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Biomarkers for In Vivo Assessment of Transporter Function

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Abstract—Drug–drug interactions are a major concern not only during clinical practice, but also in drug development. Due to limitations of in vitro–in vivo predictions of transporter-mediated drug–drug interactions, multiple clinical Phase I drug–drug interaction studies may become necessary for a new molecular entity to assess potential drug interaction liabilities. This is a resource-intensive process and exposes study participants, who frequently are healthy volunteers without benefit from study treatment, to the potential risks of a new drug in development. Therefore, there is currently a major interest in new approaches for better prediction of transporter-mediated drug–drug interactions. In particular,

researchers in the field attempt to identify endogenous compounds as biomarkers for transporter function, such as hexadecanedioate, tetradecanedioate, coproporphyrins I and III, or glycochenodeoxycholate sulfate for hepatic uptake via organic anion transporting polypeptide 1B or N¹-methylnicotinamide for multidrug and toxin extrusion protein-mediated renal secretion. We summarize in this review the currently proposed biomarkers and potential limitations of the substances identified to date. Moreover, we suggest criteria based on current experiences, which may be used to assess the suitability of a biomarker for transporter function. Finally, further alternatives and supplemental approaches to classic drug–drug interaction studies are discussed.

I. Introduction

In the last two decades, significant progress has been made in the understanding of the relevance of transporters for absorption, disposition, and effects of drugs (Giacomini et al., 2010; Zolk and Fromm, 2011; DeGorter et al., 2012; Lepist and Ray, 2012; König et al., 2013; Yoshida et al., 2013). It is therefore no surprise that transporter-mediated drug–drug interactions gained considerable attention in academia, regulatory authorities, as well as pharmaceutical industry (Fromm et al., 1999; Giacomini et al., 2010; Hillgren et al., 2013; König et al., 2013; Yoshida et al., 2013; Koepsell, 2015).

The International Transporter Consortium (ITC), consisting of members from academia, industry, and the US Food and Drug Administration, published recommendations on in vitro and in vivo assessment of drug transporters of emerging clinical relevance. These recommendations include decision trees to guide planning of clinical trials based on in vitro transport and inhibition data. Specifically, decision trees were proposed for key drug transporters, including organic anion transporting polypeptides (OATP) 1B1 and 1B3, organic anion transporters (OAT) 1 and 3, organic cation transporter (OCT) 2, P-glycoprotein, breast cancer resistance protein (BCRP),

and multidrug and toxin extrusion proteins (MATE) 1 and 2-K (Giacomini et al., 2010; Hillgren et al., 2013). In line with the emerging clinical relevance of drug transporters, regulatory authorities in Europe, Japan, and the United States now expect thorough assessment of transporters for absorption, disposition, and drug–drug interactions of a new molecular entity by means of in vitro experiments and clinical studies [European Medicines Agency (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf); FDA (<https://www.fda.gov/downloads/drugs/guidances/ucm292362.pdf> and <https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM581965.pdf>); [http://www.solvobiotech.com/documents/Japanese_DDI_guideline_\(draft\)_2014Jan.pdf](http://www.solvobiotech.com/documents/Japanese_DDI_guideline_(draft)_2014Jan.pdf)]. It is no surprise that transporter information in drug labels of new molecular entities has considerably increased (Tweedie et al., 2013).

However, it has been recognized that current in vitro–in vivo extrapolations have limitations (Giacomini et al., 2010; Brouwer et al., 2013; Tweedie et al., 2013), among several other factors caused by interlaboratory variability of determined IC₅₀ values (Bentz et al., 2013). It has been argued that the currently proposed strategies may lead to an increased number of unnecessary drug–drug

ABBREVIATIONS 2-Py, N¹-methyl-2-pyridone-5-carboxamide; 4-Py, N¹-methyl-4-pyridone-3-carboxamide; ASP⁺, 4-(4-(dimethylamino)styryl)-N-methylpyridinium; AUC, area under the plasma/serum concentration-time curve; BCRP, breast cancer resistance protein; BSEP, bile salt export pump; C_{max}, maximum plasma/serum concentration; C_{max,u}, maximum, unbound plasma/serum concentration; DHEAS, dehydroepiandrosterone sulfate; ENT, equilibrative nucleoside transporter; GWAS, genome-wide association study; HEK cells, human embryonic kidney 293 cells; ITC, International Transporter Consortium; K_i, inhibition constant; K_m, (apparent) Michaelis-Menten constant; MATE, multidrug and toxin extrusion protein; MPP⁺, 1-methyl-4-phenylpyridinium; MRP, multidrug resistance protein; NNMT, nicotinamide N-methyltransferase; NTCP, sodium/taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; OCTN, organic zwitterion/cation transporter; PAT, proton-coupled amino acid transporter; PEPT, peptide transporter; PET, positron emission tomography; po, oral administration; SNP, single-nucleotide polymorphism; SULT2A1, sulfotransferase 2A1; TauT, taurine transporter; TEA, tetraethylammonium; THTR, thiamine transporter; V_{max}, maximal transport rate.

interaction studies in humans (Prueksaritanont et al., 2013; Dong et al., 2016; Vaidyanathan et al., 2016), and measures have been proposed to reduce false-positive predictions (i.e., a drug–drug interaction is predicted based on in vitro data, but not observed in vivo in humans; e.g., Ellens et al., 2013).

In the field of drug metabolism, several endogenous compounds have been discussed or implemented in clinical trials since many years as biomarkers for activity of certain enzymes in humans (e.g., 6 β -hydroxycortisol or 4 β -hydroxycholesterol for assessment of CYP3A activity) (Diczfalusy et al., 2011; Peng et al., 2011; Shibasaki et al., 2013; Shin et al., 2013; Kasichayanula et al., 2014). In light of the current limitations in the in vitro–in vivo prediction of transporter-mediated drug–drug interactions, endogenous compounds might be valuable tools for assessment of transporter activity in humans and for the prediction of the risk for transporter-mediated drug–drug interactions (Fromm, 2012; Ito et al., 2012a; Chu et al., 2017; Rodrigues et al., 2017).

A biomarker can be defined as a “biological characteristic that is objectively measured and evaluated as an indicator of normal biological or pathological processes, or a response to a therapeutic intervention” (<http://www.nature.com/subjects/biomarkers>). This review focuses on currently available in vitro and in vivo data on specific endogenous compounds as potential biomarkers of transporter function in humans and on potential limitations of these currently proposed biomarkers. Moreover, we propose criteria to support discussions regarding whether a proposed endogenous compound may be a valid biomarker for transporter function or not or whether further studies are necessary to make that decision. Finally, potential alternatives and supplemental approaches to classic perpetrator drug/victim drug interaction studies are discussed.

II. Drug Transporters

Several uptake and efflux transporters with clinical relevance are listed in a white paper published by the ITC (Giacomini et al., 2010). In this white paper, the uptake transporters OATP1B1 and OATP1B3, both members of the SLC21/SLCO family and OAT1, OAT3, and OCT2 from the SLC22 family, as well as the export pumps P-glycoprotein (ABCB family of the ABC transporter superfamily) and BCRP (ABCG family), have been discussed in detail with regard to their clinical importance (Giacomini et al., 2010). In 2013, the export proteins of the SLC47 family MATE1 and MATE2-K were added by the ITC to be evaluated during drug development (Hillgren et al., 2013). On the basis of preclinical and clinical observations, in vitro studies on the inhibition of multi-drug resistance protein (MRP) 2- (ABCC family; for clarification of drug-induced conjugated hyperbilirubinemia) and bile salt export pump-mediated export (BSEP, ABCB family; for clarification of cholestatic issues) are

recommended by the ITC (Hillgren et al., 2013). Other transporters are acknowledged as of emerging importance, such as the equilibrative nucleoside transporter 1 (ENT1, a member of the SLC29 family), the peptide transporters 1 and 2 (PEPT1 and PEPT2, members of the SLC15 family), OCT1, OATP2B1, MRP3, and MRP4 (Giacomini et al., 2010; Hillgren et al., 2013). Knowledge in this field is rapidly expanding, and it is expected that the understanding of the role of transporters for drug absorption and disposition, as well as for therapeutic and side effects will be further refined in the coming years. For some clinically relevant transporters, the knowledge on endogenous substrates is limited (e.g., P-glycoprotein) or only few endogenous compounds have been described as substrates (e.g., for BCRP). Transporters with compelling evidence for clinical relevance and for which data are available on potential biomarkers are discussed below in greater detail.

A. Organic Anion Transporting Polypeptides 1B1 and 1B3

The closely related SLC21/SLCO family members OATP1B1 (gene symbol *SLCO1B1*) (Hsiang et al., 1999; König et al., 2000b) and OATP1B3 (*SLCO1B3*) (König et al., 2000a) are predominantly, if not exclusively, expressed in human liver mediating the uptake of endogenous substances and xenobiotics from portal venous blood into hepatocytes across the basolateral membrane of hepatocytes. Both uptake transporters share an overlapping substrate spectrum with differences described in the affinities to the respective substrates. Endogenous substances transported by OATP1B1 and OATP1B3 include bile acids such as taurocholate (Maeda et al., 2006), hormone metabolites [e.g., estradiol-17 β -glucuronide and dehydroepiandrosterone sulfate (DHEAS)] (Kullak-Ublick et al., 2001), as well as bilirubin and bilirubin glucuronides (Cui et al., 2001; Briz et al., 2003). Drugs identified in vitro as substrates for OATP1B1 and OATP1B3 include 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) such as rosuvastatin and pravastatin; anticancer drugs such as methotrexate; and antibiotics such as rifampin (for an overview about the substrate spectrum, see König and Fromm, 2013). In addition, several substances have been identified in vitro as inhibitors of OATP1B1- and OATP1B3-mediated uptake. One potent (but not selective) inhibitor of both is cyclosporine with IC₅₀ values of 0.31 μ M for OATP1B1- and 0.06 μ M for OATP1B3-mediated rosuvastatin uptake (Ho et al., 2006). Clinical data with pravastatin indicate the relevance of this inhibitory effect by cyclosporine (Hedman et al., 2004). Another example for a clinically relevant inhibitor of OATP1B is the antibiotic rifampin (Prueksaritanont et al., 2014).

In addition to drug–drug interactions, genetic variants [single-nucleotide polymorphisms (SNPs) or

haplotypes] in the *SLCO1B1* and *SLCO1B3* genes encoding OATP1B1 or OATP1B3 may affect drug disposition. A clinically relevant and frequently investigated polymorphism in the *SLCO1B1* gene is *SLCO1B1*c.521T>C encoding the variant OATP1B1*5 (OATP1B1p.V174A) (Tirona et al., 2001). This variant shows reduced transport activity (Tirona et al., 2001) leading to altered pharmacokinetics of multiple drugs, as demonstrated, for example, for ezetimibe (Oswald et al., 2008) or pravastatin (Niemi et al., 2004). Furthermore, this variant is strongly associated with an increased risk of statin-induced myopathy (Link et al., 2008). The current knowledge on the clinical relevance of *SLCO1B1* genetic variants for drug disposition is summarized in an excellent review (Niemi et al., 2011).

B. Organic Anion Transporters 1 and 3

The uptake transporters OAT1 (gene symbol *SLC22A6*) and OAT3 (*SLC22A8*) belong to the organic cation/anion/zwitterion transporter family SLC22 (Koepsell, 2013). Both transporters are expressed primarily in human kidney and are localized to the basolateral membrane of proximal tubule cells (Hosoyamada et al., 1999; Motohashi et al., 2002). Expression of both OAT1 and OAT3 was also detected in other tissues such as the choroid plexus of human brain (Alebouyeh et al., 2003) or skeletal muscle (Takeda et al., 2004). OAT1 and OAT3 most likely operate as organic anion/ α -ketoglutarate exchangers (Bakhiya et al., 2003). Endogenous substances such as α -ketoglutarate (Lu et al., 1999), urate (Sato et al., 2008), and prostaglandin E₂ (Kimura et al., 2002) have been characterized as substrates of OAT1. OAT3 has an overlapping but not identical substrate spectrum to OAT1. For example, taurocholate has been described as substrate of OAT3, but was not transported by OAT1 (Chen et al., 2008). Several drugs have been identified in vitro as substrates or inhibitors of OAT1 and OAT3. These include angiotensin-converting enzyme inhibitors (e.g., captopril), angiotensin II receptor blockers (e.g., olmesartan), antiviral drugs (e.g., adefovir), and several nonsteroidal anti-inflammatory drugs (e.g., salicylate; for a summary of OAT1 and OAT3 drug substrates, see Burckhardt and Burckhardt, 2011).

The prototypic OAT1 and OAT3 inhibitor is probenecid. Using different organic anions as substrates, OAT1-mediated transport was inhibited by probenecid with inhibition constant (K_i) values between 4.3 and 12.1 μ M (Jung et al., 2001; Takeda et al., 2001), whereas probenecid competitively inhibited OAT3-mediated transport with K_i values between 4.4 and 9.0 μ M (Jung et al., 2001; Takeda et al., 2001). Inhibition of OAT1 and OAT3 may decrease renal tubular secretion of their substrates. For instance, decreased renal clearance and increased plasma concentrations of furosemide, a substrate of both OAT1 and OAT3 (Ebner et al., 2015), were observed when given with probenecid (Smith et al., 1980).

Nonsynonymous SNPs in the genes encoding OAT1 and OAT3 have been reported (Nishizato et al., 2003; Bleasby et al., 2005; Erdman et al., 2006). However, in contrast to the well-established clinical relevance of *SLCO1B1* genetic variations, the clinical relevance of variations in the genes encoding for OAT1, OAT3, and the other transporters discussed further below appears to be considerably lower.

C. Organic Cation Transporter 2

OCT2 (gene symbol *SLC22A2*) belongs to the same family as the OATs and is expressed in human kidney, where it is localized to the basolateral membrane of proximal tubule cells (Motohashi et al., 2002). OCT2 plays an important role in the renal secretion of organic cations. *SLC22A2* transcripts were also detected in several other organs, including lung and brain (Busch et al., 1998; Lips et al., 2005). OCT2 transports positively charged compounds in an electrogenic and sodium-independent manner. The cations 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP⁺), 1-methyl-4-phenylpyridinium (MPP⁺), and tetraethylammonium (TEA) are in vitro substrates. Endogenous substrates include acetylcholine, creatinine, N¹-methylnicotinamide, and epinephrine (for a summary of substrates, see Nies et al., 2011; Koepsell, 2015). Knockout mouse models have contributed to the understanding of the role of OCTs for drug disposition. Similar to OCT1 in humans, Oct1 is localized to the basolateral membrane of hepatocytes in mice. Whereas OCT2 is the primary OCT in human kidney, both Oct1 and Oct2 are found at the basolateral membrane of renal proximal tubule cells in mice. In Oct1 knockout mice, distribution of the antidiabetic metformin and the model cation TEA to liver is decreased, indicating a role of Oct1 in hepatocellular uptake of organic cations (Jonker et al., 2001; Wang et al., 2003). In Oct1/Oct2 double-knockout mice, plasma concentrations of metformin and TEA are increased, and renal clearance is decreased to approximately the level of glomerular filtration rate, indicating an absence of renal tubular net secretion associated with deletion of Oct function (Jonker et al., 2003; Higgins et al., 2012). The oral antidiabetic drug metformin, which is not relevantly metabolized in humans, is a clinically important drug substrate of OCT2 (Kimura et al., 2005) and may be used as a victim probe in drug–drug interaction studies. Cimetidine reportedly caused a 27% decrease of renal clearance and a 50% increase of the area under the plasma/serum concentration–time curve (AUC)_{0–24} of metformin (Somogyi et al., 1987). This interaction had been attributed to OCT2 inhibition by cimetidine in the past (see also *Multidrug and Toxin Extrusion Proteins 1 and 2-K*). However, the role of OCT2 inhibition in this interaction appears to be limited, because K_i values for OCT2 inhibition by cimetidine (95–724 μ M) (Lee et al., 2009; Ito et al., 2012b) exceed maximum, unbound plasma/serum concentration ($C_{\max,u}$) of cimetidine by several fold. Dolutegravir (50 mg twice daily) increased the AUC of

metformin by 145%, and a role of OCT2 inhibition in this interaction is discussed (Song et al., 2016). A summary of variations in the *SLC22A2* gene encoding for OCT2 is provided by Stocker et al. (2013). Among these, the variant *SLC22A2c.808G>T* encoding the protein OCT2p.A270S has been studied in several in vitro and in vivo studies that reported effects on the transport and kinetics of multiple drugs (Choi and Song, 2012; Iwata et al., 2012). From studies with metformin, lower (Song et al., 2008; Wang et al., 2008) or higher (Chen et al., 2009a) renal metformin clearance in subjects carrying the *SLC22A2c.808G>T* variant as compared with the homozygous wild-type carriers has been reported. A recently published meta-analysis investigating nine candidate polymorphisms in five transporter genes (including the variant OCT2p.A270S) found that none of the investigated variants showed a significant effect on metformin response as studied in 7968 individuals with type 2 diabetes (Dujic et al., 2017).

D. Multidrug and Toxin Extrusion Proteins 1 and 2-K

MATE1 (encoded by the *SLC47A1* gene) and MATE2-K (*SLC47A2*) belong to the SLC47 family of solute carriers. MATE1 expression has been reported in kidney, liver, adrenal gland, and other tissues, whereas MATE2-K is primarily expressed in the kidney (Otsuka et al., 2005; Masuda et al., 2006; Nies et al., 2016). Both MATE1 and MATE2-K are located in the brush border membrane of renal proximal tubule cells mediating the export of organic cations out of cells, using an oppositely directed proton gradient as driving force (Otsuka et al., 2005; Masuda et al., 2006; Tanihara et al., 2007). MATE1 in hepatocytes is thought to export its substrates into bile (Nies et al., 2016). Substrate specificities of MATEs partially overlap with those of OCT2, and OCT2 and MATEs are thought to work together in renal tubular secretion. TEA and MPP⁺ are model substrates for in vitro investigations, and examples of endogenous substrates are N¹-methylnicotinamide, creatinine, and thiamine. Drug substrates include metformin, the antiarrhythmic drug procainamide, and the H₂-antihistaminic cimetidine (for an overview, see Motohashi and Inui, 2013; Nies et al., 2016). As shown in several in vitro studies, cimetidine inhibited MATE-mediated transport with higher potency as compared with OCT2, and inhibition of MATEs is thought to underlie clinically observed interactions of cimetidine with, for example, metformin or procainamide (Tsuda et al., 2009b; Hillgren et al., 2013; Nies et al., 2016). Similarly, other clinical inhibitors of renal tubular organic cation secretion such as trimethoprim or vandetanib generally exhibit higher inhibitory potency toward MATEs as compared with OCT2 (Shen et al., 2013; Müller et al., 2015). For pyrimethamine, the difference in inhibitory potency between OCTs and MATEs is particularly pronounced with *K_i* values of 77 nM and 46 nM for MATE1 and MATE2-K and of 3.8

μM and 10 μM for OCT1 and OCT2, respectively, using TEA as substrate (Ito et al., 2010). Comparable *K_i* values are reported for inhibition of TEA transport by murine isoforms by pyrimethamine with 145 nM for Mate1, 3.6 μM for Oct1, and 6.0 μM for Oct2 (Ito et al., 2010). In mice, similar to humans, Mate1 is localized on the brush border membrane of renal proximal tubule cells. An ortholog protein corresponding to human MATE2-K, however, has not been identified in mouse kidney. In mice, the relevance of Mate1 for pharmacokinetics and effects of drugs has been studied by use of pyrimethamine at doses estimated to inhibit renal Mate1 but not Octs and also by use of Mate1 knockout models (Tsuda et al., 2009a; Ito et al., 2010; Kajiwarra et al., 2012). In humans, MATE inhibition may decrease renal clearance of MATE substrates. Administration of a single 50 mg dose of pyrimethamine in humans has been estimated to achieve unbound concentrations in kidney tissue and blood plasma that inhibit renal MATE activity without affecting OCT2 (Kusuhara et al., 2011). Kusuhara et al. (2011) reported results of a four-period fixed-sequence crossover trial in eight healthy male subjects, in which metformin was given at a microdose of 100 μg [single dose, oral administration (po)] in periods 1 and 2, and at a therapeutic dose of 250 mg (single dose, po) in periods 3 and 4. In periods 2 and 4, a single dose of 50 mg pyrimethamine was given 1 hour prior to metformin (Kusuhara et al., 2011). When given with pyrimethamine, renal clearance of metformin at microdose or therapeutic dose was decreased by 23% and 34%, respectively (Kusuhara et al., 2011). AUC_{0–12} and maximum plasma/serum concentration (*C_{max}*) of the therapeutic dose of metformin were increased by approximately 40%, whereas AUC_{0–12} and *C_{max}* values of metformin given as microdose were not significantly affected (Kusuhara et al., 2011). Genetic variations, including loss-of-function variants, have been described for both MATE1 and MATE2-K (Chen et al., 2009b; Kajiwarra et al., 2009). The relevance of MATEs for drug disposition and therapeutic effects, including the impact of genetic variants, has recently been discussed in an excellent review (Nies et al., 2016).

III. Biomarkers for Transporter Function

A. Biomarkers for Function of Organic Anion Transporting Polypeptides

Coproporphyrins, fatty acid dicarboxylates, and glycochenodeoxycholate sulfate have been proposed as potential biomarkers for hepatic OATPs (Lai et al., 2016; Yee et al., 2016; Shen et al., 2017a; Takehara et al., 2017) (Tables 1 and 2). Table 1 summarizes selected disposition parameters of these endogenous compounds in humans. In vitro substrate characteristics for human transporters, formation and metabolism, as well as additional confounders, are summarized in Table 2. For these potential biomarkers, plasma/serum concentrations are

likely a suitable parameter for assessment of the likelihood of OATP-mediated drug–drug interactions (Lai et al., 2016; Yee et al., 2016; Shen et al., 2017a; Takehara et al., 2017). A recent comparative evaluation in healthy volunteers concluded, based on the effects of rifampin (administered together with rosuvastatin), that coproporphyrins and the fatty acid dicarboxylates tetradecanedioate and hexadecanedioate, but neither 13 bile acids nor DHEAS, could serve as sensitive endogenous biomarkers of OATP activity (for details, see below; Shen et al., 2017a). Shen et al. proposed that a panel of endogenous compounds, including coproporphyrins I and III, tetradecanedioate, and hexadecanedioate, may be the method of choice to detect changes in OATP1B activity in early clinical studies, as the individual biomarkers may have different sensitivities to potentially confounding factors, such as inhibition of drug transporters other than OATP1B or modulation of enzyme activity (Shen et al., 2017a). The bile acid glycochenodeoxycholate sulfate had initially been proposed as a biomarker of OAT3 activity (see *Biomarkers for Function of Organic Anion Transporters*) (Tsuruya et al., 2016). In addition, Takehara et al. (2017) recently proposed glycochenodeoxycholate sulfate as endogenous marker of OATP1B function.

1. Coproporphyrins I and III. The isomers coproporphyrin I and III have been extensively characterized in vitro as substrates of various transporters (Table 2) (Bednarczyk and Boiselle, 2016; Shen et al., 2017a). Coproporphyrins I and III are substrates of OATP1B1 with (apparent) Michaelis-Menten constant (K_m) values of 0.13 and 0.22 μM , respectively (Bednarczyk and Boiselle, 2016). Reported K_m values for OATP1B3-mediated transport were 4.0 μM for coproporphyrin I and 1.6 μM for coproporphyrin III (Bednarczyk and Boiselle, 2016). Interestingly, only coproporphyrin III, but not coproporphyrin I, was transported by OATP2B1 with a K_m value of 0.31 μM (Bednarczyk and Boiselle, 2016). Coproporphyrins I and III are not substrates of OAT1, OAT2, OAT3, OAT4, OCT1, OCT2, MATE1, MATE2-K, and sodium/taurocholate cotransporting polypeptide (NTCP) (Bednarczyk and Boiselle, 2016; Shen et al., 2017a).

Shen et al. (2016) found evidence in vitro and in cynomolgus monkeys that coproporphyrins I and III could be useful biomarkers of hepatic OATP activity in preclinical species. They showed that coproporphyrins I and III are not only substrates of human OATP1B1 and OATP1B3, but also of cynomolgus monkey OATP1B1 and OATP1B3 (Shen et al., 2016). Oatp1a/1b gene-cluster knockout mice had drastically increased coproporphyrin I and III plasma concentrations and an increased urinary excretion compared with respective wild-type animals (Shen et al., 2016). The OATP inhibitor rifampin significantly increased coproporphyrin I and III plasma concentrations and urinary excretion in cynomolgus monkeys, whereas renal clearance of both coproporphyrins was not significantly altered.

Without inhibitor, coproporphyrin III renal clearance was considerably higher compared with coproporphyrin I in the cynomolgus monkeys (63 versus 1.8 ml/min) (Shen et al., 2016).

In a follow-up study, Lai et al. (2016) investigated whether coproporphyrins I and III might be valuable biomarkers for OATP-mediated drug–drug interactions in humans. They performed a three-period fixed-sequence crossover study in 12 healthy male volunteers, in which they administered rifampin 600 mg single dose po as potent OATP inhibitor alone, rosuvastatin 5 mg single dose po as sensitive OATP probe alone, or the combination of rosuvastatin and rifampin in periods 1, 2, and 3, respectively (Lai et al., 2016). None of the subjects had *SLCO1B1**5 or *15 variations, which have previously been associated with increased plasma concentrations of OATP1B1 substrates (Seithel et al., 2008; Niemi et al., 2011). Without OATP inhibition, but in the presence of the OATP substrate rosuvastatin, renal clearance of coproporphyrin I was approximately 30 ml/min (serum protein binding: 72%; Table 1), suggesting passive renal elimination (Lai et al., 2016). In contrast, renal clearance of coproporphyrin III was approximately 190 ml/min (serum protein binding: 72%; Table 1), indicating an important contribution of active renal secretion to coproporphyrin III renal elimination (Lai et al., 2016). At present it is unclear which transporters are responsible for renal secretion of coproporphyrin III. Rifampin increased coproporphyrin I and III C_{max} $\geq$ fivefold and AUC_{0-24} $\geq$ threefold (Lai et al., 2016). Rifampin also increased the amount of coproporphyrin I excreted into urine by approximately 3.5-fold, but had only a smaller and nonsignificant effect on the amount of coproporphyrin III excreted into urine. C_{max} and AUC_{0-24} of rosuvastatin increased 13.2- and 5.0-fold, respectively, when rifampin was coadministered. No circadian changes in coproporphyrin I and III plasma concentrations were found (Lai et al., 2016). The authors concluded that coproporphyrin I and III plasma concentrations and urinary coproporphyrin I could be suitable biomarkers for drug–drug interactions with involvement of OATPs (Lai et al., 2016).

Coproporphyrins I and III are side products of heme synthesis (Shen et al., 2016). No further metabolism in the liver has been reported (Kaplowitz et al., 1972). Plasma levels, urinary excretion, and/or fecal disposition of coproporphyrins I and III differ in a number of diseases such as porphyrias, Dubin-Johnson syndrome, and Rotor syndrome (Wolkoff et al., 1976; Puy et al., 2010; Szlendak et al., 2016). Dubin-Johnson syndrome is characterized by a hereditary deficiency of MRP2 (Keppler, 2014). Rotor syndrome is a hereditary disease characterized by conjugated hyperbilirubinemia and coproporphyrinuria, caused by complete deficiency of OATP1B1 and OATP1B3 (van de Steeg et al., 2012). Because coproporphyrins I and III are substrates of OATP1B1 and OATP1B3, changes in coproporphyrin I and III urinary

elimination can nicely be explained by reduced (re-)uptake of these substances into liver, leading to a shift from biliary to urinary elimination in particular for coproporphyrin I (van de Steeg et al., 2012; Bednarczyk and Boisselle, 2016). Urinary coproporphyrins have been suggested in a small genetic association study to be potential biomarkers of MRP2 (Benz-de Bretagne et al., 2011). Accordingly, coproporphyrin I was recently identified as an MRP2 substrate ($K_m = 7.7 \mu\text{M}$) (Gilibili et al., 2017). Due to light sensitivity of porphyrins (Sobel et al., 1974), care should be taken to protect samples as much as possible from light (Lai et al., 2016).

2. Tetradecanedioate and Hexadecanedioate. The fatty acid dicarboxylates tetradecanedioate and hexadecanedioate are substrates of OATP1B1, but not of other OATPs such as OATP1A2, OATP1B3, and OATP2B1 (Yee et al., 2016). Moreover, tetradecanedioate and hexadecanedioate are substrates of OAT1 and OAT3 (Yee et al., 2016). All in vitro transport studies were performed at single substrate concentrations in nanomolar range, and no affinities of tetradecanedioate and hexadecanedioate to the respective transporters have been determined to date (Yee et al., 2016).

Yee et al. (2016) identified potential biomarkers for OATP1B1 using publicly available data from genome-wide association studies (GWAS) (Shin et al., 2014) and a metabolomic analysis of plasma samples from a pravastatin–cyclosporine drug–drug interaction study. Twenty endogenous metabolites were significantly associated with a functionally impaired OATP1B1 nonsynonymous variant (OATP1B1p.Val174Ala, *SLCO1B1*c.521T>C), among them bile acids, steroids, fatty acid dicarboxylates, and lysolipids (Yee et al., 2016). The metabolomic analysis revealed that plasma concentrations of 12 of the 20 metabolites found to be associated with the OATP1B1 nonsynonymous variant OATP1B1p.Val174Ala were increased in the healthy volunteers after coadministration of the OATP inhibitor cyclosporine (Yee et al., 2016). In vitro inhibition of OATPs and other drug transporters was tested for six compounds. Tetradecanedioate and hexadecanedioate inhibited only OATP1B1 (and OAT1 and OAT3), whereas the other tested substances inhibited multiple transporters or were not substrates of OATP1B1 (Yee et al., 2016). Interestingly, tetradecanedioate and hexadecanedioate in plasma correlated with pravastatin AUC, indicating the potential utility of tetradecanedioate and hexadecanedioate as quantitative biomarkers (Yee et al., 2016). In line with the findings by Yee et al. (2016) described above, Yu et al. (2016) also reported an association between *SLCO1B1* mutations and hexadecanedioate serum levels. In agreement with these findings, Shen et al. (2017a) recently reported a significant effect of rifampin on tetradecanedioate and hexadecanedioate plasma concentrations in healthy volunteers (2.8-fold

and 2.2-fold increase in AUC_{0-24} , respectively, when rifampin alone was given compared with a control phase with rosuvastatin alone).

The findings by Shen et al. (2017a) indicate only very modest circadian changes in tetradecanedioate and hexadecanedioate plasma concentrations in healthy volunteers. A standard meal did also not appear to have impact on tetradecanedioate and hexadecanedioate plasma concentrations in healthy volunteers (Tables 1 and 2) (Lai et al., 2016; Shen et al., 2017a). Recent data indicate an association between hexadecanedioate fasting blood concentrations and blood pressure (Menni et al., 2015). The work by Menni et al. (2015) also indicates a correlation between fasting blood concentrations of tetradecanedioate and hexadecanedioate. In the study by Yu et al. (2016) described above, the authors not only reported an association between the *SLCO1B1* splice donor mutation c.481+1G>T and hexadecanedioate serum levels, but also that one copy of the mutated T allele in *SLCO1B1*c.481+1G>T was associated with a 29% increased risk for heart failure ($P = 0.048$).

The importance of OAT1 and OAT3 for tetradecanedioate and hexadecanedioate disposition requires further studies. In GWAS described above, tetradecanedioate and hexadecanedioate were not only associated with *SLCO1B1* variants, but also with variations in *CYP4A11* (Yee et al., 2016). Moreover, Yee et al. (2016) showed that tetradecanedioate inhibits CYP4A11 function, suggesting that fatty acid dicarboxylic acids may be CYP4A11 substrates or products.

3. Glycochenodeoxycholate Sulfate. Glycochenodeoxycholate sulfate (glycochenodeoxycholate-3-sulfate) is a substrate of OATP1B1 and OATP1B3, with reported K_m values of 10 and $5.2 \mu\text{M}$, respectively (Takehara et al., 2017). In addition, the bile acid conjugate is a substrate of NTCP (Takehara et al., 2017) and of OAT3 (see *Biomarkers for Function of Organic Anion Transporters* and Table 2) (Tsuruya et al., 2016).

Glycochenodeoxycholate sulfate is a major sulfate conjugate of bile acids yielding about one fourth of total bile acid sulfate conjugates in both serum and urine (Bathena et al., 2013). Bile acid synthesis is a multi-step/multi-enzyme process starting with cholesterol, yielding amidated (conjugated with glycine or taurine) and nonamidated bile acids (Russell, 2009). Sulfation by sulfotransferase 2A1 (SULT2A1) is the major pathway for further metabolism of bile acids (Alnouti, 2009; Bathena et al., 2013). Sulfation at the 3-hydroxy position is considered the likely major metabolic step in humans (Alnouti, 2009; Bathena et al., 2013), resulting in sulfated bile acids such as the sulfated glycine conjugate glycochenodeoxycholate sulfate. Sulfation is an important step in detoxification and facilitation of excretion of bile acids, as sulfation enhances bile salt solubility, decreases their enterohepatic cycling, and increases their urinary and fecal excretion (Alnouti, 2009; Bathena et al., 2013). Desulfation may occur in

human colon, resulting in limited bile acid reabsorption (Alnouti, 2009).

In healthy volunteers, glycochenodeoxycholate sulfate plasma concentration showed moderate circadian differences (Tsuruya et al., 2016; Takehara et al., 2017). Glycochenodeoxycholate sulfate has a high plasma protein binding of 98% (Tsuruya et al., 2016). Its renal clearance in healthy volunteers is approximately 4 ml/min (Tsuruya et al., 2016). Tsuruya et al. (2016) suggested that OAT4 could be one candidate mediating tubular reabsorption of glycochenodeoxycholate sulfate. Detailed analyses on bile acid concentrations in healthy subjects and patients with liver disease have recently been published (Bathena et al., 2013, 2015b,a; Haag et al., 2015). Generally, serum bile acid concentrations were increased after food intake, whereas urinary concentrations of bile acids were slightly decreased (Bathena et al., 2015a). Effects of sex, age, body mass index, and moderate alcohol consumption on bile acid profiles in urine and serum were minimal (Bathena et al., 2015a).

Likely glycochenodeoxycholate sulfate (X-12850) showed signals in the transporter biomarker study by Yee et al. (2016) that revealed fatty acid dicarboxylates as potential markers of OATP1B1 activity, which is described in greater detail above. Likely glycochenodeoxycholate sulfate (X-12850) was significantly associated with an OATP1B1 nonsynonymous variant (OATP1B1p.Val174Ala, *SLCO1B1*c.521T>C) in a genome-wide metabolomic study, and its plasma concentrations also significantly increased in healthy volunteers treated with cyclosporine (Yee et al., 2016), which inhibits OATP1B1, but also several other drug transporters. In vitro, glycochenodeoxycholate (the sulfate could not be tested) at a concentration of 30 μ M significantly (>70%) inhibited transport of prototypical substrates by OATP1B1, but also by OATP1B3, NTCP, and BSEP (Yee et al., 2016). The results of the study by Yee et al. (2016) suggest that variation in function of OATP1B1 can affect plasma concentrations of glycochenodeoxycholate sulfate.

Takehara et al. (2017) used plasma samples from a prior fixed-sequence three-period crossover drug–drug interaction trial in eight healthy subjects. In this trial, the subjects received in all three treatment periods a cocktail consisting of subtherapeutic doses of clarithromycin, darunavir, midazolam, and the OATP1B substrates simeprevir, bosentan, and repaglinide. In the second treatment period, the subjects received in addition a single dose of the OATP inhibitor rifampin (600 mg po), and in the third treatment period, the subjects received in addition a single dose of itraconazole (Takehara et al., 2017). Plasma concentrations of glycochenodeoxycholate sulfate were determined in samples of treatment periods 1 and 2. In the second treatment period (cocktail with rifampin), the AUC_{0–24} of glycochenodeoxycholate sulfate was 20-fold higher as compared with the first treatment period (cocktail

alone) (Takehara et al., 2017). The effect of rifampin on glycochenodeoxycholate sulfate AUC was more pronounced as compared with the effect of rifampin on the three OATP1B substrate probe drugs simeprevir, bosentan, and repaglinide. The authors concluded that glycochenodeoxycholate sulfate can be used in healthy volunteers as a surrogate endogenous probe for OATP1B activity (Takehara et al., 2017).

4. Other Potential Biomarkers. Other potential biomarkers for hepatic OATPs have been proposed albeit with a weaker data basis for prediction of drug–drug interactions in humans compared with the compounds discussed above. Table 3 summarizes information on these potential biomarkers with currently limited evidence or limitations.

a. Bilirubin. Transport of unconjugated and conjugated bilirubin by OATP1B1 and OATP1B3 and the importance of these transporters for disposition of unconjugated and conjugated bilirubin have recently been reviewed (Keppler, 2014). Conjugated bilirubin is also transported by the conjugate efflux pump MRP2 localized in the canalicular membrane of hepatocytes as well as by MRP3 localized in the sinusoidal membrane of hepatocytes (for review, see Keppler, 2014).

In vitro studies as well as experiments in rats (Watanabe et al., 2015b) suggested that bilirubin glucuronides may be used as biomarkers for Oatp/Mrp2 inhibition in preclinical drug development as well as OATP/MRP2 inhibition during early clinical drug development. Chu et al. (2015) evaluated potential biomarkers for OATP1B inhibition and reported that conjugated and unconjugated bilirubin could be endogenous biomarkers for inhibition of hepatic transporters, particularly OATP1B in cynomolgus monkeys. In healthy volunteers, a single oral 600 mg dose of the OATP inhibitor rifampin increased AUC and C_{max} of total bilirubin 2.1-fold and 2.3-fold, respectively (Prueksaritanont et al., 2017). AUC and C_{max} of conjugated bilirubin were elevated 2.3-fold and 2.6-fold, respectively (Prueksaritanont et al., 2017). Prueksaritanont et al. (2017) discussed that these relatively small changes in bilirubin plasma concentrations in humans suggest a limited sensitivity of bilirubin as endogenous biomarker, in particular if weaker OATP1B inhibitors are evaluated. It was also emphasized that due to the fact that multiple enzymes and transporters are involved in bilirubin disposition (Keppler, 2014; Namjou et al., 2015), the specificity of the perpetrator should be thoroughly evaluated, if bilirubin is used to study OATP1B inhibition (Prueksaritanont et al., 2017).

b. Bile Acids and Bile Acid Conjugates. In addition to glycochenodeoxycholate sulfate (see above), further bile acids and bile acid conjugates are discussed as biomarkers of OATP1B activity. General aspects of bile acid synthesis and metabolism are discussed above in the section on glycochenodeoxycholate sulfate.

Hepatocyte transport of bile acids and regulation of bile acid synthesis have been reviewed by Wolkoff and Cohen (2003) and Fuchs (2003). Detailed analyses of bile acid concentrations in healthy subjects and patients with liver disease have recently been published (Bathena et al., 2013, 2015b,a; Haag et al., 2015). A recent study by Suga et al. (2017) investigated the OATP1B1- and OATP1B3-mediated transport of five major unconjugated human bile acids (cholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, and lithocholic acid) together with their glycine and taurine conjugates. In line with previous studies (Maeda et al., 2006; König et al., 2012), ursodeoxycholic acid was not transported by OATP1B1 and OATP1B3. Suga et al. (2017) also reported that lithocholic acid was not significantly transported by OATP1B1 and OATP1B3. Cholic acid, chenodeoxycholic acid, and deoxycholic acid, as well as the glycine- and taurine-conjugated bile acids, were transported by OATP1B1 and OATP1B3, and it was concluded that conjugated bile acids are preferred substrates of OATP1B1 (K_m 0.74–14.7 μM) and OATP1B3 (K_m 0.47–15.3 μM) compared with unconjugated bile acids (Suga et al., 2017).

Chu et al. (2015) studied the effect of the OATP inhibitor rifampin on bile acid plasma concentrations in cynomolgus monkeys. They concluded that the bile acids investigated in that study (cholic acid, chenodeoxycholic acid, deoxycholic acid, glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid, glycolithocholic acid, hyodeoxycholic acid, lithocholic acid, taurocholic acid, taurochenodeoxycholic acid, and taurodeoxycholic acid) are not sensitive biomarkers for hepatic uptake transporters in cynomolgus monkeys, although a weak increase in plasma concentrations of glycocholic acid, glycochenodeoxycholic acid, and glycodeoxycholic acid by rifampin was observed (Chu et al., 2015). In another study on the effect of rifampin on concentrations of 30 bile acids in cynomolgus monkeys, Thakare et al. (2017) found pronounced changes for sulfated bile acids. In the report by Yee et al. (2016) described above in the section on fatty acid dicarboxylates, tauroolithocholate-3-sulfate and presumably three additional conjugated bile acids [X-11529 (likely to be glycochenodeoxycholate glucuronide), X-13429 (likely to be glycodeoxycholate sulfate or isomer), and X-12850 (likely to be glycochenodeoxycholate sulfate)] were significantly associated with rs4149056 (OATP1B1p.Val174Ala, *SLCO1B1*c.521T>C) in a GWAS and also showed significant increases in plasma concentrations in healthy volunteers after administration of cyclosporine (Table 3). Glycochenodeoxycholate sulfate has been further characterized as in vitro substrate and in vivo marker of OATP1B and OAT3 activity by Takehara et al. (2017) and Tsuruya et al. (2016) (see above, *Biomarkers for Function of Organic Anion Transporters*, and Tables 1 and 2).

Further work is required to characterize the other substances as substrates of transporters. The same applies for additional metabolites also identified by the study of Yee et al. (2016) (Table 3).

Shen et al. (2017a) used plasma samples of a previous study (Lai et al., 2016), which is described in detail in *Coproporphyrins I and III*, for investigations on the suitability of 13 bile acids (deoxycholic acid, cholic acid, chenodeoxycholic acid, glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid, glyoursodeoxycholic acid, lithocholic acid, taurocholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid, taurohyodeoxycholic acid/taoursodeoxycholic acid, and ursodeoxycholic acid/hyodeoxycholic acid) as endogenous biomarkers of OATP activity. They reported for 8 of the 13 bile acids remarkable increases in plasma concentrations after the first meal. Although AUC_{0-24} for some of the bile acids was considerably higher in the treatment period with rifampin alone as compared with the period with rosuvastatin alone, e.g., for glycodeoxycholic acid (7.4-fold) and taurodeoxycholic acid (4.8-fold), the repeated measurement model yielded that increases of plasma concentrations were not significant (Shen et al., 2017a). In the study that identified glycochenodeoxycholate sulfate as potential marker of OATP1B activity in humans by use of rifampin as OATP inhibitor, Takehara et al. (2017) also investigated the effect of rifampin on plasma concentrations of other bile acids. The authors reported that the AUCs of bile acids, including cholic acid, chenodeoxycholic acid, glycocholic acid, glycochenodeoxycholic acid/glycodeoxycholic acid, taurocholic acid, taurochenodeoxycholic acid/taurodeoxycholic acid, and taoursodeoxycholic acid, were significantly higher in the treatment period with rifampin as compared with the control period without rifampin (Takehara et al., 2017). Chenodeoxycholate-24-glucuronide plasma concentrations were mostly below lower limit of quantification in the control period, but the AUC_{0-24} was ~fourfold higher than the product of the lower limit of quantification and 24 hours in the rifampin period (Takehara et al., 2017). Further research is needed to elucidate which bile acids and bile acid conjugates or which panels of these substances may be suitable biomarkers for assessment of OATP1B activity in clinical trials.

It should be noted that a single-dose administration of the NTCP inhibitor myrcludex B to healthy volunteers caused pronounced increases in plasma concentrations of glycine-conjugated and taurine-conjugated bile acids in healthy volunteers (Haag et al., 2015), indicating the potential limitations of bile acids as biomarkers for function of specific transporters (e.g., OATPs).

c. Dehydroepiandrosterone Sulfate. DHEAS is a metabolic product of the endogenous sex steroid precursor dehydroepiandrosterone (Table 3) (Haring et al., 2012; Watanabe et al., 2015a). In vitro, it is a substrate of human OATP1B1 and OATP1B3 (e.g., Cui et al., 2001).

Based on studies in cynomolgus monkeys and in rats, DHEAS was suggested as potential endogenous probe for the prediction of drug–drug interactions mediated by inhibition of hepatic OATPs (Watanabe et al., 2015a; Nishizawa et al., 2017). DHEAS serum concentrations depend on sex with significantly higher concentrations in men than in women at ages 20–69 years and strongly depend on age (Orentreich et al., 1984). SULT2A1 catalyses formation of DHEAS from dehydroepiandrosterone (Rainey et al., 2002). Zhai et al. (2011) reported in a meta-analysis of genome-wide association data with 14,846 individuals that eight common genetic variants were associated with serum DHEAS concentrations, one of which was located near the coding region of *SULT2A1*. However, a subsequent study did not find an association of SULT2A1 SNPs with DHEAS serum concentrations (Haring et al., 2012). Shen et al. (2017a) and Takehara et al. (2017) recently reported the absence of an effect of rifampin on DHEAS plasma concentrations in two independent studies in healthy volunteers. These findings argue against a usability of DHEAS as endogenous biomarker for OATP-mediated drug–drug interactions (Shen et al., 2017a; Takehara et al., 2017).

B. Biomarkers for Function of Organic Anion Transporters

Taurine, glycochenodeoxycholate sulfate, and 6 β -hydroxycortisol have been proposed as potential biomarkers for OAT1 or OAT3 (Imamura et al., 2014; Tsuruya et al., 2016) (Tables 1 and 2). Table 1 summarizes selected disposition parameters of these endogenous compounds in humans. In vitro substrate characteristics of these potential biomarkers for human transporters, their formation and metabolism, as well as additional confounders, are summarized in Table 2. For all three compounds, renal clearance has been proposed as parameter for assessment of the potential of a perpetrator drug to cause OAT1/OAT3-mediated drug–drug interactions (Imamura et al., 2014; Tsuruya et al., 2016).

The amino acid taurine is a substrate of OAT1 with a K_m value of 379 μ M (Tsuruya et al., 2016) and is not transported by OAT3 (Tsuruya et al., 2016) (Table 2). In contrast, glycochenodeoxycholate sulfate was transported by OAT3 with a K_m value of 64 μ M, but not by OAT1 (Tsuruya et al., 2016). Tsuruya et al. (2016) analyzed taurine and glycochenodeoxycholate sulfate in plasma and urine samples of a study in healthy volunteers, who received the OAT3 substrate benzylpenicillin in all four study periods of a four-period fixed-sequence crossover study. In addition, increasing doses of the OAT1 and OAT3 inhibitor probenecid (500, 750, and 1500 mg po, respectively) were administered in three of the four study periods (Tsuruya et al., 2016). Initially, peaks of nine endogenous compounds were identified by a metabolomic analysis of plasma and urine, which showed clear effects of probenecid in all volunteers. Structural identity of six of the nine peaks was unclear; one peak was identified as

inosine, and two peaks were identified as taurine and glycochenodeoxycholate sulfate, which were both chosen for further evaluation in the clinical study described above (Tsuruya et al., 2016).

Probenecid caused a dose-dependent reduction of taurine renal clearance by 45%–70% without significant alterations of taurine AUC. Moreover, probenecid caused a dose-dependent reduction of glycochenodeoxycholate sulfate renal clearance by 60%–88% without significant alterations of glycochenodeoxycholate sulfate AUC (Tsuruya et al., 2016). In the samples of this study, kinetic parameters of the OAT3 substrate 6 β -hydroxycortisol had also been determined. Mean renal clearances of the OAT substrates benzylpenicillin, 6 β -hydroxycortisol, taurine, and glycochenodeoxycholate sulfate obtained from the four study periods strongly correlated among each other (Tsuruya et al., 2016). Finally, Tsuruya et al. (2016) reported that the extent of the reduction of taurine and glycochenodeoxycholate sulfate renal clearance by probenecid could satisfactorily be explained by using geometric mean values of unbound probenecid concentrations as well as experimentally determined K_i values for inhibition of OAT1- and OAT3-mediated transport of taurine and glycochenodeoxycholate sulfate, respectively (Tsuruya et al., 2016). Tsuruya et al. (2016) concluded that renal clearance of taurine and glycochenodeoxycholate sulfate can be used for investigations on OAT1- and OAT3-mediated drug–drug interactions, respectively.

Further studies investigating glycochenodeoxycholate sulfate as potential biomarker should consider the discussion by Tsuruya et al. (2016) that possibly glycodeoxycholate sulfate was detected as glycochenodeoxycholate sulfate in their analysis, because glycodeoxycholate and glycochenodeoxycholate are isomers. Abundances of glycochenodeoxycholate sulfate and glycodeoxycholate sulfate are similar in both serum and urine (Bathena et al., 2013).

Glycochenodeoxycholate sulfate has also been proposed as a biomarker of OATP1B activity (see *Biomarkers for Function of Organic Anion Transporting Polypeptides*). Tsuruya et al. (2016) commented that, due to the minor effect of probenecid on glycochenodeoxycholate sulfate AUC, inhibition of OATP1B1 and OATP1B3 by probenecid is likely of minor relevance.

It should be noted that all in vivo data for taurine and glycochenodeoxycholate sulfate from the study in healthy volunteers by Tsuruya et al. (2016) were obtained in the presence of the OAT3 substrate benzylpenicillin. Further work should address whether benzylpenicillin affects plasma concentrations or renal elimination of taurine and glycochenodeoxycholate sulfate. Moreover, it was discussed that other bile acids might also be suitable OAT3 biomarkers (Tsuruya et al., 2016). Further details on formation and disposition of bile acids, including glycochenodeoxycholate sulfate, are described in *Biomarkers for Function of Organic Anion Transporting Polypeptides*.

TABLE 1
Potential biomarkers for transporter function and their disposition in humans

Transporter	Biomarker	Biomarker Characteristics	Molecular Weight (g/mol)	Plasma Concentration (nM)	Circadian Rhythm of Plasma Concentration	Plasma/Serum Protein Binding (%)	Renal Clearance (ml/min)	Suggested PK Parameter for DDI Biomarker	Reference
OATP1B	Hexadecanedioate	Fatty acid dicarboxylate	284.4	~30	(No)	Unknown	Unknown	Plasma concentration	Yee et al., 2016; Shen et al., 2017a
	Tetradecanedioate	Fatty acid dicarboxylate	256.3	~20	(No)	Unknown	Unknown	Plasma concentration	Yee et al., 2016; Shen et al., 2017a
	Coproporphyrin I	Porphyrin metabolite	654.7	~0.91	No	72	~30	AUC, C _{max} , A _e	Kaplowitz et al., 1972; Lai et al., 2016
	Coproporphyrin III	Porphyrin metabolite	654.7	~0.15	No	72	~190	AUC, C _{max}	Kaplowitz et al., 1972; Lai et al., 2016
OAT1 OAT3, OATP1B	Taurine Glycochenodeoxycholate sulfate	Amino acid Bile acid conjugate	125.1 529.7	~60,000 ~100	(Yes) (Yes)	0 98	~25 ~4	CL _R CL _R (OAT3); AUC (OATP1B)	Tsuruya et al., 2016 Tsuruya et al., 2016; Yee et al., 2016; Takehara et al., 2017
	OAT3	Metabolite of cortisol	378.5	~6	Yes	~80	~230	CL _R , AUC	Imamura et al., 2013, 2014; Tsuruya et al., 2016
OCT2/MATE ^a	N ¹ -Methylnicotinamide	Metabolite of tryptophan and niacin metabolism	137.2	~20–850	Yes	0	258, 403 ^b	CL _R	Somogyi et al., 1990; Weber et al., 1991; Musfeld et al., 2001; Fukuwatari et al., 2004; Menon et al., 2007; Ito et al., 2012a; Hiratsuka et al., 2014; Müller et al., 2015
Thiamine	Essential vitamin		265.4	~6	(Yes)	0	~50	CL _R	Weber et al., 1990; Ihara et al., 2005; Kato et al., 2014

CL_R, renal clearance; DDI, drug-drug interaction; PK, pharmacokinetic. Application of parentheses to “Yes” or “No” signifies uncertainty or modest/minor extent.
^aFurther data are needed to clarify whether N¹-methylnicotinamide or thiamine may act as in vivo indicators for OCT2 function.
^bExemplary mean values from selected studies in healthy volunteers (Ito et al., 2012a; Müller et al., 2015).

TABLE 2
In vitro substrate characteristics of potential biomarkers for human transporters, formation and metabolism, as well as additional confounders

Biomarker	Substrate of Human Transporter ^a	No Substrate of Human Transporter	Formation by ^b	Metabolism by ^b	Impact of Nutrition	Impact of Disease	Reference
Tetradecanedioate	OATP1B1, OAT1, OAT3	OATP1A2, OATP1B3, OATP2B1	CYP4A11 (?)	CYP4A11 (?)	(No?)	Reye syndrome, blood pressure	Tongard, 1986; Menni et al., 2015; Yee et al., 2016; Shen et al., 2017a
Hexadecanedioate	OATP1B1, OAT1, OAT3	OATP1A2, OATP1B3, OATP2B1	CYP4A11 (?)	CYP4A11 (?)	(No?)	Reye syndrome, blood pressure	Tongard, 1986; Menni et al., 2015; Yee et al., 2016; Shen et al., 2017a
Coproporphyrin I	OATP1B1 (0.13 μ M), OATP1B3 (4.0 μ M), MRP2 (7.7 μ M)	OATP2B1, OAT1, OAT2, OAT3, OAT4, OCT1, OCT2, MATE1, MATE2-K, NTCP	By-product of heme synthesis	—	(No)	Porphyrrias, Dubin-Johnson and Rotor syndrome	Kaplowitz et al., 1972; Wolkoff et al., 1976; Puy et al., 2010; Bednarczyk and Boiselle, 2016; Shen et al., 2016, 2017a; Szlendak et al., 2016; Glibili et al., 2017
Coproporphyrin III	OATP1B1 (0.22 μ M), OATP1B3 (1.6 μ M), OATP2B1 (0.31 μ M)	OAT1, OAT2, OAT3, OAT4, OCT1, OCT2, MATE1, MATE2-K, NTCP	By-product of heme synthesis	—	(No)	Porphyrrias, Dubin-Johnson and Rotor syndrome	Kaplowitz et al., 1972; Wolkoff et al., 1976; Puy et al., 2010; Bednarczyk and Boiselle, 2016; Shen et al., 2016, 2017a; Szlendak et al., 2016; Rana and Sanders, 1986; Franconi et al., 1995; Russell, 2009; McLellan and Lieberman, 2012; Lambert et al., 2015; Sagara et al., 2015; Tsuruya et al., 2016
Taurine	OAT1 (379 μ M), ^c TauT/SLC6A6 (<60 μ M), PAT1/SLC36A1 (7.5 mM)	OAT3	Cysteine via cysteine dioxygenase to cysteinesulfinate via cysteinesulfinate decarboxylase to hypotaurine via oxidation to taurine	Bile acid CoA: amino acid N-acyltransferase	High urinary taurine with meat- and fish-based diet; present in energy drinks; vegans: low urinary taurine	Decreased plasma concentrations in patients with diabetes; association of urinary taurine and cardiovascular risks	Alhouti, 2009; Russell, 2009; Bathena et al., 2013, 2015a,b; Tsuruya et al., 2016; Takehara et al., 2017
Glycochenodeoxycholate sulfate	OAT3 (64 μ M), OAT4, ^d NTCP, OATP1B1 (10 μ M), OATP1B3 (5.2 μ M)	OAT1	SULT2A1	Desulfation in large intestine	Increase of bile acid serum concentrations by food intake	Increased urinary concentrations in patients with liver diseases	Abel et al., 1992; Seidegård et al., 1998; Fuhr et al., 2002; Shibuya et al., 2003; Peng et al., 2011; Imamura et al., 2013; Esche et al., 2016
6 β -Hydroxycortisol	OAT3 (183 μ M), MATE1, MATE2-K ^d	OAT1, OCT2 (?), P-gp	CYP3A4	11 β -Hydroxysteroid dehydrogenase	Reduced urinary 6 β -hydroxycortisol to cortisol ratio in the 0- to 4-hour fraction after ingestion of grapefruit juice, but not in the 4- to 24-hour fraction; increased urinary excretion of 6 β -hydroxycortisol after ingestion of grapefruit juice; impact of diet-dependent renal acid load on urinary 6 β -hydroxycortisol in children	Reduced urinary 6 β -hydroxycortisol to cortisol ratios in patients with liver cirrhosis	Abel et al., 1992; Seidegård et al., 1998; Fuhr et al., 2002; Shibuya et al., 2003; Peng et al., 2011; Imamura et al., 2013; Esche et al., 2016
N ¹ -Methylnicotinamide	OCT2 (300 μ M), 318 μ M), MATE1 (301 μ M), MATE2-K (422 μ M), OCT1	—	Nicotinamide N-methyltransferase	Aldehyde oxidase	Formation dependent on intake of precursors tryptophan and niacin	Decreased CL _R and increased plasma concentrations in impaired kidney	Cazzullo et al., 1976; Barlow et al., 1977; Shibata and Matsuo, 1989; Rini et al., 1990;

(continued)

TABLE 2—Continued

Biomarker	Substrate of Human Transporter ^a	No Substrate of Human Transporter	Formation by ^b	Metabolism by ^b	Impact of Nutrition	Impact of Disease	Reference
Thiamine	MATE1 (3.5 μM, 0.83 mM), MATE2-K (3.9 μM), OCT1 (0.78 mM), OCT2 (59.9 μM, 0.75 mM), OCT3, ^d THTR1 (2.5 μM), THTR2 (27 nM), SLC35F3	OCTN1, OCTN2, RFC	Dephosphorylation of thiamine phosphates	Thiamine pyrophosphokinase	Essential vitamin, intake with food (mostly as thiamine phosphates) or vitamin supplements	function; increased synthesis, urinary excretion, and CL _R in liver cirrhosis; increased serum concentrations in coronary artery disease; increased plasma concentrations in idiopathic pulmonary hypertension; increased urinary excretion in burns; increased serum concentrations in obesity and diabetes mellitus; decreased urinary concentrations in depressive disorder Clinical deficiency of total thiamine may occur in diabetes mellitus, gastrointestinal surgery, chronic alcoholism, and cancer; thiamine metabolism or transport is disturbed in thiamine metabolism dysfunction syndromes such as THTR1 or THTR2 deficiency	Maiza et al., 1992; Aksoy et al., 1994; Gorboulev et al., 1997; Orlando et al., 2000; Pumpo et al., 2001; Masuda et al., 2006; Kitamura et al., 2008; Ito et al., 2012a; Hiratsuka et al., 2014; Kannt et al., 2015; Liu et al., 2015; Fedorowicz et al., 2016; van Haren et al., 2016; Zheng et al., 2016; Liu et al., 2017 Dutta et al., 1999; Said et al., 2004; Tanihara et al., 2007; Thornalley et al., 2007; Page et al., 2011; Brown, 2014; Chen et al., 2014; Kato et al., 2014; Latt and Dore, 2014; Stroh et al., 2014; Zhang et al., 2014; Isenberg-Grzeda et al., 2015

CL_R, renal clearance; P-gp, P-glycoprotein (gene: *ABCB1*); RFC, reduced folate carrier (gene: *SLC19A1*); SLC35F3, solute carrier 35F3 (gene: *SLC35F3*).
^aIn parentheses: *K_m* value.
^bThese columns highlight important steps of formation and metabolism of the potential biomarkers. No complete pathways are shown. Other pathways may exist or may not have been fully characterized.
^cSignificant uptake in HEK cells transiently expressing OAT1; no significant uptake was observed in HEK cells stably expressing OAT1 (Tsuruya et al., 2016).
^dMinor transport rate.

TABLE 3
Selected, additional potential biomarkers with limited evidence or limitations

Transporter	Biomarker	Biomarker Characteristics	In Vitro Substrate of ^a /In Vivo Association with Human Transporter	Factors Limiting Use	Reference
OATP1B	Bilirubin glucuronides	Products of heme metabolism	OATP1B1, OATP1B3, MRP2, MRP3	Impact of <i>UGT1A1</i> polymorphism; substrates of MRP2, MRP3; moderate (~twofold) effects of rifampin on AUC	Dai et al., 2013; Keppler, 2014; Namjou et al., 2015; Prueksaritanont et al., 2017
	X-11529 (likely to be glycodeoxycholate glucuronide)	Bile acid conjugate	Significant association for rs4149056 (OATP1B1p. Val174Ala; GWAS) and significant increase in plasma after CSA	In vitro transport by OATP1B1 and OATP1B3 not characterized; further in vivo characterization as biomarker necessary	Yee et al., 2016
	X-13429 (likely to be glycodeoxycholate sulfate or isomer)	Bile acid conjugate	Significant association for rs4149056 (OATP1B1p. Val174Ala; GWAS) and significant increase in plasma after CSA	In vitro transport by OATP1B1 and OATP1B3 not characterized; further in vivo characterization as biomarker necessary	Yee et al., 2016
	Taurolithocholate 3-sulfate	Bile acid conjugate	OATP1B1, BCRP, BSEP, MRP2, MRP3, SOAT; significant association for rs4149056 (OATP1B1p. Val174Ala; GWAS) and significant increase in plasma after CSA	Determination of kinetic parameters in vitro for OATP1B1-mediated transport needed; in vitro transport by OATP1B3 not characterized; further in vivo characterization as biomarker necessary	Akita et al., 2002; Sasaki et al., 2002; Hayashi et al., 2005; Geyer et al., 2007; Blazquez et al., 2012; Yee et al., 2016
	X-11905, X-14626, X-11440	Unknown	Significant association for rs4149056 (OATP1B1p. Val174Ala; GWAS) and significant increase in plasma after CSA	Compounds not identified; transporter substrate characteristics unknown	Yee et al., 2016
	4-androsten-3 β , 17 β -diol disulfate ^b	Steroid	Significant association for rs4149056 (OATP1B1p. Val174Ala; GWAS) and significant increase in plasma after CSA	In vitro transport by OATP1B1 and OATP1B3 not characterized; further in vivo characterization as biomarker necessary	Yee et al., 2016
	Octadecanedioate	Fatty acid dicarboxylate	Significant association for rs4149056 (OATP1B1p. Val174Ala; GWAS) and significant increase in plasma after CSA	In vitro transport by OATP1B1 and OATP1B3 not characterized; further in vivo characterization as biomarker necessary	Yee et al., 2016
	1,5-Anhydroglucitol	Monosaccharide	Significant association for rs4149056 (OATP1B1p. Val174Ala; GWAS) and significant increase in plasma after CSA	No in vitro transport by OATP1B1 (at 10 nM)	Yee et al., 2016
	Glycodeoxycholic acid	Bile acid conjugate	OATP1B1, OATP1B3, ASBT, BSEP, NTCP	Nonsignificant effect of rifampin on plasma concentrations in repeated measurement model; no effect of <i>SLCO1B1</i> genotype on plasma concentrations; sensitivity of plasma concentrations to food intake	Craddock et al., 1998; Mita et al., 2006; Xiang et al., 2009; Haag et al., 2015; Shen et al., 2017a; Suga et al., 2017
	Taurodeoxycholic acid	Bile acid conjugate	OATP1B1, OATP1B3, BSEP, NTCP	Nonsignificant effect of rifampin on plasma concentrations in repeated measurement model; no effect of <i>SLCO1B1</i> genotype on plasma concentrations; sensitivity of plasma concentrations to food intake	Mita et al., 2006; Xiang et al., 2009; Haag et al., 2015; Shen et al., 2017a; Suga et al., 2017
	DHEAS	Metabolite of a sex steroid precursor	OATP1B1, OATP1B3, BCRP, SOAT, OST α -OST β	Lack of effect of rifampin on plasma concentrations in humans; impact of sex and age; formation by SULT2A1	Orentreich et al., 1984; Cui et al., 2001; Rainey et al., 2002; Suzuki et al., 2003; Geyer et al., 2007; Fang et al., 2010; Zhai et al., 2011; Shen et al., 2017a; Takehara et al., 2017

(continued)

TABLE 3—Continued

Transporter	Biomarker	Biomarker Characteristics	In Vitro Substrate of ^a /In Vivo Association with Human Transporter	Factors Limiting Use	Reference
OCT2/MATE	Carnitine	Amino acid derivative	OCT2, CT2, CACT, OCTN1, OCTN2; decrease of CL _R by MATE inhibitor pyrimethamine	No MATE-mediated in vitro transport; no human in vivo data for relevance of OCT2	Tamai et al., 1998; Yabuuchi et al., 1999; Enomoto et al., 2002; Tanihara et al., 2007; Lancaster et al., 2010; Palmieri, 2013; Kato et al., 2014; Wu et al., 2015; Giangregorio et al., 2017
	Acetylcarnitine	Acetylated form of carnitine	OCTN2; decrease of CL _R by MATE inhibitor pyrimethamine	No in vitro data on OCT- or MATE-mediated transport; no human in vivo data for relevance of OCT2	Wu et al., 1999; Kato et al., 2014
	Creatinine	Degradation product of creatine and creatine phosphate	MATE1, MATE2-K, OCT2, OCT3, OAT2, OAT3 (?), OAT4 (?)	Modest contribution of secretion to renal clearance; appears less sensitive compared with classic probe drugs such as metformin	Urakami et al., 2004; Masuda et al., 2006; Tanihara et al., 2007; Imamura et al., 2011; Ciarimboli et al., 2012; Lepist et al., 2014; Shen et al., 2015, 2017b; Chu et al., 2016; Mathialagan et al., 2017
	Dopamine ^c	Catecholamine, neurotransmitter	MATE1, MATE2-K, OCT1, OCT2, OCT3, PMAT, DAT, VMAT1, VMAT2	Lack of validation in humans	Busch et al., 1998; Duan and Wang, 2010; Lawal and Krantz, 2013; Boxberger et al., 2014; German et al., 2015; Kajiwarra et al., 2016
	Tryptophan	Essential amino acid	OCT1 (?), OCT2 (?), LAT1, PAT4	Further characterization of in vitro transport by OCT2 needed; no data for MATE-mediated transport available; confirmatory in human data needed	Kanai et al., 1998; Pillai and Meredith, 2011; Song et al., 2012; Timosenko et al., 2016; Yanagida et al., 2001

ASBT, apical sodium-dependent bile acid transporter (gene: *SLC10A2*); CACT, carnitine/acylcarnitine transporter (gene: *SLC25A20*); CL_R, renal clearance; CSA, cyclosporine; CT2, carnitine transporter 2 (gene: *SLC22A16*); DAT, dopamine transporter (gene: *SLC6A3*); LAT1, L-type amino acid transporter 1 (gene: *SLC7A5*); OST α -OST β , organic solute transporter (genes: *SLC51A/SLC51B*); PAT4, proton-coupled amino acid transporter 4 (gene: *SLC36A4*); PMAT, plasma membrane monoamine transporter (gene: *SLC29A4*); SOAT, sodium-dependent organic anion transporter (gene: *SLC10A6*); VMAT1, vesicular monoamine transporter 1 (gene: *SLC18A1*); VMAT2, vesicular monoamine transporter 2 (gene: *SLC18A2*).

^aIncluding minor transport.

^bThe sulfated species could be in different site, and the exact location may not be known (Yee et al., 2016).

^cIt is unclear whether urinary excretion of dopamine may reliably reflect OCT2 activity because dopamine is synthesized in renal proximal tubule.

1. **Taurine.** Lambert et al. (2015) summarize as major determinants of taurine plasma concentrations the absorption from food, its formation from cysteine, and its renal reabsorption. Taurine can be excreted as bile salt as well as unconjugated taurine via the kidneys (Lambert et al., 2015). Urinary taurine excretion is low, with low dietary taurine intake (e.g., vegetarians) (Rana and Sanders, 1986). High taurine intake due to consumption of meat or seafood leads to a high urinary taurine excretion (Chesney et al., 2010). Energy drinks are also known to contain significant amounts of taurine (McLellan and Lieberman, 2012).

Taurine transporter (TauT; *SLC6A6*) and proton-coupled amino acid transporter 1 (PAT1; *SLC36A1*) mediate taurine uptake across the luminal membrane of enterocytes with K_m values of <60 μ M and 7.5 mM, respectively (Anderson et al., 2009; Thwaites and Anderson, 2011; Lambert et al., 2015). Taurine is formed in the body from cysteine via sequential steps. Cysteine dioxygenase catalyzes formation of cysteinesulfinate; hypotaurine is formed from cysteinesulfinate by the cysteinesulfinate

decarboxylase; and finally, hypotaurine is oxidized to taurine (Lambert et al., 2015). Bile acid CoA:amino acid N-acyltransferase is responsible for the final step of synthesis of conjugated bile salts and mediates the conjugation of taurine to bile acid CoA thioesters (Russell, 2009; Styles et al., 2016). Interestingly, TauT knockout mice have a dramatically increased renal clearance of taurine compared with TauT-expressing mice (Huang et al., 2006), indicating an important role for TauT in renal reabsorption of taurine. In humans, taurine renal clearance is approximately 25 ml/min, with no binding of taurine to plasma proteins (Tsuruya et al., 2016). In the clinical study described above by Tsuruya et al. (2016), taurine plasma concentrations in the control period without probenecid appeared to show modest circadian differences.

2. **6 β -Hydroxycortisol.** 6 β -Hydroxycortisol, a metabolite of cortisol, is a substrate of OAT3 with a K_m value of 183 μ M (Imamura et al., 2013). 6 β -Hydroxycortisol is not transported by OAT1 and P-glycoprotein (Imamura et al., 2013) (Table 2). 6 β -Hydroxycortisol is also a substrate of MATE1 and, with a low transport rate, of

MATE2-K (Imamura et al., 2013). OCT2-transfected cells also showed a moderate 6 β -hydroxycortisol uptake compared with control cells, but the net uptake could not be inhibited by quinidine (Imamura et al., 2013).

In mice, Oat3 plays a significant role in renal 6 β -hydroxycortisol elimination, because renal clearance of 6 β -hydroxycortisol was reduced by more than 50% in Oat3 knockout mice compared with mice expressing Oat3 (Imamura et al., 2014). Imamura et al. (2014) used three drug interaction studies in healthy volunteers to evaluate the roles of OAT3 and MATEs in the urinary excretion of 6 β -hydroxycortisol. In study 1, the OAT3 substrate benzylpenicillin was administered alone or in combination with the OAT inhibitor probenecid; in study 2, the OAT1 substrate adefovir was given alone or in combination with probenecid; in the third study, the MATE substrate metformin was administered alone or in combination with the MATE inhibitor pyrimethamine. No significant change in 6 β -hydroxycortisol AUC and renal clearance was detected when the MATE inhibitor pyrimethamine was added. In contrast, probenecid significantly increased 6 β -hydroxycortisol AUC in studies 1 and 2, whereas the 6 β -hydroxycortisol amount excreted in urine remained unchanged, resulting in a reduced renal 6 β -hydroxycortisol clearance by about 40% (Imamura et al., 2014). Imamura et al. (2014) concluded that these data suggest that OAT3 plays an important role in renal elimination of 6 β -hydroxycortisol and that 6 β -hydroxycortisol could be used as biomarker for drug–drug interactions mediated by OAT3. Renal clearance of 6 β -hydroxycortisol is approximately 230 ml/min, with a plasma protein binding of approximately 80% (Imamura et al., 2013, 2014), indicating a major role of renal secretion to urinary 6 β -hydroxycortisol elimination.

6 β -Hydroxylation of cortisol is mediated primarily by CYP3A4 (Abel et al., 1992; Peng et al., 2011), resulting in 6 β -hydroxycortisol. 6 β -Hydroxycortisol can be reversibly converted to 6 β -hydroxycortisone by 11 β -hydroxysteroid dehydrogenase (Abel et al., 1992; Peng et al., 2011). Various approaches have been made to estimate induction or inhibition of CYP3A4 using the endogenous probe 6 β -hydroxycortisol (e.g., Galteau and Shamsa, 2003; Peng et al., 2011). One approach uses, for example, the urinary 6 β -hydroxycortisol-to-cortisol ratio (e.g., Roby et al., 2000; Galteau and Shamsa, 2003; Peng et al., 2011). Modulation of CYP3A4 activity could confound 6 β -hydroxycortisol plasma concentration parameters as a readout for OAT3 activity. Therefore, Imamura et al. (2014) recommended use of renal clearance of 6 β -hydroxycortisol as a more suitable parameter for assessment of the risk for OAT3-mediated drug–drug interactions by drugs potentially inhibiting or inducing CYP3A4. In the study by Tsuruya et al. (2016), probenecid increased 6 β -hydroxycortisol AUC in a dose-dependent manner. If a new molecular entity is not a CYP3A4 inhibitor or inducer, 6 β -hydroxycortisol AUC might also be a quantitative predictor of OAT3 activity (Tsuruya et al., 2016). Moreover, it was proposed that clearance of

6 β -hydroxycortisol could be used to investigate hepatic CYP3A4 irrespective of OAT3 (Imamura et al., 2014).

6 β -Hydroxycortisol plasma concentrations show a clear circadian rhythm (Imamura et al., 2014). 6 β -Hydroxycortisol excreted into urine did not differ between collection intervals 0–4 hours and 4–8 hours (time relative to probe drug administration), whereas the amount of 6 β -hydroxycortisol excreted per hour into urine was lower in the collection interval 8–24 hours, resulting in a decrease of 6 β -hydroxycortisol renal clearance in this time interval, possibly due to circadian OAT3 regulation (Imamura et al., 2014).

C. Biomarkers for Function of Renal Cation Transporters (Organic Cation Transporter 2/Multidrug and Toxin Extrusion Proteins)

N¹-Methylnicotinamide and thiamine have been proposed as in vivo probes for assessment of the activity of renal MATEs and possibly also of OCT2 (Ito et al., 2012a; Kato et al., 2014, 2015; Müller et al., 2015). For both compounds, renal clearance has been proposed as a parameter to assess the potential for drug–drug interactions by inhibition of renal tubular organic cation secretion. Table 1 summarizes selected disposition parameters of these endogenous compounds in humans, and Table 2 summarizes in vitro substrate characteristics, formation, metabolism, and potential confounders.

1. *N¹-Methylnicotinamide*. N¹-Methylnicotinamide (1-methylnicotinamide, 1-methylpyridin-1-ium-3-carboxamide) is a product of tryptophan and niacin metabolism (Fukuwatari et al., 2004; Menon et al., 2007; Hiratsuka et al., 2014). It is synthesized from nicotinamide by cytosolic nicotinamide N-methyltransferase (NNMT) using S-adenosyl-L-methionine as methyl donor (Rini et al., 1990; Aksoy et al., 1994; van Haren et al., 2016) and is then further metabolized by cytosolic aldehyde oxidase to two metabolites, N¹-methyl-2-pyridone-5-carboxamide (2-Py) and N¹-methyl-4-pyridone-3-carboxamide (4-Py) (Kitamura et al., 2008). Both enzymes are predominantly expressed in the liver, but localization to other tissues, including the kidney, has been reported (Aksoy et al., 1994; Smith et al., 1998; Nishimura and Naito, 2006).

N¹-Methylnicotinamide is a substrate of OCT2, MATE1, and MATE2-K with reported *K_m* values of 300–318 μ M (OCT2), 301 μ M (MATE1), and 422 μ M (MATE2-K) in human embryonic kidney 293 (HEK) cells or *Xenopus laevis* oocytes overexpressing the respective human transporter (Gorboulev et al., 1997; Masuda et al., 2006; Ito et al., 2012a; Müller et al., 2015). In transporter-overexpressing HEK cells, the weak bases pyrimethamine and trimethoprim inhibited N¹-methylnicotinamide uptake with *K_i* values of 9.4 μ M (OCT2), 83 nM (MATE1), and 56 nM (MATE2-K) for pyrimethamine (Ito et al., 2012a) and IC₅₀ values of 134 μ M (OCT2), 29 μ M (MATE1), and 0.61 μ M (MATE2-K) for trimethoprim (Müller et al., 2015). In addition, transport of N¹-

methylnicotinamide by OCT1 has been reported (Gorboulev et al., 1997). To our knowledge, transport by other human drug transporters has not been examined.

In mouse kidney slices, N¹-methylnicotinamide uptake was inhibited by the Oct substrate TEA at excess concentration of 10 mM and by pyrimethamine at a concentration of 10 μ M, which is comparable to the K_i values for Oct inhibition by pyrimethamine (see *Multi-drug and Toxin Extrusion Proteins 1 and 2-K*), suggesting Oct-mediated N¹-methylnicotinamide uptake; however, species differences between mouse and human may exist (Ito et al., 2012a). In human kidney brush border membrane vesicles, N¹-methylnicotinamide was transported with reported K_m values of 0.36–0.44 mM, which is in the range of K_m values for MATE-mediated N¹-methylnicotinamide transport; its uptake was stimulated by an outward directed proton gradient, in line with MATE-mediated proton–substrate antiport; and MATE substrates and inhibitors such as TEA, quinine, pyrimethamine, or cimetidine inhibited its uptake (Ott et al., 1991; Ito et al., 2012a). Addition of the MATE substrate metformin increased the K_m value for N¹-methylnicotinamide transport in brush border membrane vesicles from 360 to 758 μ M, suggesting that N¹-methylnicotinamide and metformin share the same transporters at human kidney brush border membrane (Ito et al., 2012a).

In rats, similar to humans, N¹-methylnicotinamide is actively secreted into urine by the organic cation transport system. Shim et al. (1982) reported a strong positive correlation ($r = 0.97$) between renal secretion clearance of the model cation TEA and N¹-methylnicotinamide in intact and acute renal failure rats and proposed the use of N¹-methylnicotinamide as endogenous marker for renal tubular function for secretion of organic cations. Subsequently, N¹-methylnicotinamide was further explored using rat models of induced acute renal failure with glomerulo-tubular dysbalance, i.e., predominant damage to selected parts of the nephron causing a stronger reduction of tubular secretion as compared with glomerular filtration or vice versa, e.g., proximal tubular necrosis by cisplatin or glomerulonephritis using anti-glomerular basement membrane antibodies. In such acute renal failure rat models, endogenous N¹-methylnicotinamide renal clearance was found to highly correlate with renal clearance of drugs secreted in proximal tubule by the organic cation transport system, including the weak bases cimetidine and N-acetylprocainamide, and the zwitterion cephalixin, with a higher predictive value than glomerular filtration rate (Maiza and Daley-Yates, 1990, 1991, 1993; He et al., 2001).

In healthy subjects, plasma concentrations of N¹-methylnicotinamide follow a circadian rhythm, with higher plasma concentrations in the morning hours and lower concentrations in the evening (Musfeld et al., 2001; Ito et al., 2012a; Müller et al., 2015). N¹-Methylnicotinamide and its two metabolites 2-Py and 4-Py are

excreted in urine, with a typical rank order of amount excreted of 2-Py > N¹-methylnicotinamide > 4-Py in humans (Shibata and Matsuo, 1989; Shibata, 1990; Pumpo et al., 2001; Fukuwatari et al., 2004). Amounts of N¹-methylnicotinamide, 2-Py, and 4-Py excreted in urine depend on intake of tryptophan and niacin (Shibata and Matsuo, 1989; Hiratsuka et al., 2014). Physical stress in form of exposure to cold temperature has been reported to increase urinary excretion of N¹-methylnicotinamide and the sum of N¹-methylnicotinamide and its two metabolites (Okamoto et al., 2003). Bergagnini-Kolev et al. (2017) reported that renal clearance of N¹-methylnicotinamide was increased in mid and late pregnancy, and this change seemed generally more pronounced as compared with similarly observed increases of metformin and creatinine renal clearances. Moreover, N¹-methylnicotinamide kinetics may be affected by disease (Table 2).

In patients with impaired kidney function, N¹-methylnicotinamide renal clearance is decreased and its plasma concentrations are increased. In a study in healthy volunteers and patients with renal impairment in which subjects were divided into three groups according to their renal creatinine clearance, mean renal clearance of N¹-methylnicotinamide was 255 ml/min in a group of subjects with mean renal creatinine clearance of 99 ml/min, whereas renal clearance of N¹-methylnicotinamide was 137 and 38 ml/min in subject groups with mean renal creatinine clearances of 52 and 13 ml/min, respectively (Maiza et al., 1992). A limitation of that study was that renal clearance values were based on 24-hour urine collection and only a single plasma sample taken at mid-morning, which does not take into account the diurnal changes of N¹-methylnicotinamide plasma concentrations. The correlation between renal clearance and plasma concentrations of N¹-methylnicotinamide is modest. N¹-Methylnicotinamide plasma concentrations seem less sensitive to kidney function than those of creatinine, probably because of alternative elimination pathways, in particular metabolic clearance by aldehyde oxidase relevantly contributing to N¹-methylnicotinamide clearance (Maiza et al., 1992).

N¹-Methylnicotinamide is not bound to plasma proteins in humans (Weber et al., 1991), and free filtration in the glomerulus may be assumed. As N¹-methylnicotinamide is proposed as a marker for renal MATEs and possibly also OCT2, renal clearance is of particular interest. In humans, renal clearance of N¹-methylnicotinamide exceeds glomerular filtration rate indicating net secretion. For example, N¹-methylnicotinamide renal clearance in healthy volunteer studies by Ito et al. (2012a) was 403 ml/min (mean; range: 250–765 ml/min; $N = 8$) and by Müller et al. (2015) was 258 ml/min (mean; range: 92–457 ml/min; $N = 12$).

In addition to glomerular filtration and extensive secretion, renal tubular reabsorption seems to affect renal clearance of N¹-methylnicotinamide, and this

process seems to be saturable. In a study in eight healthy female volunteers, Weber et al. (1991) investigated N¹-methylnicotinamide renal clearance at physiologic conditions and upon experimental increase of plasma concentrations via combined loading bolus and i.v. infusion by approximately 20-fold. Compared with baseline, the authors reported an approximately twofold increase of renal clearance at the experimentally high plasma concentration, to a value of approximately sixfold renal creatinine clearance, which is similar to the expected value for renal plasma flow. This suggests that, at physiologic conditions, renal clearance of N¹-methylnicotinamide may be limited by renal tubular reabsorption, whereas this process seems to lose importance with increased plasma concentrations indicating saturation.

N¹-Methylnicotinamide renal clearance has been used to assess the effect of several drugs on renal tubular cation secretion. Edwards et al. (1994) reported that, in psoriasis patients, a low-dose cyclosporine regimen led to a reduction of renal clearance of N¹-methylnicotinamide with incomplete recovery 3 months after dosing of cyclosporine was stopped, despite recovery of glomerular filtration rate, which may suggest toxicity-induced persistent dysfunction of renal secretory function for organic cations. Thomas et al. (2005) described that, in diabetic patients, 2-year treatment with the angiotensin-converting enzyme inhibitor perindopril increased N¹-methylnicotinamide renal clearance by 96%, and the authors suggested that both increased renal plasma flow and enhanced cation transport function in proximal tubule may have contributed to this effect.

In another study, Tschuppert et al. (2007) assessed the effect of the antiarrhythmic dronedarone (400 mg twice daily) on kidney function, to elucidate mechanisms underlying a 10%–15% increase of serum creatinine in clinical trials. In 12 healthy male volunteers, in addition to creatinine, sinistrin was measured as marker for glomerular filtration, para-amino-hippurate as marker for both renal plasma flow and organic anion secretion, and N¹-methylnicotinamide as marker for organic cation secretion into urine. In this study, dronedarone had no effect on sinistrin or para-amino-hippurate renal clearance but decreased creatinine and N¹-methylnicotinamide renal clearance by 18% and 17%, respectively, indicating a decrease of renal tubular cation secretion without evidence for relevant change to anion secretion, glomerular filtration rate, or renal blood flow. It is interesting that dronedarone, an inhibitor of P-glycoprotein, exerted these effects on renal cation secretion, although it seems to be a rather weak inhibitor of OCT2 and MATEs in vitro with values for $C_{\max,u}/IC_{50}$ of 0.003 (OCT2), 0.013 (MATE1), and 0.001 (MATE2-K) (Chu et al., 2016). In line with these in vitro results, the dronedarone summary of product characteristics reports the absence of an interaction with the OCT and MATE substrate metformin [Sanofi-Aventis Deutschland GmbH (<https://www.fachinfo.de/pdf/012151>)].

More specific data with regard to the characteristics of N¹-methylnicotinamide as a MATE probe come from two clinical trials that were used to assess the effect of the potent MATE inhibitor pyrimethamine or of trimethoprim on N¹-methylnicotinamide kinetics in healthy volunteers. In a clinical trial in eight healthy male Japanese subjects, Kusuvara et al. (2011) investigated the effect of pyrimethamine (single dose 50 mg po) on the pharmacokinetics of the OCT and MATE probe drug metformin at a microdose of 100 μ g and at a therapeutic dose of 250 mg. At metformin microdose, pyrimethamine had no effect on metformin AUC_{0–12} and $C_{\max}$ but reduced renal clearance of metformin by 23% from 623 to 451 ml/min. N¹-Methylnicotinamide was measured in plasma and urine samples of this study, and the authors found a pronounced reduction of N¹-methylnicotinamide urinary excretion in the interval 0–12 hours from 3.12 to 0.804 mg and renal clearance from 403 to 119 ml/min upon concomitant administration with pyrimethamine (Ito et al., 2012a). Mean renal clearance of N¹-methylnicotinamide when given together with pyrimethamine was close to the expected level of glomerular filtration rate in healthy volunteers, suggesting complete inhibition of tubular net secretion (Ito et al., 2012a). Pyrimethamine unbound plasma concentration in this trial was calculated as approximately 300 nM (Kusuvara et al., 2011), which exceeds the K_i values for MATE inhibition but is much lower than the K_i value for inhibition of OCT2, suggesting that pyrimethamine decreased N¹-methylnicotinamide renal clearance primarily by reducing its MATE-mediated secretion at the brush-border membrane in the proximal tubule without relevantly affecting OCT2 (Ito et al., 2012a).

Another study investigated the effect of the MATE inhibitor trimethoprim (200 mg twice daily po) on the pharmacokinetics of metformin at a therapeutic dose of metformin (850 mg each in the evening and 12 hours later in the morning) and on N¹-methylnicotinamide kinetics in 12 healthy volunteers (Müller et al., 2015). In this trial, metformin AUC_{0–24} and $C_{\max}$ were increased by 29% and 23%, respectively, and metformin renal clearance was reduced by 26% when given with trimethoprim. N¹-Methylnicotinamide renal clearance was reduced to a similar extent by 20%. Trimethoprim $C_{\max,u}$ in this trial was estimated to be approximately 9 μ M, suggesting partial MATE inhibition as underlying reason for the reduction of N¹-methylnicotinamide renal clearance with rather limited effect on OCT2. The authors also reported that the relative extent of the effect of trimethoprim on N¹-methylnicotinamide renal clearance correlated positively ($r_s = 0.73$) with the effect of trimethoprim on metformin renal clearance, suggesting that subjects with a more pronounced decrease of N¹-methylnicotinamide renal clearance experienced also a more pronounced decrease of metformin renal clearance and vice versa. In this trial, the relative

extent of the effect of trimethoprim on metformin and N¹-methylnicotinamide renal clearance was similar (reduction by 26% and 20%, respectively). As reported by Ito et al. (2012a), pyrimethamine had a much more pronounced effect on N¹-methylnicotinamide renal clearance (reduction by approximately 70%), which may be explained by higher potency of pyrimethamine for MATE inhibition as compared with trimethoprim. Unexpectedly, the effect of pyrimethamine on renal clearance of metformin at a microdose was much weaker, with a reduction by only 23%, which was lower than expected based on the K_i values for MATE inhibition by pyrimethamine and its unbound plasma concentration (Kusuhara et al., 2011).

Despite the reduction in renal clearance, the plasma concentrations of N¹-methylnicotinamide were not increased by pyrimethamine or trimethoprim in the two studies of Ito et al. (2012a) and Müller et al. (2015). On the contrary, N¹-methylnicotinamide plasma concentrations were slightly lower when given with pyrimethamine (Ito et al., 2012a). Trimethoprim reduced N¹-methylnicotinamide $C_{\max}$ and increased its minimum plasma concentration without an effect on AUC_{0–24}, apparently reducing the diurnal changes to N¹-methylnicotinamide plasma concentrations (Müller et al., 2015). The reasons for these observations with regard to plasma concentrations are not understood. In addition to their effects on renal clearance, pyrimethamine and trimethoprim may have affected N¹-methylnicotinamide formation or metabolism. Given the impact of metabolism, nutrition, and disease on N¹-methylnicotinamide plasma concentrations and that plasma concentrations appear to be less sensitive to changes of renal function as compared with creatinine, renal clearance of N¹-methylnicotinamide is the more suitable marker for renal secretion of organic cations.

In summary, N¹-methylnicotinamide renal clearance is characterized by free glomerular filtration and tubular net secretion, and it has been used as indicator for organic cation secretory function. There is compelling evidence that MATEs are relevantly involved in renal tubular secretion of N¹-methylnicotinamide, and MATE inhibitors have reduced its renal clearance in clinical trials in healthy volunteers, with first data on a limited number of subjects suggesting a quantitative relationship between reduction of N¹-methylnicotinamide renal clearance and metformin renal clearance by a MATE inhibitor (Müller et al., 2015). PMDA draft drug–drug interaction guideline lists N¹-methylnicotinamide as in vivo MATE and OCT2 substrate [http://www.solvobio-tech.com/documents/Japanese_DDI_guideline_(draft)_2014Jan.pdf]. N¹-Methylnicotinamide plasma concentrations and urinary excretion may be influenced by NNMT-mediated synthesis and metabolism by aldehyde oxidase, food, physical stress, concomitant treatment, and disease. Renal clearance shows substantial interindividual variation and may be affected by

concomitant treatment, diseases such as impaired kidney function or liver cirrhosis, or by pregnancy. When planning to use N¹-methylnicotinamide to assess the effect of a new molecular entity on renal secretory function, it would be recommendable to control known confounders by appropriate study design, exclusion of relevant concomitant medication, and standardization of food and water intake as well as physical activity. In vitro data on the effect of a test drug on activity of NNMT and aldehyde oxidase or urinary excretion data of the metabolites 2-Py and 4-Py could be helpful for interpretation of the results of such a study.

To enhance understanding of the qualities and suitability of N¹-methylnicotinamide as a probe for renal MATE function (Fromm, 2012; Ito et al., 2012a), the impact of further MATE inhibitors such as cimetidine and also the effect of noninhibitors should be assessed. Also, investigation of the sensitivity of N¹-methylnicotinamide renal clearance to OCT2 inhibition as well as differentiation between inhibition of MATE1 and MATE2-K by use of selective inhibitors would be desirable. In vitro assessment of potential N¹-methylnicotinamide transport by other membranous transport proteins, in particular those localized in the kidney, is necessary to understand the specificity.

2. Thiamine. Thiamine (vitamin B₁) was identified in an untargeted metabolomic analysis in samples of humans and mice treated with the MATE inhibitor pyrimethamine as potential endogenous marker for assessment of drug–drug interaction risk by MATE inhibition (Kato et al., 2014).

In vitro, thiamine is a substrate of both MATEs with K_m values reported as 3.5 μ M to 0.83 mM (MATE1) and 3.9 μ M (MATE2-K) (Table 2) (Tanihara et al., 2007; Chen et al., 2014; Kato et al., 2014). For OCT1 and OCT2, one group found no transport (Dutta et al., 1999), whereas other groups identified thiamine as substrates of these transporters with K_m values of 0.78 mM for OCT1 (Chen et al., 2014) and 59.9 μ M to 0.75 mM for OCT2 (Chen et al., 2014; Kato et al., 2014). The reported K_m values for all four transporters largely exceed physiologic thiamine plasma concentrations, indicating that saturation of transport would not be expected.

Thiamine transport has also been described for OCT3, although reported uptake rates were modest (Dutta et al., 1999; Chen et al., 2014). The organic zwitterion/cation transporters (OCTN) 1 and 2 did not mediate uptake of thiamine (Dutta et al., 1999). In addition, thiamine is a substrate of thiamine transporter (THTR) 1 (*SLC19A2*) and THTR2 (*SLC19A3*) with reported K_m values of 2.5 μ M for THTR1 and 27 nM for THTR2 (Dutta et al., 1999; Said et al., 2004). Both transporters are found in various tissues, with high expression of *SLC19A2* in skeletal muscle (Dutta et al., 1999) and high expression of *SLC19A3* in the duodenum (Said et al., 2004).

Data from mouse studies are in line with a role of OCTs and MATEs in thiamine disposition. Experiments in Oct1 knockout mice and humanized OCT1 transgenic mice signal a relevant role of OCT1 in hepatic and possibly intestinal uptake of thiamine (Chen et al., 2014). In Oct1/Oct2 double-knockout mice, plasma concentrations of endogenous thiamine were 5.8-fold higher and renal clearance of exogenous thiamine (high-dose infusion to suppress the extensive renal tubular thiamine reabsorption) was lower by 79% in comparison with the wild-type (Kato et al., 2015). In wild-type mice, the MATE inhibitor pyrimethamine reduced urinary excretion of endogenous thiamine by approximately 70% (Kato et al., 2014) and renal clearance of exogenous thiamine (high-dose infusion to suppress thiamine reabsorption) by 58% (Kato et al., 2015). Taken together, mouse data indicate a role of OCT1 in hepatic thiamine uptake, and it appears that OCT1 may also be involved in uptake of thiamine by the intestine and secretion of thiamine into breast milk (Kato et al., 2015), whereas it is suggested that MATEs (Mate1 in mice) and possibly OCT2 (Oct1 and Oct2 in mice) play a role in renal tubular thiamine secretion processes.

As an essential vitamin, thiamine is found at low levels in most foods, mostly as phosphate derivatives (Brown, 2014). Thiamine phosphates taken up with food are catalyzed by intestinal phosphatases to free thiamine, which is subsequently absorbed. Intracellularly, thiamine is activated to thiamine pyrophosphate, the most abundant form of thiamine in the body, which serves as a cofactor for enzymes involved in energy metabolism (Manzetti et al., 2014). Thiamine pyrophosphate may be further converted to thiamine triphosphate or thiamine monophosphate (Manzetti et al., 2014). Both unphosphorylated thiamine and thiamine monophosphate are found in plasma, whereas only unphosphorylated thiamine is found in urine (Weber et al., 1990; Ihara et al., 2005). Deficiency of total thiamine (sum of thiamine cation and phosphate derivatives), observed for instance in chronic alcoholism, malnutrition, or impaired intestinal absorption after gastrointestinal surgery, may lead to beriberi with congestive heart failure (wet beriberi) or peripheral neuropathy (dry beriberi), lactic acidosis, Wernicke encephalopathy, or Korsakoff syndrome (Oriot et al., 1991; Latt and Dore, 2014; Stroh et al., 2014). Thiamine transporters THTR1 and THTR2 are involved in thiamine disposition (Zhao and Goldman, 2013). Based on studies in mice, THTR2 appears to be relevantly involved in intestinal thiamine absorption (Reidling et al., 2010). Both transporters are involved in distribution of thiamine to tissues, as evidenced by metabolic disorders caused by defects of these transporters. Deficiency of THTR1 results in thiamine-responsive megaloblastic anemia, a condition characterized by the triad of diabetes mellitus, sensorineural deafness, and anemia, and associated with a variety of other symptoms such as

cardiac, ophthalmologic, or platelet disorders (Brown, 2014). THTR2 deficiency is characterized by central nervous system disorders such as lesions to basal ganglia (Brown, 2014).

Under physiologic conditions and without thiamine supplementation, elimination of thiamine is mainly extrarenal. Upon thiamine supplementation resulting in increased plasma concentrations, extrarenal elimination may become saturated, and renal excretion may become the principal path of elimination (Alexander et al., 1946; Weber et al., 1990). In addition to free glomerular filtration, which may be assumed due to low molecular weight and absence of protein binding (Weber et al., 1990), both extensive tubular secretion and reabsorption processes may occur. At physiologic plasma concentrations, these processes result in net reabsorption of thiamine, with renal clearance reported as approximately 50 ml/min in one study at mean plasma concentrations of approximately 5–9 nM (Kato et al., 2014). At conditions of thiamine deficiency, renal excretion rate drops toward zero, indicating near-complete reabsorption (Mickelsen et al., 1947; Weber et al., 1990). In contrast, when thiamine plasma concentrations are increased to approximately 200 nM by i.v. administration of thiamine, renal clearance increases and may reach values close to estimated renal plasma flow, indicating saturation of reabsorption, whereas secretion processes remain unsaturated (Weber et al., 1990). In line with extensive reabsorption processes, thiamine excretion is urine-flow dependent and is increased in enhanced diuresis induced by diuretic drugs such as furosemide or fluid load (Lubetsky et al., 1999; Rieck et al., 1999).

A study in healthy volunteers examined the effect of pyrimethamine, at a dose expected to inhibit renal MATE but not OCT2 activity, on the pharmacokinetics of metformin administered as a microdose or as a therapeutic dose (100 μ g and 250 mg) (Kusuhara et al., 2011). Thiamine was investigated in urine and plasma samples from this trial. Plasma concentrations of thiamine were barely affected, but thiamine renal clearance was decreased by 70%–84%, whereas renal clearance of metformin was decreased by only 23% and 34% at microdose or therapeutic dose, respectively, when pyrimethamine was coadministered (Kusuhara et al., 2011; Kato et al., 2014). This suggests that renal clearance of thiamine may be a more sensitive parameter to pyrimethamine-mediated MATE inhibition as compared with metformin; however, it is unclear how this can be transferred to other MATE inhibitors. Kato et al. (2015) also expect thiamine to be a probe for in vivo evaluation of the inhibitory potential of drugs toward OCT1 and OCT2; however, human data supporting this hypothesis are not available. Investigations of the effect of other OCT/MATE inhibitors on thiamine disposition and comparison with drugs that are in vivo substrates of these transporters are needed.

3. *Other Potential Biomarkers.* Other potential markers have been proposed, albeit with either a weaker or uncertain data basis or with apparently limited sensitivity for inhibition of renal tubular organic cation secretion. Table 3 summarizes information on potential markers with limitations.

a. Carnitine and Acetylcarnitine. In the untargeted metabolomic analysis, in which thiamine was found as a potential *in vivo* marker for MATE activity, 10 other compounds showed substantial and consistent decrease of urinary excretion in both mice and healthy volunteers when the MATE inhibitor pyrimethamine was given. Five of the 10 compounds were identified as N¹-methylnicotinamide, 2'-deoxycytidine, carnitine, and its derivatives acetylcarnitine and propionylcarnitine, whereas the identity of the other five compounds could not be definitely established (Kato et al., 2014). 2'-deoxycytidine was not further investigated due to instability in plasma and difficulties with the *in vitro* uptake assay (high uptake in vector control cells). Renal clearance of carnitine and acetylcarnitine was decreased by 90%–94% and 87%–91%, respectively, in healthy volunteers treated with metformin at micro-dose or therapeutic dose when pyrimethamine was coadministered (Kato et al., 2014). In mice, pyrimethamine reduced carnitine renal clearance by 62% (Kato et al., 2014). However, carnitine uptake was not higher in MATE1- and MATE2-K-expressing HEK cells as compared with control cells (Masuda et al., 2006; Tanihara et al., 2007; Kato et al., 2014), and the molecular mechanisms underlying the effect of pyrimethamine on disposition of carnitine and its metabolites remain unclear. Another experimental approach such as investigation of MATE export function in vectorial, transcellular transport experiments (Müller et al., 2011) may be useful to test a potential role of MATE in carnitine export from the cell; however, this has to date not been investigated to our knowledge. Lancaster et al. (2010) found that urinary excretion of both carnitine and acetylcarnitine was lower in Oct1/Oct2 double-knockout mice in comparison with wild-type mice and that carnitine is a substrate of OCT2. These results suggest that OCT2 may be involved in renal secretion of carnitine and possibly acetylcarnitine; however, *in vivo* confirmation in humans is lacking. Both carnitine and acetylcarnitine are subject to extensive tubular net reabsorption. It was reported in two studies that the renal clearance of carnitine was approximately 40- to 80-fold lower and that the renal clearance of acetylcarnitine was approximately 20-fold lower than glomerular filtration rate (Kato et al., 2014; Nobuoka et al., 2014).

b. Creatinine. Serum creatinine concentrations are commonly employed for estimation of glomerular filtration rate in clinical practice as a marker of renal function. Although creatinine is indeed freely filtered in the glomerulus, it is not a perfect index of glomerular

filtration rate (Soveri et al., 2014) because tubular net secretion is regularly observed, albeit to a limited extent in most cases. Inhibitors of renal tubular organic cation secretion are frequently associated with decreased creatinine clearance or increased creatinine concentrations in serum, and it has been suggested that, for OCT2/MATE inhibitors, serum creatinine increases may serve as a biomarker for drug–drug interaction potential (Giacomini and Huang, 2013).

In vitro, creatinine is a low-affinity substrate of OCT2, MATE1, and MATE2-K with reported K_m values of 1.9–56 mM, 10 mM, and 22 mM, respectively (Table 3) (Urakami et al., 2004; Masuda et al., 2006; Tanihara et al., 2007; Imamura et al., 2011; Ciarimboli et al., 2012; Lepist et al., 2014; Shen et al., 2015). For OCT3, creatinine uptake was described with a K_m value of 1.3 mM (Ciarimboli et al., 2012; Lepist et al., 2014). For OAT3, either no creatinine transport (Urakami et al., 2004; Lepist et al., 2014; Mathialagan et al., 2017) or uptake with a limited uptake ratio of less than twofold was reported (Imamura et al., 2011; Ciarimboli et al., 2012). The contribution of OAT3 to creatinine clearance has been estimated to be very limited based on relative-activity factor approach using recombinant OAT3 and human kidney slices (Imamura et al., 2011). Similarly, no or only limited uptake of creatinine was found to be facilitated by OAT4 (Imamura et al., 2011; Lepist et al., 2014). No transport was detected for OCT1, OAT1, OCTN1, and OCTN2 (Urakami et al., 2004; Imamura et al., 2011; Ciarimboli et al., 2012; Lepist et al., 2014; Mathialagan et al., 2017). For OAT2, interesting results have recently been reported. For details on OAT2, the reader may refer to an excellent, recent review (Shen et al., 2017b). Ciarimboli et al. (2012) found significant OAT2-mediated creatinine uptake. Subsequently, two independent groups reported OAT2-mediated transport of creatinine with high uptake rates and K_m values of 795–986 μ M (Lepist et al., 2014; Shen et al., 2015). Notably, when maximal transport rates (V_{max}) of creatinine were normalized to mRNA copy number (Lepist et al., 2014) or protein expression determined by mass spectrometry (Shen et al., 2015) of the respective transporter, transport efficiency (V_{max}/K_m) was several-fold higher for OAT2 as compared with OCT2, OCT3, MATE1, or MATE2-K. These *in vitro* results suggest a potential role of OAT2 in renal tubular handling of creatinine, although the *in vivo* relevance of OAT2-mediated creatinine transport for creatinine disposition in humans requires further studies.

Genetic association studies in humans point to involvement of OCT2 and MATE1 in creatinine disposition. Two noncoding SNPs, rs2504954, located between *SLC22A2* (OCT2) and *SLC22A3* (OCT3) and strongly linked to several SNPs in both genes, and rs2279463, located in an intron of *SLC22A2*, were associated with serum creatinine concentrations or glomerular filtration rate estimated from serum creatinine (Köttgen

et al., 2010; Ciarimboli et al., 2012). In two further studies, coding SNP rs316019 (OCT2p.270Ala>Ser), assessed either directly or by the highly correlated tag SNP rs316009, was associated with lower glomerular filtration rate estimated from serum creatinine or with lower creatinine clearance as a fraction of [125 I]iothalamate clearance (Hinai et al., 2013; Reznichenko et al., 2013). With respect to MATE1, rs2453580, located in an intron of *SLC47A1* (MATE1), and rs111653425, a coding mutation in *SLC47A1* (MATE1p.465Ala>Val), were identified in GWAS as associated with glomerular filtration rate estimated from serum creatinine or increased serum creatinine, respectively (Pattaro et al., 2012; Sveinbjornsson et al., 2014).

Creatinine is derived from nonenzymatic, spontaneous hydrolysis of creatine and creatine phosphate, with a nearly constant formation rate corresponding to a degradation of approximately 1.7% of total creatine pool (sum of creatine and creatine phosphate) per day in humans (Wyss and Kaddurah-Daouk, 2000; Brosnan et al., 2011). More than 90% of total creatine is found in muscle tissue (Wyss and Kaddurah-Daouk, 2000). Hence, creatinine formation is proportional to muscle mass and is decreased in states of muscle wasting such as hyperthyroidism, muscular dystrophy, or glucocorticoid therapy (Levey et al., 1988). Also, due to differences in total muscle mass, creatinine formation is higher in men than in women, higher in young adults as compared with elderlies, and dependent on race. In addition to endogenous formation, creatinine may be taken up from cooked meat, as heating converts creatine to creatinine, and consumption of cooked meat may cause considerable temporary increases of serum creatinine (Jacobsen et al., 1979; Mayersohn et al., 1983; Nair et al., 2014; Pimenta et al., 2016). Also, creatine supplements, used in fitness training and bodybuilding, may be a cause of increased serum creatinine (Spillane et al., 2009; Velema and de Ronde, 2011; Williamson and New, 2014).

In subjects with normal renal function, creatinine is almost exclusively eliminated by renal excretion. Renal impairment may reflect in serum creatinine increases, and single serum creatinine values are regularly used in clinical practice for derivation of estimated glomerular filtration rate as an index of kidney function by correction for creatinine formation using surrogate factors for muscle mass such as age, sex, or race (Levey et al., 2014). Creatinine is freely filtered at the glomerulus. In addition, renal tubular secretion is regularly observed, accounting for approximately 5%–40% of renal creatinine excretion in healthy subjects (Opravil et al., 1993). In addition to secretion processes, renal tubular reabsorption of creatinine may occur, but the underlying mechanisms are poorly understood. The notion of tubular reabsorption is supported by data from a study in eight healthy volunteers that showed that creatinine-to-inulin clearance ratio varied between 1.05 in a state of

dehydration and 1.47 upon rehydration, suggesting that urine flow rate may be a determinant of creatinine clearance due to differences in the extent of reabsorption (Sjöström et al., 1988). Also, net reabsorption has been reported from healthy subjects treated with the fluoroquinolone DX-619, a potent *in vitro* inhibitor of OCT2, MATE1, and MATE2-K (Imamura et al., 2011).

Treatment with compounds that are inhibitors of OCT2 and/or MATEs such as pyrimethamine (Opravil et al., 1993), trimethoprim (Berglund et al., 1975), or dolutegravir (Koteff et al., 2013) may be associated with rapid-onset and reversible increases of serum creatinine without evidence of renal toxicity or change to glomerular filtration rate as measured by compounds such as inulin, [131 I]iothalamate, or iohexol. For individual patients receiving DX-619, physiologically based pharmacokinetic models including OCT2 alone, OCT2 and MATE1, or OCT2 and MATE2-K could reasonably predict increases of serum creatinine in individual subjects (Imamura et al., 2011). Increases of serum creatinine concentrations were also described for cimetidine. Hilbrands et al. (1991) found that high daily doses of cimetidine (2000 mg/d in subjects with normal renal function and 1000–1600 mg/d in subjects with impaired renal function) did not affect inulin or [51 Cr]EDTA clearances, but the ratios of creatinine-to-inulin or creatinine-to-[51 Cr]EDTA clearances were decreased from 1.23 or 1.19 without cimetidine to 0.96 and 1.01 with cimetidine, respectively, indicating complete inhibition of creatinine net secretion. Interestingly, no significant changes to creatinine disposition were observed in two independent studies investigating the effect of cimetidine on metformin pharmacokinetics. In the first study, a therapeutic cimetidine dose of 400 mg twice daily led to a 27% decreased renal metformin clearance and increases of metformin AUC_{0-24} and C_{max} by 50% and 81%, respectively, but no significant changes to renal creatinine clearance were observed. In addition, the metformin-to-creatinine renal clearance ratio was significantly reduced by cimetidine (Somogyi et al., 1987). Similarly, renal creatinine clearance was not affected in another study describing a significant pharmacokinetic interaction between cimetidine (400 mg twice daily) and metformin as a victim in dependency on the *SLC22A2* genotype (Wang et al., 2008). In another study with cimetidine (300 mg four times per day) as a perpetrator, renal clearance of the victim varenicline decreased by 25%, and $AUC_{0-\infty}$ increased by 29%, but estimated (Cockcroft-Gault formula) and calculated creatinine clearances were essentially unchanged (Feng et al., 2008). Similarly, in 10 ventricular arrhythmia patients treated with procainamide and receiving in addition cimetidine 300 mg four times per day, the procainamide-to-creatinine and N-acetylprocainamide-to-creatinine renal clearance ratios decreased by 33% and 21% (Bauer et al., 1990), indicating a more pronounced effect on renal clearance of procainamide and its metabolite as compared with creatinine. Similarly, in a study in 20 healthy

volunteers on the effect of pyrimethamine (two doses of 50 mg given on subsequent days) on metformin pharmacokinetics, renal clearance of metformin was decreased by 72%, whereas renal clearance of creatinine was decreased by only 31% (Oh et al., 2016). These results suggest that creatinine disposition may be less sensitive to inhibition of drug transporters involved in active renal secretion of organic cations as compared with drugs such as metformin, varenicline, or procainamide.

The association between in vitro inhibition of renal drug transporters and in vivo disposition of creatinine is illustrated in an excellent review on reversible increase in serum creatinine concentrations occasionally observed during drug development (Chu et al., 2016). In this review, Chu et al. (2016) also compared the influence of OCT2/MATE inhibitors on victim drug exposure with the effect on clearance or serum concentrations of creatinine using data obtained within the same studies. In this analysis, drug–drug interactions perpetrated by cimetidine, pyrimethamine, dolutegravir, trimethoprim, and vandetanib were associated with qualitatively corresponding changes in creatinine disposition. In contrast, two cimetidine–metformin interaction studies reported no changes in creatinine clearance (Somogyi et al., 1987; Wang et al., 2008). In the analysis by Chu et al. (2016), ranitidine showed up as false negative. This was because of a reported significant interaction with triamterene without relevant changes in renal creatinine clearance (Muirhead et al., 1988). Chu et al. (2016) summarized that changes in serum creatinine due to OCT2/MATE inhibition are weak to moderate and may be affected by confounding factors. Hence, Chu et al. (2016) concluded that serum creatinine does not appear to be a sufficiently sensitive biomarker for qualitative or quantitative prediction of OCT2/MATE-mediated drug–drug interactions.

This assessment by Chu et al. (2016) appears justified. It should be possible to control potential external confounders of creatinine disposition such as hydration state, diet, exercise, or menstrual cycle (Phipps et al., 1998). However, creatinine disposition parameters do not appear to be as sensitive indicators for inhibition of renal tubular organic cation secretion as probe drugs such as metformin. In cases of drug-associated increases of serum creatinine levels without change to true glomerular filtration rate, in vitro data on inhibition of renal drug transporters may help to understand the underlying molecular mechanisms (Chu et al., 2016; Mathialagan et al., 2017).

c. Dopamine. Dopamine is a substrate of MATE1 and MATE2-K, and in mice, *Mate1* knockout or administration of imatinib, an in vitro MATE inhibitor (Minematsu and Giacomini, 2011), decreased urinary excretion of dopamine (Table 3) (Kajiwara et al., 2016). Based on these results, urinary excretion of dopamine has been suggested as potential index of renal MATE activity (Kajiwara et al., 2016). In vitro, dopamine is also a substrate of OCT2 (Busch et al., 1998), but

dopamine is synthesized in renal proximal tubule cells, and this could limit the relationship between OCT2 activity and urinary dopamine excretion (Kajiwara et al., 2016). Exploration of dopamine as marker for MATE activity in humans is lacking.

d. Tryptophan. An untargeted metabolomic analysis in urinary samples of 21 subjects that had participated in a previous clinical trial on metformin pharmacokinetics in different *SLC22A2* variants (Song et al., 2008) revealed the essential amino acid, tryptophan, and the nucleoside, uridine, as associated with *SLC22A2* coding SNP rs316019 (OCT2p.270Ala>Ser) (Song et al., 2012). A canonical correlation analysis showed positive linear association of urinary tryptophan with metformin pharmacokinetic parameters, including renal clearance and renal secretory clearance. In vitro investigations found OCT1- and OCT2-mediated transport of tryptophan, and transport by OCT2 variant p.270Ala>Ser was lower than transport by wild-type OCT2. Based on these results, Song et al. (2012) suggested tryptophan as a biomarker candidate for OCT2 activity. However, for OCT2, no uptake ratio in comparison with control cells was reported, and for OCT1, the uptake ratio was low (<1.5), and it was not investigated whether OCT-mediated tryptophan transport could be inhibited by OCT inhibitors. Further in vitro studies are necessary to clarify characteristics of tryptophan as OCT substrate, including determination of kinetic parameters and inhibition experiments. Renal clearance of tryptophan is characterized by extensive reabsorption (Møller, 1981).

IV. Suggested Factors To Be Considered for Biomarkers of Transporter Function

As outlined above and as discussed by others (Yee et al., 2016; Chu et al., 2017; Rodrigues et al., 2017), a broad range of factors needs to be considered to characterize and validate endogenous compounds as biomarkers of transporter function. We propose to consider the following questions in further research on potential biomarkers of transporter function. The proposed factors are classified into the categories sensitivity, specificity, predictivity, robustness, and further considerations.

A. Sensitivity

- Is the endogenous compound an in vitro substrate of the transporter(s) of interest? Are K_m values available?
- Do classic modulators of the transporter(s) of interest significantly alter the kinetic endpoint of the endogenous compound in humans (positive control)?
- Is kinetics of the endogenous compound sufficiently sensitive to detect effects of known weak

or moderate modulators of the transporter(s) of interest?

- Is there a significant association between well-characterized, functionally relevant polymorphisms in genes encoding the transporter(s) of interest and kinetics of the endogenous compound in humans?
- If there is a genetic disease in humans with a total knockout of function of the transporter of interest, what is the extent of change in kinetics of the endogenous compound?

B. Specificity

- Do in vitro data indicate that the endogenous compound is not a clinically relevant substrate of transporters other than the transporter(s) of interest?
- Is the kinetic endpoint of the endogenous compound unchanged after administration of drugs [including classic modulators of transporters other than the transporter(s) of interest, modulators of drug-metabolizing enzymes] to humans, which are predicted not to affect the transporter of interest based on in vitro studies and in vivo concentrations (negative controls)?

C. Predictivity

- Do changes in kinetics of the endogenous compound when challenged with classic modulators of the transporter(s) of interest qualitatively predict the changes in kinetics of classic victim drugs due to classic modulators of the transporter(s) of interest?
- Does the extent of changes in kinetics of the endogenous compound due to classic modulators of the transporter(s) of interest correlate quantitatively with the extent of changes in kinetics of classic victim drugs due to classic modulators of the transporter(s) of interest?

D. Robustness

- Is the matrix for measurement of the endogenous compound easily accessible (e.g., plasma or urine)?
- Is the endogenous compound stable in the matrix?
- Can the biomarker concentrations be measured accurately with a sensitive and specific bioanalytical method?
- Is the kinetic endpoint of the endogenous compound stable over time without provocation by a modulator of the transporter(s) of interest in humans (low intraindividual variability)?
- What is the interindividual variability of the kinetic endpoint of the endogenous compound (e.g., within and between ethnic groups, between male and female subjects)?

- Are there qualitative or quantitative differences in the changes of the kinetic endpoint of the endogenous compound due to modulators of the transporter(s) of interest between, e.g., different ethnic groups, male/female subjects?
- Is there a circadian rhythm of the kinetics of the endogenous compound or not?
- Is there an impact of acute food intake and/or long-term nutrition on the kinetics of the endogenous compound?
- Which processes are involved in endogenous formation of the endogenous compound?
- Which processes are involved in distribution of the endogenous marker?
- Which processes are involved in clearance of the endogenous compound (e.g., by further metabolism/nonenzymatic degradation or excretion) and what are their relative contributions?
- How do alterations in endogenous formation or disposition of the endogenous compound by potential modulators of the transporter(s) of interest affect the kinetic endpoint?

E. Further Considerations

- Which is the optimal kinetic parameter (e.g., AUC, renal clearance)?
- Which are the minimum and optimal sampling time points/periods, taking into account biomarker disposition as well as perpetrator disposition?
- Ideally, the endogenous compound should have limited metabolic clearance such that the outcomes of the administration of drugs are not confounded by changes in metabolic clearance.
- Is kinetics of the endogenous compound similarly determined by an ortholog transporter in animal models allowing to test the influence of potential perpetrator drugs on the endogenous compound in animal models?
- What is the impact of disease on kinetics of the endogenous compound, in case it is intended to be used in patient populations?

V. Alternatives and Supplemental Approaches to Classic Drug-Drug Interaction Studies

In addition to the biomarkers elucidated in this manuscript, alternate means of studying transporter function in vivo have been evaluated in the recent years. Among these, some of the more promising ones, i.e., drug cocktails, microdosing, or imaging using positron emission tomography (PET), are described here.

A. Drug Cocktails

An efficient approach to drug-drug interaction assessment is the so-called cocktail study, which involves the simultaneous administration of various probe

substrates together with a new investigational drug. A probe–drug cocktail should consist of sensitive probes that do not mutually interact when given together [European Medicines Agency (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf); FDA (<https://www.fda.gov/downloads/drugs/guidances/ucm292362.pdf>); Zhang and Sparreboom, 2017]. This approach has been extensively evaluated and established in the field of cytochrome P450–based drug–drug interaction testing and has recently been applied in the field of transporter-based drug–drug interactions (Stopfer et al., 2016; Prueksaritanont et al., 2017). The use of a cocktail approach is expected to help screen the influence of a new molecular entity on several transporters in a single trial. In case the results are positive for one or more probe drugs in the cocktail, the particular pathway involved and the magnitude of effect may need to be followed up in confirmatory drug–drug interaction trials. However, if the outcome of a cocktail trial is negative, further investigation is usually not required (Zhang and Sparreboom, 2017).

Stopfer et al. (2016) reported a transporter cocktail dedicated to key drug transporters consisting of four drugs, i.e., digoxin (probe drug for P-glycoprotein), rosuvastatin (BCRP, OATP1B1 and OATP1B3), furosemide (OAT1 and OAT3), and metformin (OCT2, MATE1, and MATE2-K). In choosing drugs for the cocktail, besides picking well-established drugs whose involvement via a transporter pathway and safety were already established, attention was paid to select drugs with minimal metabolism to allow investigation of transporter activity without confounding influence of metabolism. The cocktail covers nine drug transporters for which preclinical and clinical drug–drug interaction investigations are requested by regulatory authorities, including also drug transporters involved in active transport processes in the kidney. In a first trial, mutual pharmacokinetic interactions between the cocktail compounds were assessed (Stopfer et al., 2016). It was found that, when given in the cocktail, rosuvastatin $C_{\max}$ and AUC were increased by approximately 40% as compared with when given alone (Stopfer et al., 2016). This alteration is small as compared with reported increases of rosuvastatin exposure in the presence of potent OATP inhibitors such as rifampin (Prueksaritanont et al., 2014). Results of a recent follow-up trial indicated that the increase of systemic exposure of rosuvastatin in the cocktail was most likely due to the combination with metformin and only to a minor extent to furosemide (Stopfer et al., 2017). That follow-up trial also suggested that the cocktail may be free from mutual pharmacokinetic interactions by reduction of the doses of metformin and furosemide. Further exploration of this four-component cocktail using reduced doses of metformin and furosemide is currently ongoing.

Prueksaritanont et al. (2017) tested a five-drug microdose cocktail consisting of midazolam, atorvastatin, rosuvastatin, dabigatran etexilate, and pitavastatin,

addressing CYP3A, OATP1B, BCRP, and P-glycoprotein. They validated this cocktail using the inhibitors rifampin, itraconazole, and clarithromycin. Microdosing is further discussed in the next section.

B. Microdosing

Microdosing may be defined as administration of drug at a dose of up to 100 μg , 1/100th of a no observed adverse effect level, or 1/100th of a pharmacologically active dose, whichever is lower. At such low doses, neither therapeutic nor adverse effects of a drug are generally expected; however, administration of such low doses also necessitates very sensitive bioanalytical assays such as accelerator mass spectrometry, PET, or sufficiently sensitive liquid chromatography–tandem mass spectrometry (Burt et al., 2016). Accelerator mass spectrometry and PET require radiolabeling. When given as a microdose, several drugs can be combined in a cocktail, allowing investigation of the effect on various metabolic pathways and/or transporters simultaneously, thus reducing intra-occasion variability. In addition to the microdose cocktail discussed above (Prueksaritanont et al., 2017), another example is available from a study by Maeda et al. (2011a), who used a microdose cocktail to investigate the relative importance of hepatocellular OATPs in comparison with CYP3A4 in the hepatic clearance of atorvastatin. A microdose cocktail consisting of atorvastatin, pravastatin, and midazolam was administered to healthy volunteers, and its pharmacokinetics was evaluated at baseline, after 600 mg oral rifampin as OATP inhibitor, or after 200 mg i.v. itraconazole as CYP3A4 inhibitor. Pravastatin was included in this trial as a substrate of hepatocellular OATPs, and midazolam was included as a substrate of CYP3A4. Coadministration of rifampin increased dose-normalized AUC_{0-10} of atorvastatin by 12-fold and AUC_{0-8} of pravastatin by 4.6-fold, but did not affect dose-normalized AUC_{0-10} of midazolam. Coadministration of itraconazole did not have a relevant influence on pharmacokinetics of either pravastatin or atorvastatin but increased dose-normalized midazolam AUC_{0-10} by 1.7-fold. These data indicate that the clearance of atorvastatin as a microdose was driven by the hepatic uptake process, which is mediated by OATPs and not by the metabolic clearance by CYP3A4. A concern with the use of microdosing is nonlinearity of the pharmacokinetics between therapeutic and microdosing approach (Maeda et al., 2011b). One possible reason for nonlinearity may be the saturation of CYP3A4 and/or P-glycoprotein in the small intestine due to high local drug concentration at therapeutic dose, but not at microdose, resulting in microdosing underestimating the intestinal availability of a drug. The pharmaceutical formulation can be a further limitation of microdosing, as time- and resource-intensive manufacturing processes may be involved. Another example for the use of microdosing in investigation of transporter-mediated drug–drug interactions is the investigation of the effect of the

MATE inhibitor pyrimethamine on metformin as a microdose (100 μ g) and on a therapeutic dose of 250 mg described in *Multidrug and Toxin Extrusion Proteins 1 and 2-K*. In this study, pyrimethamine caused a decrease of renal clearance of metformin at both microdose and therapeutic dose; however, the effect was less pronounced at microdose, and a significant effect on metformin $C_{\max}$ and AUC was observed only at therapeutic dose of metformin, suggesting that metformin given as a microdose may, for unknown reasons, be less sensitive to MATE inhibition by pyrimethamine (Kusuhara et al., 2011).

C. Imaging Techniques

Assessment of the function and expression of transporters and also drug–drug interactions may be improved in some cases by knowledge of the tissue concentrations in tandem to the plasma concentration of a probe substrate. This can be done noninvasively by using nuclear imaging techniques such as single-photon emission-computed tomography (Gomes et al., 2011) or PET (Wulkersdorfer et al., 2014). Using a PET or single-photon emission-computed tomography camera, radioactivity concentrations of tracer molecules in organs can be quantified and modeled. In recent years, PET has emerged as a preferred technique due to its superior sensitivity, resolution, and a possibility to quantify radioactivity in tissue (Mairinger et al., 2011).

As an example, PET was used to dynamically and noninvasively quantify hepatobiliary transport and hepatic concentration of a [11 C]rosuvastatin tracer (He et al., 2014). Male Sprague–Dawley rats, who were receiving either the OATP/Oatp inhibitor rifampin or saline, obtained [11 C]rosuvastatin tracer as an i.v. infusion. Using PET imaging of the lower abdomen of the animals, radioactivity in liver, kidney, and intestine was followed quantitatively. [11 C]Rosuvastatin radioactivity data were analyzed by noncompartmental and compartmental (five-compartment) analysis. In control animals, [11 C]rosuvastatin radioactivity was mainly distributed into the liver, whereas kidney was a minor site for rosuvastatin distribution (He et al., 2014). Rifampin caused an approximately threefold increase in [11 C]rosuvastatin radioactivity blood AUC while reducing its distribution into the liver and kidney by 54% and 73%, respectively (He et al., 2014). Rifampin decreased [11 C]rosuvastatin radioactivity clearance from blood to liver by approximately 50% and clearance from liver to intestine by approximately 30% (He et al., 2014). The decreased clearance from blood to liver was presumably due to inhibition of hepatocellular Oatp-mediated uptake by rifampin, whereas the decreased biliary clearance may be attributed to inhibition of export pumps in the canalicular membrane such as Mrp2. Also, blood-to-kidney clearance and renal efflux clearance were reduced, but not the clearance from liver to blood. The latter indicates that efflux by sinusoidal

efflux pumps such as Mrp3 or Mrp4 was not affected (He et al., 2014).

PET imaging has also been applied clinically for the assessment of transporter function. For instance, Shimizu et al. (2012) used [11 C]telmisartan as a tracer with PET imaging for studying OATP1B3 function in rats and in humans. Bauer et al. (2015) studied the inhibition of P-glycoprotein at the blood brain barrier by tariquidar (a potent P-glycoprotein inhibitor) infusion using (R)-[11 C]verapamil PET tracer in human volunteers showing P-glycoprotein inhibition at the blood brain barrier by tariquidar.

Application of PET in the field of studying transporter function is rapidly developing. PET has the advantage of allowing a dynamic and quantitative evaluation of transporter expression and function (or disruption), which can be done across various species in a non-invasive manner using a microdose of the tracer. Applied in animals, it allows confirming in vitro findings in a dynamic physiologically relevant environment and at relevant concentrations. Although in most situations an ideal transporter probe would be a molecule that is not metabolized or only to a very limited extent, this limitation may be overcome by combining PET imaging with radiochromatographic assessment of blood or tissue biopsies, thereby allowing to distinguish signals from drug molecule versus its metabolites (Schou et al., 2009; Wulkersdorfer et al., 2014). Designing of PET tracers has become easier with advancement of novel synthetic methodologies such as [11 C]-carbonylation and [18 F]-radiochemistry, allowing labeling of existing or newly developed drugs (Testa et al., 2015). Costs and the availability of the facilities to conduct PET-based work currently limit its more widespread use.

VI. Conclusions and Outlook

Carefully considered approaches are required to optimize prediction of clinical drug–drug interactions with involvement of drug transporters. Identification of endogenous compounds as biomarkers for prediction of transporter-mediated drug–drug interactions has gained considerable attention during recent years. However, as outlined in *Suggested Factors To Be Considered for Biomarkers of Transporter Function*, a number of questions need to be addressed to understand factors affecting disposition of potential biomarkers. In our opinion, currently these potential confounders are not well understood for many of the discussed endogenous compounds. There is also a major demand on larger numbers of studies in humans investigating the impact of drugs on disposition of potential biomarkers. Are the observations from these clinical studies in line with the respective in vitro data, i.e., do inhibitors of respective transporters affect the disposition of the potential biomarker in humans, and is there a lack of

effect on the disposition of the biomarker by drugs, which do not inhibit the respective transporter? Finally, endogenous functions of many transporters are still poorly characterized (Nigam, 2015). Future research should also focus on additional endogenous substrates of drug transporters, e.g., substrates of P-glycoprotein.

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Authorship Contributions

Performed data analysis: Müller, Sharma, König, Fromm.

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