Localization of P-gp (Abcb1) and Mrp2 (Abcc2) in freshly isolated rat hepatocytes.

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d) **Abbreviations**: BSA, bovine serum albumin; CDF, carboxy-2’,7’ dichlorofluorescein; CDF-DA, carboxy-2’,7’-dichlorofluorescein diacetate; Oatp1a1, Organic anion transporting polypeptide 1a1; PBS, phosphate buffered saline; P-gp, P-glycoprotein; Mrp2, multidrug resistance-associated protein 2; RT, room temperature; ZO-1, Zonula occludens 1.
Abstract

Freshly-isolated hepatocytes are widely accepted as the “gold standard” for providing reliable data on drug uptake across the sinusoidal (basolateral) membrane. However, the suitability of freshly-isolated hepatocytes in suspension to assess efflux by apical proteins or predict biliary excretion in the intact organ is unclear. Following collagenase digestion, hepatocytes rapidly lose polarity, but localization of canalicular transport proteins in the first few hours after isolation has not been well-characterized. In this study, immunostaining and confocal microscopy have provided, for the first time, a detailed examination of canalicular transport protein localization in freshly-isolated rat hepatocytes fixed within 1hr of isolation, and in cells cultured for 1hr. Oatp1a1 was expressed in all hepatocytes and distributed evenly across the basolateral membrane; there was no evidence for co-localization of Oatp1a1 with P-gp or Mrp2. In contrast, P-gp and Mrp2 expression was lower than Oatp1a1 and confined to junctions between adjacent cells, intracellular compartments and ‘legacy’ network structures at or near the cell surface. P-gp and Mrp2 staining was more predominant in regions adjacent to former canalicular spaces, identified by ZO-1 staining. Functional analysis of rat hepatocytes cultured for 1hr demonstrated that the fluorescent anion and Mrp2 substrate, carboxydichlorofluorescein (CDF), accumulated in cellular compartments; compartmental accumulation of CDF was MK571 (Mrp inhibitor)-sensitive and not observed in hepatocytes isolated from TR̅ rats (Mrp2-deficient). Drug efflux from freshly-isolated hepatocytes as an estimate of apical efflux/biliary excretion would give an inaccurate assessment of true apical elimination and, as such, should not be used to make in vivo extrapolations.
Introduction

Reliable *in vitro* models to predict hepatic accumulation, distribution, metabolism and excretion of drug candidates are important tools in pre-clinical drug development. Many of the currently available models are excellent predictors of *in vivo* hepatic and biliary clearance (Obach et al., 1997; Ghibellini et al., 2007) and several well-established models exist to investigate hepatic drug metabolism and elimination. Freshly isolated hepatocytes in suspension contain a complete complement of drug metabolizing enzymes, remain viable for several hours, and possess relevant drug transport proteins (Hengstler et al., 2000; Gebhardt et al., 2003). Although suspensions of both freshly isolated and cryopreserved hepatocytes are accepted widely as the “gold standard” for providing reliable data on drug uptake across the sinusoidal (basolateral) membrane (Sandker et al., 1994; Hirano et al., 2004; Lam et al., 2006), there is no evidence to support the suitability of this model to accurately predict apical efflux, e.g., biliary excretion. Following enzymatic and/or mechanical disruption, hepatocytes rapidly lose their polarity (Groothuis et al., 1981; Talamini et al., 1997). Examination of cultured hepatocytes 18-24 hr after isolation, indicated that canalicular proteins were internalized; the fluorescent glutathione conjugate, glutathione bimane accumulated in intracellular vesicles of hepatocytes from Wistar but not Mrp2-deficient TR rats (Oude Elferink et al., 1993), and the apical efflux transport proteins, P-glycoprotein (P-gp) and multidrug resistance associated protein 2 (Mrp2) were internalized (Hoffmaster et al., 2004; Zhang et al., 2005). Despite this, some investigators have reported that P-gp function is measurable in suspensions of isolated hepatocytes (Lam and Benet, 2004; Lam et al., 2006), although others have not reached the same conclusion (Jorgensen et al., 2007). The present study
was designed to determine whether freshly isolated, suspended hepatocytes in the first hour post-isolation can be used to assess accurately the extent of drug efflux by canalicular proteins. Data presented in this paper provide, for the first time, clear evidence that P-gp and Mrp2 are rapidly internalized following isolation, remain confined to junctions between cell couplets, or reside on ‘legacy’ canalicular networks of individual cells which represent cell regions with concentrated tight junction protein expression where there was once cell-to-cell contact and a functional canalicular network. Thus, these transporters are largely unavailable to mediate efflux from the freshly isolated hepatocyte.
Materials and Methods

Materials

Monoclonal mouse antibody against C219 (P-gp) was supplied by Covance Research Products, Inc., Denver, PA. Rabbit-anti rat Oatp1a1 antibody was kindly provided by Dr. Peter Meier, University of Zurich, Switzerland. Monoclonal antibody to MRP2 (M2III-6) was supplied by Axxora, LLC (San Diego, CA). Zymed® rabbit-anti ZO-1 and Dulbecco’s modified Eagle's medium (DMEM) without phenol red were supplied by Invitrogen (Carlsbad, CA). Type I rat collagen was supplied by BD Biosciences (San Jose, CA), carboxy-2',7'-dichlorofluorescein diacetate (CDF-DA) by Molecular Probes (Eugene, OR), and MK571 by Calbiochem (San Diego, CA). All other chemicals were obtained from commercial suppliers and were of the highest purity available.

Animals

Male wild-type Wistar (Charles River Laboratories, Raleigh, NC) or TR- Wistar (in-house breeding colony originally obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY) rats (175-300 g) were used for hepatocyte isolation from whole liver. Animals had free access to water and food before surgery. All animal procedures were compliant with the guidelines of the University of North Carolina Institutional Animal Care and Use Committee.

Isolation and culture of rat hepatocytes

Hepatocytes were isolated from male wild-type or TR- Wistar rats using a collagenase perfusion method as described previously (Liu et al., 1999). Hepatocyte viability was >85% as determined
by trypan blue exclusion. Immediately following isolation, suspended hepatocytes were centrifuged for 5 min at 50g and then re-suspended in phosphate buffered saline (PBS) at ~1 million cells/ml. Cells were allowed to attach to 35 mm glass bottom culture dishes [(glass coverslip; 14 mm diameter, 0.16-0.19 mm thickness) (MatTek Corporation, Ashland, MA) previously coated with rat tail collagen solution (75 µl Type I collagen; 1.5 mg/ml, pH 7.4)] at room temperature (RT) for 30-60 min. For the purpose of this study, cells allowed to attach to dishes at RT were described as ‘freshly isolated’. For the 1-hr culture period, hepatocytes (0.75-1.5x10⁶) were seeded in DMEM (supplemented with 5% fetal bovine serum, non-essential amino-acids, L-glutamine, penicillin/streptomycin, and 1 µM dexamethasone) on the collagen-coated 35 mm glass bottom culture dishes and cultured in a humidified incubator with 95% air/5% CO₂.

**Immunostaining**

Both freshly isolated and cultured cells were washed three times with PBS and fixed in ice-cold acetone for 10 min at 4 °C. Cells were washed three times with PBS and then blocked (30 min at RT) with PBS containing 5% goat serum and 1% BSA. Primary antibodies (P-gp, 1:10; Oatp1a1, 1:20; MRP2, 1:20; ZO-1, 1:50) were diluted in PBS containing 5% v/v goat serum and 0.1% w/v acetylated BSA, added to the cells and incubated for 1 hr at RT. Cells were washed with PBS (x3) for 10 min at RT with shaking. Secondary antibodies (diluted in PBS containing 5% v/v goat serum and 0.1% w/v acetylated BSA) were added to the cells and incubated for 1 hr at RT [Alexa Fluor® 488- goat anti-mouse IgG (H+L) (Invitrogen, Carlsbad, CA) for P-gp and Mrp2 (1:1000), and Alexa Fluor® 543- goat anti-rabbit IgG (H+L) (Invitrogen, Carlsbad, CA) for Oatp1a1 and ZO-1 (1:500)]. Following incubation, cells were washed (x3) with PBS for 10 min.
at RT with shaking. Cells were imaged using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY) with a Zeiss C-Apochromat 40x/1.2 water objective lens. The Alexa Fluor® 488, and Alexa Fluor® 543 images were acquired consecutively using, respectively, the 488-nm (Argon laser, 2 %) and 543-nm (HeNe laser, 20 %) laser lines for excitation and the 505-530-nm band pass, and 650-nm long pass filters for emission. Negative control experiments showed no signal at the settings used to image specific fluorescence.

**CDF accumulation in cultured hepatocytes**  Hepatocytes were cultured for 1 hr (0.75x10^6 cells/plate), washed three times with Hank’s balanced salt solution-HEPES (pH 7.4), incubated for 20 min with or without 10 µM MK571, then with 1 µM CDF-DA for ~20-30 min at 37 °C. The diacetate form of CDF, CDF-DA, readily diffuses across the plasma membrane where it is hydrolysed to CDF; a fluorescent Mrp2 substrate (Zamek-Gliszczynski et al., 2003). Cells were imaged with a laser scanning confocal microscope as previously described. The CDF images were collected using the 488-nm (Argon laser, 0.05 %) laser line for excitation with a 505 nm long pass filter for emission.
Results

Examination of freshly isolated hepatocytes, fixed and immunostained for P-gp (C219) or Mrp2 with Oatp1a1 (Fig 1A & B), indicated that Oatp1a1 was distributed evenly on the basolateral membrane while P-gp and Mrp2 staining was confined primarily to junctions between adjacent cells and intracellular compartments. 3D reconstructions suggested that both P-gp and Mrp2 were localized in cellular compartments at or near the cell surface and/or at junctions between cells referred to as ‘legacy’ canalicular spaces. Slices in the z-plane (iii) of freshly isolated hepatocytes showed that neither P-gp nor Mrp2 were localized with Oatp1a1 on the basolateral membrane. Examination of hepatocytes cultured for 1 hr prior to fixation (Fig 2A & B) showed similar distribution patterns, but internalization of P-gp and Mrp2 was more apparent. The expression and localization of P-gp and Mrp2 were investigated further by dual immunostaining with a ZO-1 antibody. In freshly isolated hepatocytes, P-gp (Fig 1C) and Mrp2 (Fig 1D) expression was very closely associated with ZO-1. ZO-1 expression appeared to be less organized in the cultured cells (Fig 2C & 2D) following loss of cell polarization. Cells cultured for 1 hr were examined to determine whether the regions expressing Mrp2 in single hepatocytes were sealed compartments. CDF accumulation appeared to be diffuse within cells and also concentrated in cellular compartments (regions with bright punctate staining), thought to be fragments of ‘legacy’ networks (Fig 3A). CDF accumulation was not apparent in cells pre-treated with MK571 (Fig 3B), or in TR’ (Mrp2 deficient) hepatocytes (Fig 3C). Closer examination of these cells with differential interference contrast microscopy, showed sealed structures at or near the cell surface where CDF accumulation was apparent (Fig 3D & E).
Discussion

Numerous studies have established that isolated hepatocyte suspensions are an excellent *in vitro* model to study both drug metabolism and drug uptake/accumulation. However, there is some debate regarding the suitability of this *in vitro* system to measure apical efflux/canalicular excretion and to predict *in vivo* biliary excretion. During collagenase isolation, hepatocyte cell polarity is quickly lost; returning with time under optimal culture conditions (Groothuis et al., 1981; Talamini et al., 1997). Recent studies have confirmed the apical transport proteins, P-gp and Mrp2, are expressed correctly on the canalicular membrane of sandwich-cultured hepatocytes (by day 3) and efflux drugs into sealed canalicular networks (Hoffmaster et al., 2004; Zhang et al., 2005). However, the cellular localization of apical transport proteins immediately following isolation of hepatocytes from the liver remains to be established. In the present study, using immunostaining and transport of the fluorescent Mrp2 substrate, CDF, we demonstrated that P-gp and Mrp2 (*Fig 1*) are confined to sealed compartments or to junctions between cells, immediately following isolation from rat liver. P-gp and Mrp2 (*Fig 2*) internalization appeared to be more pronounced following 1 hr of culture in accord with the previously published data for Mrp2 in cell couplets (Roelofsen et al., 1998). Sealed tight junctions between hepatocytes form the basis for the development of biliary networks *in vivo*. Following disruption of cell-to-cell contacts, tight junctions are disrupted; however, the tight junction protein, ZO-1, remained highly organized at the former tight junction site (*Fig 1*) with only a slight loss in organization apparent after 1 hr of culture (*Fig 2*). Initially, in freshly isolated hepatocytes, both P-gp and Mrp2 remained closely associated with ZO-1 (*Fig 1C & D*).
in cell couplets and ‘legacy’ canalicular networks of single cells. The staining of Oatp1a1 was diffuse across the surface of the basolateral membrane and did not appear to be co-localized with either P-gp or Mrp2 (Fig 1A & B).

Collagenase isolation of hepatocytes can result in a mixture of both single cells and cell couplets. Cell couplets have been used in multiple studies to examine biliary excretion into sealed compartments between cells (Boyer et al., 1990; Wilton et al., 1993). As shown in the present study, CDF accumulated within ‘legacy network’ compartments (Fig 3), indicating that these are sealed units expressing functional Mrp2. Following synthesis, ABC canalicular transport proteins translocate in a direct manner from golgi bypassing the basolateral membrane in immortalized cell lines (Kipp and Arias, 2000; Slimane et al., 2003). However, little work has been done on protein trafficking at early time points in freshly isolated hepatocytes, which may differ from cell lines. The apical plasma membrane marker, DPPIV (dipeptidyl peptidase IV) follows an indirect pathway to the canalicular membrane via sorting at the basolateral membrane in the polarized hepatic cell line, WIF-B (Bastaki et al., 2002). Interestingly, in a mutant hepatocyte cell line (HepG2-AJ'), cells modified to lack E-cadherin and β-catenin, DPPIV trafficked in a direct manner to either canalicular or basolateral membranes (Theard et al., 2007). In the polarized WIF-B9 cell line, BSEP (bile salt export pump; canalicular transport protein) cycles in and out of the canalicular membrane, moving into intracellular pools of rab11a-positive endosomes where it can translocate to the surface membrane as required (Wakabayashi et al., 2004). Internalized P-gp and Mrp2 in freshly isolated hepatocytes also may be held within endosomes until polarization is re-established, apparent after culturing hepatocytes for 2-3 days in a sandwich configuration (Hoffmaster et al., 2004; Zhang et al., 2005). Indeed, in hepatocytes cultured for 18-24 hr, canalicular proteins may be responsible for the intracellular accumulation
of organic anions in intracellular vesicles (Oude Elferink et al., 1993). As knowledge and understanding of protein function, expression and the mechanisms of protein trafficking increase, insight may be gained regarding why and how apical drug transport proteins are internalized following disruption of tight junctions and cell-to-cell contact with collagenase. Additionally, chemical modulation of protein trafficking pathways may allow re-direction of apical proteins to the basolateral membrane to facilitate efflux studies. Even if canalicular proteins could be redirected to the basolateral membrane, making the distinction between basolateral mediated efflux via canalicular proteins (e.g. P-gp, Mrp2, Bcrp) or basolateral proteins (e.g. Oatp1a1, Mrp3) without specific transport inhibitors would be difficult. Hepatocytes isolated from single, double or multiple knock-out transporter models might be useful for these types of studies.

Freshly isolated suspensions of hepatocytes are a well-established in vitro model to study drug transport and xenobiotic metabolism. Based on immunostaining profiles observed for the basolateral transport protein, Oatp1a1, the suspended hepatocyte model is an excellent first choice for studying initial hepatic uptake of drugs. However, results from uptake studies may be confounded by basolateral efflux transport proteins or bi-directionality of uptake transporters (Mahagita et al., 2007). Clearly, an important limitation of the suspended hepatocyte model is the localization of canalicular drug transport proteins. Based on the present data, canalicular transport proteins are internalized or confined to junctions between adjacent cells, and therefore, suspended hepatocytes are not an appropriate system to study apical efflux/canalicular excretion of drugs. Sandwich-cultured hepatocytes or canalicular membrane vesicles would be more suitable systems to estimate the contribution of apical proteins to efflux/biliary excretion (Nishida et al., 1991; Liu et al., 1999).
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Hirano M, Maeda K, Shitara Y and Sugiyama Y (2004) Contribution of OATP2 (OATP1B1) and OATP8 (OATP1B3) to the hepatic uptake of pitavastatin in humans. J Pharmacol Exp Ther 311:139-146.


taurocholic acid, the uncharged ouabain and the organic cations vecuronium and rocuronium.  


Footnotes

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Legends

**Figure 1** – P-gp or Mrp2 staining with Oatp1a1 or ZO-1 in freshly isolated rat hepatocytes. (i) 3D reconstruction of z-stack (1 μm xy slices), (ii) single internal 1 μM slice and (iii) cut through of z-stack. (A) Oatp1a1 (red) and P-gp (green), (B) Oatp1a1 (red) and Mrp2 (green), (C) ZO-1 (red) and P-gp (green) and (D) ZO-1 (red) and Mrp2 (green).

**Figure 2** – P-gp or Mrp2 staining with Oatp1a1 or ZO-1 in hepatocytes cultured for 1 hour. (i) 3D reconstruction of z-stack (1 μm xy slices), (ii) single internal 1 μM slice and (iii) cut through of z-stack. (A) Oatp1a1 (red) and P-gp (green), (B) Oatp1a1 (red) and Mrp2 (green), (C) ZO-1 (red) and P-gp (green) and (D) ZO-1 (red) and Mrp2 (green).

**Figure 3** – Accumulation of 1 μM CDF (green) in rat hepatocytes cultured for 1hr. (A) wild type cells, (B) wild type cells + 10 μM MK571, (C) TRc cells, (D) with differential interference contrast microscopy (DIC) and (E) without DIC. Representative images from multiple experiments.
Figure 1

A  Oatp1a1/P-gp

B  Oatp1a1/Mrp2

C  ZO-1/P-gp

D  ZO-1/Mrp2
Figure 2