

Effect of Cyclosporin A and Impact of Dose Staggering on OATP1B1/1B3 Endogenous Substrates and Drug Probes for Assessing Clinical Drug Interactions

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This study was designed to assess the quantitative performance of endogenous biomarkers for organic anion transporting polypeptide (OATP) 1B1/1B3-mediated drug-drug interactions (DDIs). Ten healthy volunteers orally received OATP1B1/1B3 probe cocktail (0.2 mg pitavastatin, 1 mg rosuvastatin, and 2 mg valsartan) and an oral dose of cyclosporin A (CysA, 20 mg and 75 mg) separated by a 1-hour interval (20 mg (−1 hour), and 75 mg (−1 hour)). CysA 75 mg was also given with a 3-hour interval (75 mg (−3 hours)) to examine the persistence of OATP1B1/1B3 inhibition. The area under the plasma concentration-time curve ratios (AUCRs) were 1.63, 3.46, and 2.38 (pitavastatin), 1.39, 2.16, and 1.81 (rosuvastatin), and 1.42, 1.77, and 1.85 (valsartan), at 20 mg, 75 mg (−1 hour) and 75 mg (−3 hours) of CysA, respectively. CysA effect on OATP1B1/1B3 was unlikely to persist at the dose examined. Among 26 putative OATP1B1/1B3 biomarkers evaluated, AUCR and maximum concentration ratio (C_{\max} R) of CP-I showed the highest Pearson's correlation coefficient with CysA AUC (0.94 and 0.93, respectively). Correlation between AUCR of pitavastatin, and C_{\max} R or AUCR of CP-I were consistent between this study and our previous study using rifampicin as an OATP1B1/1B3 inhibitor. Nonlinear regression analysis of $AUCR^{-1}$ of pitavastatin and CP-I against CysA C_{\max} yielded $K_{i,OATP1B1/1B3,app}$ (109 ± 35 and 176 ± 42 nM, respectively), similar to the $K_{i,OATP1B1/1B3}$ estimated by our physiologically-based pharmacokinetic model analysis described previously (107 nM). The endogenous OATP1B1/1B3 biomarkers, particularly C_{\max} R and AUCR of CP-I, corroborates OATP1B1/1B3 inhibition and yields valuable information that improve accurate DDI predictions in drug development, and enhance our understanding of interindividual variability in the magnitude of DDIs.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Cyclosporin A is a time-dependent inhibitor of OATP1B1 with long-lastingness. Endogenous OATP1B1/1B3 substrates are anticipated to serve as surrogate probes to assess OATP1B1/1B3-mediated drug-drug interactions (DDIs).

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ Our aim was to determine the relationships between maximum concentration ratio/area under the plasma concentration-time curve ratio (C_{\max} R/AUCR) of the putative OATP1B1/1B3 endogenous biomarkers and AUCR of the probe drugs when CysA was given at different doses (20 mg vs. 75 mg), and at different administration intervals (1-hour vs. 3-hours).

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ Unlike the values observed for OATP1B probe drugs, the C_{\max} R and AUCR of the selected endogenous OATP1B1/1B3

biomarkers, such as coproporphyrin I, were independent of dose staggering. The selected endogenous biomarkers yielded valuable information that may help to improve DDI prediction in drug development.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ OATP1B1/1B3-mediated DDI risk of new investigational drugs can be quantitatively assessed in the early phase of clinical development using the selected endogenous OATP1B1/1B3 biomarker(s). They could serve as a reference to support OATP1B1/1B3 activities during the study in order to gain a deeper understanding of the factors causing pharmacokinetic variation of the test compounds.

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Quantitative prediction of the pharmacokinetic drug-drug interaction (DDI) risks of new investigational drugs is a critical issue in drug development. The DDI risk assessment is based on the half-maximal inhibitory concentrations (IC_{50}) and/or the inhibition constants (K_i) and effective concentrations of perpetrators at therapeutic doses. However, there is uncertainty regarding the *in vivo* relevance of the *in vitro* K_i values.^{1,2} Together with the uncertainty of the effective concentrations of new investigational drugs in the preclinical stage, this can produce both over- and underprediction of the DDI risk.² We and others have identified endogenous biomarkers for drug transporters^{3,4} so that the DDI risks of new investigational drugs can be assessed without probe drug administration. Specifically, these are substrates of drug transporters that include endogenous metabolites, and food- or microbiota-derived metabolites whose area under the plasma concentration-time curve (AUC) and/or renal clearance (CL_R), can significantly change in response to drug transporter inhibition. These endogenous biomarkers are anticipated to serve as surrogate *in vivo* probes for drug transporters to capture the DDI risk analysis of new investigational drugs.

Organic anion transporting polypeptide 1B1 (OATP1B1) and OATP1B3 (OATP1B) play essential roles in the hepatic uptake of various drugs.^{5,6} Cyclosporin A (CysA) is a frequently used OATP1B1/1B3 perpetrator in clinical studies.^{5,6} Inhibition of hepatic uptake by CysA was supported by the most recent positron emission tomography analysis using [¹¹C]rosuvastatin as probe.⁷ CysA is a unique perpetrator that shows pre-incubation-dependent and long-lasting inhibition of OATP1B1/1B3. Pre-incubation in the presence of CysA potentiates its inhibitory effect toward OATP1B1/1B3 (ca 15 minutes), lowering its K_i value 3.8 times.^{8,9} Recovery of OATP1B1 activities to baseline after removal of CysA from the extracellular buffer required more than 18 hours.⁹ Shitara *et al.* proposed the trans-inhibition hypothesis where intracellular CysA also contributes to the OATP1B1/1B3 inhibition to account for these unique characteristics.¹⁰ AM1, a metabolite of CysA, is also an inhibitor of OATP1B1/1B3.¹¹

CysA administration (100 mg, *per os*) is accompanied by an increased AUC of various endogenous OATP1B1/1B3 substrates, such as coproporphyrin I (CP-I) and III (CP-III), hexadecanedioate (HDA), and tetradecanedioate (TDA) by a factor of 1.7–1.9 in healthy volunteers.^{7,12,13} Interestingly, the plasma concentrations of CP-I reached a peak (C_{max}) around 4 hours after CysA administration in humans¹³ suggesting that the decline of OATP1B1/1B3 inhibition occurs earlier than expected from *in vitro* data, but is consistent with physiologically-based pharmacokinetic (PBPK) model simulation.¹¹

To address these knowledge gaps, we designed this clinical study (i) to examine the dose-dependent effect of CysA (20 mg and 75 mg), and (ii) to examine the persistence of OATP1B1/1B3 inhibition by CysA by changing the dosing interval between CysA administration (75 mg) and probe drug administration (conventional 1-hour interval; 75 mg (–1 hour) vs. 3-hour interval; 75 mg (–3 hours)). Pitavastatin, rosuvastatin, and valsartan were selected as probe drugs that were given in a cocktail, as reported previously.¹⁴ The design offers a comparison of the C_{max} ratio ($C_{max,R}$) and AUC ratio (AUCR) across the endogenous OATP1B biomarkers and probe drugs in the same subjects to provide a rationale for selection of the endogenous OATP1B substrates as quantitative DDI biomarkers, and assurance of the reproducibility in the effect of CysA (75 mg) based on the AUCR of the endogenous OATP1B biomarkers. In addition to the forementioned metabolites, we included glucuronide and sulfate conjugates of glycochenodeoxycholate (GCDCA-3G and GCDCA-3S, respectively), and chenodeoxycholate-24-glucuronide (CDCA-24G), and direct bilirubins (D-Bil) as putative OATP1B1/1B3 biomarker based on our previous studies.^{14–16} The CysA effect on the AUC of these metabolites has not been examined. Comprehensive analysis of the putative 26 endogenous OATP1B1/1B3 biomarkers provides a rationale for selection of the endogenous OATP1B biomarkers in clinical DDI studies.

METHODS

Chemicals

All compounds and reagents were of a commercially available analytical grade. Commercial sources for all compounds are listed in the **Supplementary Methods**.

PBPK model simulation

A simulation was conducted to design this study using the PBPK models of CysA and pitavastatin constructed previously.¹⁷ CysA doses used in the simulation were 20, 35, 50, 75, and 100 mg, and the dosing interval between CysA and pitavastatin was 1 hour. Values of pharmacokinetic parameters of CysA and pitavastatin were identical to those optimized to account for the DDI between CysA (100 mg, *p.o.*) and pitavastatin (2 mg, *p.o.*). The simulation was conducted using Napp version 2.31¹⁸ on MacOS (version 10.14.6).

Clinical study design

This study was conducted in accordance with the Clinical Trials Act, Japan. The study protocol was reviewed by the Certified Review Board, the Graduate School of Medicine, The University of Tokyo (CRB3180024). The study is registered as a specified clinical trial in Japan Registry of Clinical Trials (<https://jrct.niph.go.jp/en-latest-detail/>)

jRCTs031200012). Written informed consent was provided by all participants prior to their inclusion in the study.

The study was an open-label, dual-sequence, four-period cross-over study in 10 healthy male volunteers (**Supplementary Methods**). Volunteers' ages ranged from 22 to 39 years old with a mean value of 33; height ranged from 164 to 182 cm with mean value of 173; and weight ranged from 53 to 76 kg with mean value of 65 kg, and body mass index ranged from 19 to 24 with mean value of 22. The probe drugs (0.2 mg pitavastatin, 1 mg rosuvastatin, and 2 mg valsartan) were administered in a cocktail. The schedule of dosing and blood sampling are both described in **Supplementary Methods**. Briefly, probe drugs were orally given at 10 AM and CysA (Neoral dose strengths available in Japan: 10, 25, and 50 mg) was given at 7 AM or 9 AM to have a 3-hour and 1-hour dosing interval, respectively (**Supplementary Methods**).

The 3-hour time interval to examine the persistence of OATP1B1/1B3 inhibition was based on time to C_{\max} (T_{\max}) of CP-I following 100 mg CysA.¹³ The lower dose of CysA (20 mg) was selected based on the simulation using the PBPK we reported previously (**Table S1**).¹⁷

Quantification of test compounds by liquid chromatography-tandem mass spectrometry

Plasma and urine samples analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) were prepared by protein precipitation, as described previously¹⁴ with minor modification. All compounds were separated and detected using the QTRAP5500 system (AB SCIEX, Toronto, Canada) equipped with a Nexera X2 LC system (Shimadzu, Kyoto, Japan), operating in electrospray ionization mode. Analyte quantification was performed using Analyst version 1.7 (Sciex, Toronto, Canada). The conditions of liquid chromatography, multiple reaction monitoring precursor/product ion transitions are summarized in **Supplementary Methods**.

Pharmacokinetic analysis in the human clinical study

AUC was calculated using the linear trapezoidal rule. For probe drugs, AUC was calculated from 0 to 24 hours (AUC_{0-24}). AUC_{-1-24} or AUC_{-3-24} of CysA was calculated from -1 hour or -3 hours to 24 hours. AUC_{0-24} of CysA was calculated by estimating the CysA concentration at time 0. Area from time 0 to 0.5 was estimated by prorating the area by calculating the time for 20 mg (-1 hour) and 75 mg (-1 hour), and by assuming first-order elimination from time -1.5 to 0.5 hours for 75 mg (-3 hours). For the endogenous compounds, AUC was calculated from -3.5 to 10 or -1.5 to 12 hours when CysA was given 3 hours or 1 hour before the cocktail administration, respectively. The C_{\max} represents the maximum concentration observed. In the calculation of C_{\max} of the endogenous OATP1B1/1B3 biomarker, the control values corresponding at T_{\max} in CysA-treated phases were selected.

Iterative nonlinear regression analysis was carried out using Prism 9 (GraphPad Software, San Diego, CA) as described previously to estimate $K_{i,app}$ and f_{CysA} below under constraint of $K_{i,app} > 0$ and $0 < f_{CysA} < 1$.

$$AUCR^{-1} = f_{CysA} (1 + C_{\max, blood, CysA} / K_{i, OATP1B1, app})^{-1} + (1 - f_{CysA})$$

where f_{CysA} , $C_{\max, blood, CysA}$, and $K_{i, OATP1B1/1B3, app}$ represent the fraction sensitive to inhibition by CysA, observed maximum blood concentrations of CysA in each subject, and apparent CysA inhibition constant for OATP1B1/1B3, respectively. This analysis is based on the assumption that the hepatic clearance changes in parallel with the intrinsic clearance and that the effect of CysA is attributable only to the hepatic uptake. The PBPK model analysis suggested that the hepatic extraction of pitavastatin and CP-I were 0.43¹⁷ and 0.28,¹⁹ respectively.

Statistical analysis

Natural log-transformed AUC values were analyzed to obtain the geometric mean and 90% confidence intervals (CIs). The control phase (probe drugs alone) was the reference treatment for all analyses except for

the assessment of the probe drugs' effect on the endogenous biomarkers in which the baseline phase (placebo) was the reference. Plasma concentrations are presented as mean \pm standard error (SEM).

RESULTS

Blood and plasma concentrations of cyclosporin A and its metabolite AM1 following oral administration

CysA was quickly absorbed, and C_{\max} was practically defined based on the observed values (T_{\max} 1.5 hours after CysA administration). The time profiles of CysA concentrations in the blood and plasma were almost identical between a 1-hour interval and 3-hour interval periods after 75 mg CysA, confirming reproducibility of the inhibitor concentrations (**Figure 1a**). Blood C_{\max} of AM-1, the metabolite of CysA, was similar to CysA, with slower elimination from the systemic circulation than CysA in the blood at both dose levels. Plasma concentrations of AM1 were 10 times lower than CysA both after 20 mg and 75 mg doses (**Figure 1a**).

The unbound fractions of CysA in plasma determined by equilibrium dialysis were reported in the range from 1.3% to 1.63% previously (**Table S2**). Using the geometric mean value of 0.0148, the unbound fraction of CysA in the blood (f_B) was estimated as 0.00491 using a blood-to-plasma ratio of CysA (3.02, observed in this study), and the unbound C_{\max} of CysA was estimated to be 0.284 and 1.54 nM at 20 mg and 75 mg, respectively. In accordance with the regulatory guidance,²⁰ hepatic inlet $C_{u, max}$ of CysA was calculated using $F_a F_g$ of 0.572, k_a of 0.1/min, and Q_h of 1.4 L/min, as 5.90 and 22.9 nM at 20 mg and 75 mg, respectively, which provided R-values using the IC_{50} values determined in this study (**Figure S1b**; **Table S3**) as 1.14–1.45 and 1.74–2.75, respectively, over the regulatory threshold (1.1).

Effect of CysA on the plasma concentrations of probe drugs

Compared with control values, plasma concentrations of all the probe drugs following their oral administration increased in a CysA dose-dependent manner in the 1-hour interval study (**Figure 1b**). The C_{\max} and AUC_{0-24} rates of the probe drugs are summarized in **Table S4**. AUCR of pitavastatin was the highest followed by rosuvastatin and valsartan; 1.64 and 3.46 for pitavastatin, 1.39 and 2.16 for rosuvastatin, and 1.43 and 1.77 for valsartan at 20 mg and 75 mg, respectively. AUCR of pitavastatin and rosuvastatin differentiated the dose-dependent effect of CysA (20 mg (-1 hour) vs. 75 mg (-1 hour)), whereas valsartan AUCR was comparable between the two CysA dose levels (**Figure 2**). Prolongation of the dosing interval from 1 to 3 hours impacted the effect of 75 mg of CysA on the AUCR of pitavastatin and rosuvastatin, but not for valsartan (**Figure 2**). It should be noted that the effect of CysA on the AUCR of the probe drugs was variable when they were given within a 1-hour interval (**Figure S2**).

The CL_R of rosuvastatin and valsartan was also determined. When administered with a 1-hour interval, CL_R of rosuvastatin was decreased (**Figure S3a**). Otherwise, there was no significant change in the CL_R of rosuvastatin and valsartan (**Figure S3a,b**). CysA also reduced CL_R of CP-III by 24% at 75 mg, but not CL_R of CP-I (**Figure S3c**).

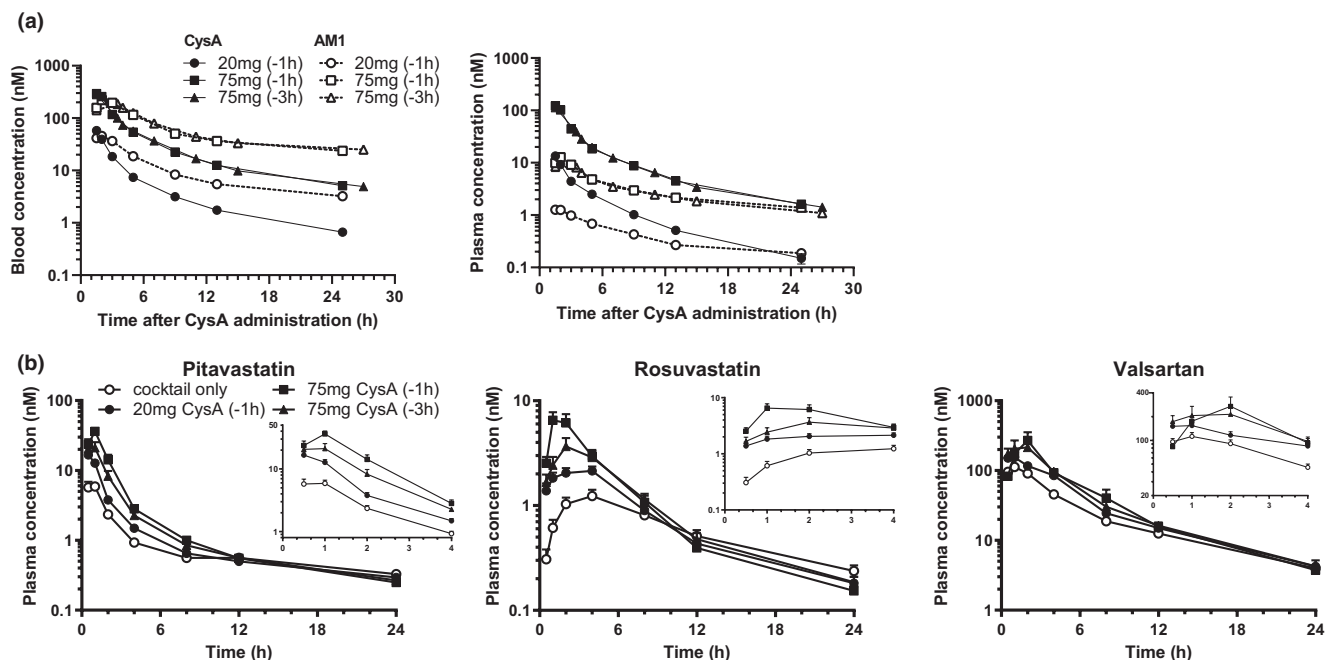


Figure 1 Blood and plasma concentration time profiles of CysA and its metabolite, AM1 (a), and the effect of CysA on the plasma concentration time profiles of pitavastatin, rosuvastatin, and valsartan (b). (a) Time 0 represents the time when the CysA was administered. (b) Time 0 represents the time when the probe cocktail was administered. The dosing interval between CysA and the probe cocktail was 1 hour for 20 mg; 1 hour (–1 hour) or 3 hours (–3 hours) for 75 mg. Symbols and bars represent the mean and SEM ($n = 10$), respectively.

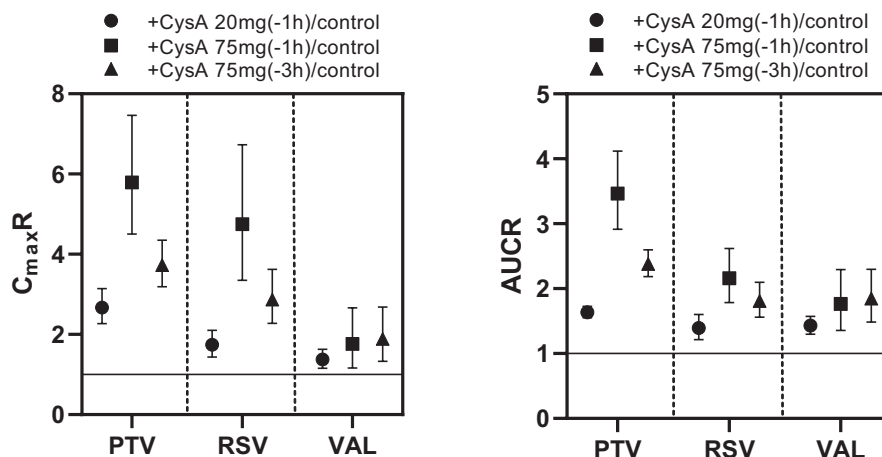


Figure 2 $C_{max}R$ and AUCR of the OATP1B probe drugs. C_{max} observed and AUC_{0-24} were used to calculate $C_{max}R$ and AUCR. Symbols and bars represent the geometric mean and 90% confidence interval. Geometric means of C_{max} and AUC_{0-24} are summarized in Table S4. AUC_{0-24} , area under the plasma concentration-time curve from zero to 24 hours; AUCR, area under the plasma concentration-time curve ratio; $C_{max}R$, maximum concentration ratio; PTV, pitavastatin; RSV, rosuvastatin; VAL, valsartan.

Effect of CysA on the plasma concentrations of the putative OATP1B endogenous biomarkers

Plasma concentrations of 28 compounds were determined (Figure 3). The compounds comprised 26 OATP1B1 endogenous substrates, the synthesis biomarker for bile acids (C4) and intermediate byproduct of heme synthesis (uroporphyrin I; UP-I). Geometric means of $AUC_{-1.5-12}$, $AUC_{-3.5-10}$, and C_{max} and AUCR and $C_{max}R$ are summarized in Table S5.

The endogenous compounds (i) whose $C_{max}R$ was more than 1.25, a threshold for the DDI, and (ii) that showed a dose-dependent

increase in $C_{max}R$ (20 mg vs. 75 mg (–1 hour) or 75 mg (–3 hours)) were CP-I, CP-III, GCDCA-S, GCDCA-G, GDCA-S, GDCA-G, and CDCA-24G (Figure 4a). $C_{max}R$ values of GCA, CDCA, GCDCA, TDA, and HDA were more than 1.25 at 20 mg CysA, and dose dependently increased at 75 mg (–1 hour) CysA, but the effect of CysA could not be differentiated at 20 mg and 75 mg (–3 hours).

AUCR was generally lower than $C_{max}R$. The endogenous compounds (i) whose AUCR was more than 1.25, and (ii) that showed a dose-dependent increase in $C_{max}R$ (20 mg vs. 75 mg (–1 hour) or 75 mg (–3 hours)) were GCDCA-S, TDA, had, and ODA. AUCR

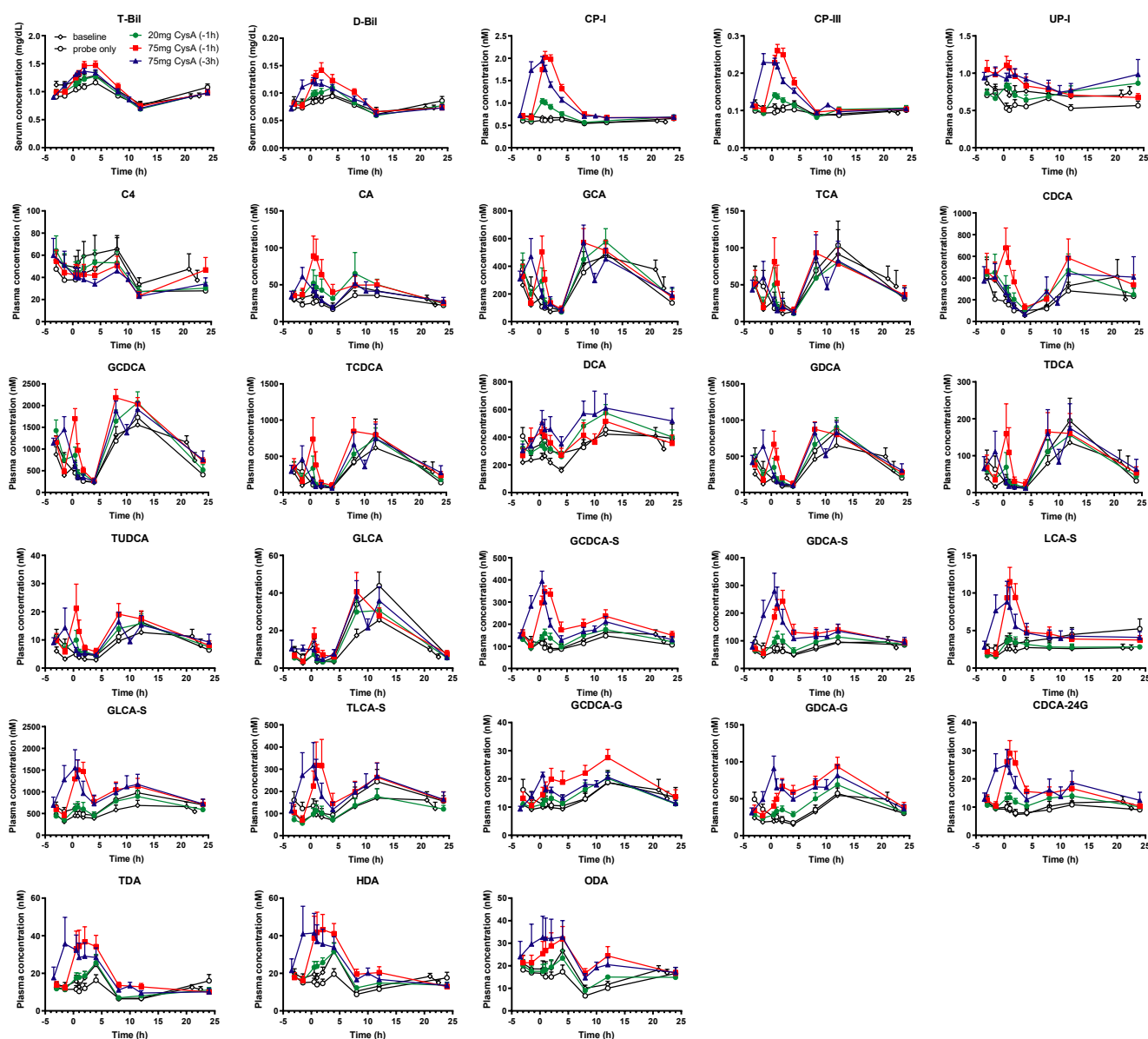


Figure 3 Plasma concentration time profiles of 26 putative endogenous OATP1B biomarkers, UP-I and C4. Time 0 was set when the probe cocktail was administered. Symbols and bars represent the mean and SEM ($n = 10$), respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

of CP-I and GDCA-S was slightly below at 20 mg CysA (1.23 and 1.24, respectively) the threshold (1.25). AUCR of GDCA-G and CDCA-24G was more than 1.25 at 20 mg CysA, and dose dependently increased at 75 mg (–1 hour) CysA, but the effect of CysA could not be differentiated at 20 mg and 75 mg (–3 hours).

Pearson's correlation coefficient between CysA AUC (AUC_{-1-24} or AUC_{-3-24} for CysA 20 mg (–1 hour) and 75 mg (–1 hour), and 75 mg (–3 hours), respectively), and values of C_{max} R or AUCR of the 28 endogenous biomarkers were calculated (Table S6). The coefficient between CysA AUC_{0-24} and AUCR of probe drugs is also shown. C_{max} R and AUCR of CP-I showed the highest correlation among the tested putative biomarkers, and higher correlation than AUCR of pitavastatin (Figure 4b). The coefficients for C_{max} R and AUCR of the putative OATP1B endogenous biomarkers are shown in Figure S4. The same analysis was conducted using

clinical data,¹⁴ where rifampicin was used as OATP1B perpetrator. C_{max} R and AUCR of CP-I and D-BIL showed high correlation (Figure S4).

Correlation between the AUCR of pitavastatin and rosuvastatin or valsartan, and relationship between pitavastatin AUCR and C_{max} R or AUCR of the endogenous OATP1B1/1B3 biomarkers

AUCR of rosuvastatin or valsartan was plotted against AUCR of pitavastatin (Figure 5a) determined previously,¹⁴ and in this study. The relationship between AUCR of pitavastatin and rosuvastatin was consistent in the two studies, whereas AUCR of valsartan was lower in this study compared with the previous rifampicin study. We selected six endogenous OATP1B1/1B3 biomarkers according to their rank (Table S6). Correlations between

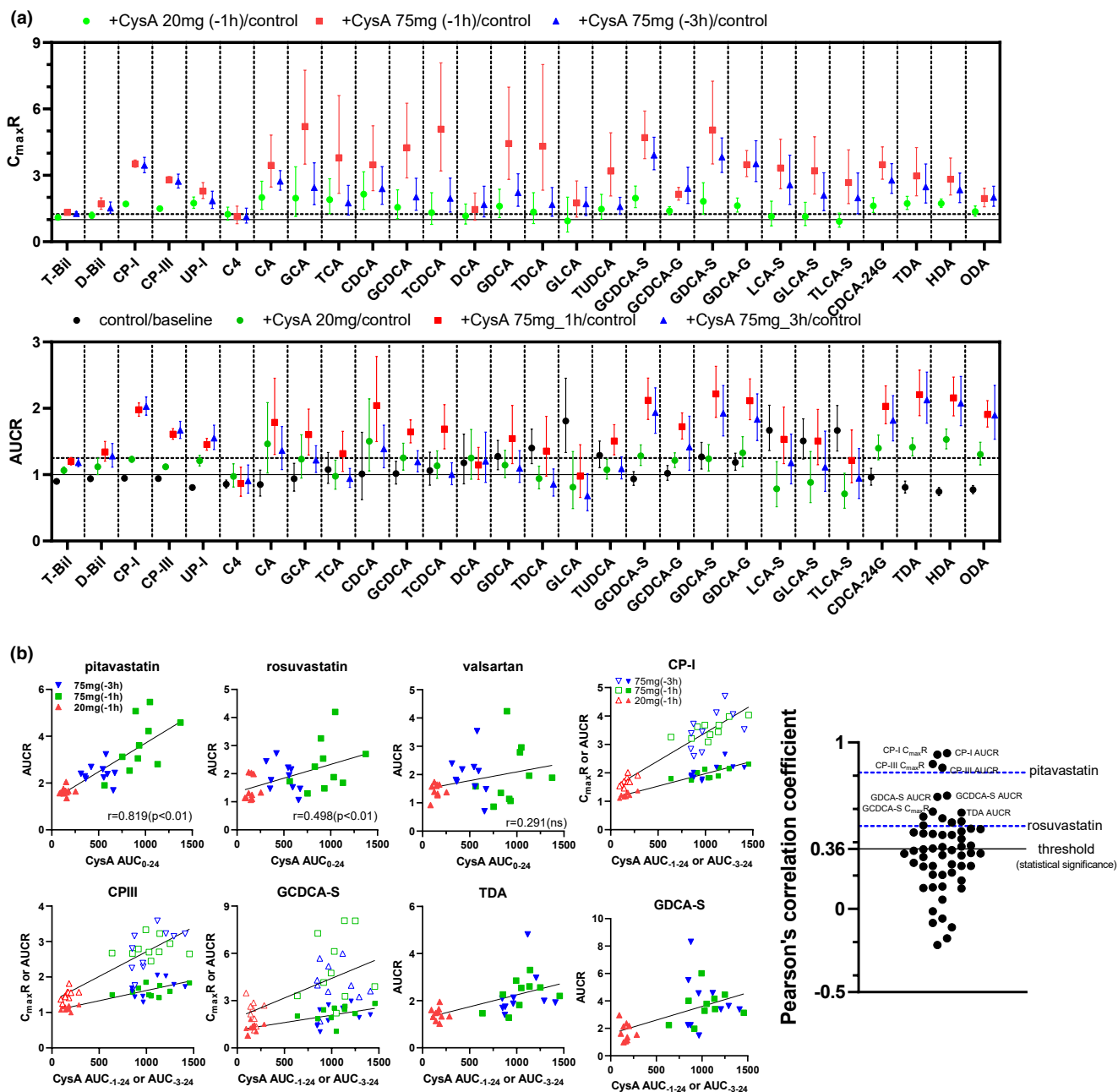


Figure 4 $C_{\max}R$ and AUCR of the 26 putative endogenous OATP1B biomarkers, UP-I and C4. **(a)** Symbols and bars represent the geometric mean and 90% confidence interval. $C_{\max}R$, $AUC_{-1.5-12}/AUC_{-3.5-10}$ are summarized in Table S5. Rigid and dotted lines represent 1 and 1.25, respectively. **(b)** Correlation between the CysA AUC and AUCR of probe drugs, or $C_{\max}R$ and/or AUCR of the endogenous OATP1B biomarkers (top eight are shown, **Table S6**) are shown. CysA AUC_{-1-24} or AUC_{-3-24} CysA were used for the endogenous biomarkers, and CysA AUC_{0-24} were used for the probe drugs. AUC, area under the plasma concentration-time curve; AUCR, AUC ratio; $C_{\max}R$, maximum concentration ratio. [Colour figure can be viewed at wileyonlinelibrary.com]

pitavastatin AUCR, and $C_{\max}R$ or AUCR of CP-I and GDCA-S were seemingly consistent across the studies (**Figure 5b**).

Estimation of $K_{i,OATP1B1/1B3,app}$ by nonlinear regression analysis

$AUCR^{-1}$ of CP-I and $C_{\max,blood,CysA}$ yielded an estimate of f_{CysA} (contribution of CysA sensitive pathway, likely OATP1B1/1B3 contribution) and $K_{i,OATP1B1/1B3,app}$ as 0.736 ± 0.069 and 176 ± 42 nM, respectively (**Figure S5**). The same approach applied to pitavastatin

AUCR provided f_{CysA} and $K_{i,OATP1B1/1B3,app}$ of 0.943 ± 0.088 and 109 ± 35 nM (1.6 times smaller than that from CP-I analysis; **Figure S5**). These $K_{i,OATP1B1/1B3,app}$ estimates were close to a $K_{i,OATP1B1/1B3}$ estimate of 107 nM obtained using a PBPK model for the DDI between CysA and pitavastatin ($K_{i,u}$ (nM)/ f_B , 11.8/0.11).¹⁷ Considering f_B of CysA (0.00491), these estimates yield 0.86 and 0.54 nM with regard to unbound CysA concentrations, which were 9.3–22 and 15–35 times smaller than the *in vitro* values determined with preincubation (8–19 nM; **Table S3**).

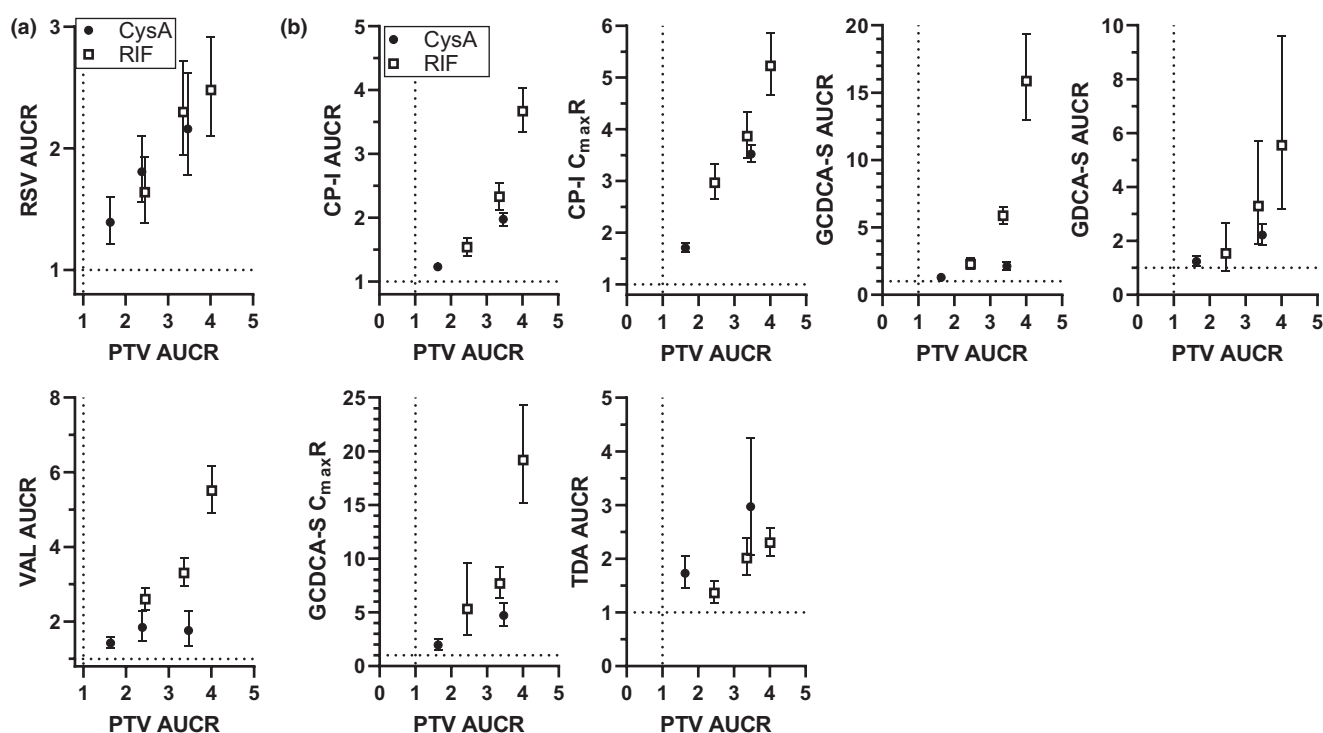


Figure 5 Comparison of AUCR of probe drugs between rifampicin and cyclosporin studies, and comparison of the profile between AUCR of pitavastatin, and C_{\max} R or AUCR of the endogenous OATP1B1/1B3 biomarker. A scatter plot of geometric means of the AUCR of rosuvastatin or valsartan with 90% confidence interval (a), or those of the endogenous OATP1B1/1B3 biomarkers (b) shown against geometric means of the AUCR of pitavastatin at three doses for rifampicin (150, 300, and 600 mg),¹⁴ and two doses for CysA (20 mg (–1 hour), and 75 mg (–1 hour)). AUCR, x area under the plasma concentration–time curve ratio; C_{\max} R, maximum concentration ratio; PTV, pitavastatin; RSV, rosuvastatin; VAL, valsartan.

DISCUSSION

The present study was designed to assess duration of OATP1B1/1B3 inhibition after CysA administration, and to identify the endogenous OATP1B1/1B3 biomarkers whose AUC showed a clear dose-dependent increase caused by OATP1B1/1B3 inhibition by CysA.

PBPK modeling was used to select the CysA doses (Table S1); however, this model should have been used to predict the effect of dosing intervals in advance to aid in the clinical study design. A CysA dose-dependent increase in the AUC of pitavastatin and rosuvastatin (Figure 1a, Figure 2) supports the concept that CysA doses were selected appropriately. This finding emphasizes the use of PBPK models for application in understanding, as well as designing appropriate transporter-mediated DDI studies. C_{\max} R and/or AUCR of the endogenous substrates have been used as metrics for detection of OATP1B1/1B3 inhibition.^{14,15,21} The endogenous substrates whose C_{\max} R satisfied the criteria, (i) greater than 1.25, and (ii) significance in the CysA dose dependence (20 mg vs. 75 mg (–1 hour) or (–3 hours)) were CP-I, CP-III, GCDCA-S, GCDCA-G, GDCA-S, GDCA-G, and CDCA-24G (Figure 4a). In terms of AUCR, GCDCA-S, TDA, HDA, and ODA satisfied the criteria (> 1.25, significance in the CysA dose dependence (20 mg (–1 hour) vs. 75 mg (–1 hour) or 75 mg (–3 hours); Figure 4a). We noted that GDCA-S and CP-I AUCR were slightly below threshold at 20 mg CysA.

C_{\max} R or AUCR of the putative OATP1B biomarkers were ranked according to the coefficient values of their correlation to CysA AUC (Table S6). AUCR and C_{\max} R of CP-I showed the highest performance followed by AUCR of GCDCA-S or GDCA-S (Figure 4b). AUCR and C_{\max} R of CP-I also showed high coefficient values in our study using rifampicin as OATP1B1/1B3 perpetrator¹⁴ (Figure S4). Provided that the coefficients of AUCR of GCDCA-S or GDCA-S were greater than for rosuvastatin, they are considered alternative candidates. Multivariate analysis was conducted to examine whether monitoring multiple biomarkers in addition to CP-I achieved significantly better performance in the DDI analysis. Among the endogenous compounds examined in this study, the AUCR for pitavastatin and rosuvastatin estimated using the C_{\max} R or AUCR of multiple endogenous metabolites provided no further information when compared with the C_{\max} R of CP-I; this finding is consistent with that of a previous study.²² It did improve our understanding of valsartan (Supplementary Analysis). Further study to confirm its robustness, and to elucidate the reason for such improvement is necessary.

To examine the duration of OATP1B1/1B3 inhibition by 75 mg CysA, 2 dosing intervals were selected (1 and 3 hours). Both blood and plasma concentrations of CysA described identical profiles in the phases of dosing intervals of 1 and 3 hours (Figure 1a). Consistent with CysA dosing times, the plasma concentration time profiles of the OATP1B1/1B3 biomarkers shifted, supporting the fact that the increment in the plasma concentrations

of the endogenous substrates depends on administration time of CysA. In addition, the C_{\max} and AUCR of the OATP1B1/1B3 biomarkers, such as CP-I and GCDCA-S, were similar between the two dose intervals (Figure 4). These findings confirm that the magnitude of OATP1B1/1B3 inhibition by 75 mg CysA was controlled appropriately in these phases. Notably, such dosing regimens could impact the C_{\max} and AUCR of bile acids (Figure S6). Their lower reproducibility, and large circadian variation are limiting factors in their use as DDI biomarkers.

Prolongation of the dosing interval between CysA and the probe cocktail attenuated the DDI for pitavastatin and rosuvastatin (Figure 2), and AUCR of these probe drugs showed a high correlation with CysA AUC₀₋₂₄ (Figure 4b). This profile was consistent with the PBPK model-based simulation based on a clinical DDI study between CysA and repaglinide.¹¹ Therefore, inhibition of OATP1B1/1B3 by CysA is unlikely to persist in clinical studies. There are several possibilities to account for this seemingly *in vitro*/animal and clinical data inconsistency. First, concentrations of CysA (1 μ M in serum-free media⁹) used in the *in vitro* experiment were more than 400 times higher than the clinical concentration (2.5 nM as a product of the total blood concentration at 75 mg and f_B ; Figure 1). Second, CysA undergoes metabolism by CYP3A4 in the liver, which might also reduce the persistence of OATP1B1/1B3 inhibition *in vivo*. It should be noted that we found that there was a relatively large variation in the pitavastatin AUCR at 75 mg CysA (–1 hour; Figure S2). Considering that the CP-I AUCR did not show such variation (Figure S2b), we speculated that the variation in AUCR of pitavastatin in this dosing condition was because of factors other than OATP1B1/1B3 inhibition by CysA. In this context, the OATP1B1/1B3 endogenous biomarkers serve as a reference to understand the factors causing variation in the pharmacokinetics of drugs in the same study. Such application was reported in investigations of the DDI mechanism between rosuvastatin and fenofibrate,²³ and also in investigations of the impact of genetic variation of OATP1B1 in the overall pharmacokinetics of iritenotecan.²⁴

We also investigated usage of OATP1B1/1B3 endogenous biomarkers for predicting the DDI risk more quantitatively. First, we compared the relationship between AUCR of the tested compounds, to examine their similarity in this study, with those in our previous published report.¹⁴ The relationship in the AUCR between pitavastatin and rosuvastatin was almost consistent across the perpetrators. However, valsartan AUCR was not consistent for an unknown reason (Figure 5a). Both C_{\max} and AUCR profiles of CP-I were likely consistent in the two studies, suggesting that C_{\max} and AUCR values of CP-I may yield a rough estimation of pitavastatin AUCR, assuming this correlation curve for new drugs. Second, nonlinear regression analysis was conducted to yield an estimate of the $K_{i,OATP1B1/1B3,app}$ values of CysA, which were similar between pitavastatin and CP-I (<2 times difference; Figure S6). The values were also similar to those yielded by PBPK model-based analysis to account for the observed DDI.¹⁷ *In vivo* relevance of the *in vitro* $K_{i,OATP1B1/1B3}$ is a critical issue for successful DDI risk assessment.^{19,25} This approach offers an easy way to examine the *in vivo* relevance of this parameter before using the PBPK model analysis except if the perpetrator does not affect the synthesis

process or other elimination pathways. Given the time profiles of UP-I (Figure 3), we speculate that CysA was unlikely to have affected the intermediate process leading to the synthesis of UP-I (Supplementary Information) or the renal elimination of CP-I (Figure S3), although it significantly reduced the CL_R of CP-III at 75 mg (Figure S3).

The difference in the effect across the OATP1B inhibitors between statins and valsartan may be explained by their fraction transported via OATP1B1/1B3 in the liver (Figure 5). OATP1B3 was suggested to mediate the hepatic uptake of valsartan and GCDCA-S together with OATP1B,^{26,27} of which CysA is a weaker inhibitor than OATP1B1 (Table S3). The effect of CysA on the AUC of the OATP1B3-specific probe needs to be confirmed. In addition, a difference in the time during which the blood/plasma concentrations were above *in vivo* $K_{i,OATP1B1/1B3}$ values (6 hours for 300 mg rifampicin,¹⁴ and 3.5 hours for 75 mg CysA) also might modulate the DDI impact. PBPK model analysis and simulation of the DDI will likely suggest the possible mechanism.

In conclusion, a persistent effect of CysA on OATP1B1/1B3 was not observed in this study, but dose staggering was shown to modulate the effect of OATP1B probe drugs on DDIs. By contrast, changes in the C_{\max} and AUCR of the selected endogenous OATP1B biomarkers were independent of dose staggering but were dependent on the dose. These results combined with those of the previous study support the use of CP-I as a quantitative tool for predicting the clinical OATP1B-mediated DDI for new molecular entities.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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CONFLICT OF INTEREST

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

T.M., M.J.Z., K.Y., J.M., K.T., H.H., X.C., Y.L., T.T., Y.S., and H.K. wrote the manuscript. T.M., M.J.Z., K.Y., J.M., K.T., H.H., X.C., Y.L., T.T., K.R., Y.Y., Y.S., and H.K. designed the research. T.M., K.F., K.M., K.F., and H.K. performed the research. T.M., M.J.Z., K.Y., J.M., K.T., H.H., X.C., Y.L., T.T., K.R., Y.Y., T.M., K.M., Y.S., and H.K. analyzed the data.

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