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Sandwich-Cultured Hepatocytes: An *In Vitro* Model to Evaluate Hepatobiliary Transporter-Based Drug Interactions and Hepatotoxicity

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Abstract

Sandwich-cultured hepatocytes (SCH) are a powerful *in vitro* tool that can be utilized to study hepatobiliary drug transport, species differences in drug transport, transport protein regulation, drug-drug interactions, and hepatotoxicity. This review provides an up-to-date summary of the SCH model, including a brief history of, and introduction to, the use of SCH, as well as methodology to evaluate hepatobiliary drug disposition. A summary of the literature that has utilized this model to examine the interplay between drug metabolizing enzymes and transport proteins, drug-drug interactions at the transport level, and hepatotoxicity as a result of altered hepatic transport also is provided.

Keywords

Sandwich-Cultured Hepatocytes; Hepatotoxicity; Drug-Induced Liver Injury; Drug-Drug Interactions; Hepatocellularity; BEI; *in vitro* Biliary Clearance

Introduction

Hepatocyte cultures are a widely accepted *in vitro* tool to evaluate mechanisms of drug uptake and metabolism, as well as cytochrome P450 induction potential. However, the rapid loss of many liver-specific functions, redistribution of canalicular membrane proteins, loss of cell polarity and architecture including bile canaliculi, and the deterioration of cell viability within several days under conventional culture conditions precludes the use of this system for long-term studies and measurement of drug excretion (Groothuis et al., 1981, Borlak and Klutcka, 2004, Luttringer et al., 2002). In contrast, when cultured between two layers of gelled collagen (i.e. sandwich-cultured configuration) hepatocytes retain more *in vivo*-like properties, including formation of intact canalicular networks and polarized excretory function. Dunn et al. first demonstrated enhanced morphology and viability of hepatocytes cultured in a sandwich configuration, including normal levels of secretion of many liver-specific proteins and organic compounds including urea, albumin and bile acids

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Conflict of Interest: Dr. Kim Brouwer is a co-founder and Chair of the Scientific Advisory Board for Qualyst, Inc., which has exclusively licensed the sandwich-cultured hepatocyte technology for quantification of biliary excretion (B-CLEAR®).

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(Dunn et al., 1992, Dunn et al., 1989, Dunn et al., 1991). Subsequent studies have demonstrated that the sandwich configuration facilitates the formation of gap junctions and functional bile canalicular networks over days in culture (LeCluyse et al., 1994, Liu et al., 1998).

Many studies have utilized freshly isolated, suspended hepatocytes to examine efflux of compounds, but this approach is unable to differentiate between sinusoidal efflux and canalicular excretion (Oude Elferink et al., 1990, Studenberg and Brouwer, 1993, Tarao et al., 1982). Therefore, hepatocyte suspensions are appropriately restricted to investigating uptake mechanisms. Canalicular excretion has been studied in hepatocyte couplets, but this approach is limited to the use of fluorescent substrates (Graf and Boyer, 1990, Mills et al., 1999). The challenges associated with isolating highly purified fractions of hepatic basolateral and canalicular liver plasma membrane vesicles with proper orientation has limited the use of liver plasma membranes as a commonly employed approach to assess hepatic drug transport. Transfected systems, which are useful for characterizing transport function, identifying driving forces, and determining substrate specificity and inhibitors, are routinely used in drug development, although the relative *in vivo* contribution of a given protein to hepatic uptake and excretion is difficult to elucidate using current methodologies. The use of whole organ or *in vivo* studies may be able to address these fundamental questions, but typically these are more labor- and animal-intensive approaches with much lower throughput and greater compound requirements.

Liu et al. were the first to demonstrate that rat sandwich-cultured hepatocytes (SCH) could be used to investigate the hepatobiliary disposition of substrates (Liu et al., 1999b, Liu et al., 1999c). Initial work to establish the SCH model system involved optimization and characterization. Liu et al. investigated the effects of calcium on tight junction integrity and taurocholate (TC) accumulation in bile canaliculi (Liu et al., 1999c). This work demonstrated that tight junctions were the major diffusional barrier between the canalicular lumen and the extracellular space in SCH; tight junctions could be disrupted rapidly (within 3 min) by depletion of extracellular calcium without altering TC transport. Further work demonstrated that relevant hepatic transport proteins were expressed and properly localized in rat SCH (Turncliff et al., 2004, Hoffmaster et al., 2004, Zhang et al., 2005b), and were induced by well-established modulators such as dexamethasone (DEX) (Turncliff et al., 2004). P-glycoprotein (P-gp) co-localized with the canalicular marker protein dipeptidyl peptidase IV in day 4 and 6 rat and human SCH, respectively (Hoffmaster et al., 2004). Zhang and colleagues demonstrated by confocal microscopy that day 4 rat SCH exhibited extensive canalicular networks and distinct canalicular and basolateral domains, as evidenced by immunofluorescent localization of multidrug resistance-associated proteins 2 (Mrp2) and Mrp3, respectively. As clearly shown in Fig. 1, Mrp2 localized to the tubular structures of the bile canaliculi (green) and did not co-localize with the red staining in the basolateral membrane (Mrp3). Results of studies suggested the involvement of glycosylation in directing the canalicular localization of Mrp2 (Zhang et al., 2005b). A plethora of work has established the function of numerous hepatic uptake and efflux proteins in rat and human SCH. *In vitro* intrinsic biliary clearance values generated for compounds in SCH, and scaled biliary clearance values, correlate well with *in vivo* biliary clearance data measured with the same compounds in rats (Abe et al., 2008, Liu et al., 1999a, Fukuda et al., 2008) and humans (Abe et al., 2009, Ghibellini et al., 2007) (Fig. 2).

Another useful application of this model is in assessing human hepatobiliary drug disposition and hepatic transporter-based drug-drug interactions (DDI). However, the lack of fresh, healthy human liver tissue suitable for hepatocyte isolation and culture is a significant limitation. Fortunately, the establishment of commercially available, cryopreserved human hepatocytes that retain properties conducive for plating and culturing has expanded the use

of this model (Bi et al., 2006). The use of cryopreserved hepatocytes is a significant advantage that allows for greater access to hepatocytes and the ability to conduct routine studies on demand. Applications of this model have been extended to include hepatocytes from other species. The predominant non-rodent species used in preclinical development are dog and monkey. Methods have been established to culture dog and monkey primary hepatocytes in a sandwich configuration (Fig. 3) (Rose et al., 2006, Zhang, 2005), which could be particularly useful in examining species differences in the hepatobiliary disposition of compounds, adverse hepatotoxic events, and/or unexpected pharmacokinetic data. Higher throughput screening systems, such as mouse SCH, may prove to be particularly useful for unraveling the physiological, pharmacological and toxicological roles of hepatic proteins utilizing hepatocytes from gene-disrupted mice, and the phenotypic diversity offered by various mouse strains such as the Collaborative Cross (Threadgill et al., 2002). Towards that end, recent efforts have focused on optimizing conditions for culturing mouse hepatocytes in a sandwich configuration (Fig. 4) (Swift and Brouwer, 2010).

Methodology

Considerable effort by many investigators has been devoted to describing the influence of numerous culture conditions on hepatocyte viability, morphology, function and growth. A complete discussion of the effects of culture media, supplemental soluble factors, chemical modulators, and extracellular matrix on hepatocytes is beyond the scope of this review [see published reviews (LeCluyse et al., 1996, Ichihara et al., 1982, Rogiers and Vercruysse, 1993)]. Detailed below is a brief review of just a few of the many factors that influence the optimization of canalicular network formation, as well as transport protein expression and function in hepatocytes that are already in a differentiated state, specifically for purposes of studying hepatobiliary disposition.

It is well understood that plating hepatocytes onto inflexible or rigid substrata such as plastic or simple protein coatings, typical of conventional cell culture conditions, is not conducive to normal expression of liver-specific and cytochrome P450 genes (Reid and Jefferson, 1984). Therefore, liver-specific extracellular matrix proteins such as collagen (types I–IV), laminin, fibronectin, and heparin sulfate proteoglycans were evaluated as potential substrata in culture (Stamatoglou and Hughes, 1994, Reid et al., 1992). Results from various studies demonstrated type I and IV collagen were superior to fibronectin and laminin (Sudhakaran et al., 1986, Bissell et al., 1986, Sawada et al., 1987). Further studies focusing on collagen demonstrated that hepatocytes plated between layers of gelled type I collagen maintained a three-dimensional cuboidal shape and distribution of cytoskeletal proteins similar to that observed *in vivo* whereas hepatocytes grown on ungelled collagen flattened and spread to confluence (Rubin et al., 1981, Lindblad et al., 1991). Other types of substrata also have been used successfully to culture hepatocytes including biomatrix (Rojkind et al., 1980), a complex, partially-purified extract of extracellular matrix material prepared from normal rat liver, and Matrigel™ (Bissell et al., 1987), an acid-urea extract prepared from Engelbreth-Holm-Swarm tumor tissue excised from lathyritic mice (Kleinman et al., 1982, Orkin et al., 1977).

In addition to extracellular matrix, investigators also have focused on determining the critical components in media that are responsible for enhancing hepatocyte function and viability. The effects of various media supplements such as amino acids, hormones, growth factors, vitamins, trace elements and other cofactors on hepatocyte function and viability in primary culture have been described extensively (Berry et al., 1991, Dich and Grunnet, 1989, Guguen-Guillouzo and Guillouzo, 1983). A large variety of commercially available media formulations have been employed for culturing primary hepatocytes including Waymouth's MB-752/1, Ham's F12, RPMI 1640, Dulbecco's modified Eagle's medium,

Williams' medium E, Leibovitz' L15 and modified Chee's medium. The use of highly enriched media such as modified Chee's medium and Leibovitz' L15, which contain amino acid concentrations that are 5–10 times higher than most standard media, are superior for maintenance of cell survival, preserving cellular protein levels and liver specific functions (Jauregui et al., 1986, Sawada et al., 1987). Higher levels of amino acids have been suggested to aid in recovery of hepatocytes following collagenase digestion, arrest lysosomal protein degradation (Jauregui et al., 1988), and inhibit the rate of autophagic protein and RNA degradation while stabilizing the activity of some liver specific enzymes (Balavoine et al., 1993, Hutson et al., 1987, Lee et al., 1992, Seglen et al., 1980). Collectively, the results of these studies emphasize the importance of supplementing standard media with various commercially-available amino acid formulations. For example, Brouwer and co-workers supplement Dulbecco's modified Eagle's medium with L-glutamine and MEM non-essential amino acids solution (Invitrogen, Carlsbad, CA).

Culture media also have been supplemented with serum, based on reported findings following early attempts to culture rat hepatocytes *in vitro* (Bissell et al., 1973, Bonney et al., 1974) demonstrating that serum improves cell attachment, survival and morphology (Williams et al., 1977). However, serum has a dedifferentiating effect on hepatocytes, inhibits the induction effects of phenobarbital, inhibits the re-establishment of bile canaliculi, and favors attachment of non-parenchymal cells (Clayton and Darnell, 1983, Enat et al., 1984, Jefferson et al., 1984, Waxman et al., 1990, Terry and Gallin, 1994). Hepatocytes cultured on an extracellular matrix such as Matrigel™, or in co-culture with other cell types that promote a differentiated state, do not exhibit the same serum effects as those maintained on simple rigid substratum (Dunn et al., 1991, Guguen-Guillouzo et al., 1983, Ichihara, 1991, Vandenberghe et al., 1988, LeCluyse et al., 1994, Chandra et al., 2001). Other important supplements include the addition of hormones such as DEX and insulin. DEX increases secretion of blood clotting factors which aggregate into fibrin meshworks that are resistant to urea, promotes an ordered arrangement of the cytoskeleton, enhances gap junction expression and function, supports cytochrome P450 activity, curtails decreased protein synthesis that is observed during the first 24 h in culture, and enhances the formation of bile canalicular networks (Arterburn et al., 1995, Forster et al., 1986, Kwiatkowski et al., 1994, Ren et al., 1994, Lambiotte et al., 1973, LeCluyse et al., 1994). Insulin is reported to improve survival and attachment, enhance amino acid transport, protein synthesis, glycogenesis and lipogenesis; insulin also inhibits protein degradation and potentiates the inhibition of RNA degradation by amino acids (Balavoine et al., 1993, Ballard et al., 1980, Chapman et al., 1973, Laishes and Williams, 1976, Dahn et al., 1993, Flaim et al., 1985, Guguen-Guillouzo and Guillouzo, 1983, Schwarze et al., 1982).

Optimization of bile canalicular network formation in SCH is critical for studying hepatobiliary disposition and accurately assessing biliary excretion. Chandra et al. refined culture conditions for determining the hepatobiliary disposition of TC in rat SCH by evaluating the time of collagen overlay, the volume of collagen (rat tail type I), media type, and media additives (Chandra et al., 2001). Results demonstrated that Williams' medium E and Dulbecco's modified Eagle's medium produced greater taurocholate accumulation and biliary excretion compared to modified Chee's medium. Other parameters, such as time and volume of collagen overlay, Dulbecco's modified Eagle's medium additives such as 5% fetal bovine serum or DEX (0.1 μM versus 1 μM) did not produce significant differences in taurocholate accumulation or biliary excretion (Chandra et al., 2001). A separate study investigated the effects of culture conditions such as extracellular substratum, culture medium, and cell density on the expression and function of the bile salt export pump (Bsep), Mrp2, and multidrug resistance 1 (Mdr1a/b, P-glycoprotein, P-gp) in rat SCH (Turncliff et al., 2006b). In general, protein expression and function were not influenced by the extracellular matrices examined (rat tail gelled collagen type I vs. Biocoat™). Similar to the

findings of Chandra et al., Williams' medium E and Dulbecco's modified Eagle's medium, compared to modified Chee's medium, resulted in greater function of Bsep, Mrp2 and Mdr1a/b based on hepatocellular accumulation and biliary excretion of taurocholate, 5(and 6)-carboxy-2',7'dichlorofluorescein (CDF), and rhodamine 123. Decreasing seeding density from 100% confluency significantly altered Mrp2 and Mdr1a/b expression and/or function (Turncliff et al., 2006b). Work by Zhang and colleagues investigated the influence of 6-, 12- and 24-well plates, three different types of media [Williams' medium E, Dulbecco's modified Eagle's medium, and hepatocyte maintenance medium (Cambrex Corporation, Baltimore, MD)], and two different extracellular matrix substrata [gelled collagen (rat tail type I) under- and overlay (GC/GC) and Biocoat™ (collagen type I, BD Biosciences, Bedford, MA) with Matrigel™ (BD Biosciences, Bedford, MA) overlay (BC/MG)] on transport protein function and expression in fresh, primary human SCH (Zhang et al., 2005a). Various media types and plate formats resulted in no difference in the biliary excretion of the probe substrates taurocholate, digoxin, estradiol-17-β-D-glucuronide and D-penicillamine^{2,5}enkephalin. BC/MG yielded higher attachment and increased levels of P-gp, Bsep and Mrp2 protein compared to GC/GC. The use of Biocoat™ plates with Matrigel™ overlay has the additional advantage of uniformity, due to the lack of interindividual differences in manual coating with gelled collagen. Further work with cryopreserved human (Bi et al., 2006) and freshly isolated mouse hepatocytes (Bi et al., 2006, Swift and Brouwer, 2010) has confirmed the superiority of the BC/MG combination. Interestingly, dog hepatocytes cultured on Biocoat™ plates and overlaid with an alkaline gelled collagen (rat tail type I; pH 9.0) resulted in increased excretion of taurocholate compared to physiologic pH 7.4 (Rose et al., 2006).

There are many other parameters in addition to media type/components and extracellular matrix to consider when culturing hepatocytes in a sandwich configuration for hepatobiliary transport studies. The extent of bile canalicular network formation and optimal levels of metabolizing enzymes and transport proteins are important factors to consider when determining the appropriate time in culture to conduct functional studies. LeCluyse et al. showed the development of bile canaliculi in rat SCH during the first 24–48 h, followed by a more uniform and homogeneous network through days 3–7; canalicular networks were maintained for at least 2–3 weeks (LeCluyse et al., 1994). The biliary excretion of TC increased and reached a maximum value four days after overlay of rat hepatocytes (Liu et al., 1999b). Likewise, the biliary excretion of [D-penicillamine^{2,5}]enkephalin, a good P-gp substrate, increased up to day 4 after overlay and then started to decrease (Hoffmaster et al., 2004). The levels of many hepatic transport proteins in rat and human SCH over days in culture have been determined (Fig. 5); proper localization of Mrp2 and Mrp3 (Fig. 1), as well as P-gp have been investigated at day 4 after overlay in rat SCH (Hoffmaster et al., 2004, Zhang et al., 2005b). The dynamic process of canalicular network formation may take longer for human SCH, which are typically cultured for up to 6–10 days after overlay. The opposite is true for mouse hepatocytes, in which optimal cell morphology and biliary excretion of TC and CDF occurred on day 3 after overlay (Swift and Brouwer).

Cell density influences the morphology of hepatocytes in culture and is important for cell-cell contact (Hamilton et al., 2001). The confluency of hepatocytes cultured in a sandwich configuration is also crucial to the induction response. Initially, cryopreserved hepatocytes were unsuitable for induction studies because of their low attachment efficiency and poor quality. A great deal of emphasis has been placed on improving cryopreservation techniques to enhance the quality of hepatocytes and increase recovery and attachment. Greuet et al. demonstrated that maintaining primary human hepatocytes at a low density in the presence of epidermal growth factor (promoting proliferation and dedifferentiation) reduced the ability of the cells to respond to the prototypical inducer rifampin (Greuet et al., 1997). Decreased seeding densities of human hepatocytes to yield confluencies of 50, 30 and 25%

of normal caused a decrease in the induction of CYP3A4 by 10 μM rifampin, as measured using the probe substrate testosterone; decreased seeding density also changed the phenotype of the hepatocytes resulting in more fibroblast-like (de-differentiated) characteristics (LeCluyse, 2001). Furthermore, Turncliff et al. demonstrated that a decrease in seeding density from 100% confluency resulted in decreased Mrp2 and Mdr1a/1b expression and/or function (Turncliff et al., 2006b). See Table 1 for a summary of recommended seeding densities based on the literature and experience by Brouwer and coworkers.

Hepatobiliary disposition is evaluated on a specific day (or range of days) in culture based on optimal canalicular network development in each species. Two different protocols using B-CLEAR® technology (U.S. Pat. No. 6,780,580, Pat. No. 7,604,934 and other US and International patents both issued and pending) to quantify hepatic uptake and biliary excretion are discussed below; calcium depletion is utilized to disrupt the tight junctions and subsequently rinse the bile canalicular networks.

Accumulation Studies

This protocol involves initial incubation of SCH with Hanks' Balanced Salts solution (HBSS) containing Ca^{2+} (standard HBSS) or Ca^{2+}-free HBSS for 10 min [to maintain tight junction integrity and bile canalicular networks, or disrupt tight junctions and open bile canalicular networks, respectively (Fig. 6)]. Accumulation of taurocholate and CDF in rat SCH was reduced within one to two minutes following incubation with Ca^{2+}-free HBSS (Liu et al., 1999b, Liu et al., 1999c). The bile canalicular space also was reduced based on phase contrast microscopy after a 10-min incubation with Ca^{2+}-free HBSS (Liu et al., 1999c). Following the 10-min preincubation, buffer is removed and all cells are incubated with substrate(s) in standard HBSS for a predetermined period of time based on the substrate (time and concentration should be within the linear range of uptake, preferably within the first 10 to 15 min). This short-term accumulation phase should be conducted in standard HBSS to prevent potential interference due to the absence of Ca^{2+} on substrate uptake in SCH. Subsequently, SCH are rinsed three times with ice-cold standard buffer, lysed, and lysate is subjected to analysis for determination of substrate accumulation, by scintillation counting, high-performace liquid chromatagraphy (HPLC) or liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) in cells+bile (hepatocytes preincubated in standard HBSS) and cells (hepatocytes preincubated in Ca^{2+}-free HBSS). Liu et al. demonstrated that rat SCH incubated for 10 min in Ca^{2+}-free HBSS followed by incubation in Ca^{2+}-containing HBSS exhibited gradual restoration of bile canalicular accumulation of taurocholate, reaching ~80% of standard HBSS-treated cells after 60 min; canaliculi re-dilated based on phase contrast microscopy (Liu et al., 1999c). Optimal study design should take into account the possibility that measurable substrate may begin to accumulate in bile canaliculi of SCH preincubated in Ca^{2+}-free HBSS within 30 min following exposure to Ca^{2+}-containing HBSS due to re-establishment of tight junction integrity. Furthermore, as discussed below, incubation of rat SCH in Ca^{2+}-free HBSS for more than 30 min causes increased cell death.

The intracellular concentration of a given substrate may be estimated from the accumulated mass and the hepatocellular volume. The average hepatocellular volume of rat SCH in 6-well and 24-well plates was 6.2×10^{-6} μl/hepatocyte, determined using 3-O-methyl-D-glucose, a metabolically stable hexose that is transported across cell membranes by facilitated diffusion [(Uhal and Roehrig, 1982), Jin K. Lee, personal communication, Qualyst, Inc., Durham, NC].

Hepatic uptake clearance of substrates may be quantified based on the total accumulation of substrate in standard HBSS in the linear range of uptake according to the following equation:

$$\textit{in vitro} \quad Cl_{uptake} = \frac{Accumulation_{Cells+Bile}}{AUC_{0-T}} \tag{1}$$

where $AUC_{0\text{-}T}$ represents the product of the incubation time (T) and the initial concentration in the medium, assuming that the concentration at T is not less than 10% different from the initial concentration. In the absence of sink conditions, the AUC should be calculated based on the log-linear trapezoidal method.

Using B-CLEAR® technology, the biliary excretion index (BEI) of substrates may be quantified based on the fraction of accumulated substrate that resides in the bile canaliculi using the following equation:

$$BEI = \frac{Accumulation_{Cells+Bile} - Accumulation_{Cells}}{Accumulation_{Cells+Bile}} \times 100 \tag{2}$$

and *in vitro* biliary clearance ($Cl_{biliary}$) may be quantified based on the total accumulation of substrate in the bile canalicular networks divided by the area under the concentration curve of the dosing medium using the following equation:

$$\textit{in vitro} \quad Cl_{biliary} = \frac{Accumulation_{Cells+Bile} - Accumulation_{Cells}}{AUC_{0-T}} \tag{3}$$

In vitro $Cl_{biliary}$ values can be scaled to per kilogram of body weight, depending on the species (see Table 2). *In vitro* $Cl_{biliary}$ values have been shown to correlate well with *in vivo* intrinsic $Cl_{biliary}$ values in rats and humans for a number of compounds (Fig. 2) (Yue et al., 2009, Abe et al., 2008, Ghibellini et al., 2007, Liu et al., 1999a, Fukuda et al., 2008). Use of the well-stirred model for scaling $Cl_{biliary}$, expressed as μL/min/million cells, to *in vivo* hepatic clearance, expressed as mL/min/kg body weight, has been shown to correlate well in rats (Abe et al., 2008).

Inhibitors may be added during the preincubation phase in standard and Ca^{2+}-free HBSS to preload the hepatocytes with inhibitor, and/or added during the substrate uptake phase in standard HBSS. However, depending on the inhibitor, modulation of substrate disposition may be due to inhibition of uptake, and/or inhibition of basolateral and/or canalicular efflux, making the results difficult to interpret. Inducers may be added to the culture medium for specified periods of time prior to conducting uptake studies. In order to avoid direct effects of the inducer or inhibitor on substrate uptake, a modulator-washout phase may be necessary, and modulators also may need to be removed from the standard HBSS buffer during the uptake phase.

Efflux Studies

Compared to the accumulation protocol, basolateral and canalicular efflux studies in SCH are defined by the reversal of substrate incubation and tight junction disruption steps. This efflux approach was first evaluated by Liu (Liu et al., 1999b), and has been utilized by Kostrubsky and colleagues as well as Jemnitz and coworkers in a number of studies (Feng et al., 2009, Kostrubsky et al., 2006, Lengyel et al., 2005, Lengyel et al., 2008). First, the

substrate of interest is incubated with SCH in standard HBSS to preload the cells. The loading phase is stopped by removing the buffer and washing hepatocytes three times with warm standard HBSS or Ca^{2+}-free HBSS. Efflux studies are initiated by incubating preloaded hepatocytes with their respective buffers; aliquots are taken at designated time points for no longer than 30 min (Fig. 7). If inhibitor(s) are included in the incubation during the efflux phase, the amount of substrate in the lysed SCH at the end of the efflux phase must be measured in addition to the amount of substrate in the aliquots in order to evaluate potential modulation of the transport of the preloaded substrate. The degree of basolateral and canalicular efflux can be determined based on the following equations:

$$\text{Basolateral Efflux} = \text{Total Mass in Efflux Medium}_{\text{Standard HBSS}} \tag{4}$$

$$\text{Canalicular Efflux} = \text{Total Mass in Efflux Medium}_{Ca^{2+}-\text{free HBSS}} - \text{Total Mass in Efflux Medium}_{\text{Standard HBSS}} \tag{5}$$

In both cases, if multiple samples of HBSS buffer are taken over a period of time, the change in volume needs to be accounted for based on the number and volume of aliquots taken. Both basolateral and canalicular efflux values can be normalized to the total amount of pre-loaded substrate using the sum of the total mass in the efflux medium and hepatocyte lysate at the end of the efflux phase.

There are several caveats that must be considered when designing efflux experiments and interpreting the results. First, it is imperative that the incubation with Ca^{2+}-free HBSS is for a limited period of time (e.g., not greater than 30 min). Omission of extracellular Ca^{2+} from cells incubated with a buffered salt solution is known to result in cell death (Reed et al., 1990); rat SCH incubated in Ca^{2+}-free HBSS exhibited a significant increase in lactate dehydrogenase (LDH) leakage within 30 min (Fig. 8). Another limitation to this method is the unknown degree of substrate accumulation in the bile canaliculi during the preloading period, which is not washed away when transport is stopped and dosing solutions are removed. This may result in an overestimate of canalicular efflux. If chemical inhibitors of active transport processes are added during the substrate preloading phase, as discussed above, decreased efflux may be due to impaired uptake and/or efflux; evaluation of the sum of the total mass in the efflux medium and hepatocyte lysate in the absence and presence of inhibitor(s) would be required to distinguish between these two possibilities. Alternatively, inhibitor(s) could be added after the preloading step to avoid potential interference with the uptake process when concomitantly incubated. However, the time required for the inhibitor(s) to reach the site of transport inhibition must be considered in this study design. As mentioned with the accumulation protocol, perturbations in substrate disposition observed during the efflux period may be due to inhibition of basolateral or canalicular efflux, as well as to inhibition of substrate re-uptake. Another consideration is that medium concentrations may be influenced significantly by basolateral efflux of substrate during the efflux phase due to the reversal of the concentration gradient. In contrast, basolateral efflux of substrate would be expected to have minimal effects on medium concentrations in the accumulation protocol due to sink conditions.

The use of chemical inhibition to investigate the role of specific transport proteins in the disposition of substrates is widely used and accepted. It is not the most precise method, however, due to incomplete knowledge about the specificity of inhibitors for individual transport proteins. Naturally occurring genetically-deficient rodents lacking a specific transport protein such as the Mrp2-deficient Wistar (TR^{-}) and Eisai hyperbilirubinemic Sprague-Dawley rats (EHBR), and the Mdr1a-deficient CF-1 mice, have been useful tools

for elucidating the role of these transport proteins in the disposition of endogenous compounds and xenobiotics; incorporation of hepatocytes from these mutants in the sandwich-cultured model has been of great utility. Abe and colleagues confirmed *in vivo* reports that olmesartan, valsartan, pravastatin and rosuvastatin were Mrp2 substrates by demonstrating that SCH from TR^- rats exhibited decreased BEI and *in vitro* $Cl_{biliary}$ relative to SCH from transport-competent Wistar controls (Abe et al., 2008). Interestingly, pitavastatin BEI was reduced despite previous data demonstrating that the *in vivo* biliary excretion of pitavastatin was similar in wild-type and EHBR rats (Hirano et al., 2005). Further investigation revealed a novel finding: breast cancer resistance protein (Bcrp) levels were considerably lower in SCH from TR^- rats compared to Wistar control rats based on immunoblot analysis (Abe et al., 2008). Many transport proteins have been knocked out alone or in combination in mice using various gene disruption techniques. However, mouse SCH have not been investigated as extensively as rat or human SCH, and mouse hepatocytes are known to be more difficult to maintain in culture.

RNA interference (RNAi) leads to post-transcriptional, sequence-specific gene silencing and is a powerful tool to study the effect of loss-of-function of genes. The application of this technology to suppress specific transport proteins in SCH (U.S. Pat. No. 6,780,580, Pat. No. 7,601,494 and other US and International patents both issued and pending) has provided another tool, in addition to knockout mouse models, to gain insight into the function of specific transport proteins. Brouwer and coworkers successfully applied this approach using small interfering RNA (siRNA) targeting Mrp2 and Mrp3; protein levels were decreased by ~50% in rat SCH, and Mrp2 and Mrp3 function also was decreased based on the hepatobiliary disposition of CDF, a probe substrate for both transport proteins (Tian et al., 2004). More recently, Brouwer and coworkers have used adenoviral vector-mediated delivery of short hairpin (sh) RNA to knock down Bcrp in rat and human SCH; decreased expression and function have been confirmed using the probe drug, nitrofurantoin (Yue et al., 2009).

Another unique tool that has been utilized with the SCH model system is incubation with fluorescent probes to study transport protein expression, localization and function. CDF and cholyl-lysyl-fluorescein (CLF), a fluorescent bile acid, have been used to visualize the development of bile canalicular networks over days in culture (Kostrubsky et al., 2003, Liu et al., 1999b, LeCluyse et al., 1994, Zhang et al., 2005b, Bi et al., 2006). CDF accumulated in the canalicular networks of SCH from C57BL/6 wild-type but not *Abcc2*$^{-/-}$ (Mrp2 knockout) mice (Fig. 6), demonstrating the important role of Mrp2 in the biliary excretion of CDF. Ruthenium red staining also has been used to demonstrate that the bile canaliculi are sealed until Ca^{2+} depletion disrupts the tight junctions (Liu et al., 1999c). An important advantage of fluorescent probe substrates is the straightforward quantification by fluorescence spectroscopy. CDF diacetate (2 μM) has been used to phenotype the function of Mrp2 and Mrp3 after siRNA knockdown in rat SCH, as mentioned above (Tian et al., 2004). Rhodamine 123, a model P-gp substrate, has been used in rat SCH to assess P-gp-mediated biliary excretion, and to examine drug interactions affecting P-gp function (Annaert and Brouwer, 2005, Annaert et al., 2001). Cholyl-glycylamido-fluoroscein (CGamF) is a fluorescent bile acid that preferentially undergoes organic anion transporting polypeptide (Oatp)-mediated hepatic uptake, as opposed to taurocholate, which is predominantly Sodium taurocholate cotransporting polypeptide (Ntcp)-mediated. CGamF was used recently to assess drug interactions in hepatic uptake with the HIV protease inhibitors ritonavir, saquinavir, atazanavir, darunavir, and amprenavir in rat SCH (Ye et al., 2008). To date, application of fluorescent probes has focused primarily on characterizing hepatobiliary transport function and transporter-based DDIs. Fluorescent probe substrates, or fluorescence-tagged xenobiotics, may be useful in the future to elucidate mechanisms of intracellular trafficking of xenobiotics. For example, the innate fluorescence of the cation,

daunorubicin, was utilized to demonstrate the intracellular sequestration of daunorubicin in vesicles in the pericanalicular region of rat isolated perfused livers (IPLs) and isolated rat hepatocyte couplets (Hayes et al., 1999). Other work has shown the expression of functional P-gp when localized to the Golgi and mitochondria of doxorubicin-resistant K562 cells (Munteanu et al., 2006). This supports the hypothesis that other transport proteins may reside at intracellular sites and play a role in xenobiotic distribution within the hepatocyte. This could be investigated with the use of the sandwich-cultured model system.

Use of Sandwich-Cultured Hepatocytes to Investigate the Interplay Between Drug Metabolism and Transport

The liver plays a major role in the biotransformation of many endogenous and exogenous compounds, and also represents an important target organ for toxicity. Hepatic cell lines, precision-cut liver slices, and primary hepatocyte cultures from various species represent predominant and well-established *in vitro* systems for conducting these studies. Advantages of intact hepatocytes include the presence of: (1) interacting enzyme systems, intracellular machinery and physiological concentrations of cofactors allowing for coupled phase I and phase II reactions to take place; (2) the entire array of hepatic transport proteins involved in hepatic uptake and excretion, some of which may limit access to the site of metabolism; and (3) nuclear receptors, which allow regulation and induction of enzymes and transport proteins. The sandwich-cultured configuration is important for maintaining the differentiated morphology of hepatocytes and longevity in culture (Tuschl and Mueller, 2006). The major limitation to using hepatocyte cultures to study drug metabolism is the decline in cytochrome P450 enzyme activity (Hoen et al., 2000, Boess et al., 2003), although medium additives can help maintain function, as detailed below. Despite this, sandwich-cultured primary hepatocytes remain a well-established model for induction studies due to the lack of phenotypic gene expression in nearly all immortalized cell lines and the short-term viability of liver slices (LeCluyse, 2001).

In order to reverse the decline of cytochrome P450 enzymes, well known inducers such as phenobarbital and DEX have been added as medium supplements, separately or in combination (LeCluyse et al., 1996, Miyazaki et al., 1998, Pichard-Garcia et al., 2002). The addition of phenobarbital, DEX and β-naphthoflavone to the culture medium of rat SCH preserves phase I and II enzyme levels almost equivalent to those in liver *in vivo*, as determined by gene expression and enzyme activity analysis (Kienhuis et al., 2007). A discussion of the expression and function of phase I and phase II biotransformation enzymes in SCH is beyond the scope of this paper, but has been reviewed by many others (Hewitt et al., 2007a, Hewitt et al., 2007b, LeCluyse, 2001, Rogiers and Vercruysse, 1993, Maurel, 1996, Ferrini et al., 1997). The current review will focus on the interplay between drug metabolism and disposition of the parent and preformed or generated metabolites in the sandwich-cultured model system.

It is not surprising that interplay exists between transport proteins and metabolizing enzymes due to the common regulatory pathways mediated by the nuclear receptors, and the substrate overlap of the parent compound and/or generated metabolites for the transport proteins. The most well-studied example of this interplay is the common regulation and substrate/inhibitor overlap between P-gp and CYP3A4 (Wacher et al., 1995, Yasuda et al., 2002). This coordinated clearance pathway has been examined in rat SCH using the drug/metabolite pair terfenadine, a known CYP3A4 substrate (Yun et al., 1993, Jurima-Romet et al., 1994), and fexofenadine, both known P-gp substrates (Hait et al., 1993, Cvetkovic et al., 1999). Turncliff et al. determined the metabolism of terfenadine as well as the basolateral efflux and biliary excretion of terfenadine and fexofenadine (both preformed and generated) in control and DEX-treated rat SCH (Turncliff et al., 2006a). Treatment with 100 μM DEX

increased the formation rates of the terfenadine metabolites, azacyclonol and fexofenadine, approximately 20- and 2-fold, respectively. Pharmacokinetic modeling of the data indicated that the rate constant for hepatocyte uptake was faster for terfenadine compared with preformed fexofenadine (2.5 vs. 0.08 h^{-1}, respectively), whereas the biliary excretion rate constant for preformed fexofenadine exceeded that of terfenadine (0.44 vs. 0.039 h^{-1}, respectively). The rate constants for basolateral excretion of terfenadine and fexofenadine were comparable (3.2 vs. 1.9 h^{-1}, respectively) (Turncliff et al., 2006a). This study emphasizes the utility of the SCH model as an *in vitro* system capable of assessing, in an integrated fashion, metabolism, biliary excretion, and basolateral transport processes.

Rat SCH have been used to compare the hepatobiliary disposition of two orally active pentamidine analogues, and pharmacokinetic modeling has been utilized to examine the rates of hepatic uptake, metabolism, biliary excretion and basolateral efflux (Yan et al., 2008). Conversion of pafuramidine to the active metabolite, furamidine, is catalyzed by cytochrome b5/NADH-cytochrome b5 and many CYP450 enzymes, notably CYP4F (Wang et al., 2006, Saulter et al., 2005). The purpose of these studies was to determine the mechanism(s) responsible for the improved efficacy of DB868, an analog of the prodrug pafuramidine, in a late-stage mouse model of African trypanosomiasis. DB829, the active metabolite of DB868, has a higher systemic exposure, due either to increased metabolism and/or increased hepatic basolateral efflux of the active metabolite. The extent of formation of the active metabolite DB829 was consistently higher than that of furamidine over time in rat SCH. In addition, the net hepatic basolateral efflux was greater for DB829 compared to furamidine, consistent with pharmacokinetic modeling in which the rate constant representing basolateral efflux was increased 6-fold for DB829 compared to furamidine (Yan et al., 2008).

The examples given above involve phase I oxidation reactions, but additional examples have focused on the formation and hepatobiliary disposition of phase II conjugates. Wolf et al. characterized the accumulation, glucuronidation and excretion of morphine in rat SCH (Wolf et al., 2008). *In vitro* data were in good agreement with *in vivo* data indicating that morphine 3-glucuronide (M3G) was the primary metabolite formed (Wolf et al., 2008). In rat SCH, M3G was eliminated across the basolateral membrane; ~99% of the drug-related mass was recovered in medium during the 120-min study as M3G. M3G reuptake in rat SCH appeared to be negligible based on pharmacokinetic modeling, consistent with findings in the rat IPL when preformed M3G was administered (Doherty et al., 2006, Ouellet and Pollack, 1995). Another example of glucuronidation in rat SCH is with the opioid antagonist naloxone (Ansede and Brouwer, 2008). Following incubation of 10 μM naloxone for 80 min, less than 1% of naloxone was recovered in the hepatocytes or bile canaliculi; 89.3% of the dose was recovered as naloxone 3-glucuronide in medium (Ansede and Brouwer, 2008). This is consistent with *in vivo* rat studies demonstrating that naloxone glucuronide is the major metabolite, with 15.4 and 1.2% of naloxone 3-glucuronide recovered in urine and feces, respectively, after 96 hr (Misra et al., 1976). SCH from both humans and rats also are capable of forming other conjugates based on data generated utilizing troglitazone (Lee et al., 2010). Troglitazone sulfate was the predominant metabolite in medium and hepatocytes, followed by troglitazone glucuronide and troglitazone quinone, over a 120-min incubation period with 10 μM troglitazone in rat and human SCH. Similar to *in vivo*, the biliary excretion of trogitazone sulfate was greater than troglitazone glucuronide, while troglitazone and troglitazone quinone underwent negligible biliary excretion in rat and human SCH (Loi et al., 1999a, Loi et al., 1999b). The impact of modulating the biliary excretion of troglitazone sulfate and troglitazone glucuronide on hepatic accumulation was assessed utilizing a Monte Carlo simulation approach. Interestingly, changes in hepatocyte concentrations were more sensitive to changes in biliary excretion than medium

concentrations, suggesting that plasma concentrations may not reflect increased liver accumulation and the potential for hepatotoxicity *in vivo* (Lee et al., 2010).

Evaluation of other substrate/metabolite pairs undergoing biotransformation by phase I and/or II enzymes, and further characterization of the biliary elimination of unchanged compounds and generated metabolites, will enhance understanding of the utility of this model system. Mass balance data detailing the parent compound and generated metabolites in medium, cells and bile canaliculi, coupled with pharmacokinetic modeling, can provide a robust evaluation of the hepatobiliary disposition of compounds, including relative rates for the hepatic uptake, metabolism, basolateral efflux and biliary excretion of the parent compound and generated metabolites. The SCH model offers the ability to quantify the impact of modulation of both hepatic transport systems and metabolism of drugs on overall disposition in a single *in vitro* system, which has significant advantages over existing methodologies.

Sandwich-Cultured Hepatoctyes as a Tool to Predict Drug Interactions in Hepatobiliary Transport

Inhibition of Bile Acid Transport by Drugs

Vectorial transport of bile acids from the blood into bile is a multistep process starting with uptake into hepatocytes, mediated predominantly by NTCP (*SLC10A1*), with a minor component mediated by OATP (*SLCO*) isoforms. Bile acids are effluxed from hepatocytes into bile, in conjugated or unconjugated form, by canalicular ATP-binding cassette (ABC) transport proteins, primarily *ABCB11* (BSEP), but also by *ABCC2* (MRP2) and others (Marion et al., 2007). Basolateral excretion of bile acids from hepatocytes into blood is mediated by MRP3 (*ABCC3*) and MRP4 (*ABCC4*), providing a compensatory pathway under certain conditions, such as cholestasis or inhibition of canalicular excretion. The organic solute transporter (OSTα/β) recently was identified as another basolateral transport protein that functions to excrete bile acids and other compounds, similar to MRP3 and MRP4 (Boyer et al., 2006, Alrefai and Gill, 2007).

The association of elevated serum bile acid concentrations with drugs known to cause hepatotoxicity led to the hypothesis that altered hepatic bile acid transport may be one mechanism of drug-induced liver injury [DILI, (Funk et al., 2001b, Fattinger et al., 2001, Funk et al., 2001a, Stieger et al., 2000, Kostrubsky et al., 2001, Roman et al., 1990, Preininger et al., 1999)]. A number of hepatotoxic drugs, including bosentan, troglitazone, and cyclosporine, inhibit BSEP-mediated bile acid transport in isolated *in vitro* systems (Stieger et al., 2000, Funk et al., 2001b, Fattinger et al., 2001, Funk et al., 2001a, Byrne et al., 2002).

TC, a model bile acid, has been used to evaluate the potential for drugs to inhibit bile acid transport in the SCH system. Kostrubsky et al. demonstrated inhibition of TC transport in human SCH with a set of structurally diverse compounds that were eliminated preferentially via bile and known to cause liver toxicity in humans (cyclosporine A, bosentan, glyburide, troleandomycin and CI-1034) (Kostrubsky et al., 2003). Kostrubsky and colleagues also established that potent inhibition of TC efflux in SCH correlated with clinical reports of liver toxicity, and elevated serum bile acids in rats (see below: Direct Assessment of Cytotoxicity in SCH from Relevant Species). In a later study, Kostrubsky and colleagues again confirmed that potent inhibition of TC efflux was predictive of the hepatotoxic potential of nefazodone compared to a pair of non-toxic structural analogues, trazodone and buspirone (Kostrubsky et al., 2006). Nefazodone was a potent inhibitor of TC transport in Sf9-derived BSEP-expressing membrane vesicles; nefazodone also inhibited biliary excretion of TC in rat SCH, and elevated serum bile acids when administered to rats *in vivo*.

Nefazodone-associated decreases in protein synthesis as a measure of cellular toxicity, and the role of phase I and phase II metabolism in the hepatotoxicity of nefazodone also were evaluated, capitalizing on the ability of SCH to maintain a complex array of liver-specific functions [see below: Hepatocyte Accumulation of Toxic Species, (Kostrubsky et al., 2006)].

The role of impaired bile acid transport in troglitazone- and bosentan-associated hepatotoxicity initially focused on BSEP-mediated canalicular efflux as the primary mechanism leading to elevated serum bile acid concentrations (Funk et al., 2001b, Kostrubsky et al., 2001, Fattinger et al., 2001, Funk et al., 2001a, Byrne et al., 2002). More recently, Kemp et al. observed that while troglitazone and bosentan both decreased the BEI of TC in SCH, consistent with Bsep inhibition, cellular accumulation of TC did not increase, as might be expected (Kemp et al., 2005). In freshly isolated rat hepatocytes, 10 and 100 μM troglitazone and bosentan each significantly decreased the uptake rate and accumulation of the probe substrates TC (1 μM) and estradiol-17β-D-glucuronide (1 μM), an Oatp substrate, consistent with inhibition of both Ntcp- and Oatp-mediated uptake mechanisms. Taken together, these results suggest that drugs known to inhibit biliary excretion of bile acids also may inhibit hepatic uptake, thus attenuating elevations of intracellular bile acid concentrations and potential toxicity that may result. This phenomenon is analogous to down-regulation of basolateral uptake mechanisms in cholestatic conditions, which effectively minimizes accumulation of toxic bile acids inside hepatocytes (Zollner et al., 2003, Lee et al., 2000, Lee and Boyer, 2000, Gartung et al., 1996, Gartung et al., 1997). Preferential inhibition of basolateral uptake, as opposed to canalicular efflux, by drugs would similarly reduce hepatocyte exposure to bile acids, while increasing bile acid concentrations in the systemic circulation. This effect was explored by Leslie et al. as an explanation for the species difference in bosentan toxicity between humans and rats [see below: Direct Assessment of Cytotoxicity in SCH from Relevant Species, (Leslie et al., 2007)].

Inhibition of bile acid transport was identified as a potential mechanism of antiretroviral-associated hepatotoxicity using multiple model systems, including human and rat SCH (McRae et al., 2006). BSEP-mediated biliary excretion, and NTCP- and OATP-mediated uptake, of TC were inhibited differentially by the antiretroviral agents ritonavir, saquinavir, and efavirenz, but not nevirapine. Potential hepatocyte accumulation of TC due to BSEP inhibition was attenuated by concomitant inhibition of NTCP- and/or OATP-mediated uptake of TC. The hepatotoxic potential of the antiretroviral agents could not be predicted by rank order of TC inhibition in isolated uptake or efflux systems or SCH, suggesting that "hepatotoxicity associated with antiretroviral therapy is likely complex and multifactorial, such that inhibition of bile acid transport may be only one of several contributing factors (McRae et al., 2006)."

An important application of the SCH model is in classifying compounds based on their relative effect on bile acid uptake and/or efflux, which may provide useful information concerning compounds that alter bile acid concentrations *in vivo*. For example, cyclosporine A and glyburide decreased total accumulation (cells+bile), increased cellular accumulation, and decreased BEI and $Cl_{biliary}$ of deuterium-labeled taurocholate (d_8-TC), suggesting that bile acid efflux was affected primarily (Ansede et al., 2009 in press). In contrast, erythromycin-estolate, troglitazone and bosentan decreased d_8-TC accumulation (both cells +bile and cells), BEI and $Cl_{biliary}$ of d_8-TC, suggesting that bile acid uptake was the predominant pathway affected. Recently, the utility of a cassette dosing approach to identify compounds that inhibit hepatic uptake and/or excretion of bile acids was demonstrated in rat and human SCH (Wolf et al., 2009).

Given the requisite overlap in substrate specificity of basolateral and canalicular hepatic transport proteins necessary to mediate vectorial transport of bile acids, it is not surprising that xenobiotics may modulate multiple hepatic bile acid transport mechanisms. The relative effect of xenobiotics on both processes should be considered in order to predict the potential consequences of modulating bile acid transport, disposition and homeostasis. SCH have been used successfully to study hepatobiliary transport kinetics and mechanisms of both hepatic uptake and biliary excretion of bile acids, simultaneously.

Drug-Drug Interactions in Hepatobiliary Transport

Hepatic clearance is an important pathway of elimination for many, if not most, drugs. Many drugs and/or generated metabolites possess limited diffusion potential and rely on transport proteins to mediate uptake into hepatocytes and/or excretion into bile. Thus, DDIs at the level of hepatic uptake or efflux processes may be responsible for significant changes in plasma or hepatocellular drug concentration-time profiles, with potential consequences for therapeutic or toxic effects. Furthermore, identifying the extent and relevance of transporter-mediated DDIs in human liver can be challenging, due to inaccessibility of the liver and bile compartments. SCH combined with probe substrates exhibiting known transport protein specificity offer the ability to identify transport proteins involved in the disposition of new chemical agents, as well as the potential for new agents to be “victims” or “perpetrators” of DDIs, in a physiologically-relevant model system.

Early work was performed in the Brouwer laboratory to optimize and validate the rat SCH model for the study of P-gp-mediated biliary excretion using substrates such as rhodamine 123 and digoxin (Annaert et al., 2001). Addition of the P-gp (Mdr1) inhibitor, GF120918, resulted in a 75% reduction in the BEI of rhodamine 123 and digoxin. Prediction of hepatic P-pg-mediated drug interactions was later established in rat SCH using the high-affinity probe substrate rhodamine 123 and various P-gp modulators (Annaert et al., 2001, Annaert and Brouwer, 2005). The classical P-gp inhibitors verapamil and progesterone significantly reduced the BEI and *in vitro* $CL_{biliary}$ ofrhodamine 123, while inhibition by 100 μM quinidine did not reach statistical significance. Quercetin, a P-gp activator, significantly increased *in vitro* $CL_{biliary}$ but not the BEI of rhodamine 123, reflecting the fact that cellular accumulation was enhanced to a greater extent than excretion into bile. Treatment of SCH with verapamil, rifampin and DEX was generally inconclusive in terms of P-gp induction. However, results from DEX treatment implicated the involvement of an inducible element to the basolateral uptake of rhodamine 123, which was supported further by inhibition of rhodamine 123 uptake by digoxin, verapamil and quinidine, all documented substrates/inhibitors of Oatp1a4. Indeed, Turncliff, et al. demonstrated that 48 h DEX treatment induced protein expression and function of CYP3A1/2, Oatp1a4 and Mrp2, but not Mdr1 [see below: Drug-Hepatic Transport Protein Interactions (Turncliff et al., 2004)]. In rat SCH, Mrp3 protein levels, determined by immunoblot analysis, were increased following incubation with 1 mM phenobarbital for 72 hr; diluted bile from Wistar wild-type and TR- rats also induced Mrp3, suggesting that one or more bile constitutents, other than glucuronide or glutathione conjugates, were responsible for Mrp3 induction (Fig. 9a). Mrp2 protein was increased after incubation of rat SCH for 72 hr with selected nuclear receptor agonists including cholic acid, chenodeoxycholic acid, phenobarbital and pregnenolone 16α-carbonitrile (Fig. 9b).

Transport of the fluorescent probe CDF in rat liver was characterized using a number of model systems (Zamek-Gliszczynski et al., 2003). Involvement of Mrp2 and Mrp3 was confirmed in Mrp-expressing Sf9 membrane vesicles and IPLs from wild-type (WT) and Mrp2-deficient TR^- rats. Saturable CDF uptake was observed in WT rat SCH and probed using nonspecific Oatp inhibitors, bromosulfophthalein (BSP) and probenecid, and specific inhibitors digoxin (Oatp2), 100 μM para-aminohippurate (PAH) (Oat2), and 1 mM PAH

(Oat3). Only BSP and probenecid inhibited uptake, suggesting CDF is taken up by an Oatp isoform(s) other than Oatp2.

Annaert and colleagues characterized the kinetics of the fluorescent bile acid analogue CGamF in rat SCH and assessed its utility as a probe for studying interactions with hepatic uptake of endogenous or exogenous compounds using seven HIV protease inhibitors [PI, (Ye et al., 2008)]. The known Oatp inhibitor, rifampin, reduced uptake of 1 μM CGamF by 70% (vs. 25% for 1 μM TC); removal of sodium produced no change in CGamF while completely abolishing TC uptake. These observations confirm the well-described routes of TC uptake (80% NTCP/20% OATPs) and suggested a complimentary role for CGamF as a more sensitive probe for Oatp-mediated inhibition (Meier et al., 1997, Kouzuki et al., 2000). Comparing the inhibitory potential of HIV PIs on the uptake of TC and CGamF in standard and sodium-free conditions, a preferential interference with sodium-independent processes (likely Oatps) emerged. Another notable observation was the striking potency of ritonavir (IC_{50} = 0.25 μM) as an inhibitor of CGamF uptake compared to other PIs, indicating that ritonavir may be a potent Oatp inhibitor, which is not detectable using TC in standard (+ sodium) conditions. The opportunity remains to elucidate the extent and possible mechanisms of CGamF biliary excretion and potential for use as a probe of overall hepatobiliary disposition.

Treijtel et al. utilized rat SCH to explore multiple processes responsible for the clearance of the frequently co-administered HIV PIs saquinavir and ritonavir (Treijtel et al., 2009). Saquinavir metabolism was significantly reduced by 5 μM ketoconazole, a known Cyp3a inhibitor, and was even more sensitive to 1 μM ritonavir, confirming the role of metabolism in the saquinavir-boosting effect of ritonavir coadministration. Biliary excretion of ritonavir was investigated in rat SCH in the presence and absence of the P-gp inhibitors verapamil, cyclosporine A and PSC833. Verapamil had little to no effect, but cyclosporine A and PSC833 decreased ritonavir cellular accumulation ~50% due to decreased biliary excretion and subsequently increased metabolism (Treijtel et al., 2009).

In a recent publication from Brouwer and coworkers, fexofenadine was evaluated as a probe for studying hepatobiliary transport using a combination of pharmacokinetic modeling/simulation of clinical and preclinical data from human SCH and rat isolated perfused livers (IPLs) (Swift et al., 2009). Simulation studies indicated that systemic exposure to fexofenadine in humans was sensitive to changes in hepatic uptake but not biliary efflux rates. Inhibition of P-gp-mediated fexofenadine efflux in human SCH using GF120918 had no effect on fexofenadine BEI or *in vitro* $CL_{biliary}$, further supporting simulation results. However, fexofenadine is a known substrate of additional canalicular efflux proteins, MRP2 and BSEP, which cannot be selectively modulated with known chemical probes, but presumably could compensate for P-gp inhibition. Of note, pharmacokinetic simulations also indicated that inhibition of fexofenadine biliary excretion may result in a significant (~20–60%) increase in hepatic exposure that is undetectable based on monitoring plasma concentrations. These studies demonstrate the utility of SCH in combination with pharmacokinetic modeling and simulation to explore and confirm mechanisms of DDIs.

The maintenance of cell polarity *in vitro* in the SCH model allows the unique ability to simultaneously evaluate hepatic uptake and efflux processes at the basolateral membrane, and biliary excretion processes at the canalicular membrane. A number of chemical probes with known transport protein specificity and modulating effects (inhibition, activation, induction) further facilitate the prediction of DDIs at the level of hepatic transport proteins. Despite previous work to establish conditions for evaluating interactions with individual or limited combinations of hepatic transport proteins, “a sufficiently diverse panel of model substrates (and inhibitors) needs to be identified and profiled with respect to transporter

affinity and interaction," to enable "rapid and reliable screening for drug interaction and cellular toxicity potential (Ye et al., 2008)."

Drug-Hepatic Transport Protein Interactions

Xeno- and endobiotics modulate transport proteins in a number of ways besides direct, competitive interactions at the active site(s) responsible for binding and translocation of substrates. Alterations in transport protein regulation at the level of transcription, translation or post-translational modifications do not require direct interactions with the transport protein. Both transcriptional and post-transcriptional regulation involves complex networks of co-activators and co-repressors that provide multiple checkpoints and ensure balanced activity of the pathways under normal conditions. The time scale of changes in transport protein expression and activity are different, however. Transcriptional regulation produces long-term changes in the total amount of transport protein that is available, while short-term changes can only result from a re-arrangement of preexisting protein, which is mobilized as a result of post-translational modifications.

A number of ligand-activated transcription factors have been associated with altered gene expression of hepatic transport proteins in various *in vitro* and *in vivo* models, including the pregnane X receptor (PXR/NR1I2), constitutive androstane receptor (CAR/NR1I3), farnesoid X-activated receptor (FXR/NR1H4), retinoid X receptor alpha (RXRα/NR2B1), aryl hydrocarbon receptor (AHR), peroxisome proliferator-activated receptors (PPARα,γ/NR1C1,3), glucocorticoid receptor (GR), small heterodimer partner (SHP), liver X receptor (LXR), nuclear factor E2-related factor 2 (Nrf2) and hepatic nuclear factor 1 alpha [HNF1α, (Kast et al., 2002, Jigorel et al., 2006, Klaassen and Slitt, 2005, Aleksunes et al., 2009, Staudinger et al., 2003, Kullak-Ublick and Becker, 2003). Similar to the transport proteins themselves, nuclear receptors (NRs) are bound by an overlapping spectrum of endogenous (hormones, bile acids) and exogenous (drugs, toxins) ligands (Parks et al., 1999, Moore et al., 2000). Thus, drug-induced NR activation may result from direct drug interactions with a given NR(s), or by altered intracellular disposition of endogenous agents, such as bile acids, that are themselves NR ligands. Examples of drugs known to directly activate transcription factors include: PXR - DEX, spironolactone; PPAR - fibrates; Nrf2 – ursodeoxycholic acid (UDCA) (Maher et al., 2005, Cheng et al., 2005, Okada et al., 2008). Other drugs, such as phenobarbital and rifampin, can alter transport protein function by both direct (competitive) and indirect (up- or down-regulation) mechanisms (Patel et al., 2003, Lam et al., 2006). Such complex and potentially confounding mechanisms present a serious challenge to differentiate between transport protein induction and inhibition.

Although mechanistic work to investigate transcriptional regulation using SCH has been minimal, routine sandwich-culture methodology takes advantage of maintained NR activity in primary hepatocytes. DEX has long been used as a media supplement due to its modulating effects on drug disposition mechanisms. DEX treatment is able to attenuate or reverse changes in gene expression observed in cultured hepatocytes and, together with a sandwich-cultured configuration, maintains expression of most relevant functions at near-physiological levels. DEX treatment of rat SCH restores physiologic expression of Mdr2 and Oatp1a4 mRNA by up-regulation, while maintaining Mdr1b and Mrp3 at baseline levels by preventing their up-regulation (Luttringer et al., 2002). However, Mrp2 mRNA is up-regulated by DEX beyond physiologic levels. The effects of DEX on protein expression and function in rat SCH have been evaluated (Turncliff et al., 2004). Forty-eight hr DEX pre-treatment increased expression of CYP3A1/2, Oatp1a4 and Mrp2, decreased expression of Ntcp, and had no effect on Oatp1a1, Mrp3, Mdr1a/b and Bsep. Modest changes in the transport of probe substrates were consistent with changes in protein expression, while patterns of protein expression generally agreed with earlier gene expression data by Luttringer et al., with the exception of Oatp1a4. Despite this example, mRNA and protein

expression levels do not always correlate, and must be evaluated on a case-by-case basis for transport proteins (Rodriguez-Antona et al., 2001, Ho et al., 2006).

Short-term regulation of hepatic transport protein trafficking to and from the plasma membrane is mediated by post-translational modifications and second messenger signaling pathways (Kipp et al., 2001, Anwer, 2004). “Signal” regions in the protein structure are necessary for proper vesicle sorting and trafficking of newly-synthesized membrane-bound proteins from the endoplasmic reticulum and Golgi network to final membrane locations. These signals can take the form of specific peptide sequences or post-translational modifications, such as glycosylation. The role of glycosylation in Mrp2 expression, localization and function was investigated in rat SCH (Zhang et al., 2005b). Although the amount of cellular Mrp2 protein did not change, a gradual increase (~10kDa by western blot) in molecular weight over days in culture correlated with increased staining at the canalicular membrane by confocal microscopy, on day 4, relative to diffuse intracellular and ER co-localization at day 0 or day 1 in culture. Molecular weight and canalicular localization were decreased by treatment with tunicamycin, an inhibitor of glycosylation.

Cellular trafficking also is mediated by other modification “signals,” such as phosphate or ubiquitin residues, on transport proteins themselves, or on the chaperone proteins that move them around within the cell (Glavy et al., 2000). The cellular machinery responsible for modifying and mobilizing proteins is under the control of a complex series of second messenger pathways. A number of second messengers have been associated with changes in transport protein expression, localization and function, including cyclic AMP (cAMP), cGMP, intracellular Ca^{2+}, phosphoinositol-3-kinase (PI3K), protein kinases A, B and C (PKA, PKB, PKC), and p38 mitogen activated protein kinase [MAPK, (Anwer, 2004, Roelofsen et al., 1998, Misra et al., 1998, Kubitz et al., 2004)]. The effect of cAMP and PKC activators, glucagon and phorbol 12-myristate 13-acetate (PMA), on Mrp3/MRP3 regulation was investigated in rat and human SCH and rat IPLs (Chandra et al., 2005). Glucagon increased CDF excretion and Mrp3/MRP3 localization at the basolateral membrane in both rat and human SCH, while PMA had no effect. Glucagon also increased CDF efflux in rat IPLs, suggesting that cAMP effects are recapitulated in the SCH model and similar across species. PMA decreased CDF efflux in rat IPLs, consistent with known down-regulation mediated by PKC activation, though it is unclear why PMA had no effect in SCH. These trafficking pathways can play a significant role in transport protein function, and may be modulated by xenobiotics, potentially including the recently developed family of kinase inhibitors (Katayama et al., 2007). SCH clearly demonstrate functional post-translational and second messenger pathways, although further characterization is needed.

Use of Sandwich-Cultured Hepatocytes to Predict Drug-Induced Liver Injury and Examine Mechanisms of Hepatotoxicity

DILI is historically one of the most common reasons that drugs fail in development or are removed from the market after being approved (Lee, 2003). Therefore, experimental models to better predict DILI are still needed. Furthermore, when DILI is predicted, it would be beneficial to understand the mechanism(s) of hepatotoxicity in order to assist in the design and testing of alternate compounds to circumvent toxic potential. In theory, a useful *in vitro* model would recapitulate relevant liver functions and mechanisms of hepatotoxicity, and resemble the *in vivo* situation as closely as possible. As detailed above, SCH are a unique *in vitro* system that maintains specific hepatic cytomorphology and function relevant to drug metabolism, disposition, and toxicity, and thus, closely resembles the *in vivo* setting.

Direct Assessment of Cytotoxicity in SCH from Relevant Species

It is an unfortunate fact that many hepatotoxic compounds go unrecognized in preclinical toxicology studies using animal models or screening for cytotoxicity. Species differences in metabolism or transport pathways can lead to marked differences in tolerance to toxic substances. In addition, *in vitro* models may not recapitulate the complex processes that often lead to gross toxicity. Therefore, the choice of preclinical toxicity models and endpoints can influence predictability of adverse events in humans.

SCH have contributed a great deal to our understanding of hepatotoxicity caused by inhibition of bile acid transport that may go unrecognized in animal studies. Kostrubsky and colleagues assessed the effects of numerous hepatotoxicants on human SCH [see above: Inhibition of Bile Acid Transport by Drugs (Kostrubsky et al., 2003, Kostrubsky et al., 2006)]. They established, for a series of macrolide antibiotics, that potent inhibition of TC efflux in human SCH correlated with clinical reports of liver toxicity, suggesting that rank order of *in vitro* TC inhibition may be predictive of hepatotoxic potential *in vivo*. Although preclinical rodent toxicity studies were previously negative, Kostrubsky and coworkers demonstrated that two inhibitors of TC transport in human SCH (CI-1034 and glyburide) caused a transient, 2.4-fold increase in serum bile acids when dosed individually *in vivo* in rats; coadministration resulted in a synergistic (6.8-fold) increase. Later, the effect of nefazodone was compared to its nontoxic structural analogues, buspirone and trazodone, in human SCH and intact rats (Kostrubsky et al., 2006). Cellular toxicity was assessed in SCH by measuring a decrease in protein synthesis after drug treatment and pulse labeling with ^{14}C-leucine. Toxicity was associated exclusively with nefazodone, and correlated with accumulation of parent drug (see below: Hepatocyte Accumulation of Toxic Species). Nefazodone, but not buspirone, elevated serum bile acids in rats 1 hr after dosing, which was resolved by 24 hr. These studies provide several preclinical alternatives with the potential to identify hepatotoxic compounds that act by different mechanisms and demonstrate species differences.

Mechanistic understanding of species differences in hepatotoxicity has the potential to identify new opportunities for predicting toxic events that previously went undetected until reaching the clinic. The hepatotoxic potential of HIV PIs was compared in human and rat SCH, with similar patterns of TC inhibition, although PIs appeared to be more potent inhibitors of rat than human TC biliary efflux (McRae et al., 2006). Rank order of inhibition of TC efflux was not able to predict PI hepatotoxicity, as it did for macrolide antibiotics (Kostrubsky et al., 2003). However, it was shown that potential accumulation of bile acids due to PI-mediated BSEP inhibition was attenuated by concomitant inhibition of TC uptake in human SCH, similar to the findings of Kemp et al. in rat SCH (Kemp et al., 2005). In another publication from Brouwer and coworkers, this effect was explored as an explanation for the species difference in bosentan toxicity between humans and rats (Leslie et al., 2007). Although bosentan inhibited uptake as well as efflux in human SCH, resulting in decreased TC accumulation, inhibition of bile acid uptake is not expected at relevant concentrations in human. However, bosentan-mediated inhibition of Na^+-dependent TC uptake was approximately 6− and 30-fold more potent in rat vs. human suspended hepatocytes and NTCP-/Ntcp-transfected HEK cells, respectively. Thus, preferential inhibition of rat Ntcp remains a plausible explanation for the observed resistance of rats to bosentan-induced hepatotoxicity observed clinically in humans.

Similarly, differential effects on TC disposition were observed in the seminal publication establishing sandwich-cultured conditions for dog and monkey hepatocytes (Rose et al., 2006). Glyburide abolished TC efflux in dog SCH, with no effect on efflux in monkey SCH. Conversely, glyburide inhibited TC uptake in monkey but not dog SCH, while cyclosporin A inhibited efflux but not uptake similarly in both dog and monkey SCH. These marked

differences in TC inhibition underscore potential differences in observed hepatotoxic potential across preclinical species.

SCH recently were evaluated for the potential to assess hepatobiliary disposition of copper (Ansede et al., 2009). Copper (Cu) is an essential nutrient necessary for the activity of various enzymes, but also can accumulate to toxic levels when Cu elimination is disrupted by drugs or genetic disorders, such as in Wilson's Disease, which is characterized by massive Cu accumulation in lysosomes and lack of excretion in bile, the major route (85%) of Cu elimination. Hepatic Cu disposition is fairly well characterized: basolateral uptake is mediated by Cu transporter 1 (CTR1), while the ATP-dependent ATP7B transports Cu into trans-Golgi Network (TGN) vesicles for incorporation into ceruloplasmin and, apical membrane vesicles for biliary excretion (via exocytosis) of excess Cu. Cu transport was evaluated in rat, dog and human hepatocytes. CTR1/Ctr1 and ATP7B/Atp7b expression was confirmed in hepatocytes from all species by western blot analysis, with slight changes in expression observed in some species following exposure to Cu-supplemented media. CTR1/Ctr1 expression was decreased slightly in human and rat SCH, while Atp7b was elevated slightly in rat SCH; all changes were consistent with a protective role of reducing hepatic exposure in the presence of excess Cu. Relative differences in endogenous Cu levels in SCH from rat, dog and human (17.2, 490 and 43.5 ng/well, respectively) were consistent with published values for Cu content in livers of intact animals from corresponding species (3.56–5.56, 57.32–173.3, 6.2 μg/g in rat, dog and human, respectively). Increased uptake and efflux were observed with increases in Cu concentration and incubation time, confirming functional Cu transport and the potential for SCH to serve as a useful model to study the role of transport in Cu homeostasis.

Various assays have been used to assess viability in cytotoxicity studies as a direct measure of toxic effects. The utility of five such assays was compared in rat SCH to measure the toxic effects of increased bile acid accumulation resulting from inhibition of bile acid efflux by troglitazone, bosentan and glibenclamide (Kemp and Brouwer, 2004). The neutral red assay was not amenable for use in SCH due to crystal formation, while the LDH, MTT, propidium iodine and alamar blue assays were useful for viability assessment, having low variability and correlating well with morphologic changes observed by light microscopy. The LDH and propidium iodide assays are the methods of choice because they are amenable to multiple time point measurements and high-throughput analysis.

Toxicogenomics is another approach to measure compound-induced changes in multiple genes and signaling pathways, with the potential to extrapolate *in vitro* toxicology experiments to the *in vivo* situation. Comparison of gene expression and traditional toxicology assays, both *in vitro* and *in vivo*, reveals that genes are affected earlier and at lower dose levels than needed to detect toxicity by traditional measures (Heinloth et al., 2004, Kienhuis et al., 2006). Thus, gene expression changes may be more sensitive indicators of potential adverse effects. The primary limitation of most hepatic *in vitro* assays used in toxicogenomics studies is the loss of liver-specific functions. Among *in vitro* systems, SCH exclusively maintain polarized morphology and the panoply of liver-specific functions reflective of the *in vivo* situation. This includes the ability to induce enzyme systems, which can be used to study induction as a mechanism of DDIs or toxicity, as well as enhance enzyme activity to study compounds for which toxicity depends on activation by P450s or other enzyme systems. In one example, SCH were maintained in standard medium or medium modified with known P450 inducers phenobarbital, DEX and β-naphthoflavone to study coumarin-induced cytotoxicity and gene expression profiles (Kienhuis et al., 2006). Coumarin metabolism and resulting cytotoxicity correlated with *in vivo* toxicity studies only in hepatocytes maintained in modified culture medium, while gene expression changes in the modified system were also much more reflective of *in vivo* studies. This demonstrates

that even when standard conditions for sandwich-culture are not entirely reflective of the desired endpoint(s), maintenance of liver-specific functions, such as induction, allows the model to be modified to recapitulate the desired effect observed *in vivo*.

Hepatocyte Accumulation of Toxic Species

Accumulation of toxic compounds within hepatocytes can occur by a number of mechanisms, including efficient uptake, inefficient or compromised excretion, and sequestration into subcellular compartments. Accumulation of bile acids to toxic levels by inhibition of canalicular efflux, including the potential attenuation of accumulation by concomitant inhibition of hepatic uptake, has been discussed extensively above.

Returning again to the study by Kostrubsky and colleagues in 2006, accumulation of parent nefazodone correlated with toxicity in human SCH (Kostrubsky et al., 2006). Total protein synthesis, determined by pulse-labeling with ^{14}C-leucine, was reported as a measure of toxicity. Nefazodone, but not buspirone or trazodone, resulted in decreased protein synthesis after treatment for 6 hr with 10, 50 and 100 μM of the antidepressants. The time course of nefazodone toxicity was evaluated by treating SCH with nefazodone (10, 50 and 100 μM) for 1, 6 and 24 hr. Decreased protein synthesis was detected at 1 hr for all doses of nefazodone. At 24 hr, protein synthesis returned to normal for 10 μM but not 50 or 100 μM nefazodone. Further analysis by LC/MS/MS for nefazodone and metabolites in media revealed a 90% decrease in parent nefazodone at 24 hr after treatment with 10 μM; no decrease in nefazodone following incubation with 50 or 100 μM suggested that metabolism was compromised or insufficient for higher doses, resulting in prolonged exposure to parent compound, a sustained decrease in protein synthesis, and morphological cell death by microscopy. This effect was confirmed by treating cells with 10 μM nefazodone + 1 mM 1-aminobenzotriazole (ABT, a P450 inhibitor), resulting in accumulation of parent nefazodone, decreased formation of conjugated metabolites, and lack of recovery in protein synthesis at 24 hr.

Trebectadin cytotoxicity and attenuation by DEX pretreatment were successfully recapitulated in rat SCH, allowing investigation of the mechanism(s) by which DEX attenuates hepatotoxicity in rats and humans (Lee et al., 2008). DEX-mediated induction of Cyp3A and Mrp2 were both postulated mechanisms of hepatoprotection. Although a 75% reduction in biliary excretion in Mrp2-deficient TR$^-$ versus wild-type rat IPLs confirmed the involvement of Mrp2 in the biliary excretion of trebectadin, TR$^-$ rat SCH were surprisingly more resistant to trebectadin-induced cytotoxicity. Increased expression and activity of P450 enzymes, glutathione (GSH) and basolateral Mrp3 in TR$^-$ rats were proposed as possible explanations. Pre-treatment with 500 μM buthionine sulfoximine (BSO), which depletes cellular GSH, resulted in an unexpected decrease in cytotoxicity. Western blot analysis of cell lysates from BSO-treated and control SCH revealed that Mrp2, Mrp3 and Mrp4 protein were increased by 1.5±0.7-, 1.9±0.4- and 3.1±0.7-fold, respectively. Results of this study suggest that trebectadin-mediated cytotoxicity can be attenuated by numerous mechanisms that reduce cellular accumulation of intact trebectadin, whether by DEX-mediated increases in CYP3A metabolism and Mrp2 excretion, or markedly increased basolateral excretion due to up-regulation of Mrp3 and/or Mrp4 in the presence of BSO. Rat SCH were used successfully to reproduce cytotoxicity of trebectadin observed in intact animals and explore the role of transport proteins in attenuation of cytotoxicity.

Human SCH were employed to determine the role of nucleoside transporters in the hepatobiliary disposition of nucleosides and nucleoside drugs in the liver (Govindarajan et al., 2008). Protein expression and localization of equilibrative and concentrative nucleoside transporters (ENTs and CNTs) in human SCH were determined, and mRNA expression was compared to human liver samples. Expression levels of ENT2 and CNT2 mRNA in SCH

format were similar to intact liver, but 2-D cultures (no overlay) resulted in significantly lower levels of ENT2 and CNT2 mRNA. Uptake into hepatocytes and elimination, by metabolism and/or vectorial transport into bile canaliculi, were demonstrated for 5 nucleosides and nucleoside analogues [guanidine, thymidine, ribavirin, formycin B and fialuridine (FIAU)]. Conditions were established to allow deconvolution of the relative contribution of individual transporters to the overall uptake of each substrate, which was varied and substrate-dependent. However, common patterns were observed, such as the significant (if not predominant) role of ENT1 for all substrates, and an inverse relationship between the extent of metabolism and excretion into bile. An example of the applicability of this system was the high extent of FIAU accumulation that, coupled with previous data detailing its mitochondrial transport and resulting cytotoxicity, may explain the significant hepatotoxicity of this drug (Lai et al., 2004). A more recent study extended the scope of ENT expression data to include ENT3, which displayed a higher level of mRNA expression than CNT1 and ENT1 in human liver samples and SCH; based on immunostaining, ENT3 was localized exclusively in the cytoplasm, co-localized with markers of both mitochondrial and non-mitochondrial compartments (Govindarajan et al., 2009).

Rat and human SCH recently were reported as a model to determine the formation of acyl glucuronides and covalent binding to cellular proteins (Dong and Smith, 2009). Glucuronidation and protein adduct formation were monitored following exposure of rat SCH to benoxaprofen, a known hepatotoxicant, and two related NSAIDs, flunoxaprofen and ibuprofen. Glucuronide and protein adducts were detected in the order: benoxaprofen > flunoxaprofen > ibuprofen. Despite this correlation, modulation of glucuronidation and oxidative metabolism pathways suggested that covalent binding to proteins was mediated only partly by reactive glucuronide formation and not at all by oxidative metabolites, leaving an unknown component of protein adduct formation. Although glucuronidation rates varied between donors of human SCH, as might be expected, glucuronide and protein adduct formation were greatest for benoxaprofen. These studies demonstrate the utility of SCH for determining relative risk of forming protein adducts from reactive acyl glucuronides.

Hepatic Transport Protein Knockdown and Knockout Models

RNA interference (RNAi) of single or multiple transport proteins is a powerful tool to explore potential rate-limiting steps in hepatic transport and consequences (“worst-case scenario”) of altered hepatic transport protein function caused by disease states, DDIs or polymorphisms. This approach is particularly useful to evaluate transport proteins for which no “specific” inhibitor has been identified. Synthetic small interfering RNA (siRNA) was transfected successfully into rat SCH to specifically knock down the efflux transporters Mrp2 and Mrp3 (Tian et al., 2004). The extent of protein modulation (~50% knockdown) agreed well with changes in activity, as determined by CDF disposition. More recently, adenoviral vectors expressing short hairpin (sh)RNA targeting Bcrp were delivered successfully into rat SCH to specifically, efficiently and sustainably suppress Bcrp protein expression and activity (Yue et al., 2009). Bcrp knockdown decreased nitrofurantoin BEI and CLbiliary to 11 and 14% of control, respectively. Digoxin, a P-gp substrate, was not affected by Bcrp knockdown; however, GF120918 significantly reduced the biliary excretion of both digoxin and nitrofurantoin, consistent with known inhibition of both P-gp and Bcrp. These studies demonstrated that knockdown of specific transport proteins in SCH is possible, and mimics the effect of inhibitors on substrate disposition.

Hepatocytes from animals with transport protein-deficient genotypes, such as Mrp2-deficient TR^{-} rats may be cultured in a sandwich configuration (Abe et al., 2008, Lee et al., 2008). Another promising source of hepatocytes for SCH studies is from genetically-modified mouse strains such as Mrp2/*Abcc2*$^{-/-}$ knockout mice. However, care must be

taken to fully characterize these models, since loss of expression or function of one transport protein may change the route and/or extent of excretion due to compensatory changes in drug metabolizing enzymes or transport proteins. For example, Nezasa et al. demonstrated that hepatic Mrp3/*Abcc3* protein levels were approximately 60% higher in Mrp2/*Abcc2*$^{-/-}$ mice compared to wild-type mice, corresponding to a 4-fold increase in the rate of basolateral CDF excretion (Nezasa et al., 2006). In addition, it was established recently that Mrp2-deficient TR^{-} rats also partially lack full Bcrp expression and activity (Abe et al., 2008). SCH, coupled with knockdown or knockout technology, represent a useful *in vitro* model to determine the contribution of specific canalicular or basolateral transport proteins to drug/metabolite disposition, and to examine the impact of loss-of-function of transport proteins on drug/metabolite disposition.

Future Challenges

Culturing hepatocytes in a sandwich configuration to maintain cell viability, polarized architecture and liver-specific function has led to significant advances in our understanding of hepatic transport processes and related interactions leading to pharmacokinetic and pharmacodynamic changes in endo- and xenobiotic disposition and hepatotoxicity. Despite these advances, a number of opportunities remain to further develop and characterize the SCH model. The limited availability of fresh hepatocytes from a variety of species, particularly human, has stimulated interest in the use of cryopreserved SCH. Unfortunately, not all batches of cryopreserved hepatocytes are suitable for culturing in a sandwich configuration, due to limitations in cell attachment and loss of transport protein expression, localization and/or function. Further research is needed to improve the cryopreservation process to yield hepatocytes amenable for SCH studies. Modifications of the culture system to incorporate sinusoidal flow and exteriorized bile flow may improve hepatocyte morphology and help retain liver-specific properties. Optimization of culture medium to maintain *in vivo*-like cytochrome 450 activity in addition to transport function would enhance utility of the model. Further use of this model as a screening tool in drug development to evaluate metabolite formation and routes of excretion, predict *in vivo* biliary clearance, and evaluate DDIs in hepatobiliary transport will undoubtedly yield a wealth of new information about species differences in hepatic transport, mechanisms of DILI, and xenobiotic-transport interactions.

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Abbreviations Used

SCH	Sandwich-cultured hepatocytes
Mrp2	Multidrug Resistance-associated protein 2
Mrp3	Multidrug resistance-associated protein 3

Bsep	Bile salt export pump
Mdr1	Multidrug resistance p-glycoprotein
CDF	5(and 6)-carboxy-2',7'dichlorofluorescein
NTCP	Sodium taurocholate cotransporting polypeptide
HBSS	Hanks' balanced salt solution
LDH	Lactate dehydrogenase
EHBR	Eisai hyperbilirubinemic Sprague-Dawley rats
Bcrp	breast cancer resistance protein
RNAi	RNA interference
siRNA	small interfering RNA
shRNA	short hairpin RNA
CLF	cholyl-lysyl-fluorescein
CGamF	Cholylglycylamido-fluoroscein
Oatp	Organic anion transporting polypeptide
DDI	Drug-drug interaction
IPL	Isolated perfused liver
OSTα/β	Organic Solute Transporter
DILI	Drug-induced liver injury
TC	Taurocholate
DEX	Dexamethasone
BSP	bromosulfophthalein
PAH	para-aminohippurate
PI	Protease Inhibitor
PXR/NR1I2	pregnane X receptor
CAR/NR1I3	constitutive and rostane receptor
FXR/NR1H4	farnesoid X-activated receptor
RXRα/NR2B1	retinoid X receptor alpha
AHR	aryl hydrocarbon receptor
PPARα,γ/NR1C1,3	peroxisome proliferator-activated receptors
GR	glucocorticoid receptor
SHP	small heterodimer partner
LXR	liver X receptor
Nrf2	nuclear factor E2-related factor 2
HNF1α	hepatic nuclear factor 1 alpha
NRs	nuclear receptors
UDCA	ursodeoxycholic acid

PI3K	phosphoinositol-3-kinase
PKA, PKB, PKC	protein kinases A, B and C
MAPK	p38 mitogen-activated protein kinase
PMA	phorbol 12-myristate 13-acetate
GSH	glutathione
DMEM	buthionine sulfoximine, BSO Dulbecco's modified Eagle's medium
BC/MG	biocoat/matrigel
GC/GC	gelled collagen/gelled collagen
M3G	morphine 3-glucuronide
IPL	isolated perfused liver
ER	endoplasmic reticulum

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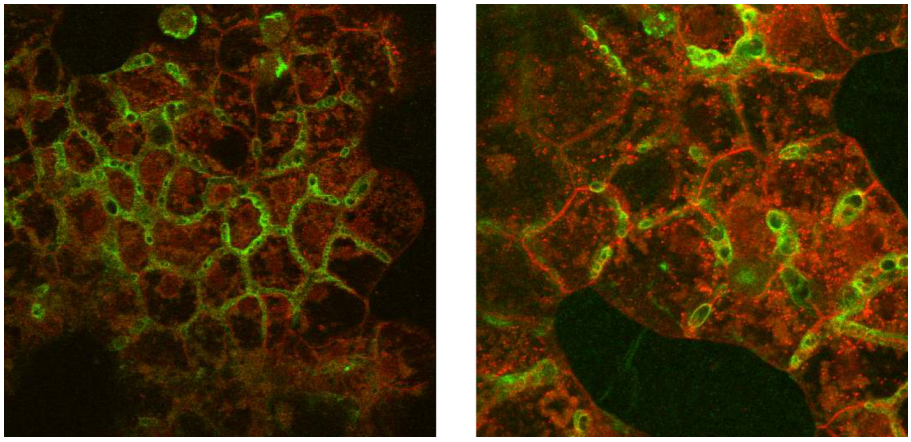

Figure 1. Confocal microscopy images revealing immunofluorescent localization of Mrp2 (green) on the canalicular domain and Mrp3 (red) on the basolateral domain in day 4 rat SCH

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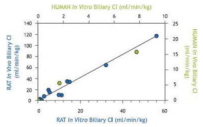

Figure 2. Relationship between *in vitro* intrinsic $Cl_{biliary}$ values generated for compounds in rat and human SCH with *in vivo* $Cl_{biliary}$ data

Rat i*n vivo* $Cl_{biliary}$ of 11 compounds vs. predicted *in vitro* $Cl_{biliary}$ data generated in rat SCH [blue; r^2=0.99; (Liu et al., 1999a)] and human *in vivo* $Cl_{biliary}$ of three compounds vs. predicted *in vitro* $Cl_{biliary}$ data generated in human SCH [green; (Ghibellini et al., 2007)].

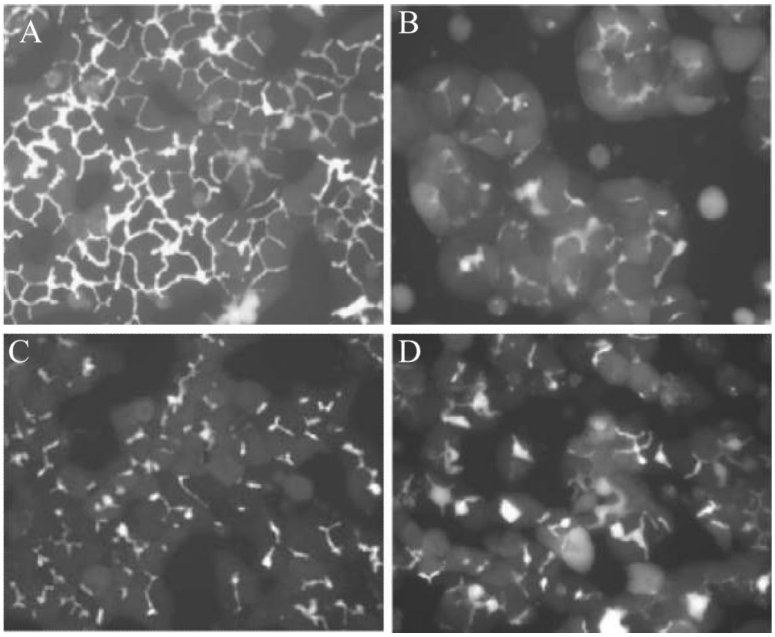

Figure 3. CDF fluorescence microscopy images of rat (A), dog (B), monkey (C), and human (D) SCH

in six-well plates (maintained in DMEM for 4–7 days). SCH were incubated with 2 μM CDF diacetate for 10 min prior to imaging.

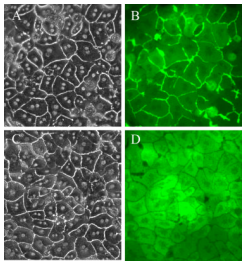

Figure 4. Light (A & C) and CDF fluorescence (B & D) microscopy images of wild-type (A & B) and*Abcc2*$^{-/-}$ [(Mrp2 knockout); C & D] mouse SCH

cultured on six-well Biocoat™ plates with rat tail gelled collagen overlay (maintained in DMEM for 4 days). Mouse SCH were incubated with 2 μM CDF diacetate for 10 min prior to imaging. Data generated by Dr. Xianbin Tian.

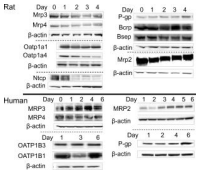

Figure 5. Transport protein levels over days in culture in rat and human SCH

Representative immunoblots of transport proteins in rat and human hepatocytes cultured in six-well Biocoat™ plates with Matrigel™ overlay and maintained with DMEM for 4 or 6 days, respectively. β-actin was used as a loading control. Immunoblots of rat Mrp3 and Mrp4 were provided by Tracy Marion; rat Ntcp immunoblots were provided by Katie Paul; rat Bcrp, Bsep, P-gp, and human MRP3 and MRP4 immunoblots were provided by Dr. Wei Yue. Select data were published previously: rat Mrp2 (Zhang et al., 2005b); rat Oatp1a1, Oatp1a4, P-gp, and for human OATP1B1, OATP1B3, MRP2 and P-gp (Hoffmaster et al., 2004); reproduced, in part, with kind permission from the American Society for Pharmacology and Experimental Therapeutics, and from Springer Science and Business Media, respectively.

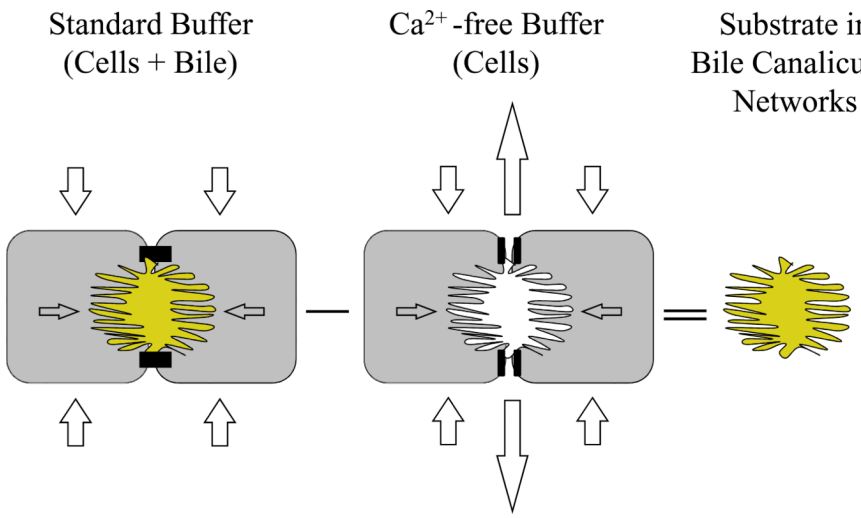

Figure 6. Schematic depicting calculation of substrate accumulation in hepatocytes and bile canalicular networks of SCH

SCH are preincubated in Ca^{2+}-containing HBSS (standard buffer) or Ca^{2+}-free HBSS (Ca^{2+}-free buffer) to disrupt tight junctions sealing the bile canalicular spaces (Liu et al., 1999b, Liu et al., 1999c). Subsequently, cultures are rinsed and substrate uptake in SCH is determined in standard buffer for a designated period of time within the linear range of uptake (typically 10 min). In SCH preincubated with standard buffer, substrate is taken up into SCH and available for excretion into the bile canalicular networks; substrate accumulation (cells+bile) is determined at the end of the uptake period. In SCH preincubated with Ca^{2+}-free buffer to open tight junctions, substrate excreted into the bile canalicular networks is released into the medium; substrate accumulation (cells) is determined at the end of the uptake period. The mass of substrate excreted into the bile canalicular networks during the uptake period is estimated as the difference in accumulation in SCH with intact and disrupted tight junctions [(cells+bile) − (cells)].

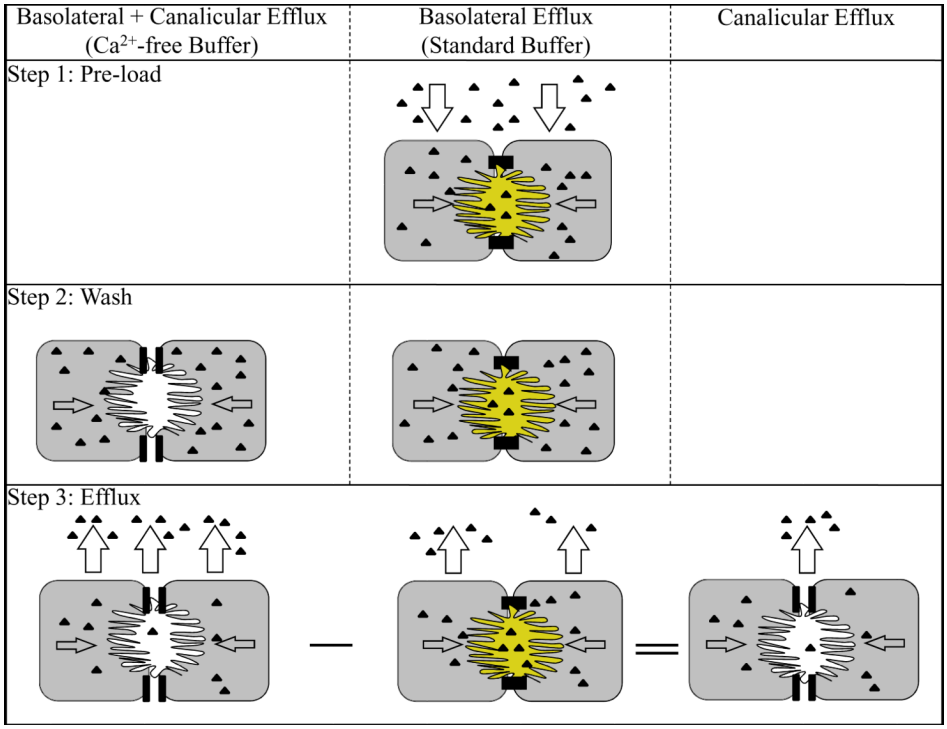

Figure 7. Schematic depicting calculation of canalicular and basolateral efflux of substrate in SCH

Step 1: SCH are preincubated with substrate (▲) in Ca^{2+}-containing HBSS (standard buffer) to preload the cells; substrate is taken up into SCH and excreted into the bile canalicular networks. Step 2: Cultures are rinsed in standard buffer, or Ca^{2+}-free HBSS (Ca^{2+}-free buffer) to disrupt tight junctions sealing the bile canalicular space. Step 3: SCH are incubated in their respective buffers, and aliquots are taken at designated time points for no longer than 30 min. In SCH rinsed and incubated with standard buffer, substrate efflux occurs across the basolateral membrane only. In SCH rinsed and incubated with Ca^{2+}-free buffer to open tight junctions, substrate efflux occurs across the basolateral and canalicular membrane. The canalicular efflux is estimated as the difference in efflux between SCH with disrupted and intact tight junctions [(basolateral + canalicular efflux) − (basolateral efflux)].

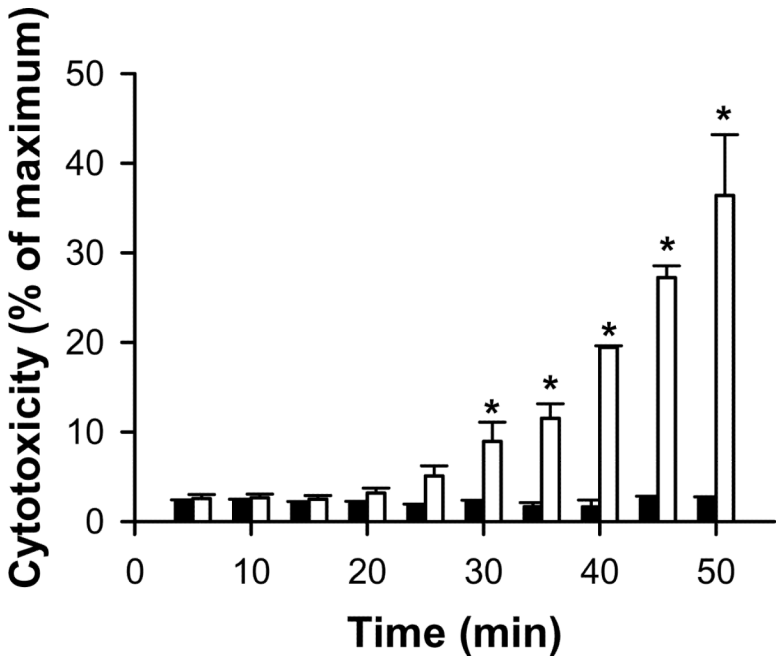

Figure 8. Effect of incubation time in standard HBSS (dark bars) and Ca^{2+}-free HBSS (white bars) on viability of rat SCH

Day 4 SCH from wild-type Wistar rats in six-well Biocoat™ plates with Matrigel™ overlay were incubated with 1.5 mL/well of warm standard HBSS for 20 min, followed by two rinses with 2 mL/well warm standard HBSS or Ca^{2+}-free HBSS, and incubation with 1.5 mL/well of the same buffer. Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release into the respective HBSS buffers after designated times using a cytotoxicity kit according to the manufacturer's instruction (Roche, Indianapolis, IN; Each bar represents the mean ± SEM; n=3 livers in triplicate; $p<0.05$).

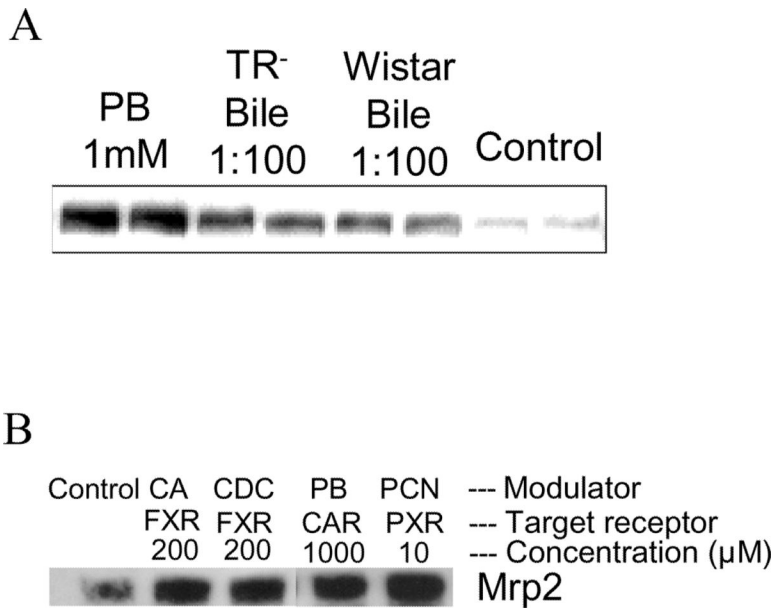

Figure 9. Immunoblot analysis of the influence of modulators on (a) Mrp3 and (b) Mrp2 protein in rat SCH

Rat SCH cultured for 72 hr with **(a)** phenobarbital (1 mM) or diluted bile (1:100) from TR^- and Wistar control rats exhibited increased Mrp3 protein. Data generated by Dr. Peijin Zhang; **(b)** Mrp2 protein was increased after incubation of rat SCH for 72 hr with selected nuclear receptor agonists including cholic acid (CA; 200 μM), chenodeoxycholic acid (CDC; 200 μM), phenobarbital (PB; 1000 μM) and pregnenolone 16α-carbonitrile (PCN; 10 μM). Data generated by Dr. Yong Hae Han.

Table 1

Sandwich-Cultured Hepatocyte Specifications for Development of Bile Canalicular Networks in Multiple Species, both Freshly Isolated and Cryopreserved

Species	Plate Format	Extracellular Substratum	Seeding Density (cells/well)	Medium Volume (mL)	Culture Day for Transport Experiments
Human	6-well	Biocoat/Matrigel	1.75×10^6	1.5	7
	24-well	Biocoat/Matrigel	0.35×10^6	0.5	7
Cryopreserved Human	24-well	Biocoat/Matrigel	0.35×10^6	0.5	5
Rat	6-well	Biocoat/Matrigel	1.75×10^6	1.5	4
	24-well	Biocoat/Matrigel	0.35×10^6	0.5	4
TR^- Wistar Rat	6-well	Biocoat/Matrigel	1.5×10^6	1.5	4
Mouse (C57BL/6)	6-well	Biocoat/Matrigel	$< 1.25 \times 10^6$	1.5	3
Monkey	6-well	Biocoat/Gelled Collagen	1.75×10^6	1.5	4
Dog (Beagle)	6-well	Biocoat/Gelled Collagen (pH 9.0)	1.75×10^6	1.5	4

Table 2

In Vitro $CL_{biliary}$ Scaling Factors

Species	Protein Concentration per Gram of Liver Tissue (mg/g)	# of Hepatocytes/Gram of Liver Tissue (10^6cell/g)	Grams of Liver Tissue/kg Body Weight (g/kg)
Human	90[3], 32[5]	139[3], 107[4], 99[5], 120[7]	25.7[1]
Rat	200[2], 112[3]	117[3], 96[6] 120[7]	40[1]
Mouse	115[3]	135[3]	87.5[1]
Monkey			30[1]
Dog	103[3]	215[3], 165[6], 240[7]	32[1]

[1](Davies and Morris, 1993)

[2](Seglen, 1976)

[3](Sohlenius-Sternbeck, 2006)

[4](Wilson et al., 2003)

[5](Barter et al., 2007)

[6](Smith et al., 2008)

[7](Bayliss et al., 1999)