The role of organic anion transporters in diagnosing liver diseases by magnetic resonance imaging

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Running title: Organic anion transporters and liver imaging markers

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Abbreviations: ATP-binding cassette (ABC), bromosulfophthalein (BSP), computed tomography (CT) focal nodular hyperplasia (FNH), gadobenate dimeglumine (BOPTA, MultiHance; Bracco Imaging SpA, Milan, Italy) and gadoxetate dimeglumine (EOB-DTPA, Primovist; Bayer HealthCare Pharmaceuticals, Berlin, Germany), hepatocellular carcinoma (HCC), indocyanine green (ICG), magnetic resonance imaging (MRI), multidrug resistance-associated protein (MRP), sodium taurocholate-cotransporting polypeptide (NTCP), organic anion transporter (OAT), organic anion transporting polypeptide (OATP), physiologically-based pharmacokinetics (PBPK), positron emission tomography (PET), single photon emission computed tomography (SPECT)
Abstract

The expression and transport function of organic anion transporters are modified in liver diseases and therefore the vascular clearances of endogenous and exogenous organic anions that are taken up by these transporters were used to assess liver diseases in patients. More recently, liver imaging with hepatobiliary contrast agents, tracers, and dyes that cross hepatocytes through the Organic Anion transporting Polypeptides - Multidrug Resistance-associated Proteins (OATPs - MRPs) pathway were developed to detect and characterize focal lesions and to assess the severity of diffuse liver diseases. The review focuses mainly on magnetic resonance imaging (MRI) and highlights the growing interest of imaging the OATPs - MRP2 pathway to better understand liver diseases. Imaging provides non-invasive measurements of tissue concentrations that result from the interplay between influx and efflux membrane transport systems in normal or injured hepatocytes. Imaging with MR hepatobiliary contrast agents improves the detection and the characterization of hepatic focal lesions and new developments of imaging to assess liver function and understand the hepatocellular concentrations of contrast agents are discussed.


**Introduction**

Endogenous organic anions (lactate, urate, citrate or bilirubin) are metabolic intermediates or end products that require transporters such as Organic Anion transporting Polypeptides – OATPs and Mutiple Resistance Proteins – MRPs to cross membranes. Bilirubin, the end product of heme degradation is removed from blood plasma, conjugated to glucuronic acid in hepatocytes and exported into bile for fecal elimination. Accumulation of bilirubin in plasma, together with an elevation of the transaminase alanine transferase, is widely used to measure liver function. Besides its role in the energy homeostasis of the body and protein synthesis, the liver is also responsible for the biotransformation of end products, excretion of xenobiotics, and bile formation. From a physiologic point of view, bile formation is one of the key functions of the liver. The major part of organic solutes in bile is bile salts which are mainly present as anions (Esteller, 2008; Hofmann, 2009). Determination of bile salt clearance from plasma was used in the past as an endogenous marker of liver function (LaRusso et al., 1975).

In addition to bile salts, other exogenous compounds such as bromosulfophthalein (BSP) and indocyanine green (ICG) assess the hepatic function of liver diseases (Sakka, 2007; Stieger et al., 2012; Hoekstra et al., 2013). These exogenous compounds are organic anions and, as such, require membrane transporters (OATPs and MRPs) to enter hepatocytes and to be excreted into bile. Because the expression and transport function of organic anion transporters are modified in liver diseases, the vascular clearance of these exogenous compounds were used to assess liver function (Stieger et al., 2012).

Additionally, liver imaging with hepatobiliary contrast agents, tracers, and dyes that cross hepatocytes through the OATPs - MRPs pathway were developed to detect and characterize focal lesions and to assess the severity of diffuse liver diseases. Thus, magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), and fluorescence imaging help clinicians to manage human liver diseases (Bae et al., 2012; Keiding, 2012; Stieger et al., 2012; Van Beers et al., 2012; Hoekstra et al., 2013; Kusuhara, 2013). Following a brief overview of the transport through OATPs and MRPs, the review highlights how MRI with hepatobiliary contrast agents is important to diagnose liver diseases. This review mainly focuses on
MR imaging because several extended and well-documented reviews were recently published by experts for positron emission tomography (PET) (Keiding, 2012; Kusuhara, 2013; Yoshida et al., 2013) and single photon emission computed tomography (SPECT) (de Graaf et al., 2010; Hoekstra et al., 2013).

**Hepatocellular uptake and efflux of organic anions**

Hepatocytes express both uptake and efflux systems for organic anions. Uptake systems are hOATP1B1 (*SLCO1B1*), hOATP1B3 (*SLCO1B3*) and hOATP2B1 (*SLCO2B1*) (Hagenbuch and Stieger, 2013) and the organic anion transporters (OATs), hOAT2 (*SLC22A7*), hOAT3 (*SLCO22A8*), hOAT7 (*SLC22A9*), hOAT5 (*SLC22A10*) (Burckhardt, 2012; Koepsell, 2013). To the best of our knowledge, the role of OATs in the hepatocellular uptake of liver function markers has not been investigated, while the role of OATPs is well established (Stieger et al., 2012; Van Beers et al., 2012). Efflux of organic anions from hepatocytes is mediated in many instances by members of the ATP-binding cassette (ABC) transporters (Wlcek and Stieger, 2013). Among them, members of the ABCC family are known to transport liver function markers (Stieger et al., 2012; Van Beers et al., 2012).

The substrates of organic anion transporters include endogenous substances such as metabolic end-products or hormones, a vast variety of xenobiotics (drugs as well as toxins) and their metabolites and have been reviewed extensively (Rizwan and Burckhardt, 2007; Hagenbuch and Gui, 2008; Nies et al., 2008; Kalliokoski and Niemi, 2009; Chen and Tiwari, 2011; Keppler, 2011; Konig, 2011; Burckhardt, 2012; Roth et al., 2012; Sodani et al., 2012; Hagenbuch and Stieger, 2013; Koepsell, 2013; Kusuhara, 2013; Shitara et al., 2013; Wang and Sweet, 2013).

Currently, no severe disease related to mutations in genes coding for OATPs and OATs have been reported. In contrast, mutations in genes coding for ABCC family members are well known and may lead to severe diseases or to benign syndromes (Chen and Tiwari, 2011). However, for many of these transporters, numerous different single nucleotide polymorphisms leading to the expression of variants are known (Cascorbi, 2006; Zair et al., 2008; Cascorbi and Haenisch, 2010; Stieger and Meier, 2011; Burckhardt, 2012). Clinical studies have evidenced that polymorphic variants of the respective transporter impact the pharmacokinetics of drugs (Zair et al., 2008; Ieiri et al., 2009;
Fahrmayr et al., 2010; Franke et al., 2010; Sissung et al., 2010; König, 2011; Niemi et al., 2011; Stieger and Meier, 2011; Yoshida et al., 2013). Such polymorphisms may among other consequences lead to altered expression levels of transporters. Considerable inter-individual variability in the expression of uptake and efflux transporters was observed in human livers (Meier et al., 2006; Ho et al., 2010; Shin et al., 2010; Deo et al., 2012; Tucker et al., 2012; Nies et al., 2013).

**Transport mechanisms of organic anion transporters**

Many substrates of OATPs are molecules with a molecular weight of more than 350 Da, anionic and amphipathic physiochemical properties (Roth et al., 2012). Furthermore, OATPs transport substances, which in plasma are tightly albumin-bound like bilirubin. BSP is a dianionic molecule that was used as lead substrate to identify the first OATP by expression cloning (Jacquemin et al., 1994). As it is transported by many OATPs, it can be considered as prototypic OATP substrate (Roth et al., 2012).

In hepatocytes, translocation of anionic OATP substrates across the membrane involves the movement of one or two negative charges against a negative membrane potential of about – 35 mV (Boyer et al., 1992). Such a process is energetically unfavorable and most likely inefficient. So far, the exact transport mechanism(s) of OATPs remain(s) elusive. Most evidence published supports an anion-exchange transport mechanism for OATPs. For example bicarbonate acts as a counter ion of rOATP1A1 and several other OATPs expressed in HeLa cells (Leuthold et al., 2009). Trans-stimulation experiments in *Xenopus laevis* oocytes identified glutathione as another intracellular counter ion for taurocholate and leukotriene uptake mediated by rOATP1A1 (Li et al., 1998). Taurocholate transport mediated by rOATP1A4 can also be stimulated by intracellular glutathione (Li et al., 2000). A comparison of rOATP1A1 and rOATP1A4 with taurocholate as substrate revealed that only rOATP1A4 can be trans-stimulated by a glutathione conjugate (Li et al., 2000). By using membrane vesicles with defined sidedness isolated from HeLa cells expressing rOATP1A1, glutathione transport was found to be asymmetric with a 2- to 3-fold higher uptake into inside-out membrane vesicles compared to outside-out membrane vesicles (Mittur et al., 2002). This suggests that rOATP1A1 may display a functional asymmetry with respect to glutathione transport with the
efflux pathway having a higher capacity. A latter study demonstrated co-transport of glutathione with taurocholate or estradiol-17β-glucuronide for hOATP1B3 (Briz et al., 2006). However, this latter finding could not be confirmed by others (Mahagita et al., 2007) and a glutathione stimulation of substrate transport by hOATP2B1 was earlier reported as absent (Nozawa et al., 2004).

Based on this information it can be concluded that OATPs work as organic anion exchangers, whereby bicarbonate is a likely counter ion in many different cell types. It should however be pointed out that a formal proof for this assumption is currently missing. So far, one study found a current to be induced by the transport of different substrates across the membrane of X. laevis oocytes expressing either hOATP1B1 or hOATP1B3 suggesting an electrogenic transport mechanism (Martinez-Becerra et al., 2011).

Considerable evidence has accumulated from kinetic experiments that OATPs display multiple binding sites. hOATP1B1 mediates the transport of estrone-3-sulfate via a high and a low affinity binding sites, while the same transporter shows simple Michaelis-Menten kinetics for estradiol-17β-glucuronide (Tamai et al., 2001). Several studies have provided evidence for multiple substrate binding sites of OATPs as recently reviewed (Hagenbuch and Stieger, 2013).

Another factor that modifies the transport of OATPs is pH. In vitro experiments demonstrated that hOATP2B1, which is expressed among other tissues in the small intestine is stimulated by an extracellular acidic pH (Kobayashi et al., 2003). This stimulation by extracellular acidic milieu was observed for 13 rat and human OATPs except for hOATP1C1 (Leuthold et al., 2009). It is important to note that this stimulation is substrate-dependent, e.g. hOATP2B1-mediated uptake of prostaglandin E2 is independent from extracellular pH, while transport of thyroxine is stimulated by a low extracellular pH (Leuthold et al., 2009). hOATP2B1 also transports pemetrexed with a higher rate when the extracellular pH decreases, which does not occur for BSP (Visentin et al., 2012). A detailed analysis of the effect of different transmembrane pH gradients on rOATP1A1 transport activity revealed a complex response of the transporter at different pH gradients (Marin et al., 2003). Taken together, pH effects on different OATPs are complex and may, at least in part, be explained by the presence of different substrate binding sites on OATPs (Stieger and Hagenbuch, submitted).

Export of solutes including organic anions from hepatocytes is often mediated by members of
the ATP-binding cassette (ABC) superfamily of transporters. The major representatives of ABC transporters expressed in hepatocytes are hABCA1, hMDR1, hMDR3, the bile salt export pump BSEP (hBSEP), hMRP2, hMRP3, hMRP4, hMRP6, CFTR, hABCG2 and hABCG5/ABCG8) (Wlcek and Stieger, 2013). Out of these transporters, hBSEP, hMRP2 and hABCG2 are expressed in the canalicular membrane and mediate efflux of organic anions into primary bile, while hMRP3 and hMRP4 are expressed in the basolateral membrane and mediate efflux of organic anions back to sinusoids (Nies et al., 2008). As these transporters hydrolyze ATP during the transport cycle, they are able to export their substrates against steep concentration gradients, i.e. under conditions where extracellular substrate concentration is considerably higher than inside the cell, which is particularly important for bile formation. Out of these transporters, hMRP2 and hMRP3 have formally been demonstrated to mediate transport of $^{99m}$Tc-mebrofenin and gadoxetate dimeglumine (EOB-DTPA, Primovist; Bayer HealthCare Pharmaceuticals, Berlin, Germany) (Ghibellini et al., 2008; Jia et al., 2014). Hence, hepatocellular handling of organic anions represents a net flux governed by simultaneously operating uptake and efflux systems.

In summary, the mechanistic understanding of hepatocellular handling of organic anions is currently incomplete, as there are still essential gaps in our knowledge on the transport mechanisms. Clearly, work is needed to obtain more detailed information on the molecular mechanisms of OATPs and also the driving force(s) of OATPs. In addition, there is currently only very limited information available on the preferred transporters for the exit of organic anions from human hepatocytes. In particular, prediction of alternate transporters in case of malfunctioning of the standard efflux pathway is currently not feasible in \textit{in vivo} situations.

Understanding the accumulation of hepatobiliary substrates in the liver is another important issue in human liver diseases. Interestingly, clinical imaging by measuring noninvasively hepatobiliary compounds in the liver may serve as biomarkers of transport function and help to assess the severity of liver diseases. The following sections focus on the role of imaging in diagnosing liver diseases. Imaging techniques include PET, SPECT, and MRI. Because several recent reviews documented the role of PET (Keiding, 2012; Kusuhara, 2013; Yoshida et al., 2013), and SPECT (de Graaf et al., 2010) in assessing the severity of human liver diseases, we focus the information on how MR imaging assess
focal lesions, diffuse liver diseases, and bile duct injury.

**Role of imaging in diagnosing liver diseases**

**Focal lesions of the liver**

In patients with benign or malignant liver tumors, the detection, characterization and accurate staging is crucial to select the appropriate treatment strategy and liver MRI following the injection of contrast agents is the method of choice. Thus, focal nodular hyperplasia (FNH) and hepatocellular adenoma are often difficult to distinguish by conventional MR imaging (MRI without contrast agent injection) (Grazioli et al., 2005). The distinction between both lesions is however clinically important, since FNH is a benign disease which does not need any therapeutic intervention, whereas adenomas have the potential for malignant transformation and profuse bleeding. The distinction is improved by injecting hepatobiliary contrast agents that accumulate differently in both types of lesions (Grazioli et al., 2005). Two hepatobiliary contrast agents for liver MRI are commercialized: gadobenate dimeglumine (BOPTA, MultiHance; Bracco Imaging SpA, Milan, Italy) and EOB-DTPA previously mentioned. Their hepatocellular transport through the OATPs – MRPs pathway will be further explained in later sections. These hepatobiliary contrast agents accumulate in FNHs because bile ducts of the lesions are not connected to the biliary tree and therefore contrast agents cannot be excreted.

Over the past years, significant progress has been made in the molecular classification of hepatocellular adenomas (Zucman-Rossi et al., 2006; Bioulac-Sage et al., 2009; Bioulac-Sage et al., 2012). Four subtypes are currently recognized: adenoma with HNF1α mutations, adenomas with β-catenin mutations, telangiectatic adenomas as well as adenomas with no specific molecular characteristics. This distinction is clinically important, because adenomas with HNF1α mutations are usually benign whereas adenomas with β-catenin mutations are at high risk for malignant transformation. The telangiectatic type has a higher propensity for bleeding. Recently it has been shown that immunohistochemical techniques as well as MRI can be used as surrogate tools to classify adenomas (Lewin et al., 2006; Paradis, 2010; Bioulac-Sage et al., 2012). Adenoma classification by MRI might even be better than routine histology and immunohistochemistry (Ronot et al., 2011).
Therefore MRI plays a crucial role in diagnosing and classifying benign liver tumors. Hepatocellular carcinoma (HCC) is the most common malignant lesions and the fifth most common cancer worldwide. It mostly occurs in patients with liver cirrhosis and therefore surveillance every 6 months by imaging (mainly by ultrasonography) is recommended in cirrhotic patients to detect the lesions at an early stage, when curative treatments are still an option. The European and American HCC guidelines rely on non-invasive radiological criteria for the diagnosis of HCC in patients with liver cirrhosis (Bruix and Sherman, 2011; 2011). When ultrasonography is not conclusive, histological confirmation is recommended as well as imaging with standard computer tomography (CT) and MRI. Interestingly, protocols with injection of hepatobiliary MR contrast agents may be used to better characterize HCC but are not available for routine use (see later sections) (Bruix and Sherman, 2011; 2011; Mullhaupt et al., 2011).

**Liver function in diffuse liver diseases**

The assessment of liver function in diffuse liver diseases is used to determine the prognosis of patients with liver cirrhosis, to define the optimal time-point for liver transplantation and to assess whether patients with liver cirrhosis can undergo major extra-hepatic surgery or tolerate partial hepatectomy. The most widely used score is the Child-Pugh score that takes into account the values of serum albumin, bilirubin and prothrombin time as well as the presence or absence of ascites and encephalopathy (Pugh et al., 1973; Conn, 1981). More recently the Model for End-Stage Liver Disease (MELD) score, which is calculated from plasma bilirubin, international normalized ratio, and plasma creatinine is also important to evaluate end-stage liver diseases (Malinchoc et al., 2000). This score is now used in many countries for graft allocation. A number of quantitative liver function tests such as galactose elimination capacity, methacetin breath test, and indocyanin (ICG) clearance were developed over the last decades (Renner, 1995; Stieger et al., 2012; Hoekstra et al., 2013). These tests have been used almost exclusively in Asia for patients with liver cirrhosis before undergoing liver surgery (Imamura et al., 2005) but recently they have gained increased attention also in Europe (Stockmann et al., 2010). However, with the recent progresses of surgical techniques, which allow various types of partial hepatectomies as well as resection of injured segments once hypertrophy of remnant segments
is high enough to avoid postoperative acute liver failure, it is no longer sufficient to determine the
global liver function and volume but rather to assess the volume as well as the functional capacity of
each segment of the liver (Schnitzbauer et al., 2012). Up to now, liver SPECT was widely used to
assess this regional liver function (Bennink et al., 2004; Yumoto et al., 2010; Bennink et al., 2012).
However, the spatial resolution of images is poor. Therefore, techniques that provide a better spatial
resolution are highly desirable and new development of MRI following the injection of hepatobiliary
contrast agents may assess not only the regional liver volume but also the regional liver function. For
example, a recent study in patients with primary sclerosing cholangitis showed how the liver disease
was heterogeneous and how the regional differences in liver function correlated to biliary obstruction
of each segment (Nilsson et al., 2013). The calculated global liver extraction fraction of the contrast
agent correlated with the PSC Mayo risk score. Thus, new MRI techniques might become an ideal tool
to assess not only the regional liver volume but also its functional capacity.

The assessment of liver fibrosis by biopsy is a standard procedure in the work-up of patients
with chronic liver diseases. Due to its invasiveness, non-invasive methods such as fibroscan, MR
elastography, and fibrotest have been extensively evaluated in the last years and may replace liver
biopsy (Poynard et al., 2011; Castera, 2012; Lee et al., 2013). Preliminary evidence exists that MRI
with hepatobiliary contrast agents might also provide some information on the degree of liver fibrosis
(Jang et al., 2013). Finally, biliary excretion of these contrast agents can visualize the bile duct system.
There is emerging evidence that this technique is superior to conventional magnetic resonance
cholangiopancreatography in certain clinical scenarios, such as in the evaluation of biliary
complications after surgery (Salvolini et al., 2012). Clearly more clinical studies are needed before
liver MRI with hepatobiliary contrast agents (EOB-DTPA or BOPTA) can be recommended as routine
procedures.

Liver imaging related to hepatic transporters

Previous sections emphasize how various compounds cross the hepatocellular membranes and
how MRI with EOB-DTPA and BOPTA is important to diagnose focal lesions and diffuse liver
diseases. This section highlights some aspects of liver MR imaging related to the hepatic transporters
OATPs and hMRP2. EOB-DTPA and BOPTA are drugs that modified the MR images following their distribution in the various compartments of the liver as previously described. These contrasts agents have rare toxic complications.

**Liver MRI and hepatobiliary contrast agents**

As previously described, MRI with EOB-DTPA or BOPTA is increasingly used to visualize the morphology of the liver and to detect and characterize focal lesions. In routine clinical practice, images are registered before the injection, during the arterial phase (entry of contrast agent in arterial vessels), the portal phase (entry of contrast agent in portal vessels), and the hepatobiliary phase (entry of contrast agent into hepatocytes) (Fig. 1). The arterial phase is registered 25 seconds and the portal phase 70 seconds after EOB-DTPA injection. EOB-DTPA within hepatocytes is recorded 20 minutes later. During the hepatobiliary phase, contrast agents are transported from sinusoidal blood to bile across hepatocytes through OATPs and hMRP2. Imaging can also visualize EOB-DTPA in bile ducts 10 min after drug injection in normal livers (Tschirch et al., 2008) (Fig. 2). Images clearly evidence the various segments of the liver and the various compartments (vessels, extracellular space, hepatic parenchyma, and bile).

**Transport of MR contrast agents**

EOB-DTPA is a substrate of hOATP1B1, hOATP1B3, and weakly of the human sodium-taurocholate-cotransporting polypeptide (hNTCP) but not of hOATP2B1 (Leonhardt et al., 2010). In HEK263 cells, the transport characteristics of EOB-DTPA are: hOATP1B1 ($K_m = 0.7 \text{ mM}$, $V_{max} = 11 \text{ pmol/mg x min}$); hOATP1B3 ($K_m = 4.1 \text{ mM}$, $V_{max} = 23 \text{ pmol/mg x min}$); NTCP (0.04 mM, 1.4 pmol/mg x min). The intrinsic uptake clearance of these transporters are: 0.015 ml/min x mg (hOATP1B1); 0.006 ml/min x mg (hOATP1B3); and 0.035 ml/min x mg (hNTCP). In this cellular model, EOB-DTPA is measured by liquid chromatography-tandem mass spectrometry. Recently, EOB-DTPA transport through hMRP2 (canalicular transporter) and hMRP3 (sinusoidal transporter) was evidenced in transport assays using vesicles isolated from MDCK cells overexpressing hMRP2 or hMRP3 (Jia et al., 2014). Indeed, human sinusoidal membranes contain efflux pumps such as hMRP3.
and hMRP4 that are normally expressed at very low levels in normal hepatocytes but can be upregulated in pathological conditions, such as cholestasis (Gu and Manautou, 2010). These pumps might favor the efflux of drugs from hepatocytes back to sinusoids for complementary clearance by the kidneys.

Competitions between several drugs and EOB-DTPA have been described. These interactions to enter into hepatocytes through hOATP1B1/B3 have clinical implications because they might impair liver imaging. Moreover, besides being a substrate, EOB-DTPA decreases in vitro the uptake of BSP by hOATP1B1 (IC$_{50}$: 0.6 mM) and hOATP1B3 (IC$_{50}$: 0.4 mM) (Leonhardt et al., 2010). Thus, EOB-DTPA is both substrate and inhibitor of hOATP1B1 and hOATP1B3. The contrast agent does not modify the uptake of taurocholate by hNTCP.

Uptake of EOB-DTPA in X. laevis oocytes injected with the rSLCO1A1 cRNA was also described (Van Montfoort et al., 1999). EOB-DTPA uptake (100 µM) through rOATP1A1 is inhibited by 100 µM rifamycin SV (97 ± 9%), 100 µM rifampicin (51 ± 15%), 10 µM BSP (45 ± 11%) and 200 µM taurocholate (92 ± 13%). During rat liver MRI with EOB-DTPA, no contrast agent uptake was measured when rifampicin was previously injected, showing that the antibiotic may interfere with clinical imaging (Kato et al., 2002).

The human transporters of BOPTA were not investigated. In rat livers, BOPTA enters into hepatocytes through the rOATP1A1, rOATP1A4, rOATP1B1 and exits into bile by rMRP2, no bile excretion being detected in rats lacking rMRP2 (Planchamp et al., 2007; Millet et al., 2011). Both MRI agents are excreted into bile without transformation.

Three factors can modify the transport of the hepatobiliary contrast agents through hepatocytes: 1) Inter-individual variability of uptake and efflux transporters in normal human livers; 2) Modifications of transporter expression in injured livers and focal lesions; and 3) Drug-drug interactions. Interestingly, several genetic variants of the human hOATP1B1/B3 modify the transport of EOB-DTPA in vitro (Nassif et al., 2012). Accordingly, MR images following EOB-DTPA in volunteers are influenced by genetic polymorphisms of hOATP1B1 (Nassif et al., 2012). Loss-of-function of the transporter is evidenced by decreased signal intensities (or decreased brightness) of the
Expression of transporters and liver diseases

The second factor that modifies the transport of hepatobiliary contrast agents through hepatocytes is the modifications of transporter expression and function in focal lesions and diffuse liver diseases.

We previously outlined that liver cirrhosis is the leading cause of end-stage liver disease worldwide and may lead to the development of hepatocellular carcinoma. Accordingly, the evaluation of hepatic dysfunction and the detection of hepatocellular carcinomas are the main issues during the course of this disease. To detect pathological nodules, MRI with EOB-DTPA injection is repeated over years (Van Beers et al., 2012). During the hepatobiliary phase (images acquired 20 min after injection), most hepatocellular carcinomas are hypointense because tumor cells do not possess hOATP1B1/B3 as shown in Fig. 3A. In contrast, surrounding hepatocytes with functional transporters take up EOB-DTPA (evidenced by the brightness of tissue). Imaging is then useful to detect small hepatocellular carcinomas and to differentiate them from dysplastic nodules, which usually do not show this hypointensity. Moreover, images of tumors obtained during the hepatobiliary phase correlate to the expression of transporters in tumor cells: the higher the severity, the lower the expression (Kitao et al., 2010; Tsuboyama et al., 2010; Kitao et al., 2011).

Assessing liver volume in patients with cirrhosis is not sufficient to perform safely partial hepatectomy and evaluation of hepatic function is also necessary during chronic liver cirrhosis to assess prognosis and to determine the timing of liver transplantation (Clavien et al., 2010). As previously highlighted, several tests are used including MRI with hepatobiliary contrast agents that evaluate the hepatic function of each segment in patients with liver diseases (Katsube et al., 2011; Yamada et al., 2011; Sommer et al., 2012). Although most studies found a decreased accumulation of EOB-DTPA in the parenchyma of cirrhotic livers, images were never related to transporter expression as done with HCC. Because MRI visualizes the entire liver, the technique offers the advantage to assess segmental function of hepatic lobes (Yamada et al., 2011). The decreased accumulation of EOB-DTPA in human cirrhotic hepatocytes results in delayed appearance of the contrast agent in bile.
duct associated with lower concentrations (Fig. 2) (Tschirch et al., 2008). In cirrhotic parenchyma surrounding focal lesions, Ogasawara et al. (Ogasawara et al., 2010) found a decreased expression of hOATP1B1/OATP1B3 and hMRP2. However, the results were partial and inhomogeneous and no liver imaging was performed. No information exists on the expression of membrane transporters in most causes of cirrhosis. Experimental studies in rats showed that the expression of rOATPs is greatly reduced by biliary cirrhosis and the signal intensities following BOPTA injection are much lower than in normal livers (Planchamp et al., 2005a).

Surgeons also use fluorescence imaging with ICG in the operating room to assess the function of liver segments. ICG is transported into human hepatocytes by hOATPs and hNTCP (de Graaf et al., 2011). The dye is likely to exit without transformation through hMRP2. The vascular clearance of ICG correlates to hepatic function of patients with liver diseases and ICG finger clip connected via an optical probe can determine the capillary ICG decay over time (de Liguori Carino et al., 2009; Levesque et al., 2009). Another application of ICG in the operating room is to better visualize the resection limits of hepatocellular carcinomas using a fluorescence camera (Gotoh et al., 2009). Such a camera also detects congestive segments (Kawaguchi et al., 2013). Thus, the authors of this recent study measured hepatic concentrations of ICG to evaluate the function of congestive hepatic segments in three groups of patients: partial hepatectomy, remnant liver of living donors, and hepatic grafts of recipients. The dye concentrations were significantly lower in congestive than in non-congestive segments. ICG hepatic uptake rates were also decreased in congestive segments. Congestive segments with poor function decrease the overall postoperative recovery, inducing complications and slowing liver regeneration. Thus, the use of a camera with fluorescence imaging in the operating room can estimate the function of congestive segments and modify the surgical procedures. However, the expression of transporters in congestive segments were not measured (Schmidt et al., 2013).

**Modifications of transport induced by drug-drug interactions**

The third factor that modifies the transport of hepatobiliary drugs through hepatocytes is drug-drug interactions. As previously emphasized, OATPs transport a broad number of compounds including drugs that may compete to enter into hepatocytes (Niemi et al., 2011; Karlgren et al., 2012).
However, few drug interactions with dyes, tracers and contrast agents have been investigated. In rat livers, BSP inhibits the entry of BOPTA into hepatocytes, with the contrast agent behaving as an extracellular contrast agent (Pastor et al., 2003). Competition between the MR contrast agent EOB-DTPA and rifampicin or BSP is found in HEK293 cells transfected with hOATP1B1/B3 (Leonhardt et al., 2010) and rifampicin impedes liver imaging with EOB-DTPA in anesthetized rats (Kato et al., 2002). In contrast, erythromycin treatment has no effect on clinical liver MRI with EOB-DTPA, but no other potentially competing drugs were investigated (Huppertz et al., 2011). In rodents, rifampicin administration blocks the rMRP2 and increases the hepatic accumulation of the SPECT tracer $^{99m}$Tc-mebrofenin (Neyt et al., 2013) and the PET tracers $^{11}$C-telmisartan (Takashima et al., 2011) and $^{[11C]}$TIC-Me (Kusuhara, 2013). Interaction of ritonavir with $^{99m}$Tc-mebrofenin was also recently investigated (Pfeifer et al., 2013a). We demonstrated that rifampicin blocks BOPTA entry into normal livers (Daali et al., 2013). In drug development, the prediction of transporter-mediated drug-drug interaction is crucial for drug efficacy and safety and the development of physiologically-based pharmacokinetic (PBPK) models is important to estimate the concentrations of competing drugs at the sites of interaction (Pfeifer et al., 2013a; Yoshida et al., 2013). Although several groups have succeeded in constructing PBPK models to predict hepatic concentration-time profiles, strategies to analyze transporter-mediated drug-drug interaction with these models are lacking (Watanabe et al., 2010; Jones et al., 2012; Rostami-Hodjegan, 2012; Pfeifer et al., 2013a). Once these PBPK models are established, the effects of various competitions could be anticipated and quantitatively estimated.

**Future developments: hepatic pharmacokinetics and hepatic concentrations**

The pharmacokinetics of a given drug was traditionally defined by the evolution over time of its absorption, distribution, metabolism, and excretion. The effects of drugs being related to their concentrations at the site of action, it would be useful to monitor such in situ concentrations. However, only drug concentrations in blood or other easily sampled fluids are available. More recently, the development of liver imaging following injection of hepatobiliary contrast agents, tracers or dyes was developed. The MR contrast agents highlighted in this review enter into hepatocytes through sinusoidal transporters OATPs and their uptake rates may be used to diagnose liver diseases. Once in
hepatocytes, their cellular clearance occurs either through biliary excretion (hMRP2) or efflux back to sinusoids (hMRP3 and hMRP4). MR imaging estimates the time-dependent changes of hepatic cellular concentrations, provides information on their distribution in the various compartments of the liver, and describes pharmacokinetic parameters related to the activity of transporters. In various diseases, imaging also confirms the putative mechanisms linked to membrane protein transport. Of note, signal intensities recorded on MR images are not linearly related to concentrations and the relation must be defined with standard curves. Evolution over time of the signal intensities in portal vein, aorta, and hepatic parenchyma contribute to the diagnosis of hepatic tumors (Fig. 1). Thus, hepatocellular carcinomas that are mainly vascularized by isolated arteries are hyperintense during the arterial phase while, in the absence of portal vessels, the lesions appear hypointense during the portal phase in comparison to surrounding normal tissues (Fig. 1). Images obtained during the hepatobiliary phase are related to the presence of transporters in tumor cells (Fig. 3).

In pharmacology, the kinetic analysis of drug behavior in the body is complex and covers several processes such as absorption, distribution, metabolism, and elimination to understand drug concentrations in tissues. To reduce the complexity of this issue, compartmental models are used (Chow and Pang, 2013). The number of compartments needed to accurately describe the drug behavior classifies the compartmental models: one-, two-, or multi-compartment models, whereby compartments do not represent specific tissues but rather tissues or fluids with similar concentration over time. Radiologists measure the evolution of contrast agent concentrations over time in the compartments of the liver (vessels, parenchyma, and bile) and determine various parameters to describe the behavior of hepatobiliary contrast agents. The first study of dynamic contrast agent-enhanced magnetic resonance imaging (DCE-MRI) was published in 1999 following the injection of extracellular contrast agents (Scharf et al., 1999). The number of images can be much higher than the acquisitions described in Fig. 1. In 2002, Bernard Van Beers used a dual-input (entry through both hepatic artery and portal vein) one-compartment model to quantify arterial and venous perfusion, as well as the hepatic distribution volume in anesthetized rabbits (Materne et al., 2002; Annet et al., 2003). The transport of BOPTA was subsequently described in perfused rat livers by pharmacokinetic modeling (Planchamp et al., 2005b; Pastor et al., 2013). In these studies, a 6-compartment model with
8 rate constants perfectly fitted the experimental data. By mathematical simulations, it was possible to determine the contribution of each transporter (uptake and exit systems) on the concentrations of BOPTA in fatty hepatocytes (Pastor et al., 2013).

Another new research topic is to understand how the transport through influx and efflux systems generates or maintains cellular concentrations as recently highlighted by Chu et al. (Chu et al., 2013; Pfeifer et al., 2013b). For several years, we have been interested by such issue. Our first study showed the transport of the hepatobiliary contrast agent BOPTA in isolated and perfused rat livers (Pastor et al., 2003). Pharmacokinetics of BOPTA in normal livers was measured in the MRI room using signal intensities. Then, BOPTA was labeled with $^{153}$Gd and measured by a gamma probe placed over rat livers. The protocol includes a perfusion period during which BOPTA uptake is measured and a rinse period that investigates the cellular clearance of BOPTA. It was found that BOPTA (200 µM perfusion over 30 min) is taken up with an extraction ratio around 25%. The maximal partition coefficient of BOPTA between perfusate (200 µM) and cellular concentrations was 2.6, BOPTA concentrations in hepatocytes being higher than the perfused concentration (Millet et al., 2011). In hepatocytes lacking rMRP2 (TR- rats), BOPTA uptake is decreased but the maximal partition coefficient of BOPTA between perfusate and cellular concentrations is higher (3.3) than in normal hepatocytes. Thus, rMRP2 transport function participates to the accumulation of the contrast agent in hepatocytes. BOPTA is not metabolized and exits mainly through rMRP2, efflux back to sinusoids being much lower (Fig. 4). Breakthrough information conveyed by the experimental model is the possibility to relate efflux rates (into bile or back to sinusoids) and cellular concentrations (Fig. 4). Thus, the function of transporters is perfectly measured in the model. In the past, when investigators measured efflux rates in cultured cells or perfused livers, little information was available on the cellular concentrations of the investigated compounds. This lack of information precludes conclusions on absolute transporter functions. For example, it a rat model with fatty livers we recently demonstrated that the high efflux back to perfusate was related to the trapping of BOPTA inside hepatocytes due to the decreased expression of rMRP2 in this group. The relationship between efflux rates to sinusoids and cellular concentrations were similar in normal and fatty livers (Pastor et al., 2013). To further illustrate this issue, the relationship between efflux rates and cellular concentrations...
in normal and TR- rats is shown in Fig. 4. The biliary excretion rates of BOPTA increase with cellular concentrations until 400 nmol/g (Fig. 4A and 4B). Over this cellular concentration, biliary excretion rates remain steady. BOPTA is not excreted from hepatocytes lacking mRP2 (Millet et al., 2011). This absence of biliary excretion was associated with higher concentrations inside cells and BOPTA efflux rates back to sinusoids were similar for higher intracellular concentrations (right shift of the relationship, Fig. 4C). Thus, BOPTA accumulates in hepatocytes lacking mRP2 and the absence of biliary excretion is not compensated by increased efflux back to sinusoids. The model was successfully applied to drug-drug interactions and a recent study found that interactions between BOPTA and rifampicin at uptake and efflux transport systems modify the cellular concentrations of the two competing substances (Daali et al., 2013).

These studies suggest that BOPTA uptake occurs against a concentration gradient because in rat livers perfused with a 200 µM concentration, concentrations in the livers were as high as 600 µM (Planchamp et al., 2007; Millet et al., 2011). ICG concentrations in rat livers are also 10-fold higher than concentrations in plasma (Horak et al., 1973). In both examples, it was however not possible to determine the free cytoplasmic concentrations which are likely to drive bile excretion. Understanding intracellular concentrations is complex. Intracellular concentrations may not only be affected by metabolism, but also regulated by the activity of uptake and export systems (Watanabe et al., 2010; Pfeifer et al., 2013b). Moreover, mRP2 is regulated by retrieval from the membrane (impeding exit into bile) or insertion into the membrane (favoring biliary excretion), which will in turn influence intracellular concentrations of substrates (Gu and Manautou, 2010). The impact of low or absent hMRP2 activity is demonstrated in patients with Dubin-Johnson syndrome, i.e. no functional hMRP2, who display a normal hepatocellular uptake but a prolonged retention of the radiotracer $^{99m}$Tc-mebrofenin (Bar-Meir et al., 1982). Accumulation of EOB-DTPA is also evidenced in HCC (Fig. 3B). The reason why these hepatocellular carcinomas are hyperintense (brighter) is that the tumours contain functional OATPs allowing for the contrast agent to enter into cells, while the contrast agent is trapped within the tumour cells by lack of exit, in contrast to surrounding liver tissue (Tsuboyama et al., 2010).

Outlook
This review outlines the lack of knowledge on the molecular transport mechanism(s) of OATPs. Such information may predict whether OATPs mediate uphill transport of substrates into hepatocytes with high intracellular accumulation of OATP substrates. The dependence of OATPs function on extracellular pH is another important issue in diffuse livers diseases and focal lesions. Answers to these questions may originate from liver imaging. Thus, the review highlights the growing interest of imaging the OATPs - MRP2 pathway to better understand liver diseases. MR images reflect the intracellular concentrations that result from the interplay between influx and efflux membrane systems in normal or injured hepatocytes. Imaging with MR hepatobiliary contrast agents improve the detection and the characterization of hepatic focal lesions. Imaging to assess liver function is developing. Finally, imaging provides non-invasive measurements of tissue concentrations in volunteers with OATP polymorphisms and in patients with liver diseases. Understanding hepatocellular concentrations will be another important research topic for the future.
Authorship contributions

Catherine M. Pastor: contributed to the writing of the manuscript
Beat Müllhaupt: contributed to the writing of the manuscript
Bruno Stieger: contributed to the writing of the manuscript
References


Hofmann AF (2009) Bile acids: trying to understand their chemistry and biology with the hope of helping patients. *Hepatology* **49**:1403-1418.


Ieiri I, Higuchi S and Sugiyama Y (2009) Genetic polymorphisms of uptake (OATP1B1, 1B3) and efflux (MRP2, BCRP) transporters: implications for inter-individual differences in the pharmacokinetics and pharmacodynamics of statins and other clinically relevant drugs. *Expert Opin Drug Metab Toxicol* **5**:703-729.


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Figure legends

Fig. 1. MRI with EOB-DTPA is increasingly used to visualize the morphology of the liver and to detect and characterize focal lesions as well as injured livers. Images are acquired before the injection of EOB-DTPA (A), during arterial phase (entry of contrast agent in arterial vessels, B), portal phase (entry of contrast agent in portal vessels, C), and hepatobiliary phase (entry of contrast agent into hepatocytes, D). The brighter the images, the higher the concentrations of EOB-DTPA. Images taken with permission from (Verloh et al., 2013).

Fig. 2. Visualization of EOB-DTPA in bile ducts and gallbladder. Normal liver (A) is bright (functional transporters) and the contrast agent appears in bile ducts and gallbladder 10 min after injection. Cirrhotic liver (B) has structural abnormalities (regenerative nodules) that take up less EOB-DTPA and no contrast agent is seen in bile ducts. Images taken with permission from (Tschirch et al., 2008).

Fig. 3. MRI with EOB-DTPA and detection of hepatocellular carcinomas (HCC) during the hepatobiliary phase. In A1, HCC is hypointense (black) in comparison to surrounding tissues because tumour cells do not possess hOATP1B3 (A2). In contrast, surrounding hepatocytes with functional transporters take up EOB-DTPA (evidenced by the brightness of tissue). In B1, HCC is hyperintense (brighter) because the tumour contains functional hOATP1B3 (B2) allowing for the contrast agent to enter into cells, while the contrast agent is trapped within tumour cells by lack of exit, in contrast to surrounding liver tissue. Alternatively, export through hMRP2 remains functional but EOB-DTPA is trapped within bile ductules, increasing signal intensities within the tumours. Images taken with permission from (Narita et al., 2009).

Fig. 4. Relationship between BOPTA cellular concentrations [nmol/g] and BOPTA excretion rates [nmol/min/liver] in CTRL (black squares) and TR - livers (hepatocytes lacking rMRP2, grey squares): A. Bile excretion rates during BOPTA perfusion period (30 min, 200 µM); B: Bile excretion rates
during rinse period with KHB solution (30 min); C: BOPTA efflux rates back to sinusoids during perfusion period with KHB solution (30 min). Livers are perfused with a non-recirculation model that allows the perfusion of constant concentrations of BOPTA (200 µM) during the perfusion period. BOPTA is labeled with $^{153}$Gd and measured on line by a gamma probe placed over livers. Flow rate is 30 ml/min. The perfusion period is important to understand BOPTA uptake into hepatocytes and the rinse period investigates how BOPTA is cleared from hepatocytes. Non-linear regression curves were statistically different in CTRL and TR- livers.
Figure 1

- A. No EOB-DTPA
- B. Arterial phase
- C. Portal phase
- D. Bile phase

EOB-DTPA at 25 sec followed by 70 sec and 20 min intervals.
Figure 2
Figure 3

MRI with EOB-DTPA

OATP1B3 expression in tumours
A. BOPTA bile efflux rates [nmol/min]
Perfusion period

B. BOPTA bile efflux rates [nmol/min]
Rinse period

C. BOPTA efflux rates back to sinusoids [nmol/min]
Rinse period

Figure 4