

The Roles of MRP2, MRP3, and OATP1B1 and OATP1B3 in Conjugated Hyperbilirubinemia

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Running title:

MRP2, MRP3, AND OATPS IN CONJUGATED HYPERBILIRUBINEMIA

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ABBREVIATIONS: ABCC (Abcc), human (non-human) ATP-binding cassette protein, subfamily C; MRP (Mrp), human (non-human) multidrug resistance protein; OATP (Oatp), human (non-human) organic anion-transporting polypeptide; *SLCO* (*Slco*), human (non-human) solute carrier gene encoding OATP; UGT (Ugt), human (non-human) UDP-glucuronosyltransferase.

ABSTRACT:

Increased concentrations of bilirubin glucuronides in blood plasma indicate hepatocellular dysfunction. Elucidation of the transport processes of bilirubin conjugates across the basolateral (sinusoidal) and the canalicular plasma membrane domains of hepatocytes has decisively contributed to our current understanding of the molecular basis of conjugated hyperbilirubinemia in human liver diseases. Under normal conditions, unconjugated bilirubin is taken up into hepatocytes by transporters of the organic anion-transporting polypeptide (OATP) family, followed by conjugation with glucuronic acid, and ATP-dependent transport into bile. This efflux across the canalicular membrane is mediated by multidrug resistance protein 2 (MRP2 or ABCC2), which is a 190 kDa glycoprotein transporting with high-affinity and efficiency monoglucuronosyl bilirubin and bisglucuronosyl bilirubin into bile. MRP2 is hereditarily deficient in human Dubin-Johnson syndrome. Under pathophysiological conditions, such as cholestatic liver injury and MRP2 inhibition, the basolateral efflux pump multidrug resistance protein 3 (MRP3 or ABCC3) is responsible for the occurrence of conjugated hyperbilirubinemia. MRP3 is a glycoprotein with a similar molecular mass as MRP2, with 48 % amino acid identity, and with overlapping substrate specificity. Human MRP3 is the only basolateral efflux pump shown to transport bilirubin glucuronides. In human and rat hepatocytes MRP3/Mrp3 is strongly upregulated under conditions of cholestasis and MRP2 deficiency. This is in line with the concept that basolateral efflux pumps of the hepatocyte compensate for impaired canalicular efflux of compounds into bile, and contribute to balance the rate of uptake or synthesis of compounds in hepatocytes with the capacity for efflux into bile.

(Introduction)

MRP2 and MRP3 are Conjugate and Drug Efflux Pumps of the MRP (ABCC) Subfamily of ATP-dependent Transport Proteins

The multidrug resistance proteins MRP2 and MRP3 (systematic names ABCC2 and ABCC3) have received particular attention because of their role in the disposition of anionic conjugates and drugs. In polarized cells, as exemplified by hepatocytes and intestinal epithelia, MRP2 is localized exclusively to the apical and MRP3 to the basolateral plasma membrane (Keppler and Kartenbeck, 1996; König et al., 1999). Note that MRP1 is neither detectable in the basolateral nor in the canalicular membrane of human hepatocytes (Keppler, 2011a). Members of the MRP subfamily are unidirectional ATP-dependent efflux pumps in the plasma membrane, as shown originally for MRP1 (Jedlitschky et al., 1994; Leier et al., 1994) and subsequently for recombinant MRP2 (Evers et al., 1998; Cui et al., 1999). Following the cloning of MRP1 in 1992 (Cole et al., 1992), human MRP2 was the second member of the MRP subfamily to be identified by molecular cloning and cellular localization in 1996 (reviewed by Nies and Keppler, 2007). MRP1 and MRP2 share 50% amino acid identity. *MRP2 (ABCC2)* is a phylogenetically very ancient gene identified not only in mammals, but also in cellular slime molds, yeast, plants, skate, zebrafish, and bony fish (National center for biotechnology information, accessed October 06, 2013, at <http://www.ncbi.nlm.nih.gov/gene?term=abcc2>). The function of human MRP2 and rat Mrp2 may be described in general terms as ATP-dependent export and detoxification of endogenous and xenobiotic substances and their conjugates (Evers et al., 1998; Cui et al., 1999; Nies and Keppler, 2007). Most MRP2 substrates are organic anions, however, millimolar concentrations of reduced glutathione (GSH), as present in living cells, broaden the

substrate spectrum, and organic cations, as illustrated by vinblastine (Evers et al., 1998), may become substrates by cotransport with GSH (Cole and Deeley, 1996). Some selected substrates for human MRP2 and MRP3, as determined with inside-out oriented plasma membrane vesicles, are listed in Table 1. More extensive lists of substrates were published previously (Zeng et al., 2000; Borst et al., 2007; Nies and Keppler, 2007). Both efflux pumps share many common substrates, most notably conjugates of lipophilic compounds with glucuronic acid (Borst et al., 2007; Nies and Keppler, 2007; Keppler, 2011a). Inhibitors, including several drugs, as well as some activators of MRP2 have been extensively characterized by use of inside-out oriented plasma membrane vesicles (Pedersen et al., 2008). Drugs which are potent *in vitro* inhibitors of MRP2 include terfenadine, fendiline, tamoxifen, in addition to inhibitory compounds such as the quinoline derivative MK-571, indocyanine green, bromosulfalein, and cyclosporin A (Pedersen et al., 2008). As exemplified by MK-571, these compounds need not be selective inhibitors for MRP2 (Keppler, 2011a).

MRP3 and MRP2 share an amino acid identity of 48 %. The localization of MRP3 to the basolateral (sinusoidal) membrane of human hepatocytes (König et al., 1999; Kool et al., 1999) and the demonstration that it transports bilirubin glucuronides (Lee et al., 2004) have been decisive for our understanding of conjugated hyperbilirubinemia in cholestasis and under conditions of impaired MRP2 function. In addition, intestinal epithelia are of interest with respect to the basolateral localization of MRP3 in extrahepatic tissues (Scheffer et al., 2002; Rost et al., 2002), since bilirubin glucuronides formed by UDP-glucuronosyltransferase 1A1 (UGT1A1) in intestinal epithelia (Tukey and Strassburg, 2000) can be effluxed via MRP3 into blood and may thus contribute to the pool of conjugated bilirubin in serum (Fujiwara et al., 2012).

Transport of Unconjugated Bilirubin into Human Hepatocytes

Pioneering studies using intravenously injected ^{14}C -labeled bilirubin in rats and humans demonstrated that the disposition of unconjugated and conjugated bilirubin is almost quantitatively determined by uptake into hepatocytes, followed by elimination into bile (Arias et al., 1961; Schmid and Hammaker, 1963). Extrahepatic, particularly intestinal, uptake and glucuronidation may play an additional role in preventing hyperbilirubinemia under some conditions, such as the impaired hepatocellular uptake in Rotor syndrome (van de Steeg et al., 2012) or inactive glucuronidation in hepatocytes associated with active glucuronidation in intestinal epithelia (Fujiwara et al., 2012). The preferential uptake of unconjugated bilirubin by hepatocytes under most conditions in the intact organism argues against an uncontrolled uptake into all cells by passive diffusion. The mode of uptake of unconjugated bilirubin into hepatocytes and other cell types has been unresolved for many years (McDonagh, 2010). The following lines of evidence indicate that unconjugated bilirubin is taken up into hepatocytes by members of the human organic anion-transporting polypeptide (OATP) family:

(i) Initially, Cui et al. showed high-affinity uptake of ^3H -labeled unconjugated bilirubin by OATP1B1 into stably transfected HEK293 cells in the presence of albumin (Cui et al., 2001b). This was not confirmed by Wang et al. (2003), whereas Briz et al. (2003) demonstrated uptake of unconjugated bilirubin into *Xenopus laevis* oocytes expressing OATP1B1 or OATP1B3.

(ii) The potent inhibition of OATP1B1 by indinavir, cyclosporin A, and rifamycin SV may lead to drug-induced unconjugated hyperbilirubinemia observed in the clinic (Campbell et al., 2004).

(iii) A genetic polymorphism in the gene encoding human OATP1B1 causing impaired transport activity was associated with a significant increase of unconjugated bilirubin in serum (Zhang et al., 2007).

(iv) Genome-wide scans demonstrated highly significant associations of the human gene encoding OATP1B3 with unconjugated hyperbilirubinemia (Sanna et al., 2009) and with total serum bilirubin (Johnson et al., 2009).

(v) Disruption of the mouse genes encoding several hepatocellular Oatp transporters of the 1a and 1b subfamilies displayed a 1.8 to 2.5-fold increase in unconjugated bilirubin and a more than 40-fold increase in total plasma bilirubin, 95% of which were conjugated (van de Steeg et al., 2010 and 2012). Transgenic mice lacking the 1a and 1b subfamilies of mouse Oatps and expressing human OATP1B1 or OATP1B3 showed a complete normalization of unconjugated and conjugated hyperbilirubinemia and a normalization of urinary excretion of bilirubin glucuronides (van de Steeg et al., 2012; van de Steeg et al., 2013).

(vi) Patients with Rotor syndrome, who are genetically deficient in OATP1B1 and OATP1B3, exhibit increased concentrations of unconjugated and of conjugated bilirubin in plasma (van de Steeg et al., 2012; Jirsa et al., 2012).

The findings summarized above support the conclusion that the uptake of unconjugated bilirubin into human hepatocytes is mediated by members of the OATP family, particularly by human OATP1B1 and OATP1B3 (Figure 1). It is not known at present to

what extent additional uptake transporters, such as human OATP2B1, further contribute to this uptake. OATP1A2, which is not expressed in hepatocytes but, among other sites, in the blood-brain barrier (Bronger et al., 2005), also seems capable of transporting unconjugated bilirubin (van de Steeg et al., 2013).

Transport of Conjugated Bilirubin by Human Hepatocytes

Four plasma membrane proteins of human hepatocytes were shown to transport monoglucuronosyl bilirubin and bisglucuronosyl bilirubin (the latter chemically correct name is preferred to bilirubin diglucuronide, which is the more conventionally used term):

(i) OATP1B1 mediates unidirectional high-affinity transport of monoglucuronosyl and bisglucuronosyl bilirubin across the hepatocyte sinusoidal membrane (König et al., 2000 a; Cui et al., 2001b).

(ii) OATP1B3, which is also localized in the sinusoidal membrane of human hepatocytes (König et al., 2000 b), transports unidirectionally monoglucuronosyl bilirubin with a K_m value of 0.5 μM (Cui et al., 2001b).

(iii) MRP3 in the sinusoidal membrane is an efficient efflux pump for bilirubin glucuronides (Lee et al., 2004).

(iv) MRP2, the human conjugate efflux pump in the hepatocyte canalicular membrane, transports bilirubin glucuronides with K_m values of 0.7 μM for monoglucuronosyl bilirubin and 0.9 μM for bisglucuronosyl bilirubin (Kamisako et al., 1999).

Under normal conditions, unconjugated bilirubin is converted in the endoplasmic reticulum to monoglucuronosyl bilirubin and bisglucuronosyl bilirubin by UGT1A1 (reviewed by Tukey and Strassburg, 2000) followed by MRP2-mediated efflux across the canalicular membrane into bile (reviewed by Nies and Keppler, 2007; Keppler, 2012). However, when the formation and/or the uptake of bilirubin glucuronides exceeds the transport capacity of MRP2 in the canalicular membrane, the sinusoidal efflux via MRP3 provides a pathway for compensation, which leads to urinary excretion. This is exemplified by the MRP2 deficiency of Dubin-Johnson syndrome (Kartenbeck et al., 1996; Keppler and Kartenbeck, 1996), which is associated with urinary excretion of bilirubin glucuronides, and an upregulation of MRP3 in the sinusoidal membrane (König et al., 1999). In accordance with the observations in human liver, Mrp2-deficient mutant rats and obstructive cholestasis in rats exhibit a strong (more than 50-fold) upregulation of sinusoidal Mrp3 protein (Ogawa et al., 2000; Donner and Keppler, 2001; Soroka et al., 2001).

Efflux from hepatocytes into sinusoidal blood via MRP3 also enables reuptake of bilirubin glucuronides by more downstream (more centrally localized) hepatocytes in the sinusoid by OATP1B1 and OATP1B3 (van de Steeg et al., 2012). Such a cycle of efflux and reuptake during percolation along the sinusoid was originally proposed for bile acids in hepatocytes and liver lobules, which can be effluxed across the sinusoidal membrane by MRP4 and re-enter hepatocytes via the sodium-dependent taurocholate-transporting polypeptide (NTCP) and OATP1B1 and OATP1B3 (Rius et al., 2006; Keppler, 2011b). The pioneering studies by van de Steeg et al. on bilirubin transport in knock-out mouse strains contribute to an understanding of the role of Mrp3 in conjugated hyperbilirubinemia by demonstrating that Mrp3 improves the efficiency of biliary bilirubin

glucuronide excretion, even though it transports its substrates initially from liver to blood, not bile (van de Steeg et al., 2012). The authors proposed that, within liver lobules, the bilirubin glucuronide effluxed by Mrp3 in upstream hepatocytes is efficiently taken up by downstream hepatocytes via Oatp1a/1b and then excreted into bile (van de Steeg et al., 2012). However, the presence of Mrp3 as well as Ugt1a1 in intestinal epithelia (Fujiwara et al., 2012) may also contribute to the pool of plasma bilirubin glucuronides, which are subsequently taken up by the kidney and by hepatocytes.

Predominantly Conjugated Hyperbilirubinemia in Rotor Syndrome

The molecular basis of the rare syndrome described by Rotor et al. (Rotor et al., 1948) was elucidated recently and shown to result from the combined hereditary deficiency of OATP1B1 and OATP1B3 (van de Steeg et al., 2012). Hyperbilirubinemia develops only in persons with biallelic inactivating mutations in both the *SLCO1B1* and the *SLCO1B3* gene. Presence of at least one wildtype (functional) allele of either *SLCO1B1* or *SLCO1B3* prevents Rotor-type hyperbilirubinemia (van de Steeg et al 2012; Jirsa et al., 2012). Remarkably, these patients are characterized by predominantly conjugated hyperbilirubinemia, associated with delayed elimination from blood of several anionic diagnostic dyes and with impaired uptake into hepatocytes (Wolpert et al., 1977; Bar-Meir et al., 1982; Jirsa et al., 2012). The exact source of this conjugated plasma bilirubin in patients with Rotor syndrome and in the knock-out mice lacking Oatp1a/1b uptake transporters has not been fully clarified (van de Steeg et al., 2012). In normal human serum, only about 4 % of bilirubin are conjugated (Muraca and Blanckaert, 1983), whereas over 50 % are conjugated in patients with Rotor syndrome (Jirsa et al., 2012). Three hypothetical pathways might be taken into consideration to explain the

predominantly conjugated hyperbilirubinemia in Rotor syndrome: (i) Entry of unconjugated bilirubin into hepatocytes via OATP2B1, which is also localized in the sinusoidal membrane of human hepatocytes (Kullak-Ublick et al., 2001), followed by glucuronidation and efflux of the conjugates from hepatocytes into blood. (ii) Uptake of unconjugated bilirubin by intestinal epithelia, followed by glucuronidation and efflux of the conjugates from intestinal epithelia into blood via MRP3. (iii) Passive diffusion of unconjugated bilirubin into hepatocytes and intestinal epithelia, followed by glucuronidation and efflux of the conjugates into blood via MRP3. The latter pathway involving passive diffusion, which was considered formerly, is most difficult to be reconciled with the currently available information, as discussed above.

The predominantly conjugated mild hyperbilirubinemia in patients with Rotor syndrome represents a steady state resulting from the absence of OATP1B1 and OATP1B3 in the hepatocyte sinusoidal membrane. In the mouse model, this hyperbilirubinemic steady state is reverted back to normal in transgenic mice lacking *Oatp1a/1b* but expressing human OATP1B1 or OATP1B3 in their sinusoidal membrane (van de Steeg et al., 2012). In patients with Rotor syndrome it is likely that much of the hepatobiliary elimination of organic anions, including anionic drugs, proceeds via OATP2B1. However, pharmacokinetic consequences may be anticipated in case of the hepatobiliary elimination of drugs which are preferentially taken up by OATP1B1 or OATP1B3, and not by OATP2B1. This aspect has been elegantly addressed with regard to methotrexate and paclitaxel elimination in humanized transgenic mice (van de Steeg et al., 2013).

Predominantly Conjugated Hyperbilirubinemia in Dubin-Johnson Syndrome

The Dubin-Johnson syndrome is a rare autosomal recessively inherited disorder characterized by a predominantly conjugated mild hyperbilirubinemia and deposition of a dark pigment in hepatocytes (reviewed by Chowdhury et al., 2001). The incidence of Dubin-Johnson syndrome ranges from 1:1,300 among Iranian Jews to 1:300,000 in a Japanese population. The absence of a functionally active MRP2 protein from the human hepatocyte canalicular membrane has been recognized as the molecular basis of this syndrome (Kartenbeck et al., 1996; Keppler and Kartenbeck, 1996). In line with the established transport function and substrate specificity of MRP2 (reviewed by Nies and Keppler, 2007), the hepatic elimination of diagnostic organic anions into bile is impaired in Dubin-Johnson syndrome (Chowdhury et al., 2001). Many sequence variants in the gene encoding human MRP2 were identified in these patients, including nonsense mutations leading to a premature stop codon (Paulusma et al., 1997), missense mutations (Toh et al., 1999; Mor-Cohen et al., 2001), and a deletion mutation leading to the loss of two amino acids from the second nucleotide-binding domain (Tsuji et al., 1999). Although all sequence variants associated with Dubin-Johnson syndrome result in the absence of a functionally active MRP2 protein from the canalicular membrane, their effects differ with regard to the biosynthesis and function of the respective MRP2 glycoprotein (reviewed by Nies and Keppler, 2007). Several MRP2 sequence variants lead to a deficient protein maturation and impaired sorting as the key mechanism (Keitel et al., 2000), or to an apically localized, but functionally inactive MRP2 protein (Mor-Cohen et al., 2001).

Uptake of unconjugated bilirubin and its conjugation with glucuronic acid in hepatocytes are apparently not affected in Dubin-Johnson syndrome (Figure 1). Accordingly, only

conjugated hyperbilirubinemia would be expected as a result of MRP2 deficiency.

However, various studies demonstrated that the relative amount of unconjugated serum bilirubin in this syndrome ranges from 24 % to 38 % of total bilirubin (Kartenbeck et al., 1996; Toh et al., 1999; Tsujii et al., 1999). Increased unconjugated serum bilirubin levels, in addition to conjugated hyperbilirubinemia, were also observed in Mrp2-deficient mutant rats (Jansen et al., 1985). So far, neither inhibition of unconjugated bilirubin uptake into hepatocytes nor deconjugation by glucuronidases have been demonstrated as possible explanations.

Dubin-Johnson syndrome (reviewed by Chowdhury et al., 2001) as well as Rotor syndrome (Rotor et al., 1948; Jirsa et al., 2012) may be considered as non-diseases.

The predominantly conjugated hyperbilirubinemia is mild because of the compensation by alternative elimination pathways leading to urinary excretion of bilirubin glucuronides. However, overloading or drug-induced inhibition of the alternative elimination pathways, such as hepatocellular MRP3-mediated efflux, may be a risk factor leading to hepatotoxicity, as discussed by van de Steeg et al., 2012 .

Authorship contribution

Dietrich Keppler wrote the manuscript and compiled the Table and the Figure

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Legend for Figure 1

Bilirubin transport into human hepatocytes, conjugation, canalicular and sinusoidal efflux, and reuptake by downstream hepatocytes. Hepatocellular uptake of unconjugated bilirubin (B) by OATP1B1 and OATP1B3, which are deficient in the Rotor syndrome (designated Rotor; van de Steeg et al., 2012), is followed by glucuronic acid conjugation catalyzed by UDP-glucuronosyltransferase 1A1 (UGT1A1), to yield bisglucuronosyl and monoglucuronosyl bilirubin (BGA). Under normal conditions, bilirubin glucuronides are predominantly transported by MRP2 into the bile canaliculus. When MRP2 is deficient, as for example in Dubin-Johnson syndrome (designated by DJS; Kartenbeck et al., 1996), or inhibited by some drugs (Pedersen et al., 2008), efflux of the conjugates by MRP3 across the basolateral membrane into sinusoidal blood enables renal uptake and excretion or reuptake by more downstream hepatocytes in the sinusoid leading to percolation and cycling along the sinusoid. Note that neither the additional uptake transporter OATP2B1 in the sinusoidal membrane (Kullak-Ublick et al., 2001) nor the additional basolateral efflux pump MRP4 (Rius et al., 2006) are indicated in this scheme. Modified from Keppler, 2012.

Table 1.

Selected substrates for recombinant human MRP2 and MRP3 assayed in vesicles

	MRP2	
	K _m (μM)	Reference
Bisglucuronosyl bilirubin	0.7	Kamisako et al., 1999
Monoglucuronosyl bilirubin	0.9	Kamisako et al., 1999
Leukotriene C ₄	1.8	Cui et al., 1999
S-Glutathionyl 2,4-dinitrobenzene	6.5	Evers et al., 1998
17β-Glucuronosyl estradiol	7.2	Cui et al., 1999
Cholyl-L-lysyl fluorescein	3.3	De Waart et al., 2010
Fluo-3	3.7	Nies et al., 1998; Cui et al., 2001a
Bromosulphophthalein	12	Cui et al., 2001a

	MRP3	
Bisglucuronosyl bilirubin		Lee et al., 2004
Monoglucuronosyl bilirubin		Lee et al., 2004
17β-Glucuronosyl estradiol	26	Zeng et al., 2000
Dehydroepiandrosterone 3-sulfate	46	Lee et al., 2004
Leukotriene C ₄	5.3	Zeng et al., 2000

