Aninat et al., 2006; Antherieu et al., 2010). Moreover, HepaRG cells exhibit notable expression of drug-sensing receptors such as the pregnane X receptor and the constitutive androstane receptor (Antherieu et al., 2012) and are consequently responsive to inducers of drug metabolism (Kanebratt and Andersson, 2008; Turpeinen et al., 2009).

In addition to drug metabolizing enzymes, hepatic drug transporters are present and functional in HepaRG cells (Kotani et al., 2012; Le Vee et al., 2006; Szabo et al., 2013). This point is likely noteworthy, because the important roles played by hepatic drug transporters in hepatic clearance of drugs and drug-induced cholestasis are now well-recognized (Giacomini et al., 2010; Pauli-Magnus and Meier, 2006). Expression of transporters in HepaRG cells remains however incompletely characterized, especially with respect to the polarization status of these transporters. Polarization into two complementary poles, i.e., a blood sinusoidal/basolateral pole and a biliary apical/canalicular pole, represents a key hallmark of differentiated hepatocytes and has important functional consequences in terms of hepato-biliary secretion of drugs (Chandra and Brouwer, 2004). Indeed, influx hepatic transporters expressed at the sinusoidal/basolateral pole of hepatocytes mediate uptake of drugs from

blood into liver, whereas canalicular transporters are responsible for secretion of drugs or drug metabolites into bile (Funk, 2008; Kock and Brouwer, 2012). In addition, some efflux transporters expressed at the sinusoidal membrane can secrete some drugs or drug metabolites back into blood, for a secondary renal elimination (Zelcer et al., 2005) (See Fig. 1 for a schematic overview of drug transporters expressed by human hepatocytes). The present study was therefore designed to carefully analyze cellular location of several major sinusoidal and canalicular hepatic transporters in HepaRG cells cultured under DMSO-based standard conditions.

2. Materials and methods

2.1 Chemicals

Taurocholate, estrone-3-sulfate (E3S) and tetra-ethylammonium (TEA) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), whereas carboxy-2,7-dichlorofluoresceine (CF) diacetate was provided by Invitrogen/Life Technologies (Villebon sur Yvette, France). [^{3}H(G)] taurocholic acid (sp. act. 1.19 Ci/mmol), [6, 7-^{3}H(N)] E3S (sp. act. 57.3 Ci/mmol) and [1-^{14}C] TEA (sp. act. 2.4 mCi/mmol) were from Perkin-Elmer (Boston, MA). All other chemicals were commercial products of the highest purity available.

2.2 *Cell culture*

Human highly-differentiated hepatoma HepaRG cells were routinely cultured in Williams' E medium supplemented with 10% (vol/vol) fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 2 mM glutamine, and 5 x 10^{-5} M hydrocortisone hemisuccinate; additional culture for two weeks in the same medium supplemented with 2 % (vol/vol) DMSO was performed in order to get a full hepatocytic differentiation of the cells (Gripon et al., 2002).

2.3 Preparation of freshly isolated human hepatocytes.

Human hepatocytes were obtained from adult donors undergoing hepatic resection for primary and secondary tumors, via the Biological Resource Center (Rennes, France), in agreement with French laws and regulations and the local ethics committee. Cells were prepared by perfusion of histologically-normal liver fragments using a collagenase solution as previously described (Jigorel et al., 2005). They were immediately used for RNA isolation.

2.4 RNA isolation and analysis

Total RNA was isolated from cells using the TRIzol reagent (Invitrogen/Life Technologies). RNA was then subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR), using the fluorescent dye SYBR Green methodology and an ABI Prism 7300 detector (Applied Biosystem, Foster City, CA, USA), as previously reported (Maubon et al., 2007). Gene primers were exactly as previously described (Le Vee et al., 2006; Moreau et al., 2011), except multidrug and toxin extrusion protein 1 (MATE1/SLC47A1) sense, GCAATCGCGGTTATCAATG, MATE1 antisense, AAGCCTGGACACATCTGGG. Amplification curves were next analysed with ABI Prism 7000 SDS software using the comparative cycle threshold method. Relative quantification of the steady-state target mRNA levels was calculated after normalization of the total amount of cDNA tested to a 18S mRNA endogenous reference using the $2^{(-\Delta Ct)}$method. This allowed to get a relative value of expression for each gene comparatively to the 18S RNA amount (20 ng) found in each RT-qPCR sample, arbitrarily set at 10^6 units (Moreau et al., 2011).

2.5 Light and fluorescence microscopy

Light microscopy and CF fluorescence were employed to monitor the presence of functional canalicular networks in HepaRG cells, as previously described (Noel et al., 2013).

For light microscopy studies, cells were observed using an Axiovert microscope (Carl Zeiss, Le Pecq, France). For fluorescence studies, HepaRG cells were incubated with 3 µM CF diacetate for 10 min at 37°C; cells were then washed with ice-cold phosphate-buffered saline and visualized using a Leica DM IRB microscope (Leica Microsystems, Wetzlar, Germany) equipped with a black/white CoolSNAP ES camera (Roper Scientific, Planegg/Martinsried, Germany). Pictures were processed using the Metaview software.

2.6 Immunolocalization studies

Immunofluorescence analyses were performed as previously described (Vee et al., 2009). HepaRG cells cultured on glass coverslips were first fixed in ice-cold acetone for 10 min. Cells were next incubated for 3 h with mouse monoclonal antibodies diluted to 1:50 in phosphate-buffered saline supplemented with 4 % (weight/weight) bovine serum albumin and directed against organic cation transporter 1 (OCT1)/SLC22A1 (Abcam, Cambridge, UK), multidrug resistance gene 1 (MDR1)/ABCB1/P-glycoprotein (Alexis Corporation, Lausen, Switzerland), multidrug drug resistance-associated protein (MRP) 2/ABCC2 or MRP3/ABCC3 (Millipore Bioscience Research Reagents, Temecula, CA), with rabbit polyclonal antibodies diluted to 1:25 in the buffer described above and directed against organic anion transporting polypeptide (OATP) 1B1/SLCO1B1 or OATP2B1/SLCO2B1 (Huber et al., 2007), or with corresponding mouse or rabbit isotypic Ig controls. After washing, goat AlexaFluor 488- or AlexaFluor 546-labeled secondary antibodies (Invitrogen/Life Technologies) were added for 1 h, and nuclei were subsequently stained with 4,6-diamidino-2-phenylindole. Immunofluorescence images were finally detected with a Zeiss Axioskop A1 microscope (Carl Zeiss) and a Nikon DS-2MBW camera (Nikon, Champigny-sur-Marne, France), using the NIS-Elements F3.2 software (Nikon).

149 *2.7 Drug transport assays*

150 For characterizing sinusoidal influx of taurocholate (a reference substrate for sodium
151 taurocholate co-transporting polypeptide (NTCP/SLC10A1)), of E3S (a reference substrate
152 for OATPs) and of TEA (a reference substrate for OCT1), HepaRG cells were incubated at
153 37°C with 43.4 nM [^{3}H] taurocholate, 3.4 nM [^{3}H] E3S or 40 µM [^{14}C] TEA in the absence
154 or presence of various concentrations of corresponding unlabelled substrates, using a defined
155 sodium- and calcium-containing transport assay medium (Jigorel et al., 2005). Incubation
156 times were 3 min (for taurocholate and E3S) or 5 min (for TEA); preliminary experiments
157 indicated that uptakes of these substrates were linear over these periods (data not shown).
158 After washing in phosphate-buffered saline, cells were lyzed and accumulation of
159 radiolabelled substrates was determined through scintillation counting. Kinetic parameters
160 (K_m and V_{max}) of taurocholate, E3S and TEA uptake in HepaRG cells were next estimated
161 though fitting data with Prism software (GraphPad software, La Jolla, CA) to Michaelis-
162 Menten plots based on the following equation:

163 $$v = (V_{max} \times [S])/(K_m + |S])$$

164 where v is the initial uptake rate of substrate, [S] is the substrate concentration in the
165 medium, K_m is the Michaelis–Menten constant, and V_{max} is the maximum uptake rate.

166 Biliary secretion of taurocholate and CF was analyzed through determination of drug
167 accumulation into bile canaliculi-like structures as previously reported (Annaert et al., 2001).
168 Briefly, HepaRG cells were first incubated for 10 min at 37°C with transport assay buffer
169 containing Ca^{2+} or with the same buffer, except that 1.8 mM $CaCl_2$ was withdrawed and 100
170 µM EGTA was added, knowing that incubation with this Ca^{2+}-free buffer promotes disruption
171 of tight junctions and opening of bile canaliculi networks. Buffers were then removed and
172 HepaRG cells were further incubated for 10 min at 37°C in transport assay medium
173 containing 43.4 nM [^{3}H] taurocholate or 3 µM CF diacetate. After washing with ice-cold

phosphate-buffered saline, accumulations of taurocholate and CF into cells + bile canaliculi (Ca^{2+}-containing conditions) and into cells only (Ca^{2+}-free conditions) were determined by scintillation counting (for taurocholate) or spectrofluorimetry (for CF) using a SpectraMax Gemini SX spectrofluorometer (Molecular Devices, Sunnyvale, CA) (excitation and emission wavelengths were 485 and 535 nm, respectively). Biliary excretion index (BEI) was finally calculated using the following equation (Liu et al., 1999):

$$BEI = \frac{\text{Accumulation (Cells+Bile canaliculi)} - \text{Accumulation (Cells)}}{\text{Accumulation (Cells+Bile canaliculi)}} \text{ X } 100$$

2.8 Statistical analysis. Quantitative data were statistically analyzed using Student's t-test or nonparametric Spearman's rank correlation method. The criterion of significance was $p < 0.05$.

3. Results

mRNA expression of sinusoidal and canalicular drug transporters in HepaRG cells was first characterized by RT-qPCR and compared to that found in freshly isolated human hepatocytes; these freshly isolated human hepatocytes were obtained from 8 donors, in order to take into consideration potential inter-individual differences in drug transporter mRNA levels. As shown in Fig. 2, expressions of sinusoidal influx solute carrier (SLC) transporters such as NTCP, OATPs, OCT1 and organic anion transporter 2 (OAT2/SLC22A7), were reduced in HepaRG cells when compared to hepatocytes. Nevertheless, when considering mRNA levels expressed in arbitrary units, substantial expressions (> 5 arbitrary units) of NTCP, OATP1B1, OATP2B1, OCT1 and OAT2 were detected in HepaRG cells, knowing that these transporters were otherwise highly expressed in freshly isolated human hepatocytes (Fig. 2). Only OATP1B3, which was markedly down-regulated in HepaRG cells when

compared to hepatocytes, was finally very poorly expressed in HepaRG cells (expression < 1 arbitrary unit). In contrast to SLC transporters, sinusoidal ATP-binding cassette (ABC) efflux transporters, except MRP6 (ABCC6), were up-regulated in HepaRG cells when compared to human hepatocytes (Fig. 2); mRNA levels of MRP1, MRP4 and MRP5, which are very poorly expressed in human hepatocytes (expression <1 arbitrary unit), remained however also low in HepaRG cells (expression < 5 arbitrary units), unlike that of MRP3 (expression = 45.0 arbitrary units). With respect to canalicular transporters, some of them, such as MRP2 and especially MDR1, were induced in HepaRG cells, when compared to human hepatocytes (Fig. 3). By contrast, the canalicular SLC transporter MATE1 remained unchanged, whereas breast cancer resistance protein (BCRP/ABCG2) and especially bile salt export pump (BSEP/ABCB11) were down-regulated (Fig. 3).

To more globally characterize drug transporter expression in HepaRG cells and human hepatocytes, sinusoidal and canalicular drug transporters were next ranked from the most expressed to the less expressed according to mRNA level of expression and the resulting expression profiles were compared. As shown in Fig. 4, expression profile of drug transporters in HepaRG cells was close to that exhibited by freshly isolated human hepatocytes, even if the correlation failed to reach a significant level (p=0.07).

DMSO-treated differentiated HepaRG cells are well-known to be polarized (Antherieu et al., 2012) and consequently exhibit bile canaliculi-like structures, which accumulated the fluorescent dye CF (Fig. 5A), indicating that they were fully functional. Immunofluorescence staining next revealed that various hepatic transporters such as OATP1B1, OATP2B1, OCT1 and MRP3, known to be physiologically present at the sinusoidal pole of hepatocytes in the liver (Funk, 2008) (Fig. 1), were similarly expressed at the sinusoidal pole of cultured HepaRG cells (Fig. 5B). In the same way, the canalicular ABC transporters MDR1/P-

glycoprotein and MRP2 were located to the bile canaliculi-like structures of HepaRG cell cultures (Fig. 5B).

HepaRG cells have previously been shown to exhibit sinusoidal transport activity, especially NTCP-mediated uptake of taurocholate, OATP-mediated uptake of E3S, estradiol-17β glucuronide and pitavastatin, and OCT1-mediated uptake of TEA (Kotani et al., 2012; Le Vee et al., 2006; Szabo et al., 2013). In order to better characterize some of these sinusoidal transport activities, we determined their kinetic parameters. As shown in Fig. 6A, uptakes of taurocholate, E3S and TEA in HepaRG cells were found to be saturable, with Km values in the μM range (for taurocholate and E3S) and in the mM range (for TEA).

Canalicular secretion of taurocholate and CF was finally quantified in HepaRG cells using the BEI approach based on Ca^{2+} withdrawal-mediated disruption of canaliculi (Liu et al., 1999). As shown in Fig. 6B, HepaRG cells pre-incubated in Ca^{2+}-free conditions exhibited decreased retention of taurocholate and CF comparatively to counterparts maintained in the presence of Ca^{2+}. This indicated that HepaRG cells secreted taurocholate and CF in bile canaliculi-like structures, in agreement, for CF, with Fig. 5A, and permitted to calculate BEI values (34.4 % and 29.2 % for CF and taurocholate, respectively).

4. Discussion

The data reported in the present study indicate that HepaRG cells exhibit polarized expression of sinusoidal and canalicular drug transporters and thus complete previous studies characterizing expression of drug transporters in these well-differentiated human hepatoma cells (Kotani et al., 2012; Le Vee et al., 2006; Szabo et al., 2013).

Most of sinusoidal and canalicular transporters were expressed at notable mRNA levels in HepaRG cells, with a global profile of transporter expression close to that displayed by freshly isolated human hepatocytes. These data fully confirm previous findings (Antherieu et al., 2012), knowing however that freshly isolated human hepatocytes were chosen as reference cells in the present study, in contrast to previous ones using primary hepatocytes or cryopreserved hepatocytes as references for investigating transporter expression in HepaRG cells (Antherieu et al., 2010; Kotani et al., 2012; Le Vee et al., 2006); this point has likely to be underlined, because placing hepatocytes in culture or hepatocyte cryopreservation are susceptible to alter drug detoxifying protein expression and activity, including those of transporters (Badolo et al., 2011; Ramboer et al., 2013). It is also noteworthy that some transporters that have not previously investigated in HepaRG cells, such as the canalicular SLC transporter MATE1 and the sinusoidal ABC transporter MRP6, were reported to be expressed by these hepatoma cells in the present study. Among transporters, some discrepancies between mRNA levels in HepaRG cells and freshly isolated human hepatocytes, were nevertheless observed; it is notably the case for OATP1B3, which was very poorly expressed in HepaRG cells, in agreement with a recent report (Kotani et al., 2012), making not appropriate the use of HepaRG cells for studying transport of specific substrates of this OATP isoform. In the same way, some ABC efflux transporters, especially MDR1, MRP1, MRP4 and MRP5, were highly expressed in HepaRG cells when compared to freshly isolated human hepatocytes. Some of these transporters such as MRP1, MRP4 and MRP5

remain however among the less expressed transporters in HepaRG cells (Fig. 4), with low absolute mRNA levels (< 5 arbitrary units). This may be consistent with a low background expression for MRP1, MRP4 and MRP5, even in HepaRG cells. Interestingly, up-regulation of the ABC efflux pumps MDR1, MRP1, MRP4 and MRP5 also occurs in primary rodent hepatocytes when compared to parental freshly isolated hepatocytes (Fardel et al., 1992; Noel et al., 2013) and it may therefore be interpreted as an adaptative/protective cellular response towards an *in vitro* unfamiliar environment.

Because expression of some transporters such as MRP2 is sometimes largely restricted to intracellular pools in hepatocytes (Roma et al., 2008) and therefore may not correlate with transport function, accurate characterization of transporter location in HepaRG cells is likely a key parameter to consider. In this context, notable mRNA expression of the major sinusoidal hepatic transporters OATP1B1, OATP2B1, OCT1 and MRP3 was associated with the detection of the corresponding proteins at the sinusoidal pole of HepaRG cells through immunofluorescence staining; in the same way, the biliary ABC transporters P-glycoprotein and MRP2 were found to be present at the canalicular pole of hepatocytes. Taken together, these data indicate a correct location of both sinusoidal and canalicular drug transporters in HepaRG cells. This point is likely important to consider for using HepaRG cells in drug transport studies, because polarized localization of transporters is crucial for *in vitro* models of hepatic drug transport, and is well-exhibited by the present gold standard of these models, i.e., sandwich-cultured hepatocytes (Swift et al., 2010).

Transporters correctly located at the sinusoidal pole HepaRG cells were moreover fully functional. In particular, uptake of taurocholate in HepaRG cells was found to be saturable, with a K_m value (6.3 μM) closed to that described for NTCP-mediated taurocholate in NTCP-transfected cells (K_m=7.9 μM) (Kim et al., 1999). E3S uptake was also saturable in HepaRG cells (Fig. 6A), with a K_m value (44.0 μM) nearly identical to that corresponding to

296 the low-affinity binding site for OATP1B1-mediated E3S transport in OATP1B1-transfected
297 cells (K_m=45.0 μM) (Noe et al., 2007). A contribution of the high-affinity binding site of
298 OATP1B1 for E3S (K_m=0.09 μM) (Tamai et al., 2001) and of OATP2B1, which is well
299 known to transport E3S (K_m=10.2 μM) (Noe et al., 2007) and is expressed in HepaRG cells at
300 substantial level (Fig. 2), has however likely to be additionally taken into consideration for
301 E3S influx in HepaRG cells. By contrast, a role for OATP1B3 may be discarded, since this
302 transporter is only present at a very low level in HepaRG cells (Fig. 2). TEA uptake was also
303 found to be saturable in HepaRG cells (Fig. 6A), with however a K_m value (1.44 mM) higher
304 than those reported for TEA uptake in OCT1-transfected cells, that ranged from 69.2 μM to
305 229.0 μM (Umehara et al., 2007; Zhang et al., 1998). The reason for such a discrepancy
306 between K_m values for TEA uptake in OCT1-expressing HepaRG cells versus OCT1-
307 transfected cells remains to be clarified; it could be linked to post-transcriptional processes
308 specifically targeting OCT1 in HepaRG cells, such as phosphorylation or glycosylation, or,
309 alternatively, to the artificially elevated levels of OCT1 in OCT1-transfected cells.

310 Like sinusoidal transporters, canalicular transporters were functional in HepaRG cells.
311 Indeed, both CF and taurocholate secretion into bile canaliculi-like structures of HepaRG cells
312 were demonstrated and quantitatively characterized in the present study, allowing to
313 determine BEI values (Fig. 6B). Interestingly, BEI value for CF in HepaRG cells (34.4 %)
314 was nearly identical to CF BEI value reported in sandwich-cultured human hepatocytes
315 (BEI=34%) (Hoffmaster et al., 2004), which suggests that MRP2 is similarly active in these
316 primary human hepatocytes and in HepaRG cells. By contrast, taurocholate BEI values in
317 sandwich-cultured human hepatocytes (around 70-75%) (Marion et al., 2007) are rather
318 higher than that found in HepaRG cells (BEI=29.2%), which likely indicates a lower BSEP
319 activity in HepaRG cells, consistent with the rather reduced BSEP mRNA expression in these
320 hepatoma cells when compared to freshly isolated human hepatocytes (Fig. 3). Interestingly,

BEI value, a useful *in vitro* parameter for predicting hepatobiliary drug disposition (Liu et al., 1999), is usually determined only from sandwich-cultured hepatocytes (De Bruyn et al., 2013), because monolayer-cultured hepatocytes failed to get polarization status and to exhibit functional bile canaliculi; the fact that BEI values can also be determined from HepaRG cells, that are not cultured on a sandwich configuration, is therefore noteworthy. Further studies are however required to fully confirm the feasibility of using HepaRG cells for *in vitro* determination of BEI for various structurally-unrelated drugs and to next determine whether such BEI determined from these cultured hepatoma cells may be really relevant for the prediction of hepatobiliary disposition of xenobiotics. In addition, it may be rather interesting to determine whether culturing HepaRG cells in a matrigel- or collagen-based sandwich configuration may improve activity of some canalicular efflux transporters, especially that of BSEP.

In summary, HepaRG cells were demonstrated to exhibit a polarized and functional expression of various major sinusoidal and canalicular drug transporters. Otherwise, HepaRG cells have been shown to be fully responsive to physiological, pharmacological or toxicological stimuli regulating hepatic transporter expression (Fardel and Le Vee, 2009; Lambert et al., 2009) and to be targeted by drugs inducing cholestasis through alteration of bile acid transporter activity (Antherieu et al., 2013; Szabo et al., 2013). Taken together, these data clearly highlight the interest of using HepaRG cells in drug transport studies as surrogates for human hepatocytes, even if some quantitative differences between HepaRG cells and human hepatocytes with respect to transporter expression levels and transport parameters exist and have likely to be kept in mind.

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493 **Legends to figures**

494

495 Fig. 1. Schematic overview of drug transporters expressed at the sinusoidal and canalicular
496 poles of human hepatocytes.

497

498 Fig. 2. Sinusoidal drug transporter mRNA expression in HepaRG cells.
499 Expression of sinusoidal drug transporter expression was determined in HepaRG cells and
500 freshly isolated human hepatocytes (FIHH) by RT-qPCR as described in Materials and
501 Methods. Data from 8 independent HepaRG cell cultures and from 8 independent hepatocyte
502 populations are expressed as boxplots, knowing that each HepaRG cell culture and each
503 hepatocyte population were analyzed in duplicate. Transporter expressions in HepaRG cells
504 expressed as % of that found in human hepatocytes (arbitrarily set at 100 %) are indicated by
505 numbers in brackets.*, $p<0.05$ when compared to hepatocytes.

506

507 Fig. 3. Canalicular drug transporter mRNA expression in HepaRG cells.
508 Expression of canalicular drug transporter expression was determined in HepaRG cells and
509 freshly isolated human hepatocytes (FIHH) by RT-qPCR as described in Materials and
510 Methods. Data from 8 independent HepaRG cultures and from 8 independent hepatocyte
511 populations are expressed as boxplots, knowing that each HepaRG cell culture and each
512 hepatocyte population were analyzed in duplicate. Transporter expressions in HepaRG cells
513 expressed as % of that found in human hepatocytes (arbitrarily set at 100 %) are indicated by
514 numbers in brackets.*, $p<0.05$ when compared to hepatocytes.

515

516

Fig. 4. Comparison of drug transporter expression profiles in HepaRG cells and freshly isolated human hepatocytes.

Drug transporters were ranked according to their level of mRNA expression; correlation between transporter expression profiles was next analyzed using the Spearman's rank correlation method. Spearman's rank coefficient (ρ) and *p* values are indicated at the top of the correlation graph.

Fig. 5. Drug transporter localization in HepaRG cells.

(A) HepaRG cell morphology was examined by light phase contrast microscopy whereas CF-labeled functional bile canaliculi were detected through fluorescence microscopy; bile canaliculi are indicated by arrows. Bar = 30 μM. (B) Immunolocalization of sinusoidal and canalicular drug transporters was performed as described in Materials and Methods. Pictures correspond to single immunolabeling, with membrane transporter-related green fluorescence, except bottom right picture, for which double immunolabeling was performed (red fluorescence for MRP2 and green fluorescence for OATP1B1); blue fluorescence corresponds to 4,6-diamidino-2-phenylindole-stained nuclei. Data shown are representative of three independent experiments. Bar = 10 μM.

Fig. 6. Sinusoidal (A) and canalicular (B) drug transport activity in HepaRG cells.

(A) Saturable uptakes of reference substrates for sinusoidal transporters (Taurocholate for NTCP, E3S for OATPs and TEA for OCT1) were analyzed as described in Materials and Methods. Data are the means ± SEM of three independent experiments, each being performed in duplicate. Kinetic parameters (K_m, V_{max}) are indicated on the top of each graph. (B) Canalicular secretions of the BSEP substrate taurocholate and the MRP2 substrate CF were determined as described in Material and Methods. Data are the means ± SEM of five

(Taurocholate) or four (CF) independent experiments, each being performed in triplicate. BEI values are indicated on the top of each graph. *, $p<0.05$ (paired Student's t-test). FAU, fluorescence arbitrary unit.