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Clinical Probes and Endogenous Biomarkers as Substrates for Transporter DDI Evaluation: Perspectives from the International Transporter Consortium

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Abbreviation List:
ADME, Absorption, distribution, metabolism, and excretion; AUC, area under the concentration-time curve; AUCR, ratio of AUC; BCRP, breast cancer resistance protein; 6βHC, 6β-hydroxycortisol; CL, clearance; CYP, cytochrome P450; CB, conjugated bilirubin; CP, coproporphyrin; Cmax, maximum concentration; CmaxR, ratio of Cmax; CLR, renal clearance; CLR,
ratio of CLr; DDI, drug-drug interaction; DE, dabigatran etexilate; GCDCA-S, glycochenodeoxycholate-3-O-sulfate; GWAS, genome wide association study; HDA, hexadecanedioate; IVIVE, in vitro to in vivo extrapolation; ITC, International Transporter Consortium; IC50, half maximal inhibitory concentration; K\textsubscript{m}, Michaelis constant; K\textsubscript{i}, inhibition rate constant; MDR, multidrug resistance; MRP, multidrug resistance protein; MATE, multidrug and toxin extrusion protein; NME, new molecular entity; NMN, N1-methylnicotinamide; NTCP, sodium taurocholate co-transporting polypeptide; OATP, organic anion transporting polypeptide; OAT, organic anion transporter; OCT, organic cation transporter; P-gp, P-glycoprotein; PBPK, physiologically-based pharmacokinetic; PK, pharmacokinetics; PPB, plasma protein binding; t\textsubscript{1/2}, half-life; THTR, thiamine transporter; TDA, tetradecanedioate; UCB, unconjugated bilirubin.
Abstract (150/150)

Drug transporters can govern the absorption, distribution, metabolism, and excretion of substrate drugs and endogenous substances. Investigations to examine their potential impact to pharmacokinetic drug-drug interactions (DDIs) are an integral part of the risk assessment in drug development. To evaluate a new molecular entity as a potential perpetrator of transporters, use of well characterized and/or clinically relevant probe substrates with good selectivity and sensitivity are critical for robust clinical DDI assessment that could inform DDI management strategy in the product labeling. The availability of endogenous biomarkers to monitor transporter-mediated DDIs in early phases of clinical investigations would greatly benefit downstream clinical plans. This article reviews the state-of-the-art in transporter clinical probe drugs and emerging biomarkers including current challenges and limitations, delineates methods and workflows to identify and validate novel endogenous biomarkers to support clinical DDI evaluations, and proposes how these probe drugs or biomarkers could be used in drug development.
Drug transporters can modulate the absorption, distribution, metabolism, and excretion (ADME) of substrate drugs and endogenous substances, ultimately determining their exposure in systemic circulation and tissues. Transporter substrate or modulator (inhibitor or inducer) drugs can become clinical victims or perpetrators of DDIs, respectively, when the transporter in question is a substantial contributor to the pharmacokinetics (PK) of the victim drug and can be inhibited or induced in the clinical setting. For example, lapatinib, a P-glycoprotein (P-gp) inhibitor, increased digoxin exposure by 2.8-fold (TYKERB labeling at Drugs@FDA), whereas tipranavir/ritonavir, a P-gp inducer, decreased saquinavir/ritonavir exposure by 76% (APTVUS labeling at Drugs@FDA). Understanding DDIs is an integral part of risk assessment in drug development considering the common practice of concomitant use of multiple medications.

Previous International Transporter Consortium (ITC) whitepapers and regulatory guidelines have proposed an integrated approach (in vitro, in vivo, in silico) to assess DDI potential and to inform safe drug use. Multiple factors contribute to the decision whether a clinical DDI study may be necessary, and if so, its prioritization during drug development, and subsequent interpretation of clinical DDI data to inform the product labeling. From the perspective of a new molecular entity (NME) as a transporter inhibitor, prediction of clinical DDI potential involves comparison of in vitro transporter inhibitory potency to various transporters that govern the victim drug’s intestinal, liver portal, systemic and tissue exposure. In this regard, in vitro transporter inhibition assays have been routinely used to determine whether an NME is an inhibitor of clinically-relevant transporters, which serve as a trigger for follow up clinical DDI studies. However, this approach may result in false positive or false negative predictions due to the limitations of in vitro transporter assays and/or gaps in in vitro to in vivo extrapolation (IVIVE). For instance, the assumptions are often made that the transporter-mediated pathway accounts for 100% of drug elimination, however, the contribution of transporter(s) to overall elimination of drugs is often not known or cannot be accurately measured.

Endogenous substances can exhibit transporter-mediated disposition, and their concentrations may be altered when transporter activity is modulated. These
endogenous substances have the potential to serve as biomarkers to study transporter function in vivo in humans. Transporter biomarkers offer the potential for evaluating NMEs as transporter inhibitors in early clinical studies, and with appropriate validation, may obviate the need for dedicated clinical DDI studies. For the purposes of drug development planning, it would be invaluable to corroborate or refute inhibition of transporters predicted from in vitro data using biomarkers, particularly in situations where in vitro prediction methods have high false positive rates. For clinical DDI studies evaluating an NME as an inhibitor, use of selective and sensitive probe substrate drugs is a key to determine clinical risk for transporter inhibition, provide a mechanistic insight, extrapolate the results to other unstudied drugs, and inform the drug labeling to provide recommendations based on the DDI results. However, this has been challenging by the multiplicity of transporter substrates and inhibitors, the presence of multiple drug binding sites in many drug transporters, and complex interplay with drug metabolizing enzymes\textsuperscript{4-6,10}. Therefore, probe drugs currently used for clinical DDI studies are largely based on the likelihood of co-administration, without comprehensive understanding and evaluation of their ADME properties/in vitro transporter profiles, which limits extrapolation of the results to other drugs. Validated clinical probes are critical for robust clinical DDI assessment that ultimately drives product labeling to inform concomitant drug use. Table 1 outlines key features of an ideal probe drug or a biomarker which can be used in vivo to assess transporter perpetrator DDIs. Notably, very few such probe substrate drugs or biomarkers have been identified to date.

This article reviews the state-of-the-art of transporter clinical probe drugs and emerging biomarkers, provides recommendations on use of relevant transporter probe substrates for DDI evaluation, and some potential transporter endogenous biomarkers for further characterization and validation, as well as current challenges and limitations. Furthermore, the article practically delineates methods and workflows to identify and validate novel endogenous biomarkers to support clinical DDI evaluation, and proposes their potential utility and application in drug development.
Clinical DDI assessment for an NME as a transporter inhibitor is typically conducted by administering an accepted probe substrate with the NME. In this section, we have conducted a critical and comprehensive analysis of probe substrate drugs commonly used for the transporters recommended by the ITC and regulatory agencies for clinical DDI evaluation based on their ADME properties, in vitro transporter profiles, and in vitro and clinical DDI data.\textsuperscript{1, 4-6, 9, 10}

**Figure 1** illustrates recommended probe substrate drugs with a relatively high selectivity (based on relative contribution of transporter-mediated pathways to overall elimination of drugs) and sensitivity (based on clinical DDI studies with known potent transporter inhibitors and/or pharmacogenomic studies in subjects carrying non- or reduced-function alleles)\textsuperscript{15, 16} with detailed ADME properties, in vitro transporter profile, and examples of clinical DDIs summarized in Table 2. These probe substrate drugs in general meet the following criteria:

- **In vitro** transporter profiles are relatively well characterized, and show higher transport activity and selectivity for the transporter of interest

- Clinical data show significant contribution of the transporter of interest in the overall elimination as indicated by

  - the ratio of area under the plasma concentration-time profile (AUCR) ≥ 2
  - and/or the ratio of plasma clearance (CL) (or renal clearance (CL\textsubscript{R}) for renal transporters) CLR or CL\textsubscript{R} ≤ 0.5

Either in the presence of a potent transporter inhibitor or in subjects with reduced function caused by a transporter polymorphism

- Are commercially available and have relatively rich clinical DDI data
In this section, potential utility and limitations of probe substrate drugs summarized in Figure 1 are discussed, together with recent examples of clinical probe drug cocktails used to simultaneously explore DDIs involving multiple drug transporters and metabolizing enzymes.\(^\text{17-20}\)

Several endogenous substrates have been identified as potential endogenous biomarkers to evaluate inhibition of several hepatic and renal transporters in humans.\(^\text{12,13,14}\) The endogenous substrates with initial clinical DDI evaluation are highlighted in Figure 1. Recent progress in identifying and evaluating these potential biomarkers are discussed below and some key preclinical and clinical data are summarized in Table 3 and Table S1.

Liver transporters: Organic Anion Transporting Polypeptide 1B1/1B3 (OATP1B) and Organic Cation Transporter 1 (OCT1)

OATP1B1/1B3

- Probe Drugs

Pitavastatin, rosuvastatin, and atorvastatin are widely used as clinical probe drugs for the evaluation of OATP1B-mediated DDIs, either following a clinically-relevant oral dose or a microdose as part of a drug cocktail\(^\text{18,21,22}\) (Figure 1, Table 2). Although considered as OATP1B substrates, the aforementioned drugs as well as other proposed “probes” have been shown to be substrates for other transporters in vitro, such as OATP2B1, sodium-taurocholate co-transporting polypeptide (NTCP), breast cancer resistance protein (BCRP), P-glycoprotein (P-gp), organic anion transporter 3 (OAT3), and multidrug resistance-associated protein 4 (MRP4) and MRP3.\(^\text{21,23,24}\)

Pitavastatin is considered as the most sensitive OATP1B1 probe,\(^\text{22}\) supported by >80% fraction transported via OATP1B1, which has been estimated from in vitro, human ADME, SLCO1B1 (OATP1B1) c. S21T>C pharmacogenomic data and DDI data with intravenous (i.v.) vs.
oraly-administered rifampin, a potent inhibitor for OATP1B. Pharmacogenomic data highlight similar sensitivity to SCL01B1 for simvastatin acid, but its complex PK (lactone-acid interconversion) and OATP1B-CYP3A interplay may limit its general use as a selective OATP1B clinical probe. In the case of rosuvastatin, the alteration of the activity of multiple non-OATP1B transporters expressed either in the intestine (BCRP and OATP2B1), liver (NTCP and OATP2B1), or kidney (OAT3) may be the driver behind the observed change in its PK following the oral administration of an inhibitor that can affect these transporters. Like rosuvastatin, atorvastatin presents a suitable OATP1B1 probe drug because of supporting pharmacogenomics data, clinical DDI data with rifampin and mechanistic insight regarding OATP1B as the rate-determining step driving its hepatic disposition. Nonetheless, the potential effect of inhibiting intestinal CYP3A4 and BCRP/P-gp efflux should also be considered when interpreting the observed changes in its systemic exposure.

- Endogenous Substrates (Potential Biomarker)

To date, the endogenous substrates of OATP1B include coproporphyrins (CPs), bile acids (BAs), sulfated and glucuronidated BAs, fatty acid dicarboxylates, bilirubin, glucuronidated bilirubin, thyroid hormones and their metabolites, steroids, and sulfated and glucuronidated steroids. Among them CPI, CPIII, glycochenodeoxycholate-3-O-sulfate (GCDCA-S), hexadecanedioate (HDA), tetradecanedioate (TDA), conjugated and unconjugated bilirubin (CB and UCB) present as potential biomarkers for clinical evaluation of OATP1B-mediated DDIs, but each has limitations (Figure 1, Table 3). In most cases, evidence for their utility as OATP1B biomarkers is based on animal data and careful profiling of plasma and urine of human SLCO1B genotyped subjects. For example, patients with Rotor’s syndrome lack expression of OATP1B1 and OATP1B3 are characterized by increased urinary excretion of CPI and higher bilirubin in the blood due to reduced secretion into the bile. Likewise, pharmacogenomic studies indicate that individuals genotyped SLCO1B1*15 and c.521T>C show increased exposure of UCB, CB and some BAs. Compelling clinical evidence supporting HDA, TDA, GCDCA-S and bilirubin as potential endogenous biomarkers for OATP1B1 has come from metabolomic and genome-wide association studies (GWAS), which show a significant association of SLCO1B1
521T>C with the plasma levels of these endogenous substances. The clinical data are consistent with the results obtained in *Oatp1a/1b<sup>−/−</sup>* mice, as they exhibit higher plasma CPI, CPIII, UCB, CB and BA concentrations (Table S1). In addition, *in vitro* studies using transporter-transfected cells and hepatocytes with an OATP1B inhibitor (e.g., rifampin) confirm that CPI, CPIII, GCDCA-S, HDA, TDA, CB and UCB are substrates for OATP1B1 (Table 3). These biomarkers are also substrates for OATP1B3 with the exception of HDA and TDA. In addition, they are also transported by other hepatic and renal transporters such as MRP2 and MRP3 (CPI, CPIII and CB), OATP2B1 (CPIII), OAT1 (HDA and TDA), OAT3 (GCDCA-S, HDA, and TDA), and NTCP (GCDCA-S) (Table 3), which may limit their selectivity.

Concerns regarding transporter selectivity aside, the above mentioned endogenous biomarkers are capable of recapitulating some clinical OATP1B DDIs. For example, a single oral dose of rifampin (600 mg) has been shown to markedly increased the AUC of GCDCA-S (10-20-fold).<sup>41</sup> This increase was significantly greater than the changes in plasma simeprevir, bosentan, and repaglinide exposures in the same study (7.2-, 3.2- and 1.9-fold, respectively).<sup>34</sup> Results of recent studies have also shown that changes in CPI plasma exposure correlate with presumably different levels of OATP1B inhibition imposed by OATP1B inhibitors that showed different in vivo inhibition (e.g., simeprevir, JNJ-A, and GDC-0810) with known OATP1B substrates.<sup>37</sup> To date, however, reported OATP1B clinical DDI studies with concomitant monitoring of both clinical probe drugs and endogenous biomarker(s) are still limited in number.<sup>38-41</sup> (Table 3).

In summary, CPI, CPIII, GCDCA-S, HDA, TDA, CB and UCB have demonstrated initial promise as clinical OATP1B biomarkers, in which, CPI appears to be the most promising biomarker with higher selectivity and sensitivity. Further characterization and validation is needed to determine their predictive value for OATP1B-related DDIs. Until other confounding factors, including overlapping substrate selectivity for transporters and enzymes, disease, diet, and circadian rhythm, are fully understood, a single OATP1B biomarker may not be sufficient for effective overall evaluation of OATP1B inhibition risk and the use of multiple biomarkers may be warranted.
Metformin is a well-known substrate of OCT1. It is also a substrate for OCT2 and multidrug and toxin extrusion protein MATE1 and MATE2K. Metformin may not be a suitable clinical OCT1 probe drug if only changes in PK is monitored, because OCT1 modulation does not impact metformin systemic PK, and it only impacts metformin hepatic distribution and pharmacodynamics (PD) response. Currently, OCT1 probe substrate drugs have only been validated clinically in pharmacogenetic studies by comparing individuals expressing inactive or reduced function of OCT1 vs. wildtype controls. Clinical OCT1 probes identified to date include sumatriptan, ondansetron, and tropisetron. Overall, sumatriptan and tropisetron are promising probe substrates of OCT1 because they exhibit a 2.2-fold and 4.9-fold increase in AUC, respectively, in subjects with a polymorphism resulting in reduced OCT1 activity vs. controls. Of the two, sumatriptan is most promising as a drug probe for OCT1 because evidence is available from a prospective study in healthy subjects (Table 2). Additional DDI studies with OCT1 inhibitors are warranted to better characterize their clinical utility as probe drugs for OCT1.

Fenoterol is also a promising clinical OCT1 probe with both notable systemic PK and PD effects of OCT1 modulation, but it is practically problematic, because it is not approved in the US. The O-desmethyl metabolite of tramadol is a sensitive OCT1 probe with both notable PK and PD effects during OCT1 modulation. However, metabolites are generally not preferred probes in the drug development setting due to additional data interpretation complexity associated with metabolite formation that may be influenced by NME and population variability (e.g., pharmacogenetic factors). Morphine may be a useful OCT1 probe, but clinical studies conducted to-date were inconsistent, and morphine cannot presently be recommended as an OCT1 probe until these findings are clarified (e.g., oral administration of the prodrug versus parenteral administration of morphine directly). Besides OCT1, morphine is a substrate for UGTs and P-gp. Moreover, morphine is a controlled substance and DDI studies with controlled substances are not practical (or safe).
Endogenous Substrates (Potential Biomarker)

Thiamine is a substrate of OCT1\textsuperscript{51} (Table S1), however, clinical evidence supporting thiamine as an endogenous OCT1 probe is lacking. Preclinical studies using Oct1\textsuperscript{-/-} mice showed that the concentrations of thiamine and its metabolites in the knockout mouse liver following i.v. administration were reduced by more than 50% compared to wild-type animals, whereas plasma levels were increased 30-40%, confirming the role of Oct1 in thiamine hepatic disposition \textit{in vivo}. However, thiamine is not selective for OCT1 because it is also a substrate for OCT2, MATE1, MATE2K, thiamine transporter 1 and 2 (THTR1 and THTR2).\textsuperscript{52, 53} Notably, up to 9% of Caucasians exhibit an OCT1 deficient phenotype, which has not been associated with markedly elevated systemic thiamine concentrations possibly due to variability associated with dietary intake. This may make thiamine an impractical clinical OCT1 biomarker.

Recently, acylcarnitines, intermediate metabolites of mitochondrial oxidation, was identified as endogenous substrates of OCT1 through GWAS, targeted metabolomics, transporter transfected cells, and Oct1 liver specific knockout mice.\textsuperscript{54} These studies have illustrated the role of OCT1 in the efflux of acylcarnitines from the liver to the circulation. Additional clinical DDI studies, however, are needed to assess the suitability of acylcarnitine as a potential endogenous biomarker of OCT1. It is worth noting that the GWAS showed that serum acylcarnitine levels are also significantly associated with organic cation/carnitine transporter 1 (OCTN1; SLC22A4), UGT1A1 and carnitine palmitoyltransferase 1 (CPT1) genes\textsuperscript{55} and acylcarnitine is also an \textit{in vitro} substrate of OCTN2, which may limit the selectivity of acylcarnitine as a biomarker for OCT1.\textsuperscript{55}

Kidney Transporters: Organic Anion Transporters OAT1/3 and Organic Cation Transporters

OCT2 and MATE1/2K

\textbf{OAT1/3}

• Probe Drugs
Despite overlapping substrate selectivity between OAT1 and OAT3, adefovir is a relatively selective OAT1 probe, because its transport by OAT3 is minimal\textsuperscript{56} \textbf{(Figure 1, Table 2)}. Adefovir is also transported by the apically localized renal efflux transporter MRP4.\textsuperscript{57} However, plasma and renal clearance of adefovir was only slightly decreased in Mrp4\textsuperscript{−/−} mice, suggesting that change in Mrp4 activity may not have significant impact on its plasma exposure.\textsuperscript{57} Probenecid, a potent inhibitor of OATs, caused a 2.1-fold increase in adefovir plasma AUC \textbf{(Table 2)}, suggesting that adefovir may be used as a probe drug for OAT1. In contrast, benzylpenicillin (also named as Penicillin G) \textbf{(Figure 1, Table 2)} is a more selective OAT3 substrate compared to OAT1.\textsuperscript{56, 58} Probenecid increased plasma AUC of benzylpenicillin by 3.3-fold\textsuperscript{56} \textbf{(Table 2)}, suggesting that it may serve as an OAT3 probe substrate. As discussed in drug probe cocktail section below, furosemide, a dual substrate of OAT1 and OAT3, could be a useful probe to study the inhibition to both OAT1 and OAT3.\textsuperscript{17}

- **Endogenous Substrates (Potential Biomarker)**

Taurine is an endogenous OAT1 substrate, whereas 6β-hydroxycortisol (6βHC) and GCDCA-S are substrates of OAT3; they may therefore potentially serve as endogenous biomarkers for assessing DDIs with these transporters\textsuperscript{12, 13} \textbf{(Figure 1, Table 3)}. In a clinical study, probenecid exhibited dose-dependent reduction of CL\textsubscript{r} of taurine and GCDCA-S without altering their plasma AUC. Moreover, the CL\textsubscript{r} values for either compound correlated strongly with benzylpenicillin (an OAT3 probe drug) and 6βHC, suggesting that either compound could be a useful biomarker for OAT1 or OAT3.\textsuperscript{59} Unlike 6βHC, the CL\textsubscript{r} of taurine and GCDCA-S was below GFR, indicating potential reabsorption from the urine in addition to tubular secretion. The selectivity of GCDCA-S as an endogenous OAT3 probe is limited since probenecid can inhibit apically-localized OAT4 and MRP2, which may be involved in the reabsorption and urinary excretion of GCDCA-S, respectively. Additionally, GCDCA-S is also known as an endogenous substrate of hepatic OATP1B\textsuperscript{60} \textbf{(Table 3)} and, as such, may confound its selectivity for OAT3. 6βHC is formed by hepatic CYP3A4 and excreted into urine. It is also known as a biomarker for induction of CYP3A4 and any change in CYP3A4 activity may therefore influence the sensitivity and selectivity of 6βHC as an OAT3 probe.\textsuperscript{61} Besides OAT3, 6βHC is a substrate for MATE1 and
MATE2K. Probenecid significantly increased plasma AUC, but reduced CL\textsubscript{r} of 6βHC, whereas pyrimethamine (a MATEs inhibitor) did not change systemic 6βHC AUC or CL\textsubscript{r} significantly. Neither probenecid nor pyrimethamine affected CYP3A4 and 11β-hydroxysteroid dehydrogenase 2 activity responsible for the formation of 6βHC and conversion of 6βHC to 6β-hydroxycortisone, respectively. Recently, pyridoxic acid and homovanillic acid have been identified as endogenous substrates of OAT1/3 using metabolomic analysis and DDI studies in cynomolgus monkeys. Additional studies are needed to validate their potential utility as OAT1/3 endogenous biomarkers in human.

OCT2, MATE1/2K

- Probe Drugs

Metformin is a well-known and broadly used probe drug to study inhibition of the renal uptake transporter OCT2, and efflux transporters MATE1/2K. For example, MATE1/2K inhibitor pyrimethamine significantly changed metformin plasma AUC and CL\textsubscript{r} by the inhibition of active renal secretion\textsuperscript{65,66} (Figure 1, Table 2). However, distribution of metformin to the liver, its primary site of action, but not clearance, is mediated by OCT1. Recent studies suggest that metformin is also a substrate of OCT3, which is a key to its skeletal muscle distribution (another site of action). The absorption and distribution of metformin is a complex and poorly understood process that involves at least several transporters, including THTR-2 and OCT1/3.\textsuperscript{64} Lack of selectivity of most transporter inhibitors complicates further the interpretation of metformin renal DDI data. For instance, famotidine (a selective MATE1 inhibitor) at a high dose (200-800 mg/day) resulted in an increase, rather than the expected decrease in metformin CL\textsubscript{r}.\textsuperscript{68} Famotidine co-administration also caused a transient enhancement of the anti-hyperglycemic effects of metformin, likely due to enhanced hepatic distribution through MATE1 inhibition.\textsuperscript{68} Due to the unique complexity of metformin mechanistic disposition, a separate commentary in this issue of CPT is dedicated to clinical DDI study design consideration for metformin.\textsuperscript{43}
• Endogenous Substrates (Potential Biomarker)

N1-methylnicotinamide (NMN) is an endogenous substrate of OCT2, MATE1/2K.\textsuperscript{69} Pyrimethamine almost completely inhibited its CL\textsubscript{r} via active secretion without altering its plasma exposure.\textsuperscript{69} Trimethoprim, another OCT2/MATEs inhibitor, also reduced NMN CL\textsubscript{r}, which correlated with the change in metformin exposure in the same healthy subjects.\textsuperscript{70} Recent studies reported increased NMN CL\textsubscript{r} in pregnant women which was positively correlated with increase in metformin CL.\textsuperscript{71} These clinical observations suggest the potential use of NMN as an endogenous probe for DDIs involving OCT2/MATEs (Figure 1, Table 3). However, it is worth noting that renal elimination of NMN also involves active tubular reabsorption.\textsuperscript{72}

Creatinine is commonly used as a biomarker for renal function. Increased serum creatinine and reduced creatinine CL\textsubscript{r} could be a result of transient inhibition of renal transporters involved in its active secretion (OCT2, OAT2, MATE1/2K), without altering GFR\textsuperscript{73} (Figure 1, Table 3). However, creatinine levels are influenced by many factors such as weight, gender, age, muscle metabolism, and diet. A retrospective analysis of the change of creatinine level and DDI data with OCT2/MATEs inhibition suggests that creatinine is not a sensitive biomarker for OCT2/MATEs inhibition, despite the fact that elevation of serum creatinine is frequently associated with inhibition of OCT2/MATEs.\textsuperscript{73} Therefore, incorporation of creatinine with other biomarkers, such as NMN may be needed to evaluate the risk for OCT2/MATEs inhibition.

Gut Transporters: P-gp and BCRP

P-gp

• Probe Drugs

Digoxin, dabigatran etexilate (DE), and fexofenadine are recommended by regulatory agencies as clinical probe drugs for P-gp inhibition (Figure 1, Table 2). However, there are a
number of limitations regarding the suitability of digoxin as a P-gp probe including the involvement of additional transporters and/or the low sensitivity of the DDI signal largely due to high oral bioavailability (60-80%). Despite these limitations, clinical DDIs with digoxin have been most extensively evaluated because digoxin exhibits a narrow therapeutic index and is commonly used in the clinical setting. The impact of NMEs on digoxin PK should therefore be carefully monitored as a safety precaution. However, DDI data obtained with digoxin cannot be readily extrapolated to other P-gp substrate drugs. The limitation of fexofenadine as a P-gp probe is the involvement of OATPs (OATP2B1, OATP1B1/1B3) and OAT3 in its absorption, hepatic and renal elimination. Fexofenadine therefore can be selected as a P-gp probe only when OATPs/OAT3 inhibition is not involved in the DDIs. DE, a prodrug of dabigatran, is a P-gp substrate, whereas its active moiety dabigatran is not. Thus, DE could be a more selective P-gp probe to study P-gp inhibition in the gut, as P-gp-mediated efflux is restricted to limiting intestinal absorption of DE. However, PK variability, conversion of DE to dabigatran via carboxylesterases, and potential saturation of P-gp in the gut at therapeutic dose may impact its sensitivity and selectivity. Given their relatively low bioavailability (Table 2), DE and fexofenadine may be more appropriate P-gp probes in comparison to digoxin when evaluating intestinal P-gp inhibition.

Endogenous Substrates (Potential Biomarker)
To date, no endogenous probe for P-gp has been identified.13

BCRP

Probe Drugs

It is challenging to identify clinical BCRP probes due to the multiplicity of substrates and inhibitors, as well as potential BCRP interactions both in the gut and liver. Following a comprehensive review of in vitro, clinical DDI, and pharmacogenomic data, sulfasalazine has been proposed as an in vivo probe for evaluating intestinal BCRP inhibition, intravenous
rosuvastatin for evaluating hepatic BCRP inhibition, and oral rosuvastatin for both intestinal and hepatic BCRP inhibition. Sulfasalazine has limited intestinal absorption due to its low permeability, low solubility, and BCRP-mediated intestinal efflux (Figure 1, Table 2). Plasma AUC of sulfasalazine in wild–type mice increased by 8-fold, following oral dosing of curcumin, a potent BCRP inhibitor, whereas no change was observed in Bcrp−/− mice. In human, curcumin increased sulfasalazine AUC by 1.8- to 3.2-fold (Table 2), suggesting that BCRP plays a significant role in limiting the intestinal absorption of sulfasalazine. Of note, sulfasalazine is a MRP2 and P-gp substrate as well, which may limit its intestinal absorption but to a much lower extent than BCRP based on the observations from transporter knockout mice. Furthermore, sulfasalazine is also transported by OATP2B1 in the gut, which is most likely saturated at sulfasalazine’s therapeutic dose, and therefore, may not have significant impact on its intestinal absorption.

As discussed above, rosuvastatin is a substrate for multiple uptake and efflux transporters, including BCRP. A polymorphism in ABCG2 (c.421C>A) results in a significant increase of rosuvastatin plasma AUC (Table S2), supporting its use as a BCRP probe. As inhibition of BCRP may alter both the intestinal absorption and hepatobiliary excretion of rosuvastatin, it can be a useful probe to assess inhibitory effects on both gut and hepatic BCRP. Differentiation between the contribution of intestinal and hepatic BCRP is possible only if the appropriate study design is used, as discussed in study design section.

- Endogenous Substrates (Potential Biomarker)

To date, no endogenous probe for BCRP has been identified.

**Transporter Probe Drug Cocktails**

Since many perpetrator drugs inhibit multiple transporters, concurrent administration of a cocktail containing multiple probe drugs at therapeutic or microdose could be an efficient and valuable tool. The cocktail approach has been successfully applied in drug development and
recognized by regulatory agencies to assess DDIs with CYP enzymes. However, the evaluation and use of dedicated drug cocktails to study transporter-related DDIs is still limited. In 2011, Maeda, et al designed a clinical study to identify the rate-determining process in hepatic elimination of atorvastatin using a microdose cocktail of atorvastatin along with probe substrates for OATP1B (pravastatin) and CYP3A (midazolam). Recently, two transporter probe drug cocktails have been proposed for studying DDIs involving transporters and their interplay with CYP3A. A transporter probe cocktail containing a conventional dose of digoxin (0.25 mg, P-gp), rosuvastatin (10 mg, OATP1B, and BCRP), furosemide (5 mg, OAT1/3), and metformin (500 mg, OCT2, MATE1/2K) has been evaluated in vitro and in clinical PK studies. In general, the PK of each probe substrate administered in a cocktail was comparable to when dosed individually, except for a small decrease in $C_{\text{max}}$ of furosemide and a moderate increase in rosuvastatin $C_{\text{max}}$ (38.6%) and AUC (43.4%). A reduction of the dose of metformin (50 mg or 10 mg) and furosemide (1 mg) is further recommended to minimize potential interactions between these probes. Recently, in a new trial where the doses of metformin and furosemide as putative perpetrators were reduced to 10 mg and 1 mg, respectively, their DDI effects on rosuvastatin were shown to be eliminated. Another microdose cocktail has recently been proposed and validated to study DDIs with OATP1B, P-gp, BCRP, and their interplay with CYP3A. This five-component cocktail contained midazolam (10 µg, CYP3A), DE (375 µg, P-gp), pitavastatin (10 µg, OATP1B), rosuvastatin (25 µg, OATP1B, BCRP) and atorvastatin (50 µg, OATP1B, BCRP, P-gp, and CYP3A). Except for DE, which showed a ~2-fold higher magnitude of DDIs with P-gp inhibitors versus a conventional dose, likely caused by saturation of gut P-gp, PK and the magnitude of DDIs with other microdosed probe drugs were generally comparable to those at their respective conventional doses. Probe drug cocktails allow the simultaneous assessment of DDI risk for different transporters/ enzymes and thus can provide a mechanistic understanding of complex DDIs involving multiple transporters and enzymes.
MECHANISTIC CONSIDERATIONS AND CHALLENGES OF USING PROBE DRUGS AND ENDOGENOUS BIOMARKERS TO ASSESS TRANSPORTER INHIBITION

This section describes mechanistic considerations and challenges associated with the use of exogenously administered probe drugs and endogenous biomarkers to study transporter inhibition. Some of the challenges, such as the lack of selectivity towards individual transporters, are common for biomarkers and probe drugs. However, there are also unique challenges. For example, for many endogenous biomarkers, the kinetic determinants of their systemic exposure are poorly understood or defined. These limitations need to be considered when designing clinical studies or extrapolating observed DDIs to new combinations of drugs.

Probe Drugs

There are multiple factors that need to be considered prior to qualifying a given drug as a useful “probe” for examining the in vivo activity of a transporter of interest, e.g., absence of additional mechanisms (i.e., selectivity) or the availability of candidate probe drugs in a form that can be safely and ethically administered to humans in a feasible manner (Table 1). The paucity of probe drugs possessing sufficient selectivity places a major limitation on the ability to demonstrate the importance of a given transporter (Table 2). As highlighted, in certain instances testing candidate probe substrates and inhibitors in preclinical species may provide valuable information to guide future clinical investigations. Ultimately, the validation of such probes requires multiple rounds of investigation to identify the appropriate study design and the relatively specific substrate/inhibitor sets that can be utilized clinically.

Overlapping Substrate Specificities of Multiple Transporters and Metabolic Enzymes

Because most transporter probes are substrates of multiple transporters and/or enzymes, there are substantial challenges in selecting a specific probe. As discussed earlier, rosvastatin is a substrate of BCRP and several other transporters (e.g., OATP1B1, -1B3, -2B1,
NTCP, and OAT3). If a perpetrator drug is an inhibitor of these transporters, observed DDIs may represent a worst-case scenario for rosuvastatin DDIs, but not inhibition by BCRP alone. In such case, a rosuvastatin clinical DDIs need to be interpreted in conjunction with in vitro and other clinical DDI studies with single or multiple transporter probes of interest or endogenous biomarkers. One promising approach to address the overlapping selectivity of transporters and enzymes, and to maximize clinical study design is to use a transporter and enzyme probe drug cocktail, as discussed in the previous section.

**In Vivo Quantitation of Transporter Activities**

Probe drugs may be used to quantitatively evaluate the effect of an NME on transporter activity in various organs. However, the nature of the biological matrices that can be easily collected (e.g., blood, urine, feces and saliva) often imposes a major limitation on the practical use of probe drugs in such clinical investigations. The use of stable- or radioisotope-labeled micro-tracers administered intravenously offers the opportunity to evaluate the effect of the perpetrator drugs on the bioavailability and total body clearance of probe drugs. However, limited ability to assess drug concentration-time profiles in tissues imposes limitations on understanding some of transporter DDIs where changes in systemic exposure do not necessarily reflect events at the tissue level. In such situations, measurement of the tissue concentration-time profiles by non-invasive approaches, such as positron emission tomography (PET), is important for quantifying transporter activities. The integrated efforts of combining in vitro, imaging and modelling approaches can improve our predictability of tissue concentrations and DDIs.

**Other Challenges and Considerations**

Taking into consideration of substrate-dependent inhibition in vitro, translation of clinical DDI data obtained using one probe to other substrate drugs remains uncertain. In vitro studies may be able to bridge the gap assuming the substrate dependence is relevant in vivo.
addition, translation of the data from healthy subjects to specific populations, such as the subjects with hepatic or renal impairment also remains challenging due to limited information on the change of transporter activity in these populations.$^{90, 91}$

**Endogenous Biomarkers**

*Mechanistic Understanding of the Formation, Disposition, and Elimination of Biomarkers*

One of the major challenges in translating response of endogenous biomarkers to the inhibition of drug transporters is the poor understanding of the mechanisms involved in their formation, disposition, and/or elimination. Using OATP1B biomarkers as an example, Figure 2 summarizes key elements that need to be considered when interpreting biomarker data. Factors potentially impacting the interpretation of renal transporter biomarker data are shown in Figure S1. They can be broadly categorized into three elements as described below: selectivity, specificity, sensitivity, and intrinsic or dietary factors. Current knowledge on some emerging biomarker candidates is summarized in Table 3, but a considerable amount of relevant information is still missing.

Selectivity and specificity of biomarkers for assessing transporter inhibition needs to be carefully considered, as highlighted in the case of probe drugs, but to date there has been limited clinical validation of the specificity and selectivity of potential biomarkers. Sensitivity of the endogenous biomarker is another key determinant of their utility in assessing transporter function, especially if the endogenous biomarker data are to be used to support the lack of an interaction between an NME and a transporter. Additionally, understanding of both intra- and inter-subject variability of biomarker levels is needed to evaluate the utility of biomarker in capturing weak or moderate transporter inhibitors/DDIs.

Some potential endogenous biomarkers, such as bile acids or bilirubin, are associated with either the development of diseases or organ injury; therefore, they are not ideal biomarkers for DDI assessment (Table 1). Additionally, some biomarkers may be derived from
dietary constituents or metabolites, and in that case the potential effects of food should be carefully ruled out. Effect of perpetrator drugs on the biosynthesis of endogenous biomarkers also needs to be considered, as illustrated recently for CPI and creatinine.\textsuperscript{92, 93, 94}

Genetic polymorphisms of drug transporters can shed a light on the mechanistic understanding of endogenous biomarker disposition. One salient example is the altered disposition and elimination of CPI and CPIII due to genetic alterations that affect MRP2 (ABCC2) and OATP1B1 (SLCO1B1) transport activities.\textsuperscript{12} In that case, the observed changes in plasma or urine concentrations of these biomarkers may provide quantitative information on the contribution of individual transporters to a given clearance pathway. It is anticipated that the incorporation of such quantitative measures can be particularly useful in verifying physiologically-based pharmacokinetic (PBPK) models to describe the PK of endogenous biomarkers.

\textit{Kinetic Consideration for Interpreting Biomarker Data}

One major difference between endogenous biomarkers and probe drugs is that the baseline concentrations of the endogenous biomarkers are determined by the rate of synthesis (as opposed to dose in the case of clinical probe) ($R_{in}$), along with the rate of degradation/elimination ($R_{out}$). When an endogenous biomarker follows first-order elimination, the concentration of the biomarker ($C$) at any given time can be described as:

\[
dC/dt = R_{in} - CL * C \quad (\text{Equation 1})
\]

Where CL represents the clearance of the biomarker. Thus, determination of $R_{in}$ and CL values is the key for quantitative, model-based analysis of biomarker kinetic profiles. When $R_{in}$ and CL are stable over time (i.e., no diurnal fluctuation), the steady-state concentration ($C_{ss}$) is described as:

\[
C_{ss} = R_{in} / CL \quad (\text{Equation 2})
\]
Unlike the probe drugs for which we can control the administered dose, it is challenging to directly estimate $R_{in}$. This has a significant implication on a model-based analysis of biomarker profile and subsequent quantitative interpretation of DDI observations. One strategy is to utilize isotope tracers of endogenous biomarker\textsuperscript{95} to determine $C_{ss}$ and CL of a biomarker, and then infer $R_{in}$ based on Equation 2. To obtain CL, kinetic profiles after i.v. administration of the biomarker are the most informative. Another approach is to administer isotopic precursor to determine synthesis rate. Alternatively, clinical data for an endogenous biomarker in the presence and absence of a transporter inhibitor can be modeled simultaneously to estimate the rate of biomarker synthesis and elimination clearances, assuming that the inhibitor only affects transporter-mediated CL, with no effect on $R_{in}$\textsuperscript{92}. Currently such information is limited to only a few transporter biomarkers\textsuperscript{96} and more research is needed to fill the gap to better characterize the kinetic behaviors of biomarkers.

Selection of descriptive PK parameters for biomarkers requires careful considerations. The simplest case is a direct evaluation of the changes in pathway-specific clearance, e.g., $CL_r$, when examining effects on renal transporters. Amount of biomarker excreted into urine over a particular time ($A_e$) can be expressed as follows:

$$A_e = CL_r \times AUC \text{ (Equation 3)}$$

Therefore, measurement of biomarker AUC and $A_e$ in a given time interval allows direct calculation of the effect of test drugs on renal clearance of the biomarker.

The systemic level of a biomarker could be used for the evaluation of transporter function in situations where the direct measurement is not possible (e.g., modulation in biliary excretion). In such cases, the time course of the biomarker after administration of a perpetrator drug is critical to allow correct interpretation of the DDI data. This time course will be influenced by the half-life ($t_{1/2}$) of not only the biomarker (which may be synthesis, absorption, or elimination rate-limited), but also the PK of the transporter inhibitor. For example, if an inhibitor has a long $t_{1/2}$, its effect on the transporter will be sustained after it reaches its maximum concentration, and the biomarker concentrations will approach new steady-state in
accordance with the $t_{1/2}$ of the perpetrator drug. In this situation, AUC would be an appropriate metric to interpret changes in transporter function. Figure 3 and Supplementary Materials show various simulations under different scenarios to illustrate these points, and the recommendations on the interpretation of systemic exposure of biomarkers.

Other Considerations

Similar to the clinical probe drugs, there is a potential substrate-dependency in the in vitro transporter $IC_{50}$ or $K_i$ values with either different biomarkers and/or biomarkers to substrate drugs. Therefore, translation of DDI data using one biomarker to predict DDI risk with NMEs needs to be fully evaluated assuming the relevance of substrate-dependent phenomenon in vivo. The importance of investigating more than one biomarker is also applicable to the evaluation of inhibitor effect on other transporters. Fully validated bioanalytical methods for reliable and reproducible measurements of biomarkers are critical (Table 1).

IDENTIFICATION AND VALIDATION OF ENDOGENOUS BIOMARKERS FOR TRANSPORTER DDI EVALUATION

The identification of novel, sensitive endogenous biomarkers to assess transporter inhibition in a clinical setting follows a series of sequential steps. Initially, a candidate biomarker can be identified from a broad scope of potential data sources and subsequently narrowed down through steps of hit identification, lead characterization and eventually clinical validation as detailed in the workflow in Figure 4.

In Vitro and Metabolomics Approaches
The process of identification may include a combination of *in vitro* and *in vivo* approaches to select a candidate endogenous molecule. For example, metabolomics approaches combined with *in vitro* assays can be used to examine the uptake of endogenous molecules into cells individually expressing transporters of interest following incubation with biological matrices. The utility of these candidate molecules can then be further challenged in systems of increasing complexity such as knockout rodent models, non-human primates and humans following the administration of a potent and relatively selective transport inhibitor. Finally, the candidate biomarker may be examined in larger populations to verify that variability in its plasma concentrations correlate with known genetic polymorphisms affecting the activity of the transporter of interest. However, there are only a few transporters with clinically meaningful genetic polymorphisms, which may limit this approach.\textsuperscript{15, 16} Table S3 provides details and examples on each experimental system. Each system will generate matching test and control samples that will be quantified using metabolomics and enable inferential comparison of dozens of endogenous and exogenous metabolites in parallel and generate candidates. The selected molecules should exhibit robust and consistent responses in all available test systems before they can be considered as biomarkers for the transporter being investigated.

Clinical Studies to Characterize and Validate Novel Endogenous Biomarkers to Study

Transporter Inhibition

The identification of an endogenous biomarker in a clinical setting is aimed at investigating the sensitivity selectivity, and specificity of candidates identified using the steps outlined above. Factors affecting the baseline concentration of a candidate biomarker such as diet, age, exercise, diurnal variation, and disease state may all confound the ability to observe meaningful changes in the biomarker level upon inhibition of an implicated transporter. Similarly, the participation of additional unidentified transporters in the systemic clearance of the candidate biomarker may also pose an additional challenge. Finally, an understanding of the synthetic and catabolic pathways of the candidate biomarker is necessary to ensure that the
test inhibitor does not acutely nor chronically interfere with either pathway. Mechanistic models that combine rates of synthesis, degradation and transport can be developed as described in previous sections to further verify the practicality of the candidate biomarker and perform power calculations to guide the design of clinical DDI studies, as illustrated recently for CPI.$^{92}$

Further validation of a candidate biomarker can be done by examining the effect of specific transport inhibitors on the biomarker exposure, preferably in a well-controlled cross-over study in subjects that have consented to genomic analysis of the suspected transporter that is known to be polymorphic. Inclusion of a validated probe drug in the study is recommended to enable parallel examination of observed effects. The number of individuals participating in a given trial and the length of treatment should be determined following the appropriate statistical and kinetic analysis of the candidate biomarker PK and extent of contribution of the implicated transporter to the overall systemic clearance. Moreover, confirmatory studies should utilize optimized analytical methods and procedures capable of detecting low levels of the biomarker in the relevant biological matrix. Study design considerations are described in detail in the next section. The results of the clinical study should be used to compare the magnitude of changes in biomarker exposure relative to the changes measured for the transporter probe drug for each of the tested inhibitors.

**CLINICAL DDI STUDY DESIGN FOR EVALUATION OF TRANSPORTER INHIBITION WITH CLINICAL PROBE DRUGS OR ENDOGENOUS BIOMARKERS**

**Probe Drugs**

Due to the complexity of transporter-mediated drug disposition and DDIs, a rational clinical DDI study design is critical to establish whether an NME can act as an *in vivo* inhibitor of drug transporter(s). Regulatory DDI guidance documents have provided some
recommendations on the design of clinical DDI studies, which in general, can be applied to transporter DDIs. Additional practical considerations for designing clinical DDI studies using the probe drugs or cocktails highlighted in previous section are discussed below.

Subjects

Transporter DDI studies are generally conducted in healthy subjects, rather than patients, unless there are safety concerns. Extrapolation of DDI results from healthy subjects to a specific and/or diseased population requires prior knowledge on alternation of transporter expression and/or activity in addition to the changes of relevant physiological parameters. Limited knowledge is currently available in this emerging area to allow direct translation of clinical DDIs to these specific populations. Carriers of certain genetic polymorphisms (e.g., SLCO1B1 c.521T>C and ABCG2 c.421C>A) will have a diminished response to transport inhibitors as previously described. In the context of this article, these variants are specifically relevant for pitavastatin for OATP1B1 (SLCO1B1) and rosuvastatin for BCRP (ABCG2). Thus, it is recommended to genotype subjects for SLCO1B1 and ABCG2 prior to enrollment or at least collect such information after the trial to help data analysis and interpretation, e.g., in case outliers are identified. Currently, genetic information may not be available in cocktail DDI studies that are conducted as a nested arm in a large clinical trial (e.g., secondary DDI endpoint in a phase I trial). However, if the DDI under investigation involves OATP1B1 and/or BCRP inhibition, the inclusion of the genetic analysis in study protocols would be helpful to interpret DDI results.

Dose and Dose Regimen

In general, the doses of probe and perpetrator drugs should be clinically relevant to appropriately characterize the DDIs. Based on this consideration, the maximum approved dose and shortest dosing interval should be used for transporter inhibitors. For probe drugs,
potential saturation of drug transporters at a given dose (e.g., intestinal P-gp) needs to be considered, as it may reduce the sensitivity of the measurements. Cocktail probes (at either microdose or therapeutic dose) need to be validated with both PK and clinical DDI studies to confirm the translation of the data to clinical DDIs and the lack of interaction between different probes. In some cases, the magnitude of DDIs at microdose level can be higher than those observed at therapeutic dose due to the saturation of transporters at therapeutic doses (especially relevant in the intestine). In such cases, microdose data could be considered a worst-case scenario for transporter DDIs and observed DDIs may not be directly translated to therapeutic dose for dosing adjustment. NMEs with complex inhibition/induction liabilities may require single-dose assessment of the inhibitory effects, and combined induction/inhibition effects following chronic administration (e.g., rifampin, an inhibitor of OATP1B and inducer of CYP3A/P-gp).

As with all DDI studies, the sequence of administration and the time interval between dosing of probe drug and perpetrator can impact the outcome. For example, when using the prodrug DE as an in vivo probe for intestinal P-gp, oral dosing of the P-gp inhibitor should precede DE administration to ensure maximum exposure considering rapid hydrolysis of DE to the non-P-gp substrate dabigatran.

In most cases, both perpetrator and probe drugs are orally administered which may reflect the clinical setting. However, use of both i.v. and oral dosing of probe drugs or perpetrators may provide additional mechanistic insight into the relative contributions of intestinal vs. hepatic transporters to the observed DDIs. For example, oral rosuvastatin is recommended to evaluate the DDIs resulting from the inhibition of both intestinal and hepatic BCRP, whereas i.v. rosuvastatin can be used to evaluate the contribution of hepatic BCRP. Similarly, i.v. and oral rifampin have been used as a perpetrator to understand the selectivity of pitavastatin (OATP1B) and rosuvastatin (BCRP/OATP1B) as DDI probe drugs for hepatic and intestinal transport activity, respectively.
Study Endpoints

Changes in systemic exposure of probe drugs (AUC and $C_{\text{max}}$ ratio with and without inhibitor) are generally used to describe the extent of transporter DDIs. Besides these parameters, $CL_r$ of probe drugs should also be measured over the PK sampling period when studying renal transporter DDIs. In such cases, urinary pH should also be measured to understand potential variability of reabsorption for probes with PKa values within urinary pH range. PD endpoints are generally not included in DDI studies. However, metformin may represent an example that including PD endpoints could help to interpret clinical DDI data and enable rational dose adjustment.\textsuperscript{42, 43}

Endogenous Biomarkers

The design of a clinical study to explore changes in the level of a validated endogenous biomarker after administration of an NME will benefit from a clear understanding of biomarker clearance(s). Characterization of such kinetic parameters may require monitoring of the disposition of an endogenous biomarker using radiolabeled or stable isotopes. The availability of quantitative data on disposition mechanisms (metabolic and renal clearance, \textit{in vitro} transporter kinetics) and rates of synthesis allows the subsequent development of biomarker PBPK models. Such models can be used to simulate the changes in exposure due to the inhibition of transporter(s) and aid in the design of clinical investigations aimed at maximizing the sensitivity of a given biomarker. In all cases, a crossover study design is preferred given the expected variability in the exposure, rate of elimination, and rate of synthesis in humans. The study design should take into consideration the appropriate time frame to collect the matched-samples based on PBPK simulations conducted in the absence or presence of the test inhibitor. The use of a standard cut-off value for the change in biomarker exposure may not be possible with all candidate transporters due to variability in the magnitude of interactions. Instead, a systematic increase in the exposure of the biomarker that corresponds to an increase in the dose of the test inhibitor is recommended as a positive indication of an interaction with
the transporter of interest. Ideally, bioanalysis of the biomarker concentration in the biological
matrices (e.g., urine, plasma, saliva) can be added onto typical first-in-human investigations
aimed at verifying the safety and tolerability of NME that have been identified as transport
inhibitors in vitro or in preclinical species. Such studies will provide a comprehensive dataset to
verify the dose-dependent change in the exposure of the biomarker for the transporter of
interest up to supra-therapeutic dose levels.

Altered transport activity due to genetic polymorphisms may complicate the ability to
detect exposure changes of a drug probe and can affect biomarker concentrations at baseline.
Therefore, understanding of this covariate on the systemic exposure of the biomarker and
associated variability is important. Moreover, the extent of change above baseline during the
course of treatment is likely to be lower in subjects with a reduced function allele than in wild
type subjects. Prior knowledge on the genetic makeup of subjects participating in the trial
would be beneficial for biomarkers of transporters with clinically relevant polymorphism, e.g.,
SLCO1B1, ABCG2, and SLC22A1 (OCT1), to explain inter-individual variability.

To address multiple sources of inter-individual variability in biomarker baseline
concentrations, a cross-over study design in a sufficiently large number of test subjects (based
on power calculations) is required to demonstrate statistical significance. Ideally, the
biomarker concentration would be monitored at the beginning of the study period and again
after 5-7 days to allow for subject acclimation under the clinical trial conditions. Moreover,
synchronization of study procedures ensures that potential diurnal fluctuations in the
biomarker level in individuals are not drastically affected in the subjects undergoing the study.
Subjects would subsequently receive the test inhibitor preferably following an 8-10 hours fast
until steady-state exposure is reached. Test inhibitor should be administered in a consistent
manner to all test subjects (e.g., oral administration followed by the consumption of 250 mL of
water) and subjects would consume other meals in a standardized fashion to rule out any
potential food effect on biomarker and inhibitor PK. In each period of the study, blood samples
(or other matrices) will be collected post-dose, the number of samples and time of collection
should closely match the PK of the test inhibitor to ensure capturing changes in the biomarker
level when the inhibitor is at $C_{\text{max}}$ and $C_{\text{trough}}$ levels. Additional treatment cycles where the dose of the inhibitor is escalated should be done in a consistent manner and additional sets of blood samples are collected at steady-state levels. Changes in the biomarker exposure that match the inhibitor dose would be assessed. Ideally changes in the biomarker exposure in individual subjects should correlate with the inhibitor dose as an indication that the examined biomarker is suitable to address the inhibition of the transport activity.

RECOMMENDATION AND PERSPECTIVES OF APPLYING PROBE DRUGS AND ENDOGENOUS BIOMARKERS TO STUDY TRANSPORTER INHIBITION

The utility of probe drugs and endogenous biomarkers to assess inhibition potential of NMEs on drug transporters are summarized in Figure 5. Monitoring plasma and/or urine exposure of endogenous biomarkers for various transporters could be incorporated in early human PK studies, such as phase I dose escalation studies. Most of the biomarkers reported thus far are not fully validated for their sensitivity, selectivity and specificity as endogenous probes of transporters of interest. Therefore, at present time (Figure 5A), in vivo biomarker data can only be used for designing DDI assessment strategy or for prioritizing the clinical DDI studies with probe substrate drugs (as individual or in a cocktail) or with the most relevant concomitant medications. The data need to be interpreted with the understanding that positive data may imply a potential DDI, but may not be specific for a particular transporter and may potentially be confounded by inhibition on the synthesis of the biomarker. Furthermore, lack of interaction between the NME and the biomarker does not ensure one did not occur and additional targeted evaluation of particular DDIs of concern using probe substrate drugs would be warranted.

Once selectivity and sensitivity of an endogenous biomarker have been validated (Figure 5B) in humans, it can be used for early assessment of transporter-based DDIs in single and multiple ascending dose phase I studies. We envisage that the use of a sensitive and selective
biomarker would potentially obviate the need to administer a probe substrate drug in a
dedicated DDI study, avoiding unnecessary drug exposure to participants and to reduce cost.
The *in vivo* biomarker data would also be useful to develop and optimize mechanistic PBPK
models for predicting unstudied scenarios for dosing recommendation or for informing
additional clinical studies. *In vivo* biomarker data together with clinical DDI results obtained
from individual and/or cocktail probe drugs and PBPK modeling can also provide a mechanistic
insight to predict complex DDIs involving inhibition of multiple transporters and enzymes. Such
an integrated approach will ensure understanding of the full dose response with respect to
transporter inhibition/induction liability of an NME as a perpetrator, while minimizing DDI
studies conducted in response to false positive predictions using *in vitro* data. It is worth noting
that continuing efforts to identify and validate new and existing biomarkers are critically
needed. Another targeted effort is to identify probe substrate drugs that are suitable for
cassette DDI assessment and exhibit high sensitivity to evaluate transporter inhibition, but with
minimal potential for mutual interactions between the components of the cocktail. Pre-
competitive efforts and dedicated consortia may be valuable in coordinating targeted efforts
and accelerating the incorporation of biomarkers and probe drug cocktails in drug
development.

In summary, multiple endogenous molecules whose exposure is modulated due to
interactions with drug transporters have been identified to support the clinical development of
safe and effective therapeutics. Ideally, suitable biomarkers would enable gauging the risk of
interactions with clinically relevant transporters. In reality, examples of promising candidate
biomarkers are currently only being identified and studied for interactions with several hepatic
(OATP1B) and renal transporters (OCT2/MATE1/2K, OAT1/3), but there is a considerable
increase in published information. There has been a clear absence of candidate biomarkers to
examine P-gp and BCRP inhibition, which is likely due to lack or limited involvement of gut P-gp,
and BCRP on *in vivo* disposition of endogenous substrates and the difficulty associated with
estimating the extent of individual contribution of gut, hepatic and renal P-gp and BCRP to the
systemic clearance of biomarkers. Continued measurement of candidate biomarkers in clinical
studies where transporter interactions are anticipated needs to become more widespread. This
is only possible if suitable language is included in early clinical study protocols to ensure adequate consent from the human subjects. This will ultimately enhance the confidence in the suitability of measuring changes in biomarker concentrations as an indicator of potential clinical transporter DDIs from pharmaceutical and regulatory perspectives.

DISCLAIMER

The contents of this article reflect the views of the authors and should not be construed to represent the FDA’s views or policies. No official support or endorsement by the FDA is intended or should be inferred. The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products by the FDA.

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FIGURE LEGENDS

Figure 1. Recommended probe drugs for clinical DDI studies to evaluate inhibitory effects of an NME on major drug transporters expressed in gut, liver and kidney and potential endogenous biomarkers for several hepatic and renal transporters.

The criteria for inclusion of probe drugs in this figure are discussed in the STATE-OF-THE ART IN CLINICAL PROBE DRUGS AND POTENTIAL ENDOGENOUS BIOMARKERS FOR CLINICAL TRANSPORTER DDI ASSESSMENT section. The endogenous substrates with clinical DDI evaluation of potent transporter inhibitor(s) have been included into this figure as potential endogenous biomarkers. Digoxin, dabigatran etexilate (DE), and fexofenadine are probe drugs for multidrug resistance protein MDR1 P-glycoprotein (P-gp, ABCB1), and sulfasalazine and rosuvastatin for breast cancer resistance protein (BCRP, ABCG2). Pitavastatin, atorvastatin, and rosuvastatin are probe drugs for the organic anion transporting polypeptides OATP1B1 (SLCO1B1) and OATP1B3 (SLCO1B3); Coproporphyrin I (CPI) and CPIII, glycochenodeoxycholate-3-O-sulfate (GCDCA-S), conjugated and unconjugated bilirubin (CB and UCB) are potential endogenous biomarkers for OATP1B1 and -1B3. Hexadecanedioate (HDA) and tetradecanedioate (TDA) are potential biomarkers for OATP1B1 (they are not substrates for OATP1B3). Sumatriptan is a probe drug for organic cation transporter 1 (OCT1; SLC22A1). Metformin is a probe drug for organic cation transporter 2 (OCT2, SLC22A2) and multidrug and toxin extrusion protein MATE1 (SLC47A1) and MATE2K (SLC47A2); N1-methylnicotinamide (NMN) and creatinine are potential endogenous biomarkers of OCT2/MATE1/2K. Adefovir is a probe drug for organic anion transporter 1 (OAT1, SLC22A6); taurine is a potential endogenous biomarker for OAT1. Benzylpenicillin is a probe drugs of organic anion transporter 3 (OAT3; SLC22A8); 6β-hydroxycortisol (6βHC) and GCDCA-S are potential endogenous biomarkers of OAT3.

a: Potential endogenous biomarkers with relatively higher selectivity and/or sensitivity for clinical evaluation based on current literature data.

b: Substrates of OATP1B1, but not -1B3.

Green colored circles: uptake transporters; Purple colored circles: efflux transporters.
Figure 2. Mechanistic challenges when developing transporter endogenous biomarker models: OATP1B1/1B3 biomarkers as an example.

Figure 3. Comparison of endogenous biomarkers and probe drugs with different t\(_{1/2}\) (A) and recommended method for interpreting systemic exposure changes in biomarkers with competitive inhibition of transporters (B-C).

A: Grey lines represent simulated inhibitor concentration and % of inhibition of the elimination pathway of a probe drug and biomarker. Red, green, and blue lines represent simulated concentration-time profiles of probe drugs and biomarkers in the absence (up to day 6) and presence (on and after day 7) of inhibitors, with colors represent different terminal t\(_{1/2}\). B-C: See Supplementary Materials for details. *C\(_{\text{max}}\) may be influenced by change in other parameters, such as distribution volume, and may not always represent change in clearance.

Figure 4: Workflow for the identification, characterization, and validation of transporter endogenous biomarkers.

a. Recombinant system may be a cellular or a vesicular system that expresses high levels of the target transporter. Transport in the overexpressing system is compared with one in a control system with non- or low transporter function.

b. One of the two data types should be a mammalian in vivo model with genetically reduced transport function.

c. Testing of multiple inhibitors or doses may be required to provide a range of inhibition potency. If a selective inhibitor is not available, multiple inhibitors with differential overlapping selectivity may be required.

d. Possible regulatory review process. For example, FDA guidance for drug development tools.\(^{100}\)
Figure 5. Examples of current (A) and future (B) considerations regarding the potential utility of endogenous biomarkers (pre-validated (A) and validated(B)) and probe drugs to assess transporter-related inhibition in drug development.

a. For transporters without identified biomarkers (e.g., gut transporters), consider to conduct clinical DDI with single or cocktail drug probe(s) at clinically relevant perpetrator dose.

b. If drugs can potentially inhibit multiple transporters, consider design a drug probe cocktail studies using *in vitro* and clinically validated probes. If no significant DDIs observed, dedicated clinical DDI studies with single drug probe at conventional doses may not be needed; If significant DDIs observed with selected transporters, dedicated clinical DDI studies with drug probes at clinically relevant exposure (both perpetrator and probes) are needed.
References:


35. van de Steeg, E., et al. Organic anion transporting polypeptide 1a/1b-knockout mice provide insights into hepatic handling of bilirubin, bile acids, and drugs. **J Clin Invest.** 120, 2942-2952 (2010).


• Supplementary Section 1
• Table S1.
• Table S2.
• Table S3.
• Supplementary Materials
• Figure S1.
• Figure S2.
Table 1. Ideal features of transported probe drugs or biomarkers for the evaluation of clinical DDIs.

- **Transport.** Active transport and transport kinetics of the clinical probe or biomarker are demonstrated using suitable *in vitro* assays. Active transport significantly contributes to the *in vitro* and *in vivo* clearance.

- **Selectivity and Specificity.** The contribution of the transporter of interest to the overall clearance of the candidate clinical probe or biomarker is clearly established kinetically. Transport represents the rate-limiting step to overall systemic clearance despite the presence of downstream disposition pathways (e.g., metabolism).

- **Sensitivity.** Active transport significantly contributes to the *in vitro* or *in vivo* clearance and can be demonstrated clinically from known genetic polymorphisms or DDI studies that affect the transport activity. The extent of change in exposure due to inhibition correlates with the potency of co-administered inhibitors and can be measured with high sensitivity and accuracy using robust validated bioanalytical methods.

- **In vivo PK.** Detailed characterization of the PK parameters including various disposition (and formation) pathways. The PK profile should provide sufficient flexibility to allow demonstration of changes in exposure preferably following common early clinical study protocols.

- **Additional desirable features**
  a. **Predictability with modeling.** The magnitude of change in PK profiles is predictable using PBPK models.
  b. **Regulatory acceptance.** The clinical probe and biomarker are accepted by regulatory agencies in support of *in vitro* data generation, IVIVE exercises, definitive clinical DDI assessment, and product labeling.
  c. **Administration to subjects.** Clinical drug probe can be administered using multiple routes and in combination with other agents as a cocktail. Changes in clinical drug PK corresponds to those of candidate biomarkers that utilize the same transport mechanism.
  d. Candidate biomarker concentrations are not significantly altered due to physiological or pathophysiological changes when compared to those when transport activity is impaired.
  e. Low likelihood of interference with rates of synthesis or catabolism of candidate biomarkers due to transporter inhibition.
Table 2: Examples of probe drugs for OATP1B, OCT1, P-gp, BCRP, OAT1, OAT3, OCT2, MATE1, and MATE2K for clinical DDI evaluation.

<table>
<thead>
<tr>
<th>Probe drugs (therapeutic class)</th>
<th>In vitro transport profile/kinetic parameters ($K_m$)</th>
<th>Key human ADME property and $C_{max}$ and $C_{maxu}$ at therapeutic dose</th>
<th>Clinical DDI with known inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibitor (dose regimen), $C_{max}$, $C_{maxu}$, $I_2$, and/or $I_{inmaxu}$</td>
</tr>
<tr>
<td>Pitavastatin (HMG-CoA reductase inhibitor)</td>
<td></td>
<td>F: 51%</td>
<td>Rivampin (iv or po 600mg SD)</td>
</tr>
<tr>
<td></td>
<td>OATP1B1: 0.81-5.53 µM</td>
<td>PPB: 99.5-99.6%</td>
<td>PO:</td>
</tr>
<tr>
<td></td>
<td>OATP1B3: 2.6-3.25µM</td>
<td>$V_d$: 148L</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OATP2B1: 1.17-25 µM</td>
<td>Metabolism: CYP2C9 (marginally), CYP2C8 (lesser), and UGT1A3 and UGT2B7 (formed major metabolites in human plasma)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OATP1A2: substrate</td>
<td>Elimination: renal (15%); feces (79%)</td>
<td></td>
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<tr>
<td></td>
<td>NTCP: 37.3-38.2 µM</td>
<td>$t_{1/2}$:12 hrs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-gp: 83.7 µM</td>
<td>$C_{max}$: 29.6 nM; $C_{maxu}$: 0.13 nM (2mg oral SD)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCRP: 1.2-5.73 µM</td>
<td>$t_{1/2}$:12 hrs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRP3: 448 µM</td>
<td>$t_{1/2}$:12 hrs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRP4: substrate</td>
<td>$t_{1/2}$:12 hrs</td>
<td></td>
</tr>
</tbody>
</table>

References
<table>
<thead>
<tr>
<th>Probe drugs (therapeutic class)</th>
<th>In vitro transport profile/kinetic parameters ($K_m$)</th>
<th>Key human ADME property and $C_{\text{max}}$ and $C_{\text{maxu}}$</th>
<th>Clinical DDI with known inhibitors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosuvastatin (HMG-CoA reductase inhibitor)</td>
<td>OATP1B1: 0.8-19.87 µM OATP1B3: 9.8-16.5 µM OATP2B1: 2.4-26.1 µM OATP1A2: 2.6-60.2 µM OAT3: 7.4 µM BCRP: 2.02-10.8 µM NTCP: 26.2-97 µM P-gp: substrate MRP4: substrate</td>
<td>F: 20% PPB: 88% $V_d$: 134L Metabolism: not extensively metabolized (~10% as metabolite). CYP2C9 (major) Elimination: renal (28%); feces (72%) $t_{1/2}$: 19 hrs $C_{\text{max}}$: 4.6 nM; $C_{\text{maxu}}$: 0.55nM (10 mg oral QD for 10 days)</td>
<td>Rifampin (iv or po 600mg SD) PO: $C_{\text{max}}$: 12.2-30.6 µM $C_{\text{maxu}}$: 3.04-7.65 µM $I_2$: 2916 µM $I_{\text{inmaxu}}$: 15.2-19.8 µM IV: $C_{\text{max}}$: 24.3 µM $C_{\text{maxu}}$: 6.08 µM</td>
<td>19, 22, 38, 103</td>
</tr>
<tr>
<td>Atorvastatin (HMG-CoA reductase inhibitor)</td>
<td>OATP1B1: 0.62-18.8 µM OATP1B3: 0.73 µM OATP2B1: 2.84 µM NTCP: 40.3-185 µM P-gp: 110 µM BCRP: substrate</td>
<td>F: 14% PPB: ≥ 98% $V_d$: 381L Metabolism: extensively metabolized. CYP3A4 (major) Elimination: primarily into bile (both parent and metabolites); renal (&lt;2%); $t_{1/2}$: 19.5 hrs $C_{\text{max}}$: 12.3 nM</td>
<td>Rifampin (iv or po 600mg SD) $C_{\text{max}}$: 9.35-22.6 µM $C_{\text{maxu}}$: 1.41-5.65 µM $I_2$: 2916 µM $I_{\text{inmaxu}}$: 8.70-17.8 µM IV: $C_{\text{max}}$: 17.1 µM $C_{\text{maxu}}$: 4.28 µM</td>
<td>19, 27, 104, 105</td>
</tr>
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</table>

References:

19, 22, 38, 103

19, 27, 104, 105
<table>
<thead>
<tr>
<th>Probe drugs (therapeutic class)</th>
<th>In vitro transport profile/kinetic parameters ($K_m^a$)</th>
<th>Key human ADME property and $C_{\text{max}}$ and $C_{\text{maxu}}$</th>
<th>Clinical DDI with known inhibitors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT1</td>
<td></td>
<td>$C_{\text{maxu}}$: 0.25 nM (20 mg oral QD for 14 days)</td>
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</tbody>
</table>
| Sumatriptan ((5-hydroxytryptamine receptor agonist for migraine treatment)) | OCT1: 55.4 µM  
OATP1A2: 94.5 µM | F: 15%  
PPB: 14-21%  
$V_d$: 2.4 L/kg  
Metabolism: extensively metabolized by monoamine oxidase type A (MAOA)  
Elimination: primarily in urine 60% as the major metabolite IAA (3% unchanged); fecal (~40%); $t_{1/2}$: 2.5 hrs | No clinical DDI studies are available with OCT1 inhibitors  
PGx study compared OCT1*1-*2 (active) to OCT1*3-*6 (deficient) | AUCR: 2.2 (higher in subjects with two deficient alleles relative to one or more active alleles)  
$C_{\text{maxR}}$: 1.8 (higher in subjects with two deficient alleles relative to one or more active alleles) | NA | 45, 106-108 |
<table>
<thead>
<tr>
<th>Probe drugs (therapeutic class)</th>
<th>In vitro transport profile/kinetic parameters ( (K_m)^a )</th>
<th>Key human ADME property and ( C_{\text{max}} ) and ( C_{\text{max u}} )</th>
<th>Clinical DDI with known inhibitors</th>
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</thead>
<tbody>
<tr>
<td><strong>P-gp</strong></td>
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<tr>
<td>Digoxin (Cardiac/digoxin glycosides)</td>
<td>P-gp: 73-220 µM</td>
<td>OATP4C1: 7.8 µM</td>
<td>F: 60-80%</td>
<td>P-gp: 0.05-9.5 µM</td>
</tr>
<tr>
<td></td>
<td>OATP4C1: 7.8 µM</td>
<td></td>
<td>P-gp: 0.05-9.5 µM</td>
<td>BCPR: 1.9-10 µM</td>
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<tr>
<td></td>
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<td></td>
<td>Itraconazole (oral 200 mg QD x 5 days)</td>
<td>AUCR: 1.68</td>
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<tr>
<td></td>
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<td></td>
<td>Verapamil (oral 80 mg TID x 2-4 weeks or 120 mg TID x 2 weeks)</td>
<td>AUCR: 1.5</td>
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<tr>
<td>Fexofenadine (Histamine H1-receptor antagonist)</td>
<td>P-gp: 25.9-150 µM</td>
<td>OATP1B1: 61.6 µM</td>
<td>F: NR</td>
<td>P-gp: 0.1-234 µM</td>
</tr>
<tr>
<td></td>
<td>OATP1B3: 108 µM</td>
<td>OATP2B1: 0.14</td>
<td>PPB: 60-70%</td>
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</table>

\(^a\) K_m values are provided for reference, actual values may vary.

References:
[109, 110, g]
<table>
<thead>
<tr>
<th>Probe drugs (therapeutic class)</th>
<th>$In vitro$ transport profile/kinetic parameters ($K_m$)</th>
<th>Key human ADME property and $C_{max}$ and $C_{maxu}$</th>
<th>Clinical DDI with known inhibitors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$M</td>
<td>renal (11%); feces (80%); $t_{1/2}$: 14 hrs; $C_{max}$: 531 nM; $C_{maxu}$: 159-212 nM (oral 60mg BID)</td>
<td>Verapamil (oral 80 mg TID x 6 days, 240 mg QD x 1day)</td>
<td>See above</td>
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<tr>
<td></td>
<td>OAT3: 70.2 $\mu$M</td>
<td>• $I_2$: 283-1134 $\mu$M</td>
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<tr>
<td></td>
<td>BSEP: substrate</td>
<td>• $I_{inmax,u}$: 9.5-38.8 nM</td>
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<tr>
<td></td>
<td>MATE1: substrate</td>
<td>• $C_{max}$: S-verapamil: 46.2 nM; R-verapamil: 154 nM (240mg QD)</td>
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<tr>
<td></td>
<td>MRP3: substrate</td>
<td>• $C_{max}$: S-verapamil: 4.62 nM; R-verapamil 15.4 nM (240mg QD)</td>
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<tr>
<td></td>
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<td>• $I_2$: 703.9-2112 $\mu$M</td>
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<tr>
<td></td>
<td></td>
<td>• $I_{inmax,u}$: 3.52 $\mu$M</td>
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<tr>
<td>Dabigatran etexilate (DE)</td>
<td>P-gp: 1 $\mu$M (predicted)</td>
<td>Dabigatran: • $F$: 3-7%; PPB: 35%; $V_d$: 50-70 L; Metabolism: carboxylesterase catalyzed hydrolysis to dabigatran, and, Itraconazole (oral 200 mg QD x 5 days)</td>
<td>Verapamil (oral 80 mg TID x 6 days, 240 mg QD x 1day)</td>
<td>See above</td>
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<tr>
<td>(Anticoagulant)</td>
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<td>• $C_{max}$: 1.9 $\mu$M</td>
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<td></td>
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<td>• $C_{maxu}$: 0.068 $\mu$M</td>
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<tr>
<td></td>
<td></td>
<td>• $I_2$: 1133.7 $\mu$M</td>
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<tr>
<td></td>
<td></td>
<td>• $I_{inmax,u}$: 0.75 $\mu$M</td>
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<td></td>
<td></td>
<td>• AUCR: 6.92</td>
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<tr>
<td></td>
<td></td>
<td>• $C_{max,R}^{d}$: 6.42</td>
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<tr>
<td></td>
<td></td>
<td>See above</td>
<td>19, 8</td>
<td></td>
</tr>
<tr>
<td>Probe drugs (therapeutic class)</td>
<td>In vitro transport profile/kinetic parameters ( (K_m)^a )</td>
<td>Key human ADME property and ( C_{\text{max}} ) and ( C_{\text{max, u}} ) then form acyl glucuronides by conjugation</td>
<td>Clinical DDI with known inhibitors</td>
<td>References</td>
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<tr>
<td></td>
<td>Elimination: ( \text{after IV dose): renal (85%) ; (after PO dose): urine (7%) and feces (86%) )</td>
<td>• ( t_{1/2} ): 12-17 hrs ( C_{\text{max}} ): 174 nM; ( C_{\text{max, u}} ): 113 nM ( (100\text{mg DE oral SD}) ) ( C_{\text{max}} ): 341 nM; ( C_{\text{max, u}} ): 222 nM ( (200\text{mg DE oral SD}) )</td>
<td>• Verapamil (oral 120 mg IR SD or oral 240 mg ER SD) ( C_{\text{max}} ): 205.5 -275 nM ( C_{\text{max, u}} ): 20.5 -27.5 nM ( I_2 ): 1056 -2112 ( \mu \text{M} )</td>
<td>See above</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• ( C_{\text{max}} ): 341 nM; ( C_{\text{max, u}} ): 222 nM ( (200\text{mg DE oral SD}) )</td>
<td>• AUCR: 1.71-2.43 ( C_{\text{max, R}} ): 1.91-2.79</td>
<td>99</td>
</tr>
</tbody>
</table>

BCRP

<p>| Rosuvastatin ( (\text{HMG Co-A reductase inhibitor}) ) | See above | See above | Fostamatinib ( ^a ) (oral 100 mg BID, x 9 days) ( C_{\text{max}} ) (active moiety R 406) : 1.6 ( \mu \text{M} ) ( C_{\text{max, u}} ) (active moiety R 406): 0.029 ( \mu \text{M} ) ( I_2 ) (fostamatinib): 691 ( \mu \text{M} ) ( I_{\text{max, u}} ) (R406): 991 ( \mu \text{M} ) | • AUCR: 1.96 ( C_{\text{max, R}} ): 1.88 | Fostamatinib: ( BCRP: 0.05 \mu \text{M} ) ( P\text{-gp}: 3.2 \mu \text{M} ) active moiety R406: ( BCRP: 0.031 \mu \text{M} ) ( OAT1B1: &gt;10 \mu \text{M} ) ( OAT3: \text{NI} ) | # |</p>
<table>
<thead>
<tr>
<th>Probe drugs (therapeutic class)</th>
<th>In vitro transport profile/kinetic parameters (K&lt;sub&gt;m&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Key human ADME property and C&lt;sub&gt;max&lt;/sub&gt; and C&lt;sub&gt;maxu&lt;/sub&gt;</th>
<th>Clinical DDI with known inhibitors</th>
<th>References</th>
</tr>
</thead>
</table>
| Rifampin (oral 600mg SD IV and PO) | 0.289µM | PO Rifampin:  
  - AUCR: 4.37  
  - C<sub>max</sub>R: 9.93  
IV Rifampin:  
  - AUCR: 3.30  
  - CmaxR:5.51 | See above | 22 |
<p>| | | | | |
| | | | | |</p>
<table>
<thead>
<tr>
<th>Probe drugs (therapeutic class)</th>
<th><em>In vitro</em> transport profile/kinetic parameters ($K_m^a$)</th>
<th>Key human ADME property and $C_{\text{max}}$ and $C_{\text{maxu}}$</th>
<th>Clinical DDI with known inhibitors</th>
<th>References</th>
</tr>
</thead>
</table>
| Sulfasalazine (Salicylate- sulfonamide) | • BCRP: 0.7 µM  
• OATP2B1: 1.73 µM  
• MRP2: substrate  
• P-gp: substrate | • F: 3-12%  
• PPB: >99.3%  
• $V_d$: 0.11 L/kg  
• Metabolism: metabolized by intestinal bacteria to 5-amino salicylic acid (5-ASA) and sulfapyridine (SP) in the intestine, and in liver to these moieties by some extent.  
• Elimination: Absorbed SP and 5-ASA and their metabolites are primarily eliminated into urine; CL$_r$: 37% of CL.  
• $t_{1/2}$: 7.6 hrs  
• $C_{\text{max}}$: 37.6-79 µM; $C_{\text{maxu}}$: 0.26-0.55 µM (3-4 g oral SD) | Curcumin (oral 2 g, SD)  
$C_{\text{avg}}$: < 0.5 ng/mL (lower limit of quantification)  
Sulfasalazine at therapeutic dose (2 g)  
$AUC_R$: 3.23  
$C_{\text{max}}$: 3.72  
Sulfasalazine at microdose (100 µg)  
$AUC_R$: 1.83  
$C_{\text{max}}$: 1.96 | • BCRP: 0.62-32 µM  
• MRP1: 16.1 µM  
• OATP1B1: 33.7 µM  
• OATP1B3: 3.81 µM  
• P-gp: 5.8-50.5 µM | 80 |
<table>
<thead>
<tr>
<th>Probe drugs (therapeutic class)</th>
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<th>Key human ADME property and $C_{max}$ and $C_{max,u}$</th>
<th>Clinical DDI with known inhibitors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adefovir f (Antivirals)</td>
<td>• OAT1: 11.4-30 µM</td>
<td>F: 59 %</td>
<td>• AUCR: 2.09</td>
<td>56, g</td>
</tr>
<tr>
<td></td>
<td>• MRP4: substrate</td>
<td>PPB: ≤ 4 %</td>
<td>• GFR ratio: 0.97</td>
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<td></td>
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<td>$V_{du}$: 352-392 ml/kg</td>
<td>• CL$_R$: 0.44</td>
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<td>Metabolism: adefovir dipivoxil is rapidly converted to adefovir</td>
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<td>Elimination: renal (45% in 24 hrs as adefovir); active secretion and glomerular filtration</td>
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<td></td>
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<td>$t_{1/2}$: 7.48 hrs</td>
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<td></td>
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<td>$C_{max}$: 0.067 µM; $C_{max,u}$: 0.065 µM (10 mg adefovir dipivoxil QD)</td>
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<tr>
<td></td>
<td>Probenecid (oral, 750mg, SD)</td>
<td>$C_{max}$: 189 µM</td>
<td>• OAT1: 3.6-27.4 µM</td>
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<tr>
<td></td>
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<td>$C_{max,u}$: 18.9 µM</td>
<td>• OAT3: 0.76-29.8 µM</td>
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<td></td>
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<td>• MRP2: 35.7-2300 µM</td>
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<td></td>
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<td>• MRP4: 8-77% inhibition at 100 µM</td>
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<td>• OAT2: 393-853 µM</td>
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<td>• OAT4: 15.5-134 µM</td>
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<td></td>
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<td></td>
<td>• OATP1B1: 39.8-740 µM</td>
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<td>• OATP1B3: 130 µM</td>
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<td></td>
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<td>• URAT1: 13.2-50 µM</td>
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<tr>
<td>Probe drugs (therapeutic class)</td>
<td>In vitro transport profile/kinetic parameters ((K_m)^a)</td>
<td>Key human ADME property and (C_{\text{max}}) and (C_{\text{maxu}})</td>
<td>Clinical DDI with known inhibitors</td>
<td>References</td>
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</tr>
</tbody>
</table>
| **Furosemide** (Diuretics) | • OAT1: 38.9 µM  
• OAT3: 21.5 µM  
• MRP4: substrate  
• MRP2: substrate  
• BCRP: substrate  
• OATP1B1: substrate  
• OATP1B3: substrate | • F: 71%  
• PPB: 98.6%  
• \(V_d\): 0.13 L/kg  
• Metabolism: gluconidation, 12% of oral dose  
• Elimination: renal (65%)  
• \(t_{1/2}\): 1.3 hrs  
• \(C_{\text{max}}\): 5.14 µM; \(C_{\text{maxu}}\): 0.07 µM (40mg oral SD) | Probencid (oral, 1g, SD)  
• \(C_{\text{max}}\): 243.9 µM  
• \(C_{\text{maxu}}\): 24.4 µM | 17, g |
| **Benzylpenicillin** (Penicillin G) (antibiotics) | • OAT3: 13.9-66 µM  
• OAT1: 884 µM, or not a substrate  
• OATP1B1: substrate  
• OATP1B3: substrate | • F%: 15-30% (fasting)  
• PPB: 45-68%  
• \(V_d\): 0.53-0.67 L/kg  
• Metabolism: 16-30% intramuscular dose is metabolized to penicilloic acid  
• Elimination: renal  
• \(t_{1/2}\): 0.4-0.6 hr  
• \(C_{\text{max}}\) and \(C_{\text{maxu}}\), NR | Probencid (oral, 1500mg, SD)  
• \(C_{\text{max}}\): 475 µM  
• \(C_{\text{maxu}}\): 47.5 µM | See Above 56, g |
<table>
<thead>
<tr>
<th>Probe drugs (therapeutic class)</th>
<th>In vitro transport profile/kinetic parameters ($K_m^a$)</th>
<th>Key human ADME property and $C_{\text{max}}$ and $C_{\maxu}$</th>
<th>Clinical DDI with known inhibitors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT2/MATE1/2K</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
| Metformin (biguanide, glucose lowering agent) | • OCT2: 235-3356 µM  
• OCT1: 83.6-6360 µM  
• MATE1: 94.7-4565 µM  
• MATE2K: 473-2986 µM  
• OCT3: 1020-3810 µM  
• PMAT: 1320 µM | • F: 52%  
• PPB: negligible  
• $V_d$: 1.12L/kg  
• Metabolism: negligible  
• Elimination: renal (~90% of the absorbed drug within the first 24 hrs)  
• $t_{1/2}$: 1.74 hrs  
• $C_{\text{max}}$: 9.7 µM;  
$C_{\maxu}$: 9.7 µM (0.5g oral SD) | • Cimetidine (oral, 400mg, bid)  
• $C_{\text{max}}$: 9.35 µM  
• $C_{\maxu}$: 7.58 µM  
• AUCR: 1.46-1.54  
• CL$_R$: 0.55-0.72  
• OCT2: 2.51-1650 µM  
• MATE1: 0.093-16.3 µM  
• MATE2K: 2.1-46.6 µM  
• OCT1: 1.4-1010 µM  
• OCT3: 9.8-111 µM  
• OAT3: 0.07-215 µM  
• OAT2: 22-102.3 µM | 65, 66 |
|                                 |                                                 |                                                 |                                |            |
| Pyrimethamine (oral, 50mg, SD) | • $C_{\text{max}}$: 2.29 µM  
• $C_{\maxu}$: 0.30 µM | • AUCR: 1.35-2.68  
• CL$_R$: 0.28-0.65 |                                |            |
| Dolutegravir (oral, 50mg QD and BID) | • $C_{\text{max}}$: 9.08-16.2 µM  
• $C_{\maxu}$: 0.1-0.18 µM | • AUCR: 1.79-2.45 |                                | 65, 66 |
|                                 |                                                 |                                                 |                                |            |
| Pyrimethamine (oral, 50mg, SD) | • $C_{\text{max}}$: 2.29 µM  
• $C_{\maxu}$: 0.30 µM | • AUCR: 1.35-2.68  
• CL$_R$: 0.28-0.65 |                                |            |
| Dolutegravir (oral, 50mg QD and BID) | • $C_{\text{max}}$: 9.08-16.2 µM  
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|                                 |                                                 |                                                 |                                |            |
| Pyrimethamine (oral, 50mg, SD) | • $C_{\text{max}}$: 2.29 µM  
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|                                 |                                                 |                                                 |                                |            |
ADME, Absorption, distribution, metabolism, and excretion; AUC, area under the concentration-time curve; AUCR, ratio of AUC; BCRP, breast cancer resistance protein; CYP, cytochrome P450; $C_{max}$, maximum concentration in plasma; $C_{max,R}$, ratio of $C_{max}$; $C_{avg}$, average plasma concentration; $CL_r$, renal clearance; $CL_{R,r}$, ratio of $CL_r$; $CL_{m,R}$, ratio of non-renal clearance; $CL/F$, apparent clearance after oral dose; DE, dabigatran etexilate; ER, extended released; F, bioavailability; IC$_{50}$, half maximal inhibitory concentration; IR, immediate released; $K_m$, Michaelis constant; $K_i$, inhibition rate constant; MDR, multidrug resistance; MRP, multidrug resistance protein; MATE, multidrug and toxin extrusion protein; NTCP, sodium taurocholate co-transporting polypeptide; NA, not applicable; NI, not inhibited; NR, not reported; NS, not significant; OATP, organic anion transporting polypeptide; OAT, organic anion transporter; OCT, organic cation transporter; P-gp, P-glycoprotein; PK, pharmacokinetics; PPB, plasma protein binding; $t_{1/2}$, half-life; THTR, thiamine transporter; $V_d$, volume distribution; $V_{dss}$, volume distribution at the steady state.

$I_2$ is gut concentration and calculated as dose/250 mL and $I_{inmax,u}$ is unbound maximal inlet concentration and is calculated as $f_{u,p}*(I_{max} + (FaFg*ka*Dose))/Qh/RB$ where $f_{u,p}$ is unbound fraction in plasma, Fa is the fraction absorbed, Fg is the intestinal availability, ka is the absorption rate constant., Qh is the hepatic blood flow rate and RB is the blood-to-plasma concentration ratio.

a: Data are obtained from the University of Washington DDI database (https://www.druginteractioninfo.org).
b: PK parameters are obtained from the University of Washington DDI database (https://www.druginteractioninfo.org) unless specified in the references; other data of ADME properties are obtained from drug labels
c: Plasma exposure of dabigatran was measured
d: DE was administered in microdose (375 µg)
e: Fostamatinib is a prodrug (R788) that is rapidly and completely metabolized by dephosphorylation in the enterocytes of the intestine to the active systemic metabolite R406.
f: Administered with adefovir dipivoxil, a diester prodrug of adefovir.
g: Refer to the University of Washington Drug Interaction and Transport Database (www.druginteractioninfo.org).
Table 3: Examples of potential clinical endogenous biomarkers of OATP1B, OAT1, OAT3, OCT2/MATE1, 2K for DDI evaluation.

<table>
<thead>
<tr>
<th>Endogenous probe</th>
<th>In vitro transport kinetics/selectivity ($K_m$)</th>
<th>Formation and elimination mechanisms</th>
<th>Change in patients with genetically altered transporter function</th>
<th>Clinical DDI with known inhibitors</th>
<th>Reference</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td>Inhibitor (Dose and administration route)</td>
<td>PK Change (inhibitor/control)</td>
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<tr>
<td>OATP1B1/OATP1B3</td>
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<tr>
<td>Coproporphyrin I (CPI)</td>
<td>OATP1B1: 0.13 (CPI) and 0.22 µM (CPIII)</td>
<td>Formation: metabolically stable byproducts of heme biosynthesis</td>
<td>Rotor syndrome (deficient in OATP1B): decreased biliary excretion and increased urinary excretion</td>
<td>Rifampin (oral 300 and 600 mg SD)</td>
<td>Rifampin 300mg:</td>
<td>38, 40, 41, 8</td>
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<td></td>
<td>OATP1B3: 3.95 (CPI) and 1.55 µM (CPIII)</td>
<td>Elimination: biliary and renal excretions</td>
<td>Dubin-Johnson syndrome (deficient in MRP2): increased urinary excretion</td>
<td></td>
<td>· CPI AUCR: 3.0</td>
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<td></td>
<td>MRP2: 7.7 µM (CPI)</td>
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<td>· CPI 600 mg:</td>
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<td>MRP3: substrate (CPI/III)</td>
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<td>· CPI AUCR: 4.0-4.6, 6.4</td>
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<td>· CPI C_{max}R: 5.4-6.4</td>
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<td>· CPIII AUCR: 3.3-3.4</td>
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<td>· CPIII C_{max}R: 3.3</td>
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<tr>
<td>Glycochenodeoxycholate-3-O-sulfate (GCDCA-S)</td>
<td>OATP1B1: 9.95 µM</td>
<td>Formation: bile acid sulfation likely by SULT2A1</td>
<td></td>
<td>Rifampin (oral 300 and 600 mg SD)</td>
<td>Rifampin 300mg:</td>
<td>34, 41</td>
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<tr>
<td></td>
<td>OATP1B3: 5.23</td>
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<td></td>
<td>· AUCR: 4.3</td>
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<td>· Rifampin 600mg:</td>
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<tr>
<td>Endogenous probe</td>
<td>In vitro transport kinetics/selectivity</td>
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<td>Change in patients with genetically altered</td>
<td>Clinical DDI with known inhibitors</td>
<td>Reference</td>
<td>Comments</td>
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<tr>
<td>µM</td>
<td>OAT3: 64.3 µM NTCP: substrate</td>
<td>Elimination: biliary excretion, renal excretions, and enterohepatic recirculation</td>
<td></td>
<td>AUCR: 10-20.3</td>
<td></td>
<td>suggested that GCDC-S was significantly associated with genetic variants in OATP1B1</td>
</tr>
<tr>
<td>Hexadecanedioate (HDA) and tetradecanoate (TDA)</td>
<td>OATP1B1: substrate (uptake ratios a 2.2 and 3.4) OAT1: substrates (uptake ratios a 2.0 and 4.1) OAT3: substrates (uptake ratios a 5.0 and 8.0)</td>
<td>Formation: CYP4A11 is involved in HDA and TDA synthesis and/or metabolism</td>
<td>HDA and TDA are significantly associated with genetic variants in OATP1B1</td>
<td>Rifampin(oral 600 mg SD) AUCR: 2.2 and 3.2</td>
<td>33, 40</td>
<td></td>
</tr>
<tr>
<td>Unconjugated and conjugated bilirubin (UCB and CB)</td>
<td>OATP1B1: 0.008-0.16 µM (UCB) and 0.1-0.28 µM OATP1B3: 0.039 and 0.50 µM</td>
<td>Formation: bilirubin glucuronidation by UGT1A1 Elimination: biliary excretion and enterohepatic recirculation</td>
<td>Rotor syndrome (deficient in OATP1B): decreased biliary excretion SLCO1B1*15/*15 genotype (decreased OATP1B1 activity): increased plasma UCB and CB levels</td>
<td>Rifampin (oral 300 and 600 mg SD) Rifampin 300mg AUCR: 1.6 (total bilirubin), 2.3 (CB) Rifampin 600 mg AUCR: 1.94 (UCB); 0.85-3.5 (CB); 1.32-2.09 (total bilirubin)</td>
<td>19, 29, 40, 41, b</td>
<td></td>
</tr>
<tr>
<td>OAT1/OAT3</td>
<td>OAT1: 379 µM</td>
<td>Formation: derived from food or produced from cysteine by cysteine dioxygenase and cysteine sulfonate Elimination: renal</td>
<td>Probenecid (oral 500, 750, and 1500 mg SD)</td>
<td>AUCR: 0.97, 0.98, and 1.02, respectively</td>
<td>39</td>
<td>Selective substrate to OAT1 over OAT3</td>
</tr>
</tbody>
</table>

59

61
<table>
<thead>
<tr>
<th>Endogenous probe</th>
<th>In vitro transport kinetics/selectivity</th>
<th>Formation and elimination mechanisms</th>
<th>Change in patients with genetically altered</th>
<th>Clinical DDI with known inhibitors</th>
<th>Reference</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>6β-hydroxyl cortisol (6βHC)</td>
<td>OAT3: substrate</td>
<td>• Formation: produced from cortisol (an endogenous substrate of OAT3) by hepatic CYP3A4 • Elimination: renal excretion</td>
<td>Probenecid (oral 750 mg SD)</td>
<td>• AUCR: 1.57-1.76 • CL\textsubscript{R}: 0.58-0.63</td>
<td>\textsuperscript{b}</td>
<td>• Amount (endogenous production of 6βHC) is also influenced by CYP3A activity</td>
</tr>
<tr>
<td>Glycochenodeoxycholate-3-O-sulfate (GCDCA-S)</td>
<td>OATP1B1: 9.95 µM OATP1B3: 5.23 µM OAT3: 64.3 µM NTCP: substrate</td>
<td>• Formation: bile acid sulfation likely by SULT2A1 • Elimination: biliary excretion, renal excretions, and enterohepatic recirculation</td>
<td>Probenecid (oral 500, 750, and 1500 mg SD)</td>
<td>• AUCR: 1.06, 1.00, and 1.37, respectively • CL\textsubscript{R}: 0.38, 0.21 and 0.11, respectively</td>
<td>\textsuperscript{59}</td>
<td></td>
</tr>
<tr>
<td>OCT2/MATEs</td>
<td>OCT2: 1.86-18.8 mM MATE1: 10.2 mM MATE2K: 21.6 mM OAT2: 795-986 µM</td>
<td>• Formation: produced mainly by muscle metabolism • Elimination: primarily filtered through the kidney through the glomeruli; 10–40% is</td>
<td>Cimetidine (oral 400 mg QD)</td>
<td>• AUCR: 1.13-1.26 • CL\textsubscript{R}: 0.80</td>
<td>\textsuperscript{60, b}</td>
<td></td>
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<tr>
<td>Endogenous probe</td>
<td>In vitro transport kinetics/selectivity</td>
<td>Formation and elimination mechanisms</td>
<td>Change in patients with genetically altered</td>
<td>Clinical DDI with known inhibitors</td>
<td>Reference</td>
<td>Comments</td>
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<td></td>
<td>actively secreted</td>
<td></td>
<td>Pyrimethamine (oral 50-100 mg SD)</td>
<td>• AUCR: 1.18-1.26</td>
<td>66, 67</td>
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<td></td>
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<td></td>
<td>• CL\textsubscript{R}: 0.73-0.75</td>
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<tr>
<td>Pyrimethamine</td>
<td></td>
<td></td>
<td>Dolutegravir (oral 50 mg QD or BID for 4 days)</td>
<td>• AUCR: 1.09-1.17</td>
<td>b</td>
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<td></td>
<td></td>
<td>• CL\textsubscript{R}: 0.86-0.90</td>
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<tr>
<td>N1-methylnicotinamide (NMN)</td>
<td>OCT2: 318 µM MATE1: 301 µM MATE2K: 422 µM</td>
<td>• Formation: a nicotinamide metabolite produced by N-methyltransferase Elimination: renal excretions and metabolism</td>
<td>Pyrimethamine (oral 50 mg SD)</td>
<td>• AUCR: 0.84</td>
<td>69</td>
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<td>• CL\textsubscript{R}: 0.30</td>
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<tr>
<td>Trimethoprim</td>
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<td>Trimethoprim (oral 200 mg BID for 5 days)</td>
<td>• AUCR: 1.00</td>
<td>70</td>
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<td></td>
<td>• CL\textsubscript{R}: 0.73</td>
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</tbody>
</table>

AUCR, ratio of AUC; CL\textsubscript{R}, ratio of CL\textsubscript{R}; BID, twice a day; C\textsubscript{max\textsubscript{R}}, ratio of C\textsubscript{max}; K\textsubscript{m}, Michaelis constant; QD, once a day; SD, single dose.

\( ^{a}\): Uptake ratio is calculated by dividing uptake activity measured in the transporter cells by that in control cells.

\( ^{b}\): Refer to the University of Washington Drug Interaction and Transport Database (www.druginteractioninfo.org).
A. Comparison of endogenous biomarkers and probe drugs with different $t_{1/2}$

Elimination  Fast  Medium  Slow

% of Control

78% inhibition (on average)

Concentration

B. Short $t_{1/2}$ of both biomarker and inhibitor

<table>
<thead>
<tr>
<th></th>
<th>With diurnal change</th>
<th>No diurnal change</th>
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</thead>
<tbody>
<tr>
<td>Single- or multiple-dose</td>
<td>Model-based analysis</td>
<td>Model-based analysis (or $C_{max}^*$)</td>
</tr>
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</table>

C. Long $t_{1/2}$ of either biomarker or inhibitor

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<tr>
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<th>With diurnal change</th>
<th>No diurnal change</th>
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</thead>
<tbody>
<tr>
<td>Multiple-dose</td>
<td>AUC or model-based analysis</td>
<td>AUC, $C_{max}^*$, or model-based analysis</td>
</tr>
<tr>
<td>Single-dose</td>
<td>Model-based analysis; may not capture full inhibition</td>
<td></td>
</tr>
</tbody>
</table>
**Data Source**

- NHP DDI
- Clinical GWAS/PGX
- Clinical disease model
- Clinical DDI
- Recombinant in vitro systems
- Rodent KO model

---

**Quantitation**

Metabolomics or targeted assay

---

**Identification**

Identify candidate biomarkers with suitable sensitivity and/or specificity:
Change from baseline is robust with statistical significance and reproducible in two or more data types

---

**Characterization**

- Clinical Selectivity towards a specific transporter:
  Compare the effects of model transport inhibitors and genetic polymorphism on the PK and elimination of candidate biomarkers

- In vitro specificity of candidate biomarkers for a transport process:
  Characterize the transport, formation, and elimination kinetics of the candidate biomarker and evaluate the effect of inhibitors on these processes

- Clinical sensitivity:
  Evaluate the magnitude of change from baseline in biological matrices at multiple time points in a sufficiently powered sample size to address physiological variability

- Mechanistic clinical deconstruction:
  Develop mechanistic PK models to optimize clinical utility. If needed, measure clinically candidate biomarker formation or elimination by administering labeled compound or parallel measurement of precursors

---

**Validation**

Optimize analytical procedures and methods

Design a dedicated prospective randomized crossover clinical DDI study with a known probe drug substrate, an inhibitor (multiple), and quantitation of the selected candidate biomarker(s)

Use suitable dosing regimen and collect appropriate matrices over a suitable time frame pre- and post-dosing

Compare magnitude of PK changes relative to probe drug substrate(s) + Determine the optimal time frame and corresponding sensitivity based on PK changes of probe drug(s) + Compare PK changes across a panel of inhibitors to determine the selectivity of the candidate biomarker and the dynamic range

PK changes demonstrate a robust trend with increasing inhibitor concentrations that mirrors those observed with probe drug(s)

---

**Application**

Incorporate in clinical programs in “first-in-human” or in DDI investigations in combination with other probe drugs. Qualify the biomarker for the regulatory intended use
A. Pre-validated endogenous biomarkers and probe drugs

- *In vitro* transporter inhibition studies with relevant probe substrates
- Apply regulatory decision criteria to assess the risk of DDIs
  - Above the cutoff values of regulatory decision criteria
    - Measure plasma and/or urine exposure of pre-validated endogenous biomarkers for relevant transporter(s) in phase I dose escalation studies
      - Significant and dose-dependent change of endogenous biomarkers
        - Prioritize clinical plan/DDI strategy (patient enrollment, co-medication, timing of clinical DDI studies) and conduct clinical DDI studies
      - No significant change of endogenous biomarkers at all dose levels
        - Conduct clinical DDI studies with drug probes and/or co-medication at clinically relevant dose in late stage trials (e.g., phase III or postmarketing commitments)
  - Below the cutoff values of regulatory decision criteria
    - Risk for DDIs is minimal

B. Validated endogenous biomarkers and probe drugs

- *In vitro* transporter inhibition studies with relevant probe substrates
- Apply regulatory decision criteria to assess the risk of DDIs
  - Above the cutoff values of regulatory decision criteria
    - Measure plasma and/or urine exposure of validated single or multiple endogenous biomarkers for relevant transporter(s) in phase I dose escalation studies
      - Significant and dose-dependent change of endogenous biomarkers
        - Develop mechanistic PBPK models for endogenous biomarkers and quantitatively translate biomarker data to drug probes to predict the magnitude of DDIs
      - No significant change of endogenous biomarkers at all dose levels
        - Risk for DDIs is minimal
  - Below the cutoff values of regulatory decision criteria
    - Risk for DDIs is minimal

Inform drug label and/or dose adjustment