

# Investigation of Endogenous Compounds for Assessing the Drug Interactions in the Urinary Excretion Involving Multidrug and Toxin Extrusion Proteins

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## ABSTRACT

**Purpose** Multidrug and toxin extrusion proteins (MATEs) are multispecific organic cation transporters mediating the efflux of various cationic drugs into the urine. The present study aimed at identifying endogenous compounds in human plasma and urine specimens as biomarkers to evaluate drug interactions involving MATEs in the kidney without administration of their exogenous probe drugs.

**Methods** An untargeted metabolomic analysis was performed using urine and plasma samples from healthy volunteers and mice treated with or without the potent MATE inhibitor, pyrimethamine. Plasma and urinary concentrations of candidate markers were measured using liquid chromatography-mass spectrometry. Transport activities were determined in MATE- or OCT2-expressing HEK293 cells. The deuterium-labeled compounds of candidates were administered to mice for pharmacokinetics study.

**Results** Urinary excretion of eleven compounds including thiamine and carnitine was significantly lower in the pyrimethamine-treatment group in humans and mice, whereas no endogenous compound was noticeably accumulated in the plasma. The renal clearance of thiamine and carnitine was decreased by 70%–84%

and 90%–94% ( $p < 0.05$ ), respectively, in human. The specific uptake of thiamine was observed in MATE1-, MATE2-K- or OCT2-expressing HEK293 cells with  $K_m$  of  $3.5 \pm 1.0$ ,  $3.9 \pm 0.8$  and  $59.9 \pm 6.7 \mu\text{M}$ , respectively. The renal clearance of carnitine- $d_3$  was decreased by 62% in mice treated with pyrimethamine.

**Conclusions** Our findings indicate that MATEs account for the efflux of thiamine and perhaps carnitine as well as drugs into the urine. The urinary excretion of thiamine is useful to detect drug interaction involving MATEs in the kidney.

**KEY WORDS** drug interaction · metabolomics · multidrug and toxin extrusion protein · organic cation · tubular secretion

## ABBREVIATIONS

AUC	area under the plasma concentration–time curve
BBM	brush border membrane
BBMV	brush border membrane vesicles
$CL_R$	renal clearance
$CL_{tot}$	total plasma clearance
dCyd	2'-deoxycytidine
GFR	glomerular filtration rate

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LC-MS	liquid chromatography-mass spectrometry
MATE	multidrug and toxin extrusion protein
NMN	<i>N</i> -methylnicotinamide
OCT	organic cation transporter
PYR	pyrimethamine
TEA	tetraethylammonium
$X_{\text{urine}}$	urinary excretion amount

## INTRODUCTION

Multidrug and toxin extrusion proteins (MATEs) facilitate the exchange of organic cations and  $H^+$ . Human MATEs are comprised of two isoforms, MATE1/*SLC47A1* and MATE2-K/*SLC47A2*, both of which are expressed on the brush border membrane (BBM) of renal proximal tubules in the kidney (1,2) and exhibit overlapping substrate specificities for organic cations such as tetraethylammonium (TEA) and metformin (3,4). To date, MATEs are considered to account for the secretion of various cationic drugs into the urine from the blood circulation in conjunction with the basolateral uptake transporter, OCT2/*SLC22A2*. This concept has been supported in an animal study using MATE1 knockout mice which showed a marked delay in the elimination of metformin from the systemic circulation, accompanied by an elevation of the metformin concentration in the kidney (5). Also, the renal secretory clearance of varenicline was reduced by 55% in the MATE1 knockout mice, compared with that in normal mice (6). Furthermore, a genetic disruption in MATE1 potentiated the nephrotoxicity of the anti-cancer platinum agent cisplatin and of the herbicide paraquat by the marked accumulation of these compounds in the kidney (7,8).

We previously reported that, among the drugs in the market, cimetidine and pyrimethamine (PYR) are potent inhibitors of MATE1 and MATE2-K (9–11). PYR completely inhibited the uptake of MATEs substrates such as metformin and *N*-methylnicotinamide (NMN), by human-kidney BBM vesicles (BBMV), driven by the  $H^+$  gradient imposed across the membrane, with  $K_i$  values similar to those observed in cDNA transfectants expressing MATE1 and MATE2-K. It is of important to note that a comparison of the  $K_i$  values of cimetidine and PYR for MATEs and OCT2 with their maximum unbound plasma concentrations achieved at their usual clinical doses suggested that both drugs can significantly inhibit both MATE1 and MATE2-K without affecting OCT2. Indeed, cimetidine and PYR caused an elevation in the kidney concentrations of MATE substrates in mice (11,12). Cimetidine has been used to investigate the urinary excretion mechanisms of drugs in humans in drug development (12). Recently, we demonstrated that PYR significantly inhibited the renal elimination of metformin in healthy subjects (13). Furthermore, in addition

to drugs, we also found that MATEs are involved in the urinary excretion of endogenous metabolites: PYR reduced the renal clearance of creatinine by ~20% (13) and of NMN by ~70% (14). This finding led the concept of prediction of transporter-mediated drug-drug interactions without administration of probe drugs. However, the role of MATEs in the urinary excretion of endogenous compounds needs more investigation.

The purpose of this study was to identify endogenous MATEs substrates in the human, plasma level and/or urinary excretion of which is highly sensitive to the activities of MATEs in the kidney. Untargeted metabolomics is a useful analytical approach for discovering novel endogenous or food-derived substrates (15–17). In this study, metabolomic analyses of plasma and urine specimens from eight healthy male volunteers treated with or without PYR in a crossover fashion were conducted using liquid chromatography-mass spectrometry (LC-MS). Consequently, we identified candidate endogenous substrates for MATEs, the renal clearances of which were markedly decreased by PYR. Our results offer a way of evaluating the inhibitory potency of drugs and drug candidates against MATEs in humans without administration of probe substrates in drug development.

## MATERIALS AND METHODS

### Materials

PYR was purchased from Wako Pure Chemicals (Osaka, Japan). Thiamine- $d_3$  hydrochloride was purchased from Toronto Research Chemicals (North York, Canada). Carnitine- $d_3$ , acetylcarnitine- $d_3$ , and 2'-deoxycytidine- $^{15}N_3$  were purchased from Cambridge Isotope Laboratories (Andover, MA). Carnitine- $d_9$  was purchased from CDN Isotopes (Quebec, Canada). [ $^{14}C$ ]TEA bromide (3.5 mCi/mmol) and [ $^3H$ ]thiamine hydrochloride (20 Ci/mmol) were purchased from PerkinElmer Life and Analytical Science (Waltham, MA) and American Radiolabeled Chemicals (St. Louis, MO), respectively. All other reagents that were used were commercially available and of analytical grade.

### Clinical Samples

The study protocol was approved by the Ethics Review Boards of both the Graduate School of Pharmaceutical Sciences of the University of Tokyo and the Clinical Investigation Center, Kitasato University East Hospital.

Plasma and urine specimens were obtained from healthy subjects participating in a pharmacokinetic interaction study, which was registered with the UMIN Clinical Trials Registry at [www.umin.ac.jp/ctr/index.htm](http://www.umin.ac.jp/ctr/index.htm) (UMIN000002739) (13). No clinically undesirable signs or symptoms possibly attributable to the administration of metformin or pyrimethamine

were identified throughout the study, except for a slight increase in the plasma creatinine level that did not result from kidney injury. All the subjects completed the study successfully according to the following protocol: briefly, eight male subjects were enrolled in the study after they had provided their written informed consent. The subjects were physically normal upon clinical examination and routine clinical testing, and none of the subjects had any history of significant medical illness or hypersensitivity to any drugs. The mean  $\pm$  SD values of the age, body weight and serum creatinine level of the volunteers were  $28.0 \pm 4.5$  years (range 22–34 years),  $63.9 \pm 6.9$  kg (range 54.1–76.6 kg) and  $0.82 \pm 0.10$  mg/dL (range 0.66–0.94 mg/dL), respectively.

The pharmacokinetic interaction study consisted of four periods and was conducted in a cross over fashion. Subjects received a microdose (100  $\mu$ g) of metformin after an overnight fast during Periods 1 and 2 and received a therapeutic dose (250 mg) of metformin during Periods 3 and 4. During Periods 2 and 4, the volunteers ingested a Daraprim® tablet (50 mg pyrimethamine/tablet) 1 h before the administration of metformin. Blood samples were taken by direct venipuncture (sodium heparin anticoagulant) and were centrifuged to isolate the plasma, which was then stored at  $-80^\circ\text{C}$  until analysis.

### Animal Experiments

All the animal studies were approved by the Taisho Pharmaceutical Animal Care Committee. Male ddY mice were purchased from Japan SLC (Shizuoka, Japan). They were housed in an air-conditioned room set to a 12-h light/dark cycle, given access *ad libitum* to water and a standard laboratory diet (MF; Oriental Yeast, Tokyo, Japan), and used at the age of 7–8 weeks for experiments. In the PYR-treated group, a bolus dose of PYR (0.5 mg/kg in 0.9% NaCl and 10% ethanol) was administered via the tail vein 30 min before the injection of carnitine- $d_3$  (0.25 mg/kg). The vehicle was administered in the control group. The animals were anesthetized with isoflurane, and blood and urine samples were collected from the postcava and bladder, respectively, at predose and at 5, 15, 30, 60, and 120 min after the administration of carnitine- $d_3$ . After the animals were sacrificed, their kidneys were quickly removed and weighed, then homogenized in 4 volumes of ice-cold water. The blood sample was collected in a tube containing EDTA as an anticoagulant and was centrifuged to obtain the plasma.

### Metabolomic Analysis

Metabolomic analyses were performed using the following samples: human urine and plasma obtained 7 h after PYR dosing during Periods 1 and 2 and mouse urine, plasma and kidney samples obtained 120 min after dosing with carnitine- $d_3$ . Cold solvent (acetonitrile: methanol=9:1, 200  $\mu$ L) was

added to 50  $\mu$ L of a biological sample. The mixture was vortexed and centrifuged for 5 min at  $10,000 \times g$ , and the supernatant was subjected to an LC-MS analysis. The LC system used was an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) equipped with an XBridge HILIC column (3.5  $\mu$ m,  $4.6 \times 50$  mm; Waters, Milford, MA). The mobile phase consisted of 10 mM ammonium acetate (pH 5.0) as solvent A and acetonitrile as solvent B, and was delivered at 1.0 mL/min using 95% solvent B between 0 and 1 min followed by a linear gradient to 70% B at 10 min and 50% B at 11 min, then isocratic to 12 min with 50% B. The MS data were acquired using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The samples were analyzed using positive ion-heated electrospray ionization. The MS conditions were set as follows: spray voltage, 2,500 V; scanning range,  $m/z$  100–800; resolving power, 30,000; and normalized collision energy (CID), 35%. The freely available MZmine software (18) was used to align the data in the time domain and to integrate the peak area automatically. The HMDB (19) and KEGG (20) databases were used for structural searching based on accurate masses.

### Quantification Using LC-MS/MS

Thiamine- $d_3$ , dCyd- $^{15}\text{N}_3$ , carnitine- $d_9$ , and acetylcarnitine- $d_3$  were used as internal standards for the quantification of the corresponding analytes. The sample-pretreatment methods, LC system, and solvents for the mobile phase were the same as those used for metabolomic analysis conditions. The composition of the mobile phase for the quantification of thiamine and 2'-deoxycytidine (dCyd) was 90% solvent B (0–0.5 min), followed by a linear gradient to 80% B at 2.2 min, 60% B at 2.7 min, 50% B at 4.5 min and then isocratic to 5 min with 50% B. The composition of the mobile phase for carnitine and acetylcarnitine was 75% B (0–0.2 min), followed by a linear gradient to 50% B at 2.5 min, and then isocratic to 3 min with 50% B. MS data were acquired using an API4000 tandem mass spectrometer (AB Sciex, Foster City, CA). The analytes were detected by exploiting the transitions of  $m/z$  265  $\rightarrow$  122 for thiamine,  $m/z$  228  $\rightarrow$  112 for dCyd,  $m/z$  268  $\rightarrow$  125 for thiamine- $d_3$ ,  $m/z$  231  $\rightarrow$  115 for dCyd- $^{15}\text{N}_3$ ,  $m/z$  162  $\rightarrow$  103 for carnitine,  $m/z$  204  $\rightarrow$  85 for acetylcarnitine,  $m/z$  171  $\rightarrow$  103 for carnitine- $d_9$  and  $m/z$  207  $\rightarrow$  85 for acetylcarnitine- $d_3$ . The lower limit of quantification was 1 ng/mL except for dCyd (0.3 ng/mL). The PYR levels were analyzed by exploiting the transitions of  $m/z$  249  $\rightarrow$  177 and using propranolol as an internal standard.

### In Vitro Transport Study Using cDNA Transfectants

HEK293 cells stably expressing OCT2, MATE1 or MATE2-K were constructed previously (9,21). Cells were

seeded 72 h before the transport assay in poly-L-lysine- and poly-L-ornithine-coated 12-well plates at a density of  $1.5 \times 10^5$  cells per well. The cell culture medium was replaced with culture medium supplemented with 5 mM sodium butyrate 24 h before the transport assay to induce the expression of the transporters. The *in vitro* transport study was conducted as described previously (22). Briefly, the cells were washed twice and preincubated with Krebs–Henseleit buffer at 37°C for 15 min. The Krebs–Henseleit buffer consisted of 118 mM NaCl, 23.8 mM NaHCO<sub>3</sub>, 4.8 mM KCl, 1.0 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM CaCl<sub>2</sub> and was adjusted to pH 7.4. Uptake was initiated by the addition of [<sup>14</sup>C]TEA or [<sup>3</sup>H]thiamine in the presence or absence of TEA (5 mM) at 37°C. For evaluation of MATE-mediated transport, cells were preincubated in the presence of NH<sub>4</sub>Cl (20 mM) for intracellular acidification. Uptake was terminated at the designated times by the addition of ice-cold Krebs–Henseleit buffer after the removal of the incubation buffer. The cells were solubilized with NaOH overnight at 4°C, then neutralized with HCl. The radioactivity in the aliquots was measured using liquid scintillation counting. The protein concentration was determined using the Lowry method (23).

### Kinetic Analyses

Specific uptake was calculated by subtracting the uptake by the vector-transfected cells from the uptake by the cDNA-transfected cells. The kinetic parameters were calculated using the following equations:

$$v = V_{\max} \times S / (K_m + S) + P_{\text{dif}} \times S \quad (1)$$

where  $P_{\text{dif}}$  is the nonsaturable uptake clearance,  $v$  is the uptake velocity of the substrate,  $V_{\max}$  is the maximum uptake rate,  $K_m$  is the Michaelis constant, and  $S$  is the substrate concentration in the medium. Fitting was performed using a nonlinear least-squares method using the MULTI program (24).

### Pharmacokinetic Analysis

The area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal rule. The renal clearance ( $CL_R$ ) was calculated by dividing the cumulative urinary excretion ( $X_{\text{urine}}$ ) by the corresponding AUC. The total plasma clearance ( $CL_{\text{tot}}$ ) was calculated by dividing the dose by the corresponding AUC.

### Statistical Analysis

Data are presented as the mean  $\pm$  S.E.M. A Student two-tailed paired *t*-test was used to identify significant differences between the two sets of data in the clinical study. A Student

unpaired *t*-test for equal variances or the Welch test for unequal variances was used for the animal experiment. A value of  $P < 0.05$  was considered statistically significant. All the statistical analyses were performed using SAS 9 (SAS Institute, Cary, NC).

## RESULTS

### Untargeted Metabolomic Analysis

An untargeted metabolomic analysis was performed using urine samples obtained from humans and mice treated with or without PYR. An LC-MS analysis detected approximately 2,000 and 3,000 peaks in the human and mice specimens, respectively. Eleven compounds of interest were selected based on the following three criteria: 1) the relative urinary excretion (the product of the peak area and the urine volume) was decreased by more than half of the control value in both humans and mice; 2) the decrease was seen in at least 7 of the 8 volunteers; and 3) the peak intensity was strong enough for obtaining the product ion spectrum. The chromatographic and mass spectral data of these compounds are shown, along with the fold changes, in Table I. Online databases, HMDB (19) and KEGG (20), were searched according to the accurate mass-to-charge ratio ( $m/z$ ) to suggest structural candidates. The LC/MS data of six compounds agreed with those of authentic standards: NMN, thiamine, dCyd, carnitine and the acyl-derivatives of carnitine, acetylcarnitine and propionylcarnitine. The structures of U146 and U160 were speculated to be 4-*N*-trimethylaminobutyrate and its CH<sub>2</sub>-elongated analogue by comparing their spectral data with that of carnitine. The structures of the other three unknown compounds have yet to be identified.

Plasma and kidney samples were also subjected to metabolomic analyses. No fold-changes greater than 2-fold were observed with the exception of PYR ( $m/z$  249.09043) and its metabolites, the oxidized form ( $m/z$  265.08527) and glucuronide ( $m/z$  425.12233). The plasma levels in the PYR-treatment group of the eleven compounds described above were comparable to those in the control group (Table I). Their accumulations in mouse kidney reached a maximum of only 1.7-fold.

### Effect of PYR on Plasma Levels and Urinary Excretion of MATE Substrate Candidates

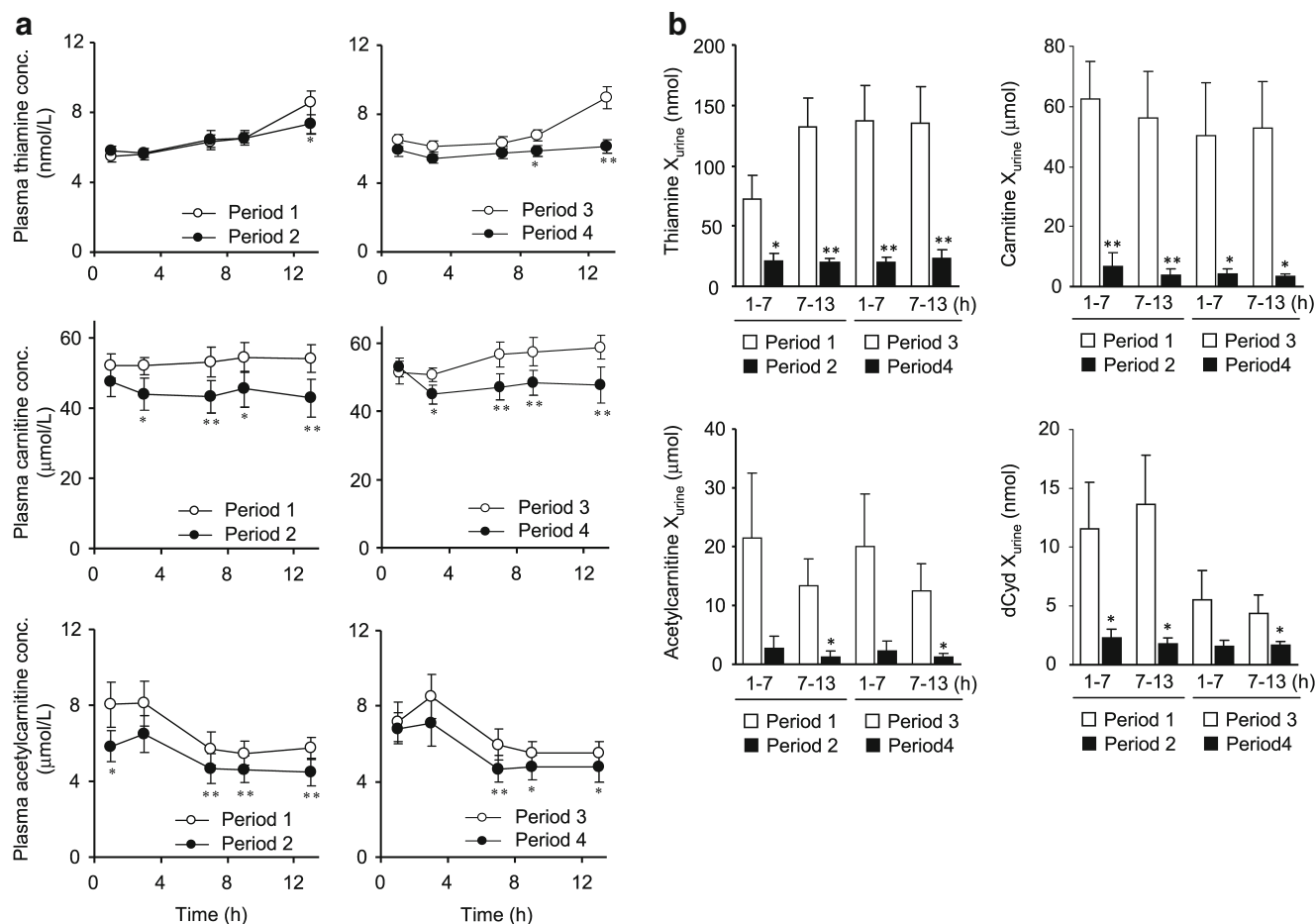
The plasma and urinary concentrations of thiamine, carnitine, acetylcarnitine and dCyd were quantified using the corresponding stable isotopes as internal standards. The plasma concentration–time profiles of thiamine, carnitine and acetylcarnitine are shown in Fig. 1a. Their AUCs and  $CL_R$  are listed in Table II. The dCyd concentration in the

**Table 1** List of Endogenous Compounds of Interest in the Metabolomic Analyses Performed in Healthy Male Volunteers and Mice Treated With or Without PYR

Peak number	Identity	Retention time (min)	Protonated molecule ( <i>m/z</i> )	Product ion ( <i>m/z</i> )		Fold Change (PYR-treatment/control)					
				Major	Others	Human <sup>b</sup>		Mouse <sup>c</sup>		Plasma	Urine
1	2'-deoxycytidine	3.1	228.09777	112.05058	189.10353	203.04364	0.5 ± 0.1	N.D.	0.2 ± 0.1	N.D.	N.D.
2	U196 <sup>a</sup>	6.4	196.07310	114.12756	152.08356		0.5 ± 0.3	1.2 ± 0.2	0.5 ± 0.1	0.8 ± 0.2	0.9 ± 0.2
3	<i>N</i> -methylnicotinamide	7.2	137.07066	94.06507			0.6 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
4	U132 <sup>a</sup>	7.4	132.13782	114.09116	72.08055		0.3 ± 0.1	N.D.	0.4 ± 0.1	N.D.	1.4 ± 0.5
5	<i>L</i> -Propionylcarnitine	7.7	218.13863	159.06537	85.02841	144.10213	0.2 ± 0.1	0.8 ± 0.05	0.3 ± 0.1	0.8 ± 0.2	1.3 ± 0.2
6	Acetyl carnitine	8.0	204.12310	85.02834	145.04955	60.08047	0.3 ± 0.1	0.9 ± 0.1	0.3 ± 0.1	1.0 ± 0.1	1.3 ± 0.2
7	Carnitine	8.4	162.11245	103.03891	60.08050	85.02836	0.4 ± 0.2	1.0 ± 0.1	0.5 ± 0.1	1.0 ± 0.02	1.0 ± 0.03
8	Thiamine	8.8	265.11148	122.07133	144.04787	156.04790	0.3 ± 0.1	N.D.	0.3 ± 0.1	0.9 ± 0.3	1.0 ± 0.1
9	U171 <sup>a</sup>	9.4	171.11242	124.08679	85.07588	109.07594	0.2 ± 0.1	1.1 ± 0.3	0.4 ± 0.2	N.D.	1.7 ± 0.6
10	U146 <sup>a</sup>	9.7	146.11742	87.04407	60.08052		0.3 ± 0.1	0.9 ± 0.1	0.4 ± 0.2	1.0 ± 0.1	1.1 ± 0.1
11	U160 <sup>a</sup>	10.0	160.13317	101.05978	60.08054		0.2 ± 0.1	0.7 ± 0.1	0.4 ± 0.2	1.2 ± 0.2	1.1 ± 0.2

N.D., not detected

<sup>a</sup> structurally unidentified<sup>b</sup> Mean fold change and standard errors were calculated in humans using the individual fold change of the peak area, as the human study was performed in a crossover fashion<sup>c</sup> The mean fold change in the mouse was represented as the ratio of the mean peak area in control and PYR-treated groups, and standard errors were estimated by propagation of errors



**Fig. 1** Effect of PYR on plasma concentration and cumulative urinary excretion of thiamine, carnitine, acetylcarnitine, and dCyd in healthy subjects. Metformin was administered orally at a dose of 0.1 mg (Periods 1 and 2) or 250 mg (Periods 3 and 4) 1 h after the oral administration of PYR. **(a)** The plasma concentrations and **(b)** urinary excretion amount ( $X_{urine}$ ) in subjects in the control groups (Periods 1 and 3; open circles and open bars) and the PYR-treatment groups (Periods 2 and 4; closed circles and closed bars) were measured using LC-MS/MS. Each point and vertical bar represent the mean  $\pm$  S.E.M. ( $n = 8$ ). \*  $P < 0.05$ ; \*\*  $P < 0.01$  versus control.

plasma was below the lower limit of quantification (0.3 ng/mL). This result is presumably due to its unstable nature in plasma. The stability of dCyd in human plasma was determined *in vitro* using cryopreserved human plasma. The residual amount of exogenous dCyd added to plasma (48 ng/mL) was decreased to 2.2% at an ambient temperature, and even to 28% on ice after 2 h. The plasma concentration of thiamine remained constant until 8 h during both the control and the PYR-treatment periods (Fig. 1a). The 12 h-samples from the control showed a slight elevation. The AUC did not differ between the control and the PYR-treatment periods except during the interval of 7–13 h falling within Periods 3 and 4 (Table II). No significant diurnal rhythms in the plasma carnitine level were observed (Fig. 1a). The inter-individual variation in the carnitine level (CV, 20%) was larger than the diurnal rhythm (CV, 6%). The plasma acetylcarnitine level tended to be higher in the morning. The AUCs of plasma carnitine and acetylcarnitine during the PYR-treatment periods were 15% lower than

those during the untreated periods (Table II). The  $X_{urine}$  of the four compounds including dCyd is shown in Fig. 1b. The  $X_{urine}$  of all four compounds were markedly lower during the PYR-treatment periods (Periods 2 and 4) compared with that during the untreated periods (Periods 1 and 3); these results agree with the results of the metabolomic analysis. The  $CL_R$  of thiamine, carnitine and acetylcarnitine were significantly reduced by 70%–84%, 90%–94% and 87%–91%, respectively, during the PYR-treatment periods, compared with the corresponding values during the untreated periods (Table II).

#### Uptake of Thiamine by HEK293 Cells Expressing MATE1 or MATE2-K

The uptakes of [ $^3$ H]thiamine (10 nM) and [ $^{14}$ C]TEA (a prototypical MATE substrate; 36  $\mu$ M) in MATE1- or MATE2-K-expressing cells were significantly greater than those in vector-transfected control cells (Fig. 2a). These

**Table II** Area Under the Plasma Concentration Time Curve and Renal Clearance of Thiamine, Carnitine and Acetylcarnitine in Controls and in Pyrimethamine-Treated Healthy Volunteers

Compound Parameter	Interval (h)	Period	Control	Period	PYR-treatment		
Thiamine							
AUC (nmol·h/L)	1–7	1	35.0±2.0	2	35.8±1.8		
		3	37.6±2.1	4	33.7±1.8		
	7–13	1	43.1±3.0	2	40.8±2.2		
		3	44.6±2.3	4	35.6±2.0	**	
	CL <sub>R</sub> (mL/min)	1–7	1	32.3±7.4	2	9.54±2.46	**
			3	58.6±9.4	4	9.55±1.85	**
7–13		1	48.6±6.2	2	8.14±0.92	**	
		3	48.1±8.3	4	10.7±3.1	**	
Carnitine							
AUC (μmol·h/L)	1–7	1	315±19	2	266±27	**	
		3	317±17	4	282±19	*	
	7–13	1	326±24	2	266±30	**	
		3	347±23	4	288±25	**	
	CL <sub>R</sub> (mL/min)	1–7	1	3.31±0.65	2	0.333±0.196	**
			3	2.49±0.75	4	0.242±0.076	*
7–13		1	2.70±0.56	2	0.172±0.087	**	
		3	2.37±0.57	4	0.180±0.042	**	
Acetylcarnitine							
AUC (μmol·h/L)	1–7	1	43.8±6.5	2	34.5±5.0	*	
		3	44.6±6.0	4	37.4±5.8	*	
	7–13	1	33.6±4.0	2	27.5±4.3	**	
		3	33.4±4.1	4	28.6±4.1	**	
	CL <sub>R</sub> (mL/min)	1–7	1	6.67±2.04	2	0.881±0.506	**
			3	6.13±2.08	4	0.746±0.314	*
7–13		1	6.01±1.31	2	0.535±0.266	**	
		3	5.55±1.22	4	0.645±0.145	**	

Healthy subjects received microdose metformin (100 μg, po) in period 1, microdose metformin plus pyrimethamine (50 mg, po) in period 2, therapeutic dose of metformin (250 mg, po) in period 3, and therapeutic dose of metformin and pyrimethamine in period 4. The kinetic parameters were calculated as described in the [MATERIALS AND METHODS](#)

AUC the area under the plasma concentration-time curve, CL<sub>R</sub> renal clearance

Data are presented as the mean ± S.E.M. (n=8)

\*P < 0.05, \*\*P < 0.01 versus control group

uptakes were decreased or saturated in the presence of an excess amount of non-labeled TEA. The uptake of thiamine by MATE1- or MATE2-K-expressing cells consisted of a saturable uptake and nonsaturable uptake (Fig. 2b and c). The kinetic parameters obtained by fitting were as follows: K<sub>m</sub> 3.54±1.02 and 3.94±0.79 μM; V<sub>max</sub> 105±25 and 50.7±9.3 pmol/2 min/mg protein; and P<sub>diff</sub> 3.87±0.66 and 3.55±0.27 μL/2 min/mg protein for MATE1 and MATE2-K. [<sup>3</sup>H]Carnitine uptake by MATE1- and MATE2-K-expressing cells was similar to that by mock-vector transfected cells (data not shown). Because of high uptake of dCyd in mock-vector transfected cells, there was no significant difference in the uptake of dCyd between mock-vector transfected and MATE expressing cells.

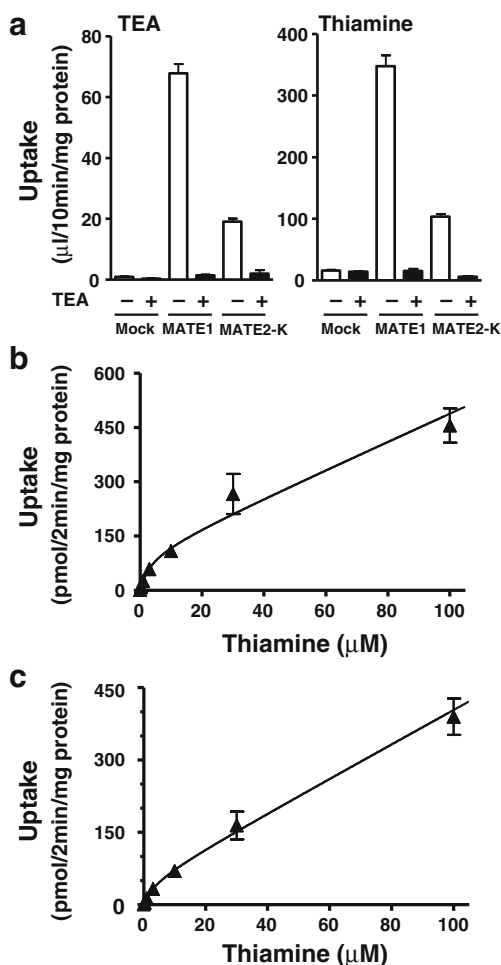
### Uptake of Thiamine by HEK293 Cells Expressing OCT2

The uptakes of [<sup>3</sup>H]thiamine (10 nM) in OCT2-expressing cells were significantly greater than those in vector-transfected

control cells (Fig. 3a). The uptake of [<sup>3</sup>H]thiamine by OCT2-expressing cells was markedly decreased in the presence of an excess amount of non-labeled TEA (5 mM). The uptake of thiamine by OCT2 was saturable (Fig. 3b), and the K<sub>m</sub> and V<sub>max</sub> values were 59.9±6.7 μM and 1.09±0.01 nmol/2 min/mg protein, respectively.

### Effect of PYR on Disposition of Carnitine in Mice

PYR (0.5 mg/kg) was administered to mice in the PYR-treatment group 30 min before dosing with deuterium-labeled carnitine (carnitine-d<sub>3</sub>). The unbound PYR concentration in the kidney was comparable to the K<sub>i</sub> value for mouse Mate1 (145 nmol/L) (11). The plasma concentration of carnitine-d<sub>3</sub> did not differ significantly between the control group and the PYR-treatment group except at 120 min after carnitine-d<sub>3</sub> dosing (Fig. 4a). The CL<sub>tot</sub> were 17.0 and 15.5 mL/min/kg in the control group and the PYR-treatment group, respectively. The cumulative X<sub>urine</sub> of carnitine-d<sub>3</sub> was significantly reduced by the PYR-treatment



**Fig. 2** Uptake of TEA and thiamine by HEK293 cells expressing MATE1 and MATE2-K. **(a)** HEK293 cells expressing MATE1 or MATE2-K were incubated with [ $^{14}\text{C}$ ]TEA (36  $\mu\text{M}$ ) or [ $^3\text{H}$ ]thiamine (10 nM) at 37°C for 10 min in the presence (closed bars) or absence (open bars) of TEA (5 mM) after intracellular acidification by preincubation in the presence of  $\text{NH}_4\text{Cl}$  (20 mM). The concentration dependence of the transporter activities in HEK293 cells expressing **(b)** MATE1 or **(c)** MATE2-K is shown. The solid lines represent the fitted line obtained by nonlinear regression analysis based on Eq. 1 as described under the “Materials and Methods” section. Data are expressed as the mean  $\pm$  S.E.M. ( $n = 3$ ).

(Fig. 4b). The  $\text{CL}_R$  was reduced by 62% in the PYR-treatment group (0.28 mL/min/kg), compared with that in the control group (0.74 mL/min/kg). Acyl forms derived from carnitine- $d_3$  in the samples collected at 120 min were detected by the metabolomic analysis. The relative urinary excretions of acetylcarnitine- $d_3$ , propionycarnitine- $d_3$ , and butyrylcarnitine- $d_3$  in the PYR-treatment group were reduced by more than half of the value in the control, in addition to the exertion of free carnitine- $d_3$  (Fig. 4c). On the other hand, the relative plasma and kidney levels of acylcarnitines- $d_3$  were slightly higher in the PYR-treatment group or were similar in both groups (Figs. 4d and e). Thiamine- $d_3$  was also administered to the mice as a continuous infusion at rate of 1.86 nmol/kg/min, but its urinary excretion level was below

the lower limit of quantification. PYR did not affect the plasma-concentration time profiles of thiamine- $d_3$  (data not shown).

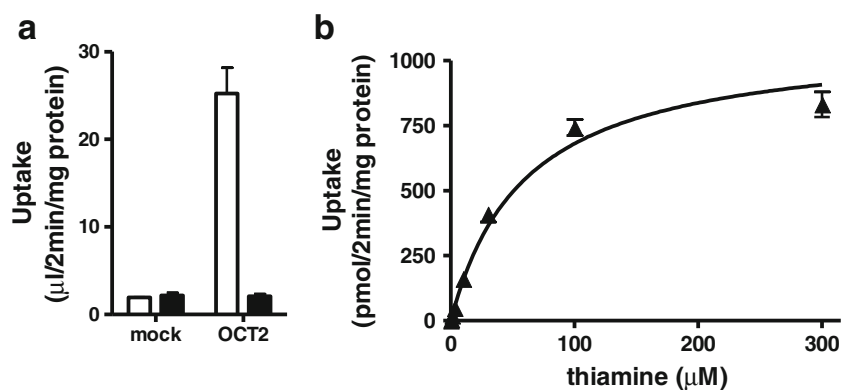
## DISCUSSION

There is growing concern on the probe substrate and *in vivo* inhibitors for drug transporters since the U S Food and Drug Administration has just released draft guidance on drug interaction studies which proposes to conduct pharmacokinetic interaction studies for drug candidates in humans with *in vivo* inhibitors and probe drugs to evaluate the magnitude of the interaction during drug discovery and development when the risk is suggested by preclinical studies (25). The present study was designed to elucidate the endogenous substrates of MATEs using metabolomic analyses of clinical samples obtained from pharmacokinetic interaction studies of metformin with PYR in healthy subjects, where  $\text{CL}_R$  of metformin was decreased by  $\sim 34\%$  (13).

Untargeted metabolomic analyses of urine specimens from healthy subjects and mice treated with or without PYR picked out 11 compounds as candidates for MATE substrates; PYR treatment reduced the  $X_{\text{urine}}$  of each of these compounds by 50% or more (Table I). We were able to identify six of these compounds as NMN, thiamine, carnitine, acetylcarnitine, propionycarnitine and dCyd. Thiamine is a vitamin (vitamin B1) that is essential for carbohydrate metabolism and neural function (26), and carnitine is indispensable in the transport of long chain fatty acids into mitochondria for the production of cellular energy by  $\beta$ -oxidation (27). Carnitine exists in the body as its free form or as acylcarnitines, which are conjugates produced from carnitine and acyl-CoA by carnitine acyltransferase. dCyd, a pyrimidine deoxyribonucleoside, is recycled in nucleotide synthesis via a salvage pathway or is biotransformed into deoxyuridine, followed by catabolism (28).

The plasma concentration-time profiles and the  $X_{\text{urine}}$  of thiamine, carnitine, acetylcarnitine, and dCyd were quantified (Fig. 1). The plasma concentration and  $\text{CL}_R$  obtained in the control groups were comparable to previously reported values (29–31). The plasma concentration of dCyd was undetectable presumably because of its rapid conversion by cytidine deaminase in plasma (32). The plasma concentrations of thiamine and acetylcarnitine showed weak rhythmic patterns, increasing or decreasing along with time, respectively, while that of carnitine was constant at least during the first 12 h. The  $X_{\text{urine}}$  of these compounds, including dCyd, was markedly lower in the PYR treatment group. Since the plasma concentrations of thiamine, carnitine and acetylcarnitine were slightly lower in the subjects treated with PYR, the reduced urinary loss may partly result from lower plasma concentrations. However, the fact that PYR-treatment

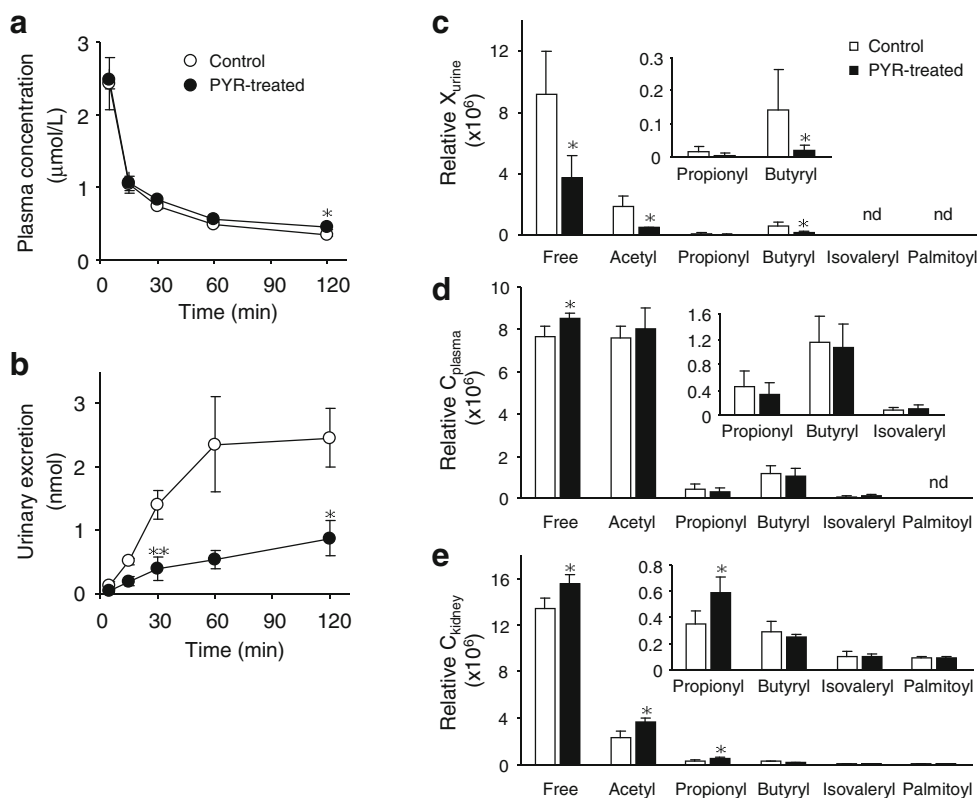




**Fig. 3** Uptake of thiamine by HEK293 cells expressing OCT2. **(a)** HEK293 cells expressing OCT2 were incubated with [ $^3$ H]thiamine (10 nM) at 37°C for 2 min in the presence (closed bars) or absence (open bars) of TEA (5 mM). **(b)** The concentration dependence of the uptake by OCT2 is shown. The solid lines represent the fitted line obtained by nonlinear regression analysis based on Eq. 1 as described under the “Materials and Methods” section. Data are expressed as the mean  $\pm$  S.E.M. ( $n=3$ ).

reduced the  $CL_R$  of thiamine, carnitine and acetylcarnitine by approximately 80%, 90% and 90%, respectively, indicates that PYR inhibits the urinary excretion of these compounds (Table II).

Thiamine was reported to be a substrate of both MATE1 and MATE2-K in an *in vitro* transport study that used MATE1- and MATE2-K-expressing cells (3). This finding was reproduced in the present study (Fig. 2). In another study

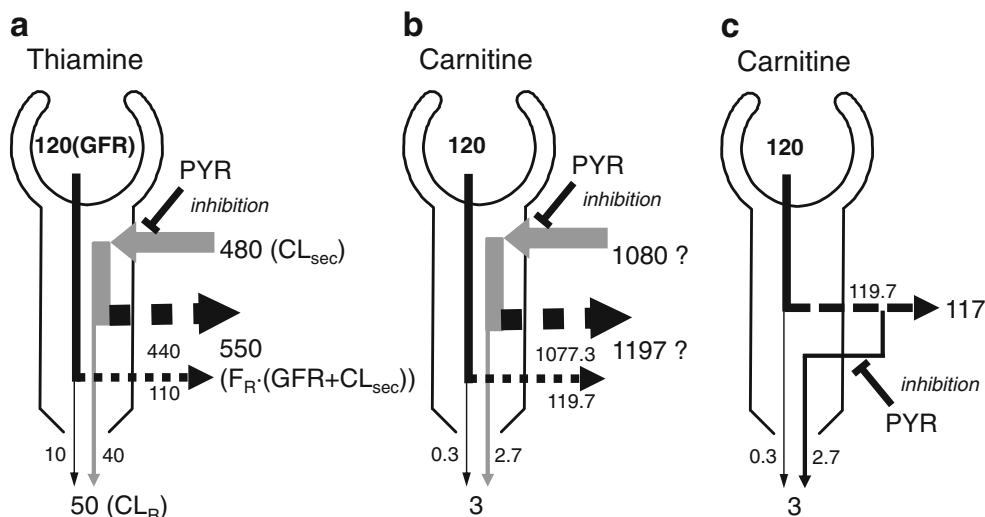


**Fig. 4** Effect of PYR on the disposition of carnitine- $d_3$  in mice after dosing with carnitine- $d_3$ . PYR (0.5 mg/kg) was administered to mouse intravenously 30 min before a bolus injection of carnitine- $d_3$  (0.25 mg/kg). **(a)** The plasma concentrations and **(b)** urinary excretion amount ( $X_{urine}$ ) of carnitine- $d_3$  were measured using LC-MS/MS in the control group (open circles) and the PYR-treatment group (closed circles) at designated time points. The bladder urine **(c)**, plasma **(d)**, and kidney **(e)** samples obtained 120 min after dosing with carnitine- $d_3$  were subjected to metabolomic analysis. The bladder urine **(c)**, plasma **(d)** and kidney **(e)** samples obtained 120 min after dosing with carnitine- $d_3$  were subjected to the metabolomic analysis. **(c)** The relative  $X_{urine}$  for carnitine- $d_3$  and its acyl forms represents the product of the urinary volume (mL) and each peak area expressed in an arbitrary unit. **(d)** The relative plasma concentration ( $C_{plasma}$ ) and **(e)** relative kidney concentration ( $C_{kidney}$ ) represent each peak area. Data are expressed as the mean  $\pm$  S.E.M. of three or four mice. \*  $P < 0.05$ ; \*\*  $P < 0.01$  versus control.

that used the BBMV of the rat kidney, thiamine/ $H^+$  antiport was observed (33). These *in vitro* studies also showed that thiamine can be used as a biomarker for evaluating the drug interactions in the urinary excretion that involves MATEs. Conversely, whether carnitine, acylcarnitine, and dCyd are MATE biomarkers remains controversial. The specific uptake of carnitine was not observed in either MATE1- or MATE2-K-expressing cells (data not shown), which agrees with the results of a previous study (3). However, consistent with the clinical observation, the  $CL_R$  of deuterium-labeled carnitine was reduced by PYR, and the amount of acetylcarnitine excreted into the urine was also decreased by PYR, whereas PYR had no effect on the plasma acetylcarnitine level and on the ratio of acetylcarnitine to free carnitine in mice (Fig. 4). Therefore, it is most likely that the reduced  $CL_R$  of carnitine and acetylcarnitine is attributable to the effect of PYR on MATE1 in the mouse, but not on the biosynthesis of carnitine and its interconversion between carnitine and acetylcarnitine in mouse. Previously, we reported that the specific uptake of cephalixin was not observed in cDNA-transfected cells; however, both PYR and cimetidine significantly elevated the kidney concentration of cephalixin (12). A marked delay in the urinary excretion of cephalixin was observed in a MATE1 knockout mouse, which supports the predominant role of MATE1 in the urinary excretion of cephalixin (34). That is, the absence of specific uptake of carnitine in the cDNA-transfected cells does not necessarily rule out the possibility that MATEs mediate its efflux into the urine. As for dCyd, there are no additional

data supporting its application as a MATE biomarker because of its instability.

The kidney plays an important role in regulating the body homeostasis of thiamine, carnitine, and acetylcarnitine by extensive reabsorption from the urine after filtration in glomeruli. In fact, the  $CL_R$  of thiamine, carnitine, and acetylcarnitine ( $\sim 50$ ,  $\sim 3$ , and  $\sim 6$  mL/min, respectively) was below the glomerular filtration rate (GFR), given that the GFR is 120 mL/min. The transporters involved in the process of reabsorption of thiamine and carnitine/acetylcarnitine are the thiamine transporters 1 and 2 (THTR1/*SLC19A2* and THTR2/*SLC19A3*) (35) and a high-affinity  $Na^+$ /L-carnitine cotransporter, OCTN2/*SLC22A5* (36), respectively; these transporters are expressed in the BBM of the proximal tubules. Similarly, an equilibrative nucleoside transporter (ENT1/*SLC29A1*) and concentrative nucleoside transporters (CNT1/*SLC28A1*, CNT2/*SLC28A2*, and CNT3/*SLC28A3*) (37) may mediate the reabsorption of dCyd for nucleoside salvage in the human BBM. Given that PYR completely inhibits the efflux across the BBM in the proximal tubules, but does not affect the reabsorption process, and that biosynthesis and metabolism in the kidney are negligible, the fractions of reabsorption can be estimated by comparing the renal clearance when PYR is given with GFR (Fig. 5a). Based on the estimated fraction of reabsorption, it is suggested that the clearance of thiamine via tubular secretion is 4-fold greater than the GFR. In fact, together with the increase in its plasma concentration, the  $CL_R$  of thiamine increases and becomes close to the renal plasma flow rate in humans because of the



**Fig. 5** Proposed schematic diagrams showing the disposition of thiamine and carnitine in the kidney in humans. The renal clearance ( $CL_R$ ) of (a) thiamine and (b) carnitine was defined as follows:  $CL_R = (GFR + CL_{sec}) \cdot (1 - F_R)$ , where GFR,  $CL_{sec}$ , and  $F_R$  represent the glomerular filtration rate, tubular secretion clearance, and fraction of reabsorption from the urine to the blood, respectively. For carnitine (c), another hypothetical diagram was also designed assuming negligible uptake of the compound from the basolateral side. The diagram includes rough estimates of the contribution of the glomerular filtration (black arrows), tubular secretion (gray arrows), and reabsorption (dotted line) under the following assumptions: PYR does not affect GFR and reabsorption [ $F_R \cdot (GFR + CL_{sec})$ ]. The units of GFR and  $CL_{sec}$  are mL/min.

saturation of its reabsorption (38). Of note, it has been reported that specific uptake of thiamine by OCT2 was not observed in cDNA-transfected HeLa cells (39); in contrast, we demonstrated that thiamine is a substrate of OCT2 (Fig. 3). The reason for this discrepancy remains unknown. However, this finding supports the efficient tubular secretion of thiamine upon saturation of reabsorption reported previously. We propose that the removal of thiamine from the systemic circulation when it is present in the blood circulation at high concentrations, to saturate its reabsorption, is one of the physiological roles of OCT2 and MATE in the kidney. This concept needs further verification by examining the effect of PYR in individuals receiving thiamine supplementation. Similarly, carnitine was estimated to be secreted at 9-fold the GFR (Fig. 5b). However, unlike what was observed for thiamine, there is no clinical evidence that carnitine undergoes tubular secretion in the kidney. Even when a high dose of carnitine is administered, the renal carnitine clearance is below the GFR (40). Therefore, another hypothetical diagram assuming negligible uptake from the basolateral side (Fig. 5c) may be appropriate for carnitine. In addition, taking the biosynthesis and metabolism of carnitine in the kidney into consideration will improve the estimates of clearance in the diagram. Further investigations are needed to propose a kinetic model of carnitine disposition in the kidney.

Evaluating the DDI risk involving MATEs is an important issue in the avoidance of severe DDIs since the inhibition of MATEs causes an elevation of the tissue concentrations of the victim drugs, and consequently potentiates its effect in the kidney. Several endogenous compounds with different kinetic characteristics are now available for this purpose. NMN and creatinine are secretion type, whereas thiamine is reabsorption type. The plasma levels of NMN show circadian rhythm for 12 h (14), whereas that of thiamine showed weak circadian rhythm. The fact that the  $CL_R$  of NMN and thiamine is more susceptible to the changes in the activities of MATEs, which showed a 70–80% reduction, than is metformin (34%, (13)) supports their usefulness as MATE biomarkers. It is obvious that use of endogenous compounds as probe can save time and the expense of additional clinical studies to evaluate the risk of DDIs during drug development. Further studies are necessary to establish such endogenous substrates for other drug transporters.

## CONCLUSION

Our findings suggest that MATEs account for the efflux of thiamine, and perhaps carnitine, into the urine. Urinary excretion of thiamine is a useful biomarker for assessing the inhibitory potential of investigational drugs toward MATEs in preclinical and early clinical studies of drug development.

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