Title: Progress in the quantitative assessment of transporter-mediated drug–drug interactions using endogenous substrates in clinical studies

Authors: Tatsuki Mochizuki, Ph.D.¹ and Hiroyuki Kusuhara, Ph.D.²

Affiliation:
¹Pharmaceutical Science Department, Translational Research Division, Chugai Pharmaceutical Co., Ltd.
²Laboratory of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, the University of Tokyo
2. **Running Title Page.** The running title page should contain the following:

**Running title:** Application of endogenous biomarkers for drug transporters

**Corresponding author:**
Name Hiroyuki Kusuhara  
Address 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan  
Telephone 811358414700  
Fax numbers 81358414766  
e-mail address kusuhara@mol.f.u-tokyo.ac.jp

The number of text pages:  
The Number of  
  Tables:1  
  Figures:1  
  References:72  
The number of words  
  Abstract:211  
  Introduction:711  
  Discussion:4620

**Abbreviations**  
ABC, ATP-binding cassette  
AUC, area under the plasma concentration time curve  
AUCR, AUC ratio  
BCRP, breast cancer resistance protein  
CGNM, Cluster Gauss–Newton method  
CysA, cyclosporin A  
CP-I, coproporphyrin I  
CP-III, coproporphyrin III  
DDI, drug–drug interaction  
GCDCA-S, glycochenodeoxycholate sulfate  
GFR, glomerular filtration rate  
GWAS, genome-wide association study  
IBC, isobutyryl-L-carnitine
MATE1, multidrug and toxin extrusion protein 1
MATE2-K, multidrug and toxin extrusion protein 2-K
MRP, multidrug resistance -associated protein
NASH, nonalcoholic steatohepatitis
NME, new molecular entity
NMN, N-methylnicotinamide
OAT1, organic anion transporter 1
OAT3, organic anion transporter 3
OATP1B1, organic anion transporting polypeptide 1B1
OATP1B3, organic anion transporting polypeptide 1B3
OCT1, organic cation transporter 1
OCT2, organic cation transporter 2
P-gp, P-glycoprotein
PBPK, physiologically based pharmacokinetic
SNP, single nucleotide polymorphisms
Abstract
Variations in drug transporter activities, caused by genetic polymorphism and drug–drug interactions (DDIs), alter the systemic exposure of substrate drugs, leading to differences in drug responses. Recently, some endogenous substrates of drug transporters, particularly the solute carrier family transporters such as OATP1B1, OATP1B3, OAT1, OAT3, OCT1, OCT2, MATE1, and MATE2-K, have been identified to investigate variations in drug transporters in humans. Clinical data obtained support their performance as surrogate probes in terms of specificity and reproducibility. Pharmacokinetic parameters of the endogenous biomarkers depend on the genotypes of drug transporters and the systemic exposure to perpetrator drugs. Furthermore, the development of physiologically based pharmacokinetic models for the endogenous biomarkers has enabled a top-down approach to obtain insights into the effect of perpetrators on drug transporters and to more precisely simulate the DDI with victim drugs, including probe drugs. The endogenous biomarkers can address the uncertainty in the DDI prediction in the preclinical and early phases of clinical development and have the potential to fulfill regulatory requirements. Therefore, the endogenous biomarkers should be able to predict disease effects on the variations in drug transporter activities observed in patients. This mini-review focuses on recent progress in the identification and use of the endogenous drug transporter substrate biomarkers and their application in drug development.

Significance Statement
Advances in analytical methods have enabled the identification of endogenous substrates of drug transporters. Changes in the pharmacokinetic parameters ($C_{\text{max}}$, AUC, or $\text{CL}_R$) of these endogenous biomarkers relative to baseline values can serve as a quantitative index to assess variations in drug transporter activities during clinical studies and thereby provide more precise DDI predictions.
Introduction

Drug transporters are membrane proteins that can accept diverse drugs as substrates and play essential roles in the tissue distribution and subsequent elimination of drugs. Among the drug transporters, this mini-review focuses on the solute carrier (SLC) family transporters that mediate tissue distribution and elimination of drugs from the blood circulation, including organic anion transporting polypeptide 1B1 (OATP1B1) and OATP1B3, and organic anion transporter 1 (OAT1) and OAT3 for the uptake of anionic drugs in the liver and kidney, respectively; and organic cation transporter 2 (OCT2) and multidrug and toxin extrusion proteins (MATE1 and MATE2-K) for the uptake and subsequent efflux of water-soluble cationic drugs in the kidney, and OCT1 for the uptake of cationic drugs in the liver, respectively. The importance of these transporters in the body has been extensively described elsewhere (Patel et al., 2016; Yee et al., 2018; Gessner et al., 2019; Koepsell, 2020; Nozaki and Izumi, 2020; Kolz et al., 2021; Zou et al., 2021).

Drug transporters also serve as a site of drug–drug interactions (DDIs), which alter the pharmacokinetic properties of their substrate drugs due to the presence of coadministered drugs. The underlying mechanisms are reversible inhibition and induction of the expression of the transporters via nuclear receptors such as NR1I2 (Pregnane X Receptor), NR1I3 (constitutive androstane receptor), and aromatic Hydrocarbon Receptor. Indirect inhibitory mechanisms also include inhibition of the phosphorylation of membrane transporters (OCT2 and OATP1B1) (Sprowl et al., 2016; Hayden et al., 2021). Assessment of DDI risks for new molecular entity (NME) drugs is indispensable to avoid adverse events caused by DDIs in the post-market process. DDI risk for NME drug candidates is routinely and systematically assessed in the preclinical and clinical stages of drug development against the major drug transporters, according to regulatory guidelines and guidance. Decisions to conduct the clinical DDI risk assessment using recommended probe drugs depend on the DDI predictions determined at the preclinical stage. However, these predictions often include some uncertainty, leading to over- or underestimation of the DDI impact in clinical studies. Therefore, it is important to improve DDI predictions during drug development both at the preclinical stage and in clinical DDI studies.

Administration of a substrate drug that serves as a probe, together with the investigational drug, is a straightforward approach to assessing transporter-mediated DDIs in humans. Recently, some endogenous substrates of drug transporters were proposed to represent endogenous DDI biomarkers that could serve as probes to investigate transporter-mediated DDIs (hepatic and renal elimination) (Mochizuki et al., 2021). Inhibition of drug transporter function in the liver and kidney leads to accumulation of the endogenous substrates in the circulation or a reduction in the clearance from the kidney (renal clearance or tubular secretion clearance), where the transporter makes a significant contribution. The magnitude of change depends on the strength of the inhibition caused by the test drug and the contribution of the transporter to the clearance of probe drugs. However, endogenous
biomarkers are attracting increased attention because they have advantages over probe drugs. First, clinical DDI studies using biomarkers no longer require the administration of probe drugs, which can increase opportunities to assess the DDI risks of investigational drugs. For example, the pharmaceutical industry can address uncertainty in DDI predictions in phase 1 studies, where administration of a drug other than an investigational drug is prohibited, by monitoring an endogenous substrate. A second advantage is that the endogenous biomarkers are synthesized within the body, and the plasma concentrations or urinary excretion rates return to baseline levels along with the decline of the inhibition. Thus, in addition to the strength of the inhibition, endogenous biomarker data can provide information about the duration of inhibition after the test drug administration (Mochizuki et al., 2022a). Finally, multiple endogenous biomarkers can be monitored to determine the effect of investigational drugs on multiple drug transporters in the same subjects. To meet these expectations, considerable effort has been made to explore metabolites in the biological fluids of humans that display appropriate specificity, sufficient dynamic range, and a small diurnal variation (Mochizuki et al., 2021). The goal of this mini-review is to discuss the application of clinical DDI assessments using endogenous substrates to determine the level of inhibition of drug transporter function and support their proposed use in the clinical development of NME drugs.

Identification of endogenous biomarkers by clinical studies

Great efforts have been made to identify the substrates of drug transporters for understanding their roles in the body. They have highlighted broad substrate range of the drug transporters from endogenous and exogenous ones. Creatinine, a metabolite produced from creatine, is monitored in clinical settings as a laboratory test to assess kidney function. It has long been known that creatinine clearance is greater than glomerular filtration rate, and the creatinine and inulin ratio (1.4) display saturation along with increased plasma concentrations (Shannon, 1935). Consistent with these clinical data, creatinine was identified as the endogenous substrate of renal organic cation transporters (OCT2, MATE1, and MATE2-K) and OAT2 (Shen et al., 2017). Because of its significance as a biomarker of kidney function, drug-induced changes in the serum creatinine concentration or creatinine clearance have been captured in clinical investigations, which were unlikely to be due to drug-induced kidney injury (Andreev et al., 1999). The perpetrator drugs responsible for such changes include cimetidine, trimethoprim, and pyrimethamine, and these drugs have been identified as MATE1 and MATE2-K inhibitors (Ito et al., 2010; Ito et al., 2012b; Muller et al., 2015). Cimetidine is known to significantly reduce the renal clearance of metformin in healthy subjects, and it has been also confirmed that both pyrimethamine and trimethoprim cause DDIs with metformin (Kusuhara et al., 2011; Grun et al., 2013; Muller et al., 2015). The fluoroquinolone, DX-619, causes a mild serum creatinine elevation during repeated administration without affecting the renal clearance of iohexol, another glomerular filtration rate (GFR) biomarker (Imamura et al.,
2013). DX-619 is a potent inhibitor of OCT2, MATE1, and MATE2-K and likely inhibits these transporters at the doses tested, although the DDI using metformin as a surrogate has not been tested (Imamura et al., 2013). However, due to a small contribution of tubular secretion of creatinine to the urinary excretion, changes in pharmacokinetic parameters induced by inhibition of OCT2, MATE1, and MATE2-K are mild, at least after a single dose of perpetrator drugs (Kusuhara et al., 2011; Ito et al., 2012a; Miyake et al., 2021; Muller et al., 2023). N-methylnicotinamide (NMN), a metabolite produced from nicotinamide, is an endogenous organic cation that undergoes tubular secretion and is also a substrate of OCT2, MATE1, and MATE2-K (Ito et al., 2012a). Unlike creatinine, NMN displays a significant diurnal change in plasma concentrations, with a decline from the morning to the evening and an increase during the night (Ito et al., 2012a). Pyrimethamine and trimethoprim significantly reduced CL_R of NMN, but did not cause an accumulation of NMN in the plasma (Ito et al., 2012a; Muller et al., 2015). Instead, AUC was decreased in the pyrimethamine- or trimethoprim-treated phase (Ito et al., 2012a; Muller et al., 2015). Similar effects were also observed for other drug transporter inhibitors, including verapamil, cimetidine, and probenecid, although neither verapamil nor probenecid affected CL_R of NMN (Muller et al., 2023). The underlying mechanism for the lowering of plasma NMN concentrations by these drugs remains unknown.

The development of an analytical method for comprehensive analysis of serum metabolites (metabolomics) has contributed to the identification of endogenous substrates. A genome-wide association study (GWAS) of human serum metabolites identified new endogenous substrates of the drug transporters, OATP1B1, OCT1 and OCT2, based on the association of genetic polymorphisms (single nucleotide polymorphism, SNP) in drug transporter genes with serum concentrations of endogenous metabolites (Yee et al., 2016; Kim et al., 2017; Miyake et al., 2019; Kim et al., 2017). Regarding OATP1B1, eight metabolites were identified by this approach, namely X-11529 (likely to be glycochenodeoxycholate glucuronide), X-13429 (likely to be glycodeloxycholate sulfate or isomer), tetradecanediol, hexadecanediol, X-11905, X-14626, 4-Androsten-3beta,17beta-diol disulfate, and octadecanediol (Yee et al., 2016). Coproporphyrin I (CP-I) was identified as a metabolite with a differing urine concentration ratio in patients with Rotor syndrome (Wolkoff et al., 1976), which is caused by dysfunction of both OATP1B1 and OATP1B3. A GWAS also reported an association of OATP1B1 SNP [*5(rs4149056) or *15 (rs2306283 and rs4149056)] with plasma concentrations of CP-I (1.6-2.0-fold higher in *5/*15 or *15/*15 subjects compared with heterozygotes and homozygotes for the reference allele) (Mori et al., 2019; Neuvonen et al., 2021; Suzuki et al., 2021), and a further association of minor OAT1B1 SNPs such as *14 (rs2306283 and rs11045819) with lower plasma concentrations of CP-I than the reference value was also reported (Neuvonen et al., 2021). Amino acid substitution at two positions, N130D and P155T, to generate OATP1B1 *14 likely increases the overall transport activity of OATP1B1 using flavopiridol as test substrate in vitro (Ni W et al. 2010). But it remains to be elucidated whether this effect was common
among OATP1B1 substrates. These data support a significant contribution of OATP1B1 to the hepatic elimination of CP-I. Consistent with the results of the GWAS, the CP-I AUC could clearly discriminate *15 homozygotes from heterozygotes (*1a/*15) and *1a homozygotes like probe drug AUC (Mori et al., 2019; Yee et al., 2019). However, the CP-I AUC analysis failed to account for the interindividual variation in the AUC of the probe drugs in the same genotype group. Note that the OATP1B1 genotypes only weakly affected the DDI impact caused by cyclosporine A (CysA) (Yee et al., 2019). In the case of OCT2, although there is a SNP (rs2450975) that displays an association with the serum concentration of N1-methyleladenosine, which was confirmed as a substrate of OCT2 and MATE2-K in vitro (Miyake et al., 2019), the SNP has not been characterized to elucidate its impact on OCT2 expression or CLR of metformin. Regarding OCT1, one of the biomarker candidates is isobutyryl-L-carnitine (IBC), which was originally identified from the GWAS study as other transporter biomarkers (Kim et al., 2017). In the following clinical study, it was revealed that the plasma level of IBC is declined (not increased) when OCT1 inhibitor is administrated (Luo et al., 2020), and that subjects with OCT1 active allele show high IBC plasma level compared to that of wild type allele (Jensen et al., 2021). Contrary to the accumulation of clinical data, IBC itself is not transported by human OCT1 in vitro, but mouse Oct1 can mediate its efflux (Kim et al., 2017).

The endogenous substrates of drug transporters were also investigated using metabolomics and clinical DDI samples. Metabolomic analysis identified thiamine, CLR of which was significantly reduced in pyrimethamine-treated samples (Kato et al., 2014), and glycochenodeoxycholate sulfate and taurine, CLR of which was significantly reduced in probenecid studies (Tsuruya et al., 2016). Thiamine is an OCT2, MATE1, and MATE2-K substrate (Kato et al., 2014), and glycochenodeoxycholate sulfate and taurine are OAT3 and OAT1 substrates, respectively (Tsuruya et al., 2016). Unfortunately, the CLR of these compounds is below their glomerular filtration rates, indicating that they also undergo significant reabsorption from the urine. Shen and colleagues also identified endogenous OAT1 and OAT3 substrates, focusing on plasma concentration time profiles, using probenecid as a typical OAT1 and OAT3 inhibitor (Shen et al., 2019; Tang et al., 2021). They reported that the standardized effect size of kynurenic acid was higher than furosemide, among the metabolites that were determined (kynurenic acid, xanthurenic acid, p-cresol sulfate, pantothenic acid, indole-3-acetic acid, homovanillic acid, and pyridoxic acid) and 6β-hydroxycortisol (Tang et al., 2021). The AUC ratios (AUCRs) caused by probenecid (1,000 mg) were as follows: kynurenic acid, 2.5-fold; xanthurenic acid, 1.4-fold; p-cresol sulfate, 1.9-fold; pantothenic acid, 1.2-fold; indole-3-acetic acid, 2.2-fold; homovanillic acid, 1.8-fold; pyridoxic acid, 3.1-fold. The CLR values of pyridoxic acid and homovanillic acid were reported to be reduced to 0.73 and 0.37, respectively, of their control values.

**Appropriate biomarkers for DDI studies**
To date, various academic and industrial research studies involving clinical trials have been conducted to validate and apply endogenous biomarkers (Table 1). Among them, the association of CP-I (and CP-III) and OATP1B has been evaluated thoroughly, including aspects such as investigational drug-exposure dependency, comparison to the profile of the co-administrated probe drugs, OATP1B specificity using non- or weak inhibitors, application to NME drug development, reproducibility in other subjects, and genetic and ethnicity effects. Physiologically based pharmacokinetic (PBPK) model analysis also has been reported frequently, and several clinical trials have been analyzed together in a single report (Yoshida et al., 2018; Takita et al., 2021; Kimoto et al., 2022; Yoshikado et al., 2022).

To select the best endogenous biomarker for OATP1B1 and OATP1B3 among the candidate compounds, the AUC change produced by 28 compounds was determined using the OATP1B1 and OATP1B3 perpetrators, rifampicin, and CysA (Mori et al., 2020b; Mochizuki et al., 2022b). Rifampicin was given orally at two or three doses to compare the dose-response between endogenous compounds and probe drugs (Takehara et al., 2018; Mori et al., 2020b). Based on the AUCR, four metabolites, namely direct bilirubins, CP-I, and glycochenodeoxycholate sulfate and glucuronide, produced a rifampicin dose-dependent increase in the AUCR (Mori et al., 2020b). The AUCR was the highest for glycochenodeoxycholate sulfate and glucuronide, followed by CP-I and direct bilirubins. Furthermore, the AUC of these metabolites displayed a high correlation with that of probe drugs administered to the same subjects. The diurnal change was minimal for direct bilirubin and CP-I (16–37% from the mean values throughout the study), but very high for bile acids (45–220%) and their conjugated metabolites (40–153%) except for lithocholate sulfate which showed 7-12% from the mean value (Mori et al., 2020b). In the CysA study, subjects received two doses (20 and 75 mg po), and the fold change in the AUCR of GCDCA-S was similar to that of CP-I (Mochizuki et al., 2022b). These differences in the AUCR may be due to the shorter duration of OATP1B1 and OATP1B3 inhibition after CysA administration, compared with that after rifampicin administration, considering that the CP-I concentrations declined to basal levels at 24 hours after rifampicin administration (600 mg) (Mori et al., 2020b), while they declined to basal levels at 12 hours after CysA administration (75 mg) (Mochizuki et al., 2022b). Similar findings were reported for paclitaxel, which caused a shorter duration of inhibition, returning to baseline values ca 8 hours after the end of paclitaxel infusion (Mori et al., 2020a). Correlation coefficients between the AUCR and C\textsubscript{max} ratio of the endogenous biomarkers to the CysA AUC were ranked as follows: AUCR and C\textsubscript{max} ratio of CP-I and CP-III (r = 0.85–0.94) and AUCR of GCDCA-S (r = 0.68). Reproducibility of the C\textsubscript{max} ratio and AUCR was also confirmed by administering the same dose of CysA (75 mg) with a one-week washout. The reproducibility was high for both the AUCR and C\textsubscript{max} ratio of CP-I.

Although multiple endogenous OATP1B1 and OATP1B3 substrates are accumulated after inhibitor administration, analysis using multiple OATP1B1 and OATP1B3 biomarkers did not improve the
interpretation of the observed DDI data of probe drugs (pitavastatin and rosuvastatin) compared with a single metabolite of CP-I (Barnett et al., 2019; Mochizuki et al., 2022b), indicating that quantification of a single metabolite is enough to assess OATP1B1- and OATP1B3-mediated DDI. Based on these studies, CP-I is the most promising quantitative biomarker to assess OATP1B1- and OATP1B3-mediated DDI. Perpetrator AUC-dependent change in the AUC of CP-I was also reported for glecaprevir (Kalluri et al., 2021). Formulations cause a variation in the plasma concentrations of glecaprevir, and this leads to variation in the effect of glecaprevir on CP-I plasma concentrations. In addition to hepatic transporters, dose-dependent data are available for pyrimethamine (three-dose study) (Miyake et al., 2021) for MATE1 and MATE2-K, and probenecid (three-dose study) for OAT1 and OAT3 (Tsuruya et al., 2016). In such studies, dose-dependent changes in CL_R were observed: NMN and N1-methyladenosine for pyrimethamine, and 6β-hydroxycortisol, GCDCA-S, and taurine for probenecid.

The specificities of the endogenous biomarkers can provide insights into complex DDI mechanisms. In the most recent case, to distinguish the inhibition effect of ritlecitinib and its metabolite on hepatic OCT1 and renal OCT2/MATE1/MATE2-K, IBC and NMN were employed as biomarkers for OCT1 and OCT2/MATE1/MATE2-K, respectively (Wang et al., 2023). As a result, NMN renal clearance was not altered, but the AUC of IBC was decreased by 15% after administration of ritlecitinib. In addition, the AUC of sumatriptan, an OCT1 probe drug, was increased by 30-50% after coadministration of ritlecitinib. From these results, authors suggested ritlecitinib has a DDI potential via hepatic OCT1, not renal OCT2/MATE1/MATE2-K. In the case of rosuvastatin, for which the AUC is determined by breast cancer resistance protein (BCRP) in the small intestine and OATP1B in the liver, CP-I was employed to assess OATP1B inhibition after administration of fenebrutinib in the overall DDI (Jones et al., 2020). Administration of fenebrutinib caused a significant change in the AUC of rosuvastatin, but it did not affect the plasma concentration of CP-I. Therefore, DDI in the small intestine, likely inhibition of BCRP, is considered the primary site. This study also shed light on the advantages of CP-I over probe drugs, as the plasma concentration of CP-I is independent of the DDI in the small intestine, at least inhibition of BCRP. Generally, probe drugs are given orally, and a DDI in the small intestine is therefore inevitable. In a clinical DDI study using CysA (Neoral®), where the same dose of CysA was given to two groups, the DDI impact on pitavastatin revealed an intergroup difference when pitavastatin was given at a one-hour interval after CysA administration, but such an effect was not apparent when the interval was extended to 3 hours (Mochizuki et al., 2022b). By contrast, the effect of CysA on the plasma concentration time profile of CP-I was almost identical in these two groups, suggesting the intergroup difference in the pitavastatin response is not due to OATP1B1 inhibition. There is a possibility that CysA or excipients affected the absorption process of pitavastatin when these drugs were given at a one-hour interval.
Model analysis to improve DDI prediction
Endogenous biomarkers suggest the occurrence of transporter inhibition after investigational drug administration, but extrapolation is required to calculate the DDI risk with the substrate drugs, since the DDI between the investigational drug and victim drugs depends on the contribution of the transporter, dosing interval, and dose of drugs, in addition to the time profile of the inhibition. Mathematical model analysis is considered a promising research platform for this purpose. It is essential to estimate the key parameter in the DDI, inhibition constants of NME drugs against the target transporters ($K_{i,in\text{ v}ivo}$), based on clinical data on the endogenous biomarker.

Static and dynamic approaches have been proposed. For pyrimethamine, with a long elimination half-life, nonlinear regression analysis of $CL_R$ of NMN and N1-methyladenosine with average plasma concentrations of pyrimethamine yielded a reasonable estimate of $K_{i,in\text{ v}ivo}$ for MATE1 and MATE2-K, but $CL_R$ of creatinine could not be analyzed, possibly because of a small contribution of tubular secretion (Miyake et al., 2021). For CP-I, a simple method that approximates $K_{i,in\text{ v}ivo}$ for OATP1B1 is to use the ratio of $C_{max}$ of CP-I and inhibitor concentration in the blood at $T_{max}$ of CP-I ($[I]_{T_{max}}$) (Mochizuki et al., 2022b).

$$C_{max\text{ ratio}} = 1 + [I]_{T_{max}}/K_{i,in\text{ v}ivo}$$

This approximation was derived from a differential equation considering the constant synthesis and first-order elimination of CP-I from the blood circulation. Note that $[I]_{T_{max}}$ is the inhibitor concentration observed at $T_{max}$ of CP-I, but not the maximum plasma concentration of the perpetrator drug. This approximation successfully yielded reasonable $K_{i,in\text{ v}ivo}$ values of two perpetrators, rifampicin and CysA (Mori et al., 2020b; Mochizuki et al., 2022b). The $K_{i,in\text{ v}ivo}$ values of rifampicin and CysA were estimated at each dose in the two or three doses studies (i.e., multiple estimates in the one study), and the estimated values were almost identical. Importantly, the estimated values were comparable to those obtained by PBPK model analysis (Mochizuki et al., 2022a; Yoshikado et al., 2022). The accuracy of the $K_{i,in\text{ v}ivo}$ estimation by this method depends on the extraction of inhibitor in the liver (difference in concentration in the venous blood for sampling and capillary) and sampling time points, to identify the maximum change in CP-I concentration after inhibitor administration.

The other approach is to use PBPK models where time profiles of the plasma concentrations of both the endogenous biomarker and perpetrator are considered (Yoshida et al., 2018; Yoshikado et al., 2018; Barnett et al., 2019; Cheung et al., 2019; Takita et al., 2021; Kimoto et al., 2022; Mochizuki et al., 2022a; Tess et al., 2022; Turk et al., 2022; Yoshikado et al., 2022). We have developed a validated PBPK model of CP-I, which comprised 18 compartments (blood, muscle, skin, adipose, capillary in the liver and hepatocytes, and intestine) with biliary elimination with enterohepatic circulation, considering that CP-I is synthesized in hepatocytes (Yoshikado et al.,
2018; Mochizuki et al., 2022a; Yoshikado et al., 2022). Connection of the PBPK model of CP-I and that of inhibitor, based on the inhibition mechanism, can allow optimization of $K_{i,\text{in vivo}}$ values by an iterative nonlinear regression algorithm to explain the clinical observations (plasma concentration time profiles of the endogenous biomarker with or without inhibitor, and plasma concentration time profiles). Comparing $K_{i,\text{in vivo}}$ with the inhibition constant determined by in vitro analysis ($K_{i,\text{in vitro}}$) elucidated an in vitro–in vivo gap in $K_{i}$ ($K_{i,\text{in vitro}}$ versus $K_{i,\text{in vivo}}$) for CysA and rifampicin (Figure 1). $K_{i,\text{in vitro}}$ generally overestimates $K_{i,\text{in vivo}}$ of these two inhibitors for unknown reasons, causing underestimation of the DDI impact. An overestimation was also observed for GDC-0810 (Yoshida et al., 2018; Kimoto et al., 2022). The presence of such inhibitors strongly supports the usefulness of endogenous biomarkers to address more accurate DDI predictions for NME drugs in the early phase of clinical development. Note that $K_{i,\text{in vivo}}$ was corrected by the unbound fraction to compare their values with $K_{i,\text{in vitro}}$ since this parameter was originally defined with regard to the total concentration. $K_{i,\text{in vivo}}$ of CysA differs depending on the reports (Figure 1). One possible reason is the variety of the unbound fraction of CysA across the reports. This parameter in the PBPK model analysis was different likely due to differences in measuring methods. The ultracentrifugation method yields greater values than equilibrium dialysis (Legg et al., 1988; Gertz et al., 2013). Even considering the difference in the unbound fraction, $K_{i,\text{in vivo}}$ was not identical. Meanwhile, a substrate dependence was found in $K_{i,\text{in vivo}}$ of rifampicin between probe drugs and CP-I, and it is likely clinically relevant, since the correction of such a substrate will produce better DDI predictions (Yoshikado et al., 2018; Barnett et al., 2019). Yoshikado et al. propose a correction of such substrate dependence based on in vitro data to use $K_{i,\text{in vivo}}$ in the DDI simulation with PBPK models (Yoshikado et al., 2018). Our PBPK model-based analysis was conducted by simultaneous parameter optimization across multiple doses. Dose–response data are not a prerequisite to estimate $K_{i,\text{in vivo}}$ appropriately, but analysis of the ascending dose data can support the reliability of the estimated $K_{i,\text{in vivo}}$ (i.e., reproducibility and accuracy of the value of this parameter) (Mochizuki et al., 2022a).

When estimating parameters including $K_{i,\text{in vivo}}$ in the PBPK model, we often face difficulties caused by the many parameters to be estimated and the complexity of the PBPK model, which contains a model of the perpetrator, CP-I, and sometimes victim drugs. To overcome these difficulties, we employ the Cluster Gauss–Newton method (CGNM) algorithm (Mochizuki et al., 2022a; Yoshikado et al., 2022). The algorithm can find multiple approximate solutions for nonlinear least-squares problems with low computational cost and short time. It is also suitable for estimating a large number of parameters without initial value dependency. In the two CysA dose studies, CP-I clearance ($CL_{\text{int,all}}$) and the synthesis rate ($v_{\text{syn}}$) of CP-I and the $K_{i,\text{in vivo}}$ values were converged in the narrow range, whereas other parameters were not identifiable because of insufficient data for their optimization (Mochizuki et al., 2022a). The estimated $K_{i,\text{in vivo}}$ was consistent with the results of the analysis using a conventional, iterative nonlinear regression algorithm. The inherent values of CP-I,
CL_int,all and \( v_{syn} \) were also consistent with the results of the CGNM analysis using two and three rifampicin dose studies (Yoshikado et al., 2022). These results indicate the usefulness of the CGNM algorithm to estimate \( K_i, \text{in vivo} \) values in PBPK analysis.

The PBPK model analysis using \( K_i, \text{in vivo} \) yields reasonable estimates of the DDI caused by rifampicin and CysA for the probe drugs tested (pitavastatin and rosuvastatin). Therefore, an appropriate DDI study can be designed using probe drugs in healthy subjects based on the improved predictions, and it may be able to extrapolate the DDI impact when repeated or higher doses are administered. We simulated the effect of dose staggering using \( K_i, \text{in vivo} \) and PBPK models of CysA (perpetrator) and pitavastatin and rosuvastatin (victims) (Mochizuki et al., 2022a). In the case of pitavastatin, CysA caused a greater DDI impact when pitavastatin was given simultaneously or 1 hour after CysA administration, whereas in the case of rosuvastatin, it caused a greater DDI impact when rosuvastatin was given ~1–2 hours before CysA administration. Furthermore, the PBPK model analysis was able to suggest the dosing interval required between perpetrator and victim drugs to reduce the DDI impact or avoid the DDI.

**Current challenges and knowledge gaps**

To date, endogenous biomarkers are limited for the SLC families, OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1, and MATE2-K. However, endogenous biomarkers cannot be applied to isoform-specific DDI risk assessments for isoforms, such as OATP1B1 and OATP1B3 in the liver, and MATE1 and MATE2-K in the kidney, but no probe drugs have been identified for isoform-selective assessments either. ABC transporters, such as P-glycoprotein and BCRP, limit absorption in the small intestine, and DDIs caused by induction or inhibition of these transporters have been reported. However, endogenous biomarkers for these transporters have not yet been established, although several promising candidates have been identified in animal studies.

With regard to CP-I, MPR2 is likely responsible for the canalicular efflux in hepatocytes (Wolkoff et al., 1976; Gilibili et al., 2017). Both rifampicin and CysA are capable of inhibiting canalicular efflux of \([^{11}\text{C}]\text{dehydropravastatin}, [^{18}\text{F}]\text{pitavastatin}, \text{and} [^{11}\text{C}]\text{rosuvastatin} \) at clinically relevant doses (Shingaki et al., 2015; Billington et al., 2019; Nakaoka et al., 2022), and the effect of these perpetrators on the AUC of CP-I could include such effects in addition to inhibition of OATP1B1 and OATP1B3. However, it is not practical to assess the effect on hepatic uptake and canalicular efflux separately based on the plasma concentrations of CP-I alone. Indeed, \( K_i, \text{in vivo} \) for MRP2 could not be converged into a narrow range by comprehensive parameter optimization using CGNM algorithms (Mochizuki et al., 2022a). Additionally, considering CP-I is also the substrate of MRP3, which is a sinusoidal efflux transporter (Kunze et al., 2018), CP-I might receive complex transport including uptake by OATP1Bs and efflux by MRP3, so-called “hepatocyte hopping” in the liver (Iusuf et al., 2012). The impact of MRP2 or MRP3 activities on the CP-I AUC remains to be
Endogenous biomarkers are used to study the activity of drug transporters under disease conditions. For example, CP-I concentrations were monitored after kidney transplantation, chronic kidney disease, liver injury, and rheumatoid arthritis (Suzuki et al., 2019; Ono et al., 2021; Lin et al., 2022; Takita et al., 2022). Note that CP-I plasma concentrations were found to increase with the severity of liver injury (Lin et al., 2022). The AUC change induced by modest and severe injury showed a good correlation across ten OATP1B substrate drugs with the corresponding AUC change induced by rifampicin administration (OATP1B inhibition) in healthy subjects. Therefore, CP-I will be useful for dose adjustment for such OATP1B substrate drugs under diseased conditions, considering OATP1B1 activities and/or overall liver ability to remove xenobiotics. However, a limitation of endogenous biomarker use is that the mass balance cannot be assessed during the study. It remains a challenge to assess the synthesis rate by monitoring other metabolites or utilizing other new technologies. Under diseased conditions, it is also possible that the functional change of other transporters such as MRP2, MRP3 and MRP4 may also affect the CP-I exposure by modulating contribution of the biliary excretion to the net efflux in the liver. In nonalcoholic steatohepatitis (NASH) model rodents, although CP-I could be a NASH progression marker, its plasma level seems to be altered caused by the change of Mrp transporter expression rather than OATP1B expression (Chatterjee et al., 2021). The analysis reviewed in this mini-review assumed that the synthesis rate is unchanged during the study. However, heme synthesis could be modulated by PXR agonists, since the rate-limiting enzyme of heme synthesis, ALAS1, is a target gene of PXR (Podvinec et al., 2004).

By contrast, repeated doses of rifampicin, which activate PXR, did not appear to affect the plasma concentrations of CP-I at a time just before the last dose (Kunze et al., 2018). According to the PBPK model analysis, the effect of rifampicin includes induction and inhibition of OATP1B (Asaumi et al., 2019). Evaluation may be necessary after sufficient time has passed since the last dose, to conclude the effect of repeated doses of rifampicin. In monkeys, repeated rifampicin treatment affected neither OATP1B expression nor CP-I level (Zhang et al., 2020), while CP-I plasma level was increased following the OATP1B repression by chenodeoxycholic acid (Zhang et al., 2022). These reports represented CP-I has a potential to indicate the degree of repression of OATP1Bs as well as its inhibition. Recently, a PBPK model of NMN was reported that included a diurnal change in NMN synthesis, using a sine function, in addition to a constant baseline synthesis (Turk et al., 2022). Interestingly, inhibition of NMN synthesis was incorporated into the PBPK model to reproduce the clinical data, and K_i of trimethoprim and pyrimethamine for this synthesis was comparable to that of OCT2 reported previously (Turk et al., 2022).

When the effects on endogenous biomarkers are compared across perpetrator drugs, attention must be paid to the time window used to calculate AUC and CL. When using probe drugs, the AUC is converged to the total body clearance (CL_tot) for injection drugs and CL_tot/F for oral drugs, along
with an extension of the time. By contrast, the plasma concentrations and urinary excretion of endogenous biomarkers return to baseline values along with recovery from transporter inhibition. Therefore, an extension of the time window will reduce the ratio of AUC or $CL_R$ after the administration of perpetrator drugs. Unlike probe drugs, this may preclude comparison of the DDI impact across drugs, or even across doses.

**Perspective on future directions**

As clinical data are accumulated, it is anticipated that it will be possible to waive clinical DDI studies, based on DDI risk assessments completed using endogenous biomarker data and model analysis. There is considerable progress in the regulatory acceptance of the use of endogenous biomarkers. The latest ICH-M12 Drug interaction studies endorsed on May 2022 stated in Section 2.2.2 that “the inhibition potential of a drug can be evaluated using mechanistic static models, PBPK modeling, or endogenous biomarkers” (U.S. Food and Drug, 2022). However, endogenous biomarkers and the threshold for changes in the pharmacokinetic parameters are not yet recommended, and further accumulation of evidence (both positive and negative data) is required.

Clinical DDI studies are generally conducted on healthy volunteers. If multiple blood sampling and urine collection are allowed to calculate pharmacokinetic parameters, the endogenous biomarkers can address the DDI impact in this specific population, e.g., possible liver and kidney injury, and assist in choosing appropriate regiments for clinical settings. According to an analysis by Lin et al. (2022), changes in CP-I concentrations follow the severity of the liver injury. Therefore, endogenous biomarkers can aid individualized therapy by determining the variation in drug transporter activities.

**Conclusions**

Studies of endogenous biomarkers yield quantitative information about the time profile of the drug transporter inhibition after administration of a test compound that improves the DDI prediction during drug development. This quantitative information also facilitates the acquisition of DDI risk in patients.
Data Availability Statement
The authors declare that all the data are collected from UW Drug Interaction Solutions (https://www.druginteractionsolutions.org/). All the data are literature information.

Authorship Contributions
Participated in research design: NA.
Conducted experiments: NA.
Contributed new reagents or analytic tools: NA.
Performed data analysis: Mochizuki T; Kusuhara, H
Wrote or contributed to the writing of the manuscript: Mochizuki T; Kusuhara, H
References


(MRP2) Suitable for Vesicle-Based MRP2 Inhibition Assay. Drug Metab Dispos 45:604-611.


Downregulation in Cynomolgus Monkeys Treated with Chenodeoxycholic Acid. *Drug Metab Dispos* 50:1077-1086.

Acknowledgments.
The authors would like to thank Asahi Kasei Pharma, Genentech, Gilead Sciences, GlaxoSmithKline, Incyte Research Institute, Merck & Co., Inc., Ono Pharmaceuticals, Pfizer, and Takeda Pharmaceuticals for their support to the clinical investigation of the endogenous biomarkers for drug transporters.
Footnotes
This work received no external funding.

The name and full address (with street address or P.O. box and postal code) and e-mail address of person to receive reprint requests.
Name Hiroyuki Kusuhara
Address 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan
Telephone 811358414700
Fax numbers 81358414766
e-mail address kusuhara@mol.f.u-tokyo.ac.jp

Conflict-of-interest statement
Tatsuki Mochizuki is an employee of Chugai Pharmaceutical Co., Ltd. Hiroyuki Kusuhara declares no conflicts of interest associated with this manuscript.
Legend to Figure

Figure 1. Comparison in vitro and in vivo Ki values against OATP1B of Cyclosporine A and Rifampicin.

Each symbol of Ki, in vitro represents were collected the University of Washington Metabolism and Transport Drug Interaction Database. In vivo Ki were referred to those determined by PBPK model analysis.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Biomarker</th>
<th>Transporter</th>
<th>Evaluated compound</th>
<th>Category</th>
<th>Exposure dependence</th>
<th>Coadministration of probe drugs</th>
<th>No/weak effect by non-/weak-inhibitor</th>
<th>Application to development of NMEs</th>
<th>Reproducibility</th>
<th>Genetic effect</th>
<th>Ethnicity</th>
<th>PBPK model analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mori D, et al.</td>
<td>CP-I, billirubs, BAs, FAs</td>
<td>OATP1Bs</td>
<td>Rifampicin</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓ (Yoshikado T,</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year</td>
<td>Study Design</td>
<td>Biomarkers</td>
<td>Drugs</td>
<td>Interactions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>------</td>
<td>--------------</td>
<td>------------</td>
<td>-------</td>
<td>-------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yee SW, et al.</td>
<td>2019</td>
<td>CP-I,CP-III, FAs</td>
<td>OATP1B1s</td>
<td>Cyclosporine A</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mochizuki T, et al.</td>
<td>2022</td>
<td>CP-I,CP-III, bilirubins, BAs, FAs</td>
<td>OATP1B1s</td>
<td>Cyclosporine A</td>
<td>✓ ✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shen H, et al.</td>
<td>2018</td>
<td>CP-I, CP-III</td>
<td>OATP1B1s</td>
<td>Rifampicin, Itraconazole, Diltiazem</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This article has not been copyedited and formatted. The final version may differ from this version.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Phase(s)</th>
<th>Transporter(s)</th>
<th>Compound(s)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mori D, et al.</td>
<td>2020</td>
<td>CP-I, CP-III, BA</td>
<td>OATP1Bs</td>
<td>Paclitaxel</td>
<td>✓</td>
</tr>
<tr>
<td>Zhang Y, et al.</td>
<td>2020</td>
<td>CP-I, CP-III, FA</td>
<td>OATP1Bs</td>
<td>Frosemide, Probenecid</td>
<td>✓</td>
</tr>
<tr>
<td>Kunze A, et al.</td>
<td>2018</td>
<td>CP-I, CP-III</td>
<td>OATP1Bs</td>
<td>JNJ-A (Pimodovir), Rifampicin</td>
<td>✓ ✓ ✓</td>
</tr>
<tr>
<td>Liu L, et al.</td>
<td>2018</td>
<td>CP-I</td>
<td>OATP1Bs</td>
<td>GDC-0810</td>
<td>✓ ✓</td>
</tr>
<tr>
<td>Cheung KWK, et al.</td>
<td>2018</td>
<td>CP-I</td>
<td>OATP1Bs</td>
<td>GDC-0810</td>
<td>✓ ✓</td>
</tr>
</tbody>
</table>

Note: ✓ indicates the compound is a substrate for the transporter(s) listed.
<p>| Sane RS, et al. J Pharmacol Exp Ther. 2021 | CP-I, CP-III | OATP1Bs | Ipatasertib, Itraconazole | ✓ | ✓ |</p>
<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Journal</th>
<th>Substrates</th>
<th>Transporters</th>
<th>Drugs</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Müller F, et al.</td>
<td>2023</td>
<td>Eur J Drug Metab Pharmacokinet</td>
<td>NMN (CLr)</td>
<td>OCT2/MA TE</td>
<td>Trimethoprim</td>
<td>✓</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------------------------------------------</td>
<td>-----------</td>
<td>-------------</td>
<td>----------------------------------------------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>Imamura Y, et al. Drug Metab Dispos. 2014.</td>
<td>6β-Hydroxycortisol</td>
<td>OAT3</td>
<td>Probenecid, Pyrimethamine</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>----------</td>
<td>--------</td>
<td>-----------</td>
<td>---</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Population pharmacokinetic analysis

BAs, bile acids; CP-I, coproporphyrin I; CP-III, coproporphyrin III; FAs, fatty acids; GCDCA-S, glycochenodeoxycholate sulfate; HVA, homovanillic acid; NMN, N-methylnicotinamide; IBC, isobutyryl-L-carnitine PDA, pyridoxic acid; CLr, renal clearance.
Figure 1

Cyclosporine A

- No preincubation
- 5 min
- 10 min
- 30 min
- 60 min
- 120 min

in vitro

preincubation time

in vivo unbound $K_i$

Rifampicin

- No preincubation
- 5 min
- 10 min
- 30 min
- 60 min
- 120 min

in vitro

preincubation time

in vivo unbound $K_i$