Organic Anion Transporter 2 (OAT2): An Enigmatic Human Solute Carrier

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Abbreviations: CMD, cimetidine; cGMP, guanosine 3,5-cyclic monophosphate; $C_{\text{max}}$, total maximum plasma concentration; $C_{\text{max,unb}}$, unbound maximum plasma concentration; DHEA, dehydroepiandrosterone; E3S, estrone-3-sulfate; GFR, glomerular filtration rate; $IC_{50}$, inhibitor concentration causing 50% inhibition; IMC, indomethacin; $K_{i}$, inhibition constant; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MATE, multidrug and toxin extrusion protein; MPP+, 1-methyl-4-phenylpyridinium; MRP, multi
drug resistance protein; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; PAH, \textit{para}-aminohippuric acid; PBS, phosphate-buffered saline; PCV, penciclovir; PROB, probenecid; RPTC, renal proximal tubule cell; SLC, solute carrier.
ABSTRACT

As a member of the solute carrier 22A (SLC22A) family, OAT2 (SLC22A7) is emerging as an important drug transporter because of its expression in both the liver and kidney, two major eliminating organs, and its ability to transport not only a wide variety of xenobiotics, but also numerous physiologically important endogenous compounds, like creatinine and guanosine 3,5-cyclic monophosphate (cGMP). However, OAT2 has received relatively little attention when compared to other OATs and solute carriers (SLCs) like organic cation transporters, sodium-dependent taurocholate co-transporting polypeptide, multidrug and toxin extrusion proteins, and organic anion-transporting polypeptides. Overall, the literature describing OAT2 is rapidly evolving, with numerous publications contradicting each other regarding the transport mechanism, tissue distribution, and transport of creatinine and cGMP, two important endogenous compounds of OAT2. Notably, despite its status as a liver and kidney SLC, tools for assessing its activity and inhibition are lacking and its role on drug disposition and elimination remains to be defined. The current review will focus on the available and emerging literature describing OAT2. It is envisioned that OAT2 will gain more prominence, as its expression, substrate and inhibitor profile is investigated further and compared to other SLCs.
1 INTRODUCTION

It has long been established that an important function of the kidney and liver is the secretion of organic anions into the urine and bile, respectively. Importantly, it has been recognized that such secretion is an active and saturable process that can be modulated by competitive inhibitors. Seven human organic anion transporters (i.e., OAT1, SLC22A6; OAT2, SLC22A7; OAT3, SLC22A8; OAT4, SLC22A11; OAT7, SLC22A9; OAT10, SLC22A13; and URAT1, SLC22A12) have been identified and characterized functionally (Burckhardt, 2012, Nigam, 2015, Nigam et al., 2015). They are part of the organic ion (solute carrier, SLC) transporter superfamily (i.e., SLC22). What is unique about these organic anion transporters is the ability to mediate the exchange (extracellular against intracellular) of an impressive number of endogenous molecules, anionic drugs, metabolites, toxins and signaling molecules with differing molecular composition, polarity and charge. The second intriguing feature relates to their principal expression in renal proximal tubule cells (RPTCs) and hepatocytes, the major sites of active compound secretion in the body.

OAT2 was actually the first mammalian organic anion transporter to be cloned. It was originally called novel liver-specific transporter (NLT) because of the dominant mRNA expression in the rat liver (Simonson et al., 1994). Later on, NLT was found to be expressed in the liver and kidney at comparable levels and renamed OAT2 because of its close homology with OAT1 and its interaction with organic anions (Sekine et al., 1998). OAT1 was the next organic anion transporter identified, which was initially cloned from a mouse kidney cDNA library (Lopez-Nieto et al., 1997). It was described as novel kidney transporter (NKT) because it was almost exclusively expressed in kidney.
In subsequent work, the OAT1 orthologs from other species including human, rat, monkey, pig, and rabbit were cloned. In 1999 cloning and characterization of rat OAT3 was reported (Kusuhara et al., 1999). The human OAT3 gene named SLC22A8 was cloned in the same year (Race et al., 1999). Functional characterization of OAT3 from human and other species was soon followed. Recently, other organic anion transporters in the family, OAT4 (Cha et al., 2000), OAT7 (Shin et al., 2007), OAT10 (Bahn et al., 2008), and URAT1 (Enomoto et al., 2002a), have been cloned from a human cDNA library. The gene expression, physiologic function and substrate specificity of these transporters is just beginning to be investigated and characterized.

OAT1 and OAT3 have been considered the primary organic anion transporters mediating renal secretion of anionic compounds. They are expressed at the basolateral membrane of RPTCs. Subsequent to the initial discovery of rodent OAT1 and OAT3, orthologs were also identified in humans, monkey, pig and rabbits and intensively characterized with respect to their tissue distribution, membrane localization, substrate specificity and functional characteristics. This knowledge has been summarized in several excellent reviews (Burckhardt, 2012, Emami Riedmaier et al., 2012, Koepsell, 2013, Wang and Sweet, 2013, Nigam et al., 2015). Over the past few years, OAT2 has emerged as the primary transporter for many endogenous and exogenous organic anions and cations, including a number of therapeutic drugs. However, conflicting data exist with regard to subcellular expression, substrate specificity and driving force of OAT2. In addition, despite numerous publications, the physiological, pharmacological and toxicological importance of OAT2 remains ambiguous. In this review, with a focus on
OAT2, we explore the implications of recent in vitro and in vivo data pertinent to OAT2 in comparison to OAT1 and OAT3.

2 RELATIVELY WELL-UNDERSTOOD ASPECTS OF OAT2

2.1 Splice Variant, Genomic and Protein Structure

OAT2 shares many structural similarities with organic anion transporters including OAT1 and OAT3; however, there are also some differences to note as well. The human OAT proteins vary in size from 541 to 563 amino acids (Emami Riedmaier et al., 2012). While the OAT1 and OAT3 genes encode 563- and 542-amino acid proteins, respectively, OAT2 proteins comprise 546, 548 and 539 amino acids, depending on splice variants (Table 1). OAT2 share 37.3% identical amino acids with OAT1 and has 35.9% identity to OAT3. Overall, the most divergent region between OAT2, OAT1 and OAT3 is found on the C-terminal end. While OAT2, OAT1 and OAT3 have high homology between species (about 80% amino acid identity in rat, rabbit, human and mouse), the homology between these three transporters for a given species is low (about 40% to 50%) (Sun et al., 2001, Jacobsson et al., 2007).

Cropp et al. reported that OAT2 had two splice variants that differ in terms of two amino acids (OAT2-546aa, NM_006672 and OAT2-548aa, NM_153320) when cloning OAT2 gene from a human kidney cDNA library (Cropp et al., 2008). The difference between the two variants is a 6-bp insertion (TCCCAG) between exons 1 and 2 of the OAT2 gene. OAT2 detected in liver, kidney and pancreas contained almost identical levels of the mRNA species of the two splice forms. However the protein localization of these two forms is found to be different; OAT2-546aa protein, detected by immunohistochemistry and immunoblotting studies, was localized to the plasma...
membrane, while OAT2-548aa protein was found in the intracellular compartment (Table 1). The former was found to be able to transport cGMP, suggesting that it may be involved in cGMP signaling and play an important role in many physiological processes such as inflammation, angiogenesis, and cardiac hypertrophy. In contrast, OAT2-548aa exhibited a complete loss of transport function in terms of cellular uptake (Cropp et al., 2008, Hotchkiss et al., 2015). When expressed in three different cell lines [i.e., human embryonic kidney (HEK), Madin-Darby canine kidney (MDCK), and Chinese hamster ovary (CHO) cells], OAT2-546aa was able to transport guanosine analogue antiviral drug penciclovir, which is a structurally similar molecule to cGMP. Furthermore, many previously identified substrates of OAT2-548aa such as para-aminohippurate (PAH), estrone-3-sulfate (E3S), glutarate, succinate, paclitaxel and dehydroepiandrosterone (DHEA) sulfate were not transported by OAT2-546aa, suggesting different substrate specificity between the splice variants. The OAT2-546aa-mediated uptake of penciclovir was inhibited by various organic anions, cations, and neutral compounds with different molecular weight, suggesting the broad ligand selectivity of OAT2-546aa.

Another variant OAT2-539aa was originally cloned from human kidney tissue by Enomoto and his colleagues (Enomoto et al., 2002b) and assigned a GenBank accession number (AF210455) (Table 1). OAT2-539aa shares 97% amino acid sequence identity with OAT2-546aa and OAT2-548aa. This sequence differs greatly from OAT2-546aa and OAT2-548aa primarily in the C-terminal end that varies significantly among species (Hotchkiss et al., 2015). Human OAT2-539aa shares lesser homology to other species including rat, cattle, horse, rabbit, pig, opossum and chicken Oat2 than OAT2-546aa. Unfortunately, the OAT2-539aa sequence could not be found in the NHLBI Exome...
Sequencing Project Exome Variant Server database (http://evs.gs.washington.edu/EVS/). Moreover, Hotchkiss et al. failed to amplify cDNA of OAT-539aa from human kidney cDNA after several attempts (Hotchkiss et al., 2015). Taken together, the tissue expression of the OAT2-539aa splice variant requires confirmation. Overall, the prevalence of 3 splice variants in different ethnic groups, which may be critical to understand the physiological, pharmacological and toxicological importance of OAT2, is not known.

The gene SLC22A7 for human OAT2 is located on chromosome 6p21.1 (Kok et al., 2000) and that of murine Oat2 on chromosome 17qB3 (Table 1) (Kobayashi et al., 2002). OAT2 is not paired with any other gene from SLC22 family and is phylogenetically distinct from OAT1 and OAT3 (Eraly et al., 2003, Cropp et al., 2008). Often those transporter pair-members are phylogenetically related and functionally similar. For example, OAT1 and OAT3 are tightly linked pair with respect to genomic locus, and appear similar functionalities serving as broad-spectrum anion transporters with overlapping groups of substrates. In this regard, OAT2 is the most distantly related to OAT1, OAT3 and other human organic anion transporters (Sun et al., 2001, Sweet et al., 2001, Eraly et al., 2003).

There are few reports describing the polymorphic nature of human OAT2. Xu et al. conducted analyses of coding region polymorphisms in OAT2 together with other organic anion transporters OAT1, OAT3, OAT4, and URAT (Xu et al., 2005). Four OAT2 SNPs were identified. Of those, three were described as nonsynonymous. The synonymous OAT2 SNP (C12697) is commonly found in all ethnic groups. Later, Shin et al. added four new OAT2 SNPs (Shin et al., 2010). But no amino acid alteration was
associated with the SNPs. In total, human OAT2 genomic sequences showed low variability. Although the authors found that there is a 10-fold variation in OAT2 protein expression in the liver specimens, genetic polymorphisms may not be a significant determinant to variation in the OAT2 expression and transport activity.

Since the crystal structures for mammalian OAT members are not available, structural information is still mainly based on hydropathical analysis of amino acid sequences. Similar to other organic anion transporter proteins, OAT2 contains 12 transmembrane domains (TMDs), both N- and C-termini localized intracellularly (OAT-548aa; Figure 2). A large extracellular loop is found between TMD1 and TMD2 of OAT2, OAT1 and OAT3 proteins, carrying 2-5 N-glycosylation sites which are important for targeting of transporter protein onto the plasma membrane (Tanaka et al., 2004). Cropp et al. reported that the addition of Ser and Gln, between Glu131 and Trp132 in the large extracellular loop 1 of OAT2-546aa, results in reduced stability and improper trafficking of OAT2-548aa. The OAT2-548aa-GFP-transfected cells exhibited much weaker fluorescent signals compared to those transfected with OAT2-546aa-GFP. The amino acid sequence near Glu131 of OAT2-546aa is highly conserved among members of OAT subfamily, suggesting that this region may contain motifs that are critical to the stability and trafficking of OAT2 (Cropp et al., 2008). There are several conserved cysteine residues for the formation of disulfide bridges which is important for stabilizing the tertiary structure. A second large loop located intracellularly between TMD6 and TMD7, carries consensus sites for phosphorylation by several protein kinases (2 for PKA and 2-6 for PKC), which indicates that the transporters could be regulated by reversible phosphorylation (Srimaroeng et al., 2008).
While the transport modes of OAT1 and OAT3 have been extensively studied and clarified over the past two decades, the transport mechanism of OAT2 is not yet fully understood. Located on the basolateral membrane of RPTCs, OAT1 and OAT3 function as tertiary active transporters and operate as organic anion-dicarboxylate exchangers (Figure 1A; Table 1). These co-transported dicarboxylates then drive the exchange of dicarboxylates with anions into the cell via OAT1 and OAT3 because the gradient favors outward movement of dicarboxylates, such as α-ketoglutarate, to the peritubular capillary (Sekine et al., 1997). As a preferred physiological counter ion of OAT1 and OAT3, the plasma concentration of α-ketoglutarate in humans is in the 8-12 µM range (Wagner et al., 2010, Halamkova et al., 2012), while the intracellular concentration of α-ketoglutarate, in rat RPTCs is much higher (approximately 265 µM). The gradient of α-ketoglutarate provides a driving force for taking up anions from blood (Pritchard, 1995, Sweet et al., 1997). Once inside the cell, organic ions may undergo export as intact molecules via the brush border membrane transporters OAT4, OAT2, and urate anion exchanger 1 (URAT1) (Miyazaki et al., 2005, Shen et al., 2015). These transporters work in concert to mediate the renal secretion of anionic endogenous solutes and xenobiotics, although some anionic compounds that are taken up by these SLCs could be metabolized by intracellular enzymes.

Assumed to be an organic anion-dicarboxylate exchanger similar to OAT1 and OAT3, the interaction of OAT2 with α-ketoglutarate and other dicarboxylates as a potential physiological counter ion of OAT2 have been investigated in a few studies. The conflicting results have been published with respect to the driving force for OAT2. α-ketoglutarate, a dicarboxylate of 5 carbons, was initially identified as a substrate of
human OAT2 expressed in EcR 293 cells that are derived from HEK 293 cells (Sun et al., 2001). However, the transport of PAH by OAT2 was not blocked by α-ketoglutarate, in concert with the finding that α-ketoglutarate does not trans-stimulate OAT2-mediated E3S and cGMP uptake (Henjakovic et al., 2015). Consistently, although rat Oat2 mediates the uptake of α-ketoglutarate (Sekine et al., 1998), 1 mM α-ketoglutarate does not inhibit the uptake of salicylate mediated by rat Oat2 (Morita et al., 2001). These results suggest that OAT2 transport is unlikely to occur via exchange with α-ketoglutarate. On the other hand, 5 mM fumarate and succinate (4-carbon dicarboxylates), but not glutarate (5-carbon dicarboxylates), trans-stimulate OAT2-mediated uptake of E3S (Kobayashi et al., 2005a), suggesting that OAT2 may be an organic anion-dicarboxylate exchanger with preference for 4-carbon dicarboxylates. However, Sato et al. failed in their attempt to repeat the trans-stimulation of OAT2-mediated urate uptake in HEK293 cells pre-loaded with succinate up to 100 mM (Sato et al., 2010). In addition, Hotchkiss et al. investigated the cis-inhibitory effect of various Kreb’s cycle intermediates, including fumarate, succinate and α-ketoglutarate, on OAT2 transport. However, none of those Kreb’s cycle intermediates inhibited OAT2-mediated penciclovir uptake (Hotchkiss et al., 2015). They also showed that, indeed, succinate is not a substrate for OAT2. Likewise, Henjakovic et al. did not observe cis-inhibition of OAT2-mediated cGMP uptake by succinate and other dicarboxylates, while in the same study, 4 out of 6 dicarboxylates (i.e., glutarate, adipate, pimelate and suberate) were shown to inhibit OAT1- and OAT3-mediated transport (Henjakovic et al., 2015). Moreover, they showed none of the 7 short-chain monocarboxylates inhibit OAT2-mediated cGMP transport. Given the high intracellular concentration of glutamate (2 to
20 mM) (Newsholme et al., 2003) and the outward direction of the glutamate gradient (Fork et al., 2011), glutamate may be the major physiological substrate that enables OAT2-mediated translocation. However, although human OAT2 has been shown to transport glutamate, cGMP-glutamate exchange by OAT2 has not been demonstrated (Henjakovic et al., 2015). Furthermore, while OAT1 and OAT3 exhibit pH- and chloride-dependence (higher transport at acidic pH and lower transport in the absence of chloride), no pH- and chloride-dependent transport has been observed with OAT2 (Henjakovic et al., 2015). Moreover, OAT2-mediated cGMP uptake is not affected by depolarization of membrane potential by high potassium. Taken together, the counter ion for OAT2 transport remains to be identified.

### 2.2 Potential Role of OAT2 in Drug Disposition

Like OAT1 and OAT3, OAT2 can accommodate a variety of organic anions, and indeed has also been shown to transport a number of pharmacologically active agents. OAT2 has been increasingly recognized in terms of its role in drug disposition (Table 2). Well-characterized drug substrates of OAT2 include numerous antibiotics (cefotaxime, erythromycin and tetracycline) (Babu et al., 2002, Kobayashi et al., 2005b, Yee et al., 2013), antivirals (acyclovir, penciclovir and ganciclovir) (Cheng et al., 2012), antimetabolites (5-fluorouracil and methotrexate) (Sun et al., 2001, Kobayashi et al., 2005a), H-2 receptor antagonists (cimetidine (CMD) and ranitidine) (Tahara et al., 2005), diuretics (bumetanide) (Kobayashi et al., 2005a), nonsteroidal anti-inflammatory drug (diclofenac) (Zhang et al., 2016), topoisomerase inhibitor (irinotecan) (Marada et al., 2015), and endogenous prostaglandins and hormones (e.g., prostaglandin E2, prostaglandin F2, DHEA sulfate and E3S) (Enomoto et al., 2002b, Kobayashi et al., 2005a).
2005a, Kobayashi et al., 2014, Jia et al., 2015) (Table 2). OAT2 has also been identified as a candidate diclofenac β-D-glucuronide transporter (Zhang et al., 2016). Similarly, theophylline has been identified as an OAT2 substrate. The apparent $K_m$ value for the OAT2-mediated transport of theophylline was reported as 12.6 µM and the OAT2-mediated theophylline uptake is inhibited by erythromycin, suggesting that hepatic OAT2 may be serve as the locus of the well described theophylline-erythromycin interaction (Table 2). Furthermore, it has been reported that OAT2 can transport a large variety of purine and pyrimidine nucleobases, nucleosides, and nucleotides, including cGMP, (Cropp et al., 2008). Many substrates of OAT2 are also substrates of OAT1 and (or) OAT3 (Table 2). It is worth noting that three OAT2 slice variants, OAT2-546aa, OAT-548aa, and OAT2-539aa, with different transport specificity, have been used by different laboratories and has led to contradictory results.

A great number of drugs that inhibit transport by OAT1, OAT2, and OAT3 have been identified and the inhibitor concentration causing 50% inhibition ($IC_{50}$) or inhibition constant ($K_i$) determined (Burckhardt, 2012, Nigam et al., 2015). Although OAT2 has distinct inhibitor specificity compared to OAT1 and OAT3, they also share overlapping specificities for numerous inhibitors (Figure 3) (Supplemental Table 1). The basolateral OAT1 and OAT3 transporters generally share comparable affinities with various inhibitors ($p > 0.05$), while the inhibition potential of these inhibitors against OAT2 is significantly low (Figures 3A and 3B) ($p < 0.0001$) (Supplemental Table 1). For example, probenecid (PROB) and ketoprofen are less potent inhibitors of human OAT2 compared to OAT1 and OAT3 ($IC_{50}$ values of 393 µM vs. 4.57 and 10.9 µM; and 400 µM vs. 4.34 and 5.98 µM, respectively) (Khamdang et al., 2002, Jia et al., 2015). However,
indomethacin (IMC) exhibited similar inhibitory potency against OAT2 (IC$_{50}$ values 2.1 to 6.5 $\mu$M) (Shen et al., 2015, Zhang et al., 2015), OAT1 (IC$_{50}$ values 3 to 10 $\mu$M), and OAT3 (IC$_{50}$ values 0.61 to 5.95 $\mu$M) (Khamdang et al., 2002). It is envisioned that inhibitors with marked differences in inhibition potency may be used to determine the relative contributions of each OAT to organic anion uptake and support transporter phenotyping employing isolated primary RTPCs.

2.3 Role of OAT2 in Transport of Endogenous Compounds

OAT2 facilitates the transport of various endogenous compounds across biological membranes in the liver and kidney, and thus regulates the intracellular concentrations of transported substances. Cyclic nucleotides cAMP and cGMP are OAT2 substrates (Sun et al., 2001). Human OAT2 transports endogenous nucleobases, nucleosides and nucleotides such as adenine, cytidine, adenosine, inosine and guanidine, but not thymine, thymidine and cytosine. It also transports GMP, GDP and GTP (Cropp et al., 2008). OAT2 also mediates the low K$_m$ (K$_m$ of 0.71 uM) transport of prostaglandin E2 (Kimura et al., 2002). Conflicting results have been published in OAT2 mediated transport of sulfated steroid hormones. For example, Sun et al (2001) reported DHEA sulfate is not an OAT2 substrate (Sun et al., 2001), while Kobayashi et al (2005) showed that OAT2 does transport the compound (Kobayashi et al., 2005b) (Table 2).

cGMP plays a critical role in many physiological processes involved in mediating cellular response to various stimuli and is a key regulator of biological processes including cell proliferation, differentiation, and apoptosis, through the activation of cGMP-dependent protein kinase signaling pathway (Sager, 2004). Several multidrug resistance proteins such as MRP4, MRP5 and MRP8 have been identified as efflux
pumps mediating the extrusion of cGMP from cells (Sager, 2004). On the other hand, OAT2 has been identified as a highly efficient, bidirectional facilitative transporter that can regulate both intracellular and extracellular levels of cGMP and may be involved in cGMP signaling pathway for gene regulation (Cropp et al., 2008); however, how OAT2 inhibition modulates cGMP intracellular concentrations has not been studied in great detail.

Creatinine is actively secreted into urine from blood, and the recent investigations have shown that the efficiency of creatinine uptake by OAT2 is greater than that of other renal transporters such as OCT2, MATE1, and MATE2-K (Lepist et al., 2014, Shen et al., 2015). A more complete overview of the role of OAT2 in creatinine elimination is presented in the following section.

3 ENIGMATIC ASPECTS OF OAT2

Despite the fact that singularly transfected OAT2 mediates the active transport of numerous xenobiotics and endobiotics, its expression profile in human tissues has received relatively little attention versus other SLCs. Importantly, OAT2 scaling factors (relative activity and relative expression) have not been developed and there are virtually no reports of in vitro-in vivo extrapolation exercises involving the transporter.

3.1 Under-Investigated Liver Solute Carrier

3.1.1 Uncertain OAT2 Expression in the Liver

A high level of OAT2 mRNA expression is observed in the liver versus other tissues such as testis, choroid plexus, stomach, ileum, and uterus in humans (Fork et al., 2011). The higher level of OAT2 mRNA expression in the liver than kidney was
observed in humans and rats by others (Sekine et al., 1998, Sun et al., 2001, Hilgendorf et al., 2007) (Table 1). However, surprisingly, Oat2 mRNA expression was reported at highest level in kidney rather than in liver in rats by another group (Buist et al., 2002).

Regarding the expression level of OAT2 relative to other SLC transporters in the liver, in fact, OAT2 mRNA levels in the liver are comparable to those of organic anion-transporting polypeptide (OATP)1B1, OATP1B3, OATP2B1, sodium-dependent taurocholate co-transporting polypeptide (NTCP), and organic cation transporter 1 (OCT1). Ohtsuki et al. simultaneously quantified protein amount, mRNA expression and activity of 19 drug-metabolizing enzymes and 22 drug transporters in 17 human liver samples. They compared the protein expression levels with corresponding mRNA expression levels and activities, and found that the OAT2 protein expression did not correlate with the corresponding mRNA expression (Ohtsuki et al., 2012). The differences between mRNA and protein expression are thought to be due to post-transcriptional modification, intracellular trafficking and membrane sorting. As a result, mRNA expression may not serve as a surrogate for transporter function, and actually be rather misleading since transmembrane proteins are partly localized to the membrane of intracellular organelles. Vildhede et al. quantified various SLCs and compared the membrane proteomic signature of freshly isolated hepatocytes to that of human liver tissue (Vildhede et al., 2015). The expression of OAT2 protein was 30- to 100-fold lower than other hepatic uptake transporters such as OATP1B1, OATP1B3 and OCT1. The low expression of OAT2 may contribute to its low activity in human liver described in the section below. More recently, Nakamura et al (2016) employed an even larger scale proteomics approach and determined that the hepatic expression of OAT2 was more
comparable to that of the OATPs (~1 to 2 fmol per μg of microsomal protein) (Prasad et al., 2016).

In regard to OAT2 liver tissue distribution, the expression of rat Oat2 mRNA is homogeneous across periportal and pericentral regions (Fork et al., 2011). Rat Oat2 protein has been specifically localized to the basolateral (sinusoidal) membrane of hepatocytes (Simonson et al., 1994), like other hepatic uptake transporters such as OATP1B1, OATP1B3 and OCT1. However, species-dependent localization of OAT2 in the liver is possible. Surprisingly, although it is assumed that human OAT2 is localized in the sinusoidal membrane of hepatocytes, the immunohistochemistry of OAT2 protein in human liver, which is the main organ of its expression, has not been demonstrated.

### 3.1.2 Limited Evidence for OAT2 Function in Liver

Limited information is available regarding the role of OAT2 in the uptake of compounds into hepatocytes and drug disposition due to the lack of experimental tools and reagents for studying OAT2. Specific substrates for human OAT2 that are not transported by other hepatic uptake transporters, likely have not yet been identified. A compound that can selectively inhibit the basolateral influx mediated by OAT2 without blocking the uptake processes mediated by OCT1 and OATP1B may be important, with respect to validating the function of OAT2 in primary hepatocytes. OAT2 expressed in *Xenopus* oocytes and HEK 293 cells is known to mediate transport of E3S (Kobayashi et al., 2005a, Kobayashi et al., 2014). But E3S, an organic anion, is also substrate for OATP1B1 and OATP2B1. Several early studies showed that PAH is a human OAT2 substrate, but Cropp et al. observed only appreciable uptake of PAH by HEK 293 cells stably transfected with OAT2 compared to the control cells (Cropp et al., 2008). Also
Hotchkiss et al. failed to observe transport of PAH by HEK-OAT2 and CHO-OAT2 cells although the uptake of PAH was approximately 2-fold higher in MDCK-OAT2 cells compared to parental cells (Hotchkiss et al., 2015). Because PROB is known to be a potent inhibitor of OAT1 and OAT3 with IC$_{50}$ values ranging from 3 to 28 µM (Chu et al., 2007, Juhasz et al., 2013, Shen et al., 2013), Enomoto et al. examined it’s inhibitory effect on PGF2α uptake by mouse second segment (S2) proximal tubule cells stably expressing OAT2 (S2-OAT2). PROB inhibited the OAT2-mediated uptake in concentration-dependent manner with an IC$_{50}$ value of 393 to 766 µM, which is almost equivalent to that against OATP1B1 (IC$_{50}$ of 79.4 to 740 µM) (Enomoto et al., 2002b, Izumi et al., 2013, Jia et al., 2015). Recently it was reported that IMC is a potent inhibitor of OAT2-mediated creatinine-d3 uptake with apparent IC50 of 2.1 ± 0.4 µM (Shen et al., 2015). Although IMC had no effect on MATE1 and OCT1 (Badolo et al., 2010, Shen et al., 2015), 20 µM IMC reduced OATP1B1-mediated estradiol-17-β-glucuronide uptake by 88.6% (Karlgren et al., 2012). After careful review of existing literature, it is apparent that tools for assessing OAT2 inhibition and activity in human primary hepatocytes are lacking.

3.2 Conflicting Literature Describing Renal OAT2

3.2.1 Expression and Localization of OAT2 in Kidney

Up to now, only a few reports have described the localization of OAT2 protein in human renal tissue. However, it is appreciated that OAT2 is unique in having different expression patterns in the kidney when compared to OAT1 and OAT3, in addition to exhibiting species- and sex-differences in the expression.
Enomoto et al. developed a custom antibody against the 14 amino acids of the carboxyl terminus of human OAT2 in rabbit and showed that human OAT2 was expressed at the basolateral membrane of RPTCs (Enomoto et al., 2002b). Cheng et al. also localized OAT2 protein to the basolateral membrane of RPTCs in the human renal cortex (Cheng et al., 2012). However, OAT2 has been recently reported to show a different expression pattern: while OAT1 and OAT3 are expressed similarly along the basolateral membrane of RPTCs, the expression of OAT2 is weaker at the basolateral membrane. In fact, OAT2 immunoactivity is more diffuse across the cytoplasm and even luminal membrane of renal tubule epithelial cells (Ikarashi et al., 2013). The localization of OAT2 limited to the basolateral membrane of RPTCs has been further challenged by the results of a recent study showing that the transporter is located at the basolateral, cytoplasmic and apical side of RPTCs of both humans and monkeys. In contrast, rat Oat2 are localized at the apical membrane RPTCs only (Shen et al., 2015). The expression of rodent Oat2 at the luminal membrane in RPTCs, or even the connecting duct, has been reported previously by a few laboratories (Kojima et al., 2002, Ljubojevic et al., 2007, Zlender et al., 2009). These results suggest species difference in localization of human, monkey, and rodent OAT2. The species-dependent cellular localization of OAT2 in RPTCs may be related to its driving force(s), which has not been fully understood as described previously. Although the simultaneous presence of a transporter at both apical and basolateral membranes of cells is not uncommon (Farrell et al., 1992, Harris et al., 1992, Gu et al., 2001, Godoy et al., 2014), the exact physiological role and function of OAT2 apically and basolaterally expressed in the tubule cells remains to be elucidated.
OAT2 expression appears to be sex-dependent, at least in the rat and mouse. In adult male Sprague-Dawley rat, significantly higher Oat2 levels were observed in the liver as compared with the kidney. In contrast, Oat2 mRNA expression is kidney-predominant as considerably higher Oat2 levels were seen in the female kidney as compared with liver (Buist et al., 2002). However, no sex difference in hepatic Oat2 mRNA expression has been reported for the rat (Buist et al., 2002). The female growth hormone secretion pattern is likely responsible for the Oat2 mRNA gender difference in the kidney as gonadectomy decreased renal Oat2 expression and growth hormone increased it in female rats (Buist et al., 2003). The gender differences and sex hormone regulation of Oat2 have been also observed at protein level, as female rats exhibit more immunoreactive protein in the brush border membrane of the proximal tubule S3 segment as compared with male rats. (Ljubojevic et al., 2007). Female rats exhibited more immunoreactive protein in the brush border membrane of the proximal tubule S3 segment as compared with male rats. The OAT2 protein in castrated male rats exhibit an approximately 6-fold increase compared to sham-operated male while the protein was decreased by 28% following ovariectomy in female rats. Testosterone treatment caused a remarkable decrease of Oat2 protein in castrated male rats to the levels observed in sham-operated, whereas estradiol and progesterone treatment caused a limited additional upregulation of the protein (Ljubojevic et al., 2007). In mice, the expression pattern largely resembles that of rats (Ljubojevic et al., 2007) (Buist and Klaassen, 2004, Cheng et al., 2008). Because species differences may exist, more information is needed regarding sex difference in human kidney OAT2 expression. Unfortunately, the recent studies of Prasad et al (2016) and Nakamura et al (2016) did not include a proteomic
analysis of human male versus female kidney cortex membrane preparations. However, both groups did conclude that OAT2 protein is present and expressed at lower levels versus OAT1, OAT3 and OCT2; 5.3 (OAT1), 0.9 (OAT2), 3.5 (OAT3), and 7.4 (OCT2) pmol/mg membrane protein (mean of N = 41 different organ donors) (Prasad et al., 2016); and 10 (OAT1), 1.5 (OAT2), 5 (OAT3), and 5 (OCT2) pmol/mg membrane protein (pool of kidney samples) (Nakamura et al., 2016).

### 3.2.2 Role of OAT2 in Creatinine Renal Clearance

It has been a common practice to use glomerular filtration rate (GFR) as an index of kidney function and the measurement of creatinine concentration in the plasma or serum is most widely used to estimate GFR (eGFR). Although creatinine, an organic cation with small molecular weight, is freely filtered through the renal glomerulus, active secretion accounts for 10 to 40% of total creatinine output in urine of human subjects (Levey et al., 1988). In RPTCs, the tubular secretion of creatinine from blood into urine is an active process, and is thought to be mediated (vectorially) by basolateral OCT2 and apical MATEs (Lepist et al., 2014, Shen et al., 2015). In fact, drugs that are inhibitors of renal organic cation transporters (i.e., OCT2/MATEs), including CMD, dolutegravir, pyrimethamine, trimethoprim, and vandetanib, inhibit creatinine secretion, resulting in increased creatinine concentrations in the blood, without causing kidney injury. Recently, Chu et al. collected OCT2, MATE1 and MATE2-K inhibition data for 16 drugs and conducted in vitro-in vivo extrapolation analysis by comparing total ($C_{\text{max}}$) and unbound maximum plasma concentrations ($C_{\text{max,u}}$) with in vitro IC$_{50}$ values (Chu et al., 2016). Because the relative contribution of individual transporters to the renal active tubular secretion of creatinine is unknown, OCT2, MATE1 and MATE-2K were assumed to
OAT2 can also transport creatinine, although reports have been rather contradictory. For example, using S2-OAT2 cells, the OAT2-mediated transport of creatinine was low and the authors concluded that creatinine was not an OAT2 substrate (Imamura et al., 2011). In contrast, Ciarimboli et al. employed OAT2 expressed in HEK 293 cells and showed that creatinine uptake was increased 2.3-fold (versus mock HEK 293 cells). Therefore, they concluded that OAT2 may play a role in creatinine clearance, although OCT2 plays a major role in creatinine tubular secretion (Ciarimboli et al., 2012). Similarly, Lepist et al. reported that creatinine was a high Km ($K_m = 986 \mu M$) OAT2 substrate in MDCK cells with the highest maximal velocity ($V_{max}=117 \times 10^4 \mu$pmol/min per mRNA copy number) versus OCT2 and OCT3 (Lepist et al., 2014). The transport rate at a physiological creatinine concentration (100 $\mu$M) for OAT2 was 4.7- and 2.3-fold higher than that estimated for OCT2 and OCT3, respectively. Recently, we confirmed the findings of Lepist et al. and showed that OAT2 is a more efficient
creatinine transporter compared to OCT2, MATE1 and MATE-2K (Shen et al., 2015); the creatinine transport efficiency by OAT2, the intrinsic transport clearance when normalized to the transporter protein, was significantly greater than that of OCT2 and MATEs (37- to 1850-fold). Moreover, potent OAT2 inhibitors (i.e., 50 µM BSP and 100 µM IMC) significantly inhibited (~50%) the intracellular uptake and trans-epithelial transport of creatinine by human RPTCs. These data suggest that OAT2 may play an important role in creatinine renal excretion. IMC was demonstrated to be a potent inhibitor for OAT2 over other known creatinine transporters OCT2, MATE1, and MATE-2K (IC\textsubscript{50} values of 2.1 µM for OAT2 versus > 200 µM for OCT2, MATE1 or MATE-2K) (Shen et al., 2015). Notably, IMC decreased creatinine tubular secretion from blood to urine, which was accompanied by the increase in creatinine plasma concentration in various clinical studies (Berg and Talseth, 1985, Al-Waili, 2002). In addition, the inhibitory effects disappeared after the cessation of IMC therapy in neonates (Kang et al., 1999, Walker et al., 2011). IMC is an inhibitor of prostaglandin synthesis, however, the transient effects might be caused by other mechanism(s) in addition to renal transporter inhibition. There is a least one report that IMC does not affect creatinine renal secretion clearance in humans, although the results of the study may be confounded by the consumption of a high-protein meal (Levey et al., 1988). The inhibition of renal OAT2 may help explain the false negative in vitro-in vivo extrapolations using OCT2 and MATE inhibition data for dronedarone, cobicistat, rilpivirine, and telaprevir (Chu et al., 2016). Unfortunately, the vitro OAT2 inhibition data for dronedarone, rilpivirine, and telaprevir are currently not available. But cobicistat showed weak inhibition of OAT2
compared with MATEs and/or OCT2. Taken together, additional clinical investigations regarding the inhibition of creatinine clearance by other OAT2 inhibitors are needed.

4 CONCLUSIONS AND OUTLOOK

Although the expression of OAT2 has been confirmed in the liver, kidney and many other tissues, its role in the disposition of drugs and endogenous molecules, species and gender differences, and genotype-phenotype associations requires further study. Such a lack of knowledge is best typified by the conflicting results obtained with creatinine in vitro and the conclusions of various investigators after attempting to localize the SLC within RTPCs. Unfortunately, the lack of selective OAT2 substrates and inhibitors, the paucity of freshly prepared human primary RPTCs, and limited information regarding the genetic polymorphisms of the human OAT2 gene and their relationship to OAT2 function have hindered progress to date. On the other hand, there is a consensus that OAT2 can mediate cGMP transport, thus potentially regulating the intracellular and extracellular levels of the important signaling cyclic nucleotide. How OAT2 functions coordinately with various multi drug resistance proteins (e.g., MRP4, MRP5, and MRP8) and the impact of OAT2 inhibition on cGMP signaling is not known. Despite the lack of knowledge, the fact that the in vitro OAT2-mediated transport efficiencies of certain drugs (i.e., acyclovir and ganciclovir) and important endogenous substrates (i.e., cGMP and creatinine) are relatively high, in addition to OAT2 expression in both liver and kidney, provides additional impetus for continued study of this enigmatic SLC. Such issues, in addition to efforts to sort out the roles of other uncharacterized OAT
transporters redundantly expressed on the plasma membrane of RPTCs and hepatocytes, are challenges for the future.
Acknowledgments

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Authorship Contributions

Participated in research design: Not applied

Conducted experiments: Not applied

Contributed new reagents or analytic tools: Not applied

Performed data analysis: Shen, Lai, and Rodrigues.

Wrote or contributed to the writing of the manuscript: Shen, Lai, and Rodrigues.

Other: None.
4 REFERENCES


Figure Legends

Figure 1. Model of OAT1/OAT3 transport in a single epithelial cell of the kidney (A) and localization of OATs in renal proximal tubule cells (RPTCs) (B) and hepatocytes (C). Question marks indicate that the membrane localization of OAT2 in RPTCs and hepatocytes are unknown.

Figure 2. Amino acid sequence and predicted membrane topology of human OAT2. Amino acid residues are designated by the single letter code. Topological structure of OAT2-548aa was generated using Protter version 1.0 (Omasits et al., 2014). Hydropathic analysis shows 12 transmembrane segments with intracellular NH2 and COOH termini. The large extracellular loop between transmembrane segments 1 and 2 contains one asparagine-X-serine/threonine (N-X-S/T) motif for potential N-linked glycosylation (N in green). Putative protein phosphorylation sites are located in the transmembrane segment 12 and large intracellular loop between transmembrane segments 6 and 7 (G and R in orange).

Figure 3. Comparison of IC50 values of dual inhibitors of human OAT1 and OAT3 (A), OAT1 and OAT2 (B) and OAT2 and OAT3 (C), respectively. The diagonal is the line of identity. The IC50 data are collected from the University of Washington Metabolism and Transport Drug Interaction Database™ (DIDB™) and listed in Supplemental Table 1. To test for statistically significant differences among different transporters for IC50, one-way analysis of variance was performed. When the F ratio showed that there were significant
differences among groups, the Turkey’s post-test was used to determine which groups differ.
Table 1. Properties of human organic anion transporter (OAT) 1-3s (NKT, novel kidney transporter; NLT, novel liver-specific transporter; ROCT, reduced in osteosclerosis transporter)

<table>
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<tr>
<th>Transporter</th>
<th>Gene Symbol</th>
<th>Alternative Name</th>
<th>Chromosomal Localization</th>
<th>Protein Length (aa)</th>
<th>mRNA Tissue Distribution</th>
<th>Protein Tissue Distribution</th>
<th>Membrane Localization</th>
<th>Transporter Mechanism</th>
<th>Major References</th>
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<td>OAT1</td>
<td>SLC22A6</td>
<td>NKT</td>
<td>11q12.3</td>
<td>563</td>
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<td>Kidney and choroid plexus</td>
<td>Basolateral</td>
<td>organic anion-dicarboxylate exchanger</td>
<td>Burckhardt, 2012; Emami Riedmaier et al., 2012; Nigam et al., 2015</td>
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<td>OAT2</td>
<td>SLC22A7</td>
<td>NLT</td>
<td>6p21.1</td>
<td>546, 548, 539</td>
<td>Liver &gt;&gt; brain, Stomach, ileum, uterus, mammary gland, etc.</td>
<td>Liver and kidney</td>
<td>Basolateral and apical in the kidney, and unknown in the liver</td>
<td>unknown</td>
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<td>542</td>
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<td>Kidney, choroid plexus and testes</td>
<td>Basolateral</td>
<td>organic anion-dicarboxylate exchanger</td>
<td>Burckhardt, 2012; Emami Riedmaier et al., 2012; Nigam et al., 2015</td>
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### Table 2. In vitro substrates of human OAT2

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<th>Cell System</th>
<th>Variant</th>
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<th>OAT1 Substrate (Km)</th>
<th>OAT1 Substrate (Km)</th>
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<td>Yes (416 to 772 µM)</td>
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<td>p-Aminohippuric acid (PAH)</td>
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<td>Yes (7.4 and 87.2 µM)</td>
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Figure 1
Figure 2
Figure 3