

# Identification of Appropriate Endogenous Biomarker for Risk Assessment of Multidrug and Toxin Extrusion Protein-Mediated Drug-Drug Interactions in Healthy Volunteers

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Endogenous biomarkers are emerging to advance clinical drug-drug interaction (DDI) risk assessment in drug development. Twelve healthy subjects received a multidrug and toxin exclusion protein (MATE) inhibitor (pyrimethamine, 10, 25, and 75 mg) in a crossover fashion to identify an appropriate endogenous biomarker to assess MATE1/2-K-mediated DDI in the kidneys. Metformin (500 mg) was also given as reference probe drug for MATE1/2-K. In addition to the previously reported endogenous biomarker candidates (creatinine and *N*<sup>1</sup>-methylnicotinamide (1-NMN)), *N*<sup>1</sup>-methyladenosine (*m*<sup>1</sup>A) was included as novel biomarkers. 1-NMN and *m*<sup>1</sup>A presented as superior MATE1/2-K biomarkers since changes in their renal clearance ( $CL_r$ ) along with pyrimethamine dose were well-correlated with metformin  $CL_r$  changes. The  $CL_r$  of creatinine was reduced by pyrimethamine, however, its changes poorly correlated with metformin  $CL_r$  changes. Nonlinear regression analysis ( $CL_r$  vs. mean total concentration of pyrimethamine in plasma) yielded an estimate of the inhibition constant ( $K_i$ ) of pyrimethamine and the fraction of the clearance pathway sensitive to pyrimethamine. The *in vivo*  $K_i$  value thus obtained was further converted to unbound  $K_i$  using plasma unbound fraction of pyrimethamine, which was comparable to the *in vitro*  $K_i$  for MATE1 (1-NMN) and MATE2-K (1-NMN and *m*<sup>1</sup>A). It is concluded that 1-NMN and *m*<sup>1</sup>A  $CL_r$  can be leveraged as quantitative MATE1/2-K biomarkers for DDI risk assessment in healthy volunteers.

## Study Highlights

### WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Drug-drug interactions (DDIs) involving renal MATE1/2-K inhibition, impacting metformin pharmacokinetics, are important. Therefore, regulators mandate clinical metformin DDI studies when investigational drugs trigger conservative agency DDI risk thresholds (e.g., projected peak plasma concentration ( $C_{max,u}$ )/*in vitro* MATE1/2-K half-maximal inhibitory concentration ratio > 0.1).

### WHAT QUESTION DID THIS STUDY ADDRESS?

☑ Could an endogenous MATE1/2-K substrate serve as a clinical biomarker to support inhibition assessment, de-risk metformin DDI, and obviate the need for a formal DDI study?

### WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ Beyond creatinine, two endogenous MATE1/2-K substrates (*N*<sup>1</sup>-methyladenosine (*m*<sup>1</sup>A) and *N*<sup>1</sup>-methylnicotinamide

(1-NMN)) present as superior urinary biomarkers. Both support MATE1/2-K inhibition assessment and the generation of *in vivo* inhibition constant ( $K_i$ ) values.

### HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ Perpetrator dose-dependent inhibition of renal MATE1/2-K can be assessed using *m*<sup>1</sup>A and/or 1-NMN. Once generated, *in vivo*  $K_i$  values could be input for physiologically-based pharmacokinetic models describing metformin DDI. The output of such models can drive the data translation (e.g., *in vitro* to *in vivo* for MATE1/2-K inhibition) and enable decision making (e.g., the need for/design of a metformin DDI study).

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To ensure safety in drug therapy upon concomitant use of drugs, drug-drug interaction (DDI) risks of new chemical entities with major drug-metabolizing enzymes and transporters are routinely assessed in drug development by mechanism-based approaches. Typically, such approaches involve the prediction of DDI based on *in vitro* data and clinical assessment using recommended probe drugs.<sup>1–3</sup> However, it is generally accepted that the use of *in vitro* data can render both significant false-positive and, to a lesser degree, false-negative predictions.<sup>4</sup> Recently, endogenous biomarkers, generally physiological substrates of drug-metabolizing enzymes and transporters, are emerging as useful tools to advance DDI risk assessment of investigational drugs in early phases of clinical development before conducting the clinical DDI study using the recommended probe drugs.<sup>2,4,5</sup> Application of endogenous biomarkers has been expanded to encompass complex DDIs involving multiple potential interaction sites with investigational drug.<sup>6</sup> Although useful, biomarkers require validation and characterization in terms of their ease of analysis, sample requirements, sensitivity, dynamic range, significant correlation with pharmacokinetic parameters of probe drugs, as well as variability (e.g., diurnal, interindividual and intra-individual differences).<sup>4</sup>

The kidneys are essential organs for eliminating drugs and metabolites into the urine from the blood circulation. In addition to glomerular filtration, drugs are eliminated into the urine by drug transport systems in the proximal tubules, which consist of OCT2, and MATE1 and MATE2-K mediating the uptake and subsequent efflux of water-soluble cationic compounds, respectively.<sup>7–9</sup> An oral antidiabetic, metformin, is a well-characterized substrate of these transporters, and serves as a typical probe for assessing potential OCT2 and/or MATE1/2-K associated DDIs.<sup>10</sup> In addition, endogenous metabolites, such as creatinine and *N*<sup>1</sup>-methylnicotinamide (1-NMN), have been also identified as substrates of OCT2 and MATEs.<sup>11–15</sup> Creatinine clearance serves as an index of kidney function.<sup>16</sup> On the other hand, drugs known to be inhibitors of OCT2 and MATEs have been shown to cause reversible elevation of serum creatinine without kidney injury,<sup>17,18</sup> such as cimetidine, trimethoprim, and pyrimethamine that also reduce the renal clearance of metformin at their therapeutic doses.<sup>19–22</sup> The 1-NMN, an endogenous metabolite of nicotinamide (also known as vitamin B3 or niacin) formed by *N*-methylation,<sup>23</sup> is also known to be secreted into the urine by renal organic cation transporters.<sup>15,24</sup> Additionally, based on the genomewide association study of serum metabolite profiles,<sup>25</sup> we recently identified *N*<sup>1</sup>-methyladenosine (*m*<sup>1</sup>A), which is considered derived from transfer RNA,<sup>26</sup> as a novel endogenous substrate of OCT2 and MATE1/2-K that undergoes significant tubular secretion in the kidneys of humans.<sup>27</sup> Administration of DX-619, an inhibitor of OCT2 and MATE1/2-K,<sup>28</sup> delayed the elimination of *m*<sup>1</sup>A in monkeys,<sup>27</sup> supporting that it can serve as a DDI biomarker.

Correlation of the pharmacokinetic parameters, such as clearance or area under the plasma concentration time curve (AUC), between endogenous substrates and the typical probe drug when transporters are inhibited in the presence of a perpetrator in a dose-dependent manner is an important criterion to convince investigators of the appropriateness of a particular biomarker.<sup>29,30</sup> To

date, however, no such data are available for biomarkers of renal OCT2 and MATE1/2-K. Therefore, we designed a clinical study to examine the dose-dependent effect of pyrimethamine on the pharmacokinetic parameters of MATE1/2-K substrates in a crossover fashion. Pyrimethamine is a potent MATE1/2-K inhibitor characterized by its long elimination half-life (ca. 100 hours) in humans, which provides constant inhibition of MATE1/2-K during a metformin DDI study.<sup>19</sup> In earlier clinical studies with healthy subjects, the pyrimethamine dose was 50 mg (single dose), which caused a slight reduction in renal clearance ( $CL_r$ ) of metformin (at most, 35% inhibition),<sup>19</sup> but a reduction of  $CL_r$  by 70% that represented an almost complete inhibition of 1-NMN renal secretory clearance.<sup>15</sup> In a later study by Oh *et al.*,<sup>20</sup> two doses of pyrimethamine 50 mg administered 12 hours apart resulted in stronger impact on metformin AUC and  $CL_r$ . Based on these clinical studies, pyrimethamine was given orally at 3 different doses, 10, 25, and 75 mg to explore the dose-dependent inhibition of renal organic cation transporters. In this regard, the use of biomarkers to assess the dose-dependent inhibition of liver (OATP1B by rifampicin) and renal (OAT by probenecid) transporters has already been described.<sup>29–31</sup> In addition to MATE1/2-K substrates, the AUC and  $CL_r$  of pyridoxic acid (PDA) was also determined to examine the effect of pyrimethamine on renal organic anion transporters.

## METHODS

### Chemicals

All authentic compounds, reagents, and organic solvents were of a commercially available analytical grade. Purchase sources of authentic compounds are listed in **Supplementary Methods**.

### Clinical study design

This study was conducted following the Clinical Trials Act, Japan. The study protocol was reviewed by Certified Review Board, the Graduate School of Medicine, The University of Tokyo (CRB3180024). The study is registered as the specified clinical trial in Japan Registry of Clinical Trials (<https://jrct.niph.go.jp/en/latest-detail/jRCTs031180125>). Written informed consent was provided by all participants prior to their inclusion in the study. The study was an open-label, dual-sequence, four-phase crossover study in 12 healthy Japanese male participants. The schedules of dosing and sampling are both described in **Supplementary Methods**.

### Quantification of test compounds by liquid chromatography with tandem mass spectrometry

Plasma and urine samples analyzed by liquid chromatography with tandem mass spectrometry were prepared by protein precipitation as described previously.<sup>27</sup> All compounds were separated and detected using the QTRAP5500 system (AB SCIEX, Toronto, Canada) equipped with the Nexera X2 LC system (Shimadzu, Kyoto, Japan), operated in electrospray ionization mode. Analyte quantification was performed using Analyst version 1.7 (Sciex, Toronto, ON, Canada). Creatinine and 1-NMN were analyzed using creatinine-*d*<sub>3</sub> and 1-NMN-*d*<sub>3</sub> as internal standards, respectively. The conditions of liquid chromatography, multiple reaction monitoring precursor/product ion transitions are summarized in **Supplementary Methods**.

### Pharmacokinetic analysis in the human clinical study

The area under the plasma concentration–time curve from zero to 24 hours ( $AUC_{0-24}$ ) was determined with the linear trapezoidal rule. However, the AUC of 1-NMN was calculated from time zero to 12 hours

postdose given that plasma concentrations of 1-NMN showed significant intraday variation, and AUC during 12–24 hours was determined only at 2 timepoints (12 and 24 hours), thus potentially impacting interpretation of  $CL_r$ .

The  $CL_r$  regarding plasma concentration of test compounds was determined by dividing the amount excreted into the urine from 0 to 24 hours ( $X_{urine}$ ) by the AUC value. For 1-NMN, AUC and the amount excreted into the urine from 0 to 12 hours were used.

$$CL_r = \frac{X_{urine}}{AUC}$$

$K_{i,app}$  (apparent inhibition constant of pyrimethamine as the total concentration) and  $f_{PYR}$  (the fraction sensitive to inhibition by pyrimethamine) were obtained by iterative nonlinear least squares method<sup>30</sup> (Prism8 version 8.4.1; GraphPad Software, La Jolla, CA) using the following equation under constraint of  $0 < f_{PYR} \leq 1$  and  $K_{i,app} > 0$ , where  $C_{av}$  represents the mean plasma concentrations of pyrimethamine in each subject:

$$CL_r \text{ ratio} = \frac{f_{PYR}}{1 + C_{av}/K_{i,app}} + (1 - f_{PYR})$$

$C_{av}$  was obtained by dividing AUC pyrimethamine with the duration time (12 hours for 1-NMN, otherwise 24 hours).

### Statistical analysis

Natural log-transformed of all parameters were analyzed by cohort using a mixed effect model with treatment as a fixed effect and subject as a random effect. Estimates of the adjusted mean differences (test-reference) and corresponding 90% confidence intervals (CIs) for AUC and  $CL_r$  were obtained from the model. The adjusted mean differences and 90% CIs for the differences were exponentiated to provide estimates of the ratio of adjusted geometric means (test/reference) and 90% CI for the ratios. The control phase (metformin alone) was the reference treatment for all analyses except for the assessment of metformin's impact on biomarkers, in which the baseline phase (placebo) was the reference. The values of plasma concentration and  $CL_r$  are presented as the mean  $\pm$  SEM.

## RESULTS

### Participant demographics

Twelve healthy male Japanese participants were enrolled into the study. The range for age, body mass index, height, and weight was 23–38 years, 20.9–24.1 kg/m<sup>2</sup>, 165–183 cm, and 59.5–75.8 kg, respectively. The participants did not manifest any abnormality by medical examination or blood or biochemical tests at baseline.

### Pyrimethamine plasma concentrations

Time profiles of the plasma concentrations of pyrimethamine are shown in **Figure S1**. The mean plasma concentrations ( $C_{av}$ ) were  $0.287 \pm 0.024$ ,  $0.781 \pm 0.031$ , and  $2.47 \pm 0.18$   $\mu$ M (mean  $\pm$  SEM) when the volunteers received 10, 25, and 75 mg single doses of pyrimethamine, respectively. The maximum plasma concentrations ( $C_{max}$ ) were  $0.390 \pm 0.047$ ,  $1.06 \pm 0.14$ , and  $3.15 \pm 0.072$   $\mu$ M (mean  $\pm$  SEM), respectively.

### Effect of pyrimethamine on the pharmacokinetics of metformin

The effect of pyrimethamine on the AUC geometric mean ratio ( $\pm$  90% CI) showed clear separation for metformin (**Table 1**).

Pyrimethamine 25 mg and 75 mg were shown to increase metformin AUC by 24% and 69%, respectively, although no impact was observed with pyrimethamine 10 mg (**Table 1**). However, pyrimethamine 10 mg, 25 mg, and 75 mg decreased metformin  $CL_r$  (**Figure 1b**) by 12%, 34%, and 55%, respectively (**Table 1**). The greatest impact on the urinary excretion of metformin occurred over 0–4 hours postdose (**Figure 1a**). Effect of pyrimethamine on other pharmacokinetic parameters are summarized in **Table S1**.

### Effect of pyrimethamine on the pharmacokinetics of endogenous compounds

Plasma concentration profiles and urinary excretion of m<sup>1</sup>A, creatinine, and 1-NMN following each dose of pyrimethamine are summarized in **Figure 2**. Under control conditions (metformin alone), plasma concentrations of m<sup>1</sup>A and creatinine were almost identical throughout the day, whereas those of 1-NMN exhibited marked intraday variation. Differences in m<sup>1</sup>A, creatinine, and 1-NMN concentrations were also observed between the baseline and control phases of the study (**Table 1**; **Figure 2**). The AUC ratio was unchanged or slightly higher in the pyrimethamine-treated groups for m<sup>1</sup>A and creatinine, respectively, whereas the AUC ratio of 1-NMN was rather lower in the pyrimethamine-treated groups (**Table 1**; **Figure 3a**). The  $CL_r$  of m<sup>1</sup>A, 1-NMN, and creatinine were decreased in a dose-dependent manner, although creatinine  $CL_r$  showed no further decrease beyond the 25 mg dose level (**Table 1**; **Figure 3a**).

The  $CL_r$  of 1-NMN was calculated using the plasma and urine data from time 0 to 12 hours because of sparse blood sampling between 12 and 24 hours to obtain reliable AUC. Correlation of  $CL_r$  calculated from time 0 to 12 or 24 hours are shown in **Figure S2**.

### Correlation on the pharmacokinetics of metformin and endogenous compounds

The  $CL_r$  ratio (+PYR/control) of endogenous compounds was compared with that of metformin and pairwise Pearson correlation coefficients ( $r$ ) were calculated (**Figure 3b**). The m<sup>1</sup>A and 1-NMN showed positive and good correlations with metformin ( $r^2 > 0.5$ ), whereas correlation between creatinine and metformin was not observed ( $r^2 = 0.11$ ).

### Nonlinear regression analysis of $CL_r$ ratio and pyrimethamine $C_{av}$

The relationship between  $CL_r$  ratio and pyrimethamine  $C_{av}$  (total concentration in plasma) was analyzed to obtain estimates of apparent inhibition constant ( $K_{i,app}$ ) and fraction sensitive to inhibition by pyrimethamine ( $f_{PYR}$ ; **Figure 4**). Curve fitting-based estimates of  $K_{i,app}$  and  $f_{PYR}$  are shown in **Table 2**. The  $CL_r$  of metformin, m<sup>1</sup>A, and 1-NMN appeared to be decreased according to pyrimethamine  $C_{av}$ . The  $f_{PYR}$  of metformin, m<sup>1</sup>A, and 1-NMN was almost identical ( $\sim 0.8$ ), whereas that of creatinine was much smaller (0.26).

### Determination of *in vitro* $K_i$ values of pyrimethamine

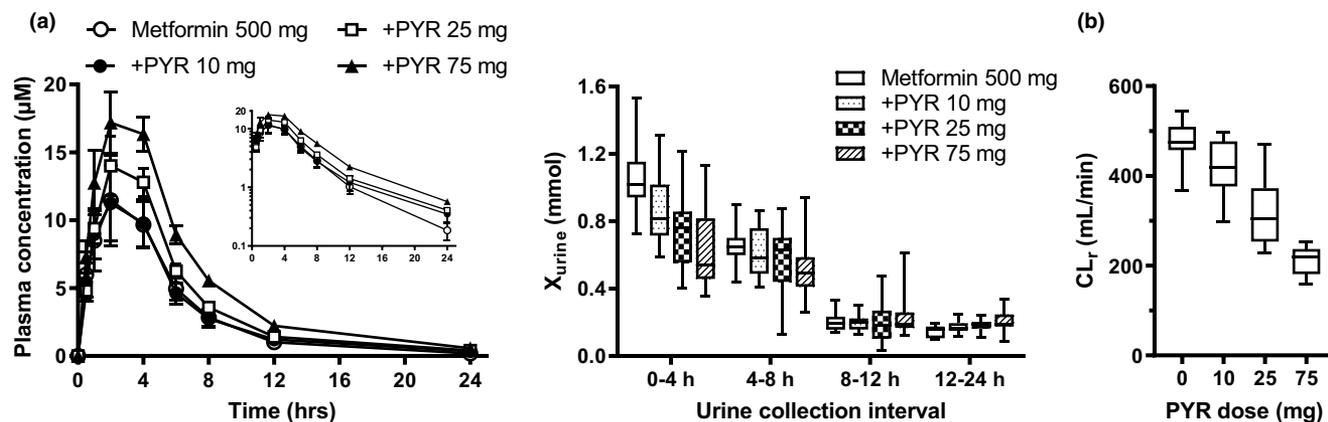
For the comparison with *in vivo*  $K_{i,app}$  values, the *in vitro*  $K_i$  values of pyrimethamine for OCT2 and MATE1/2-K-mediated substrate uptake were determined (**Table 2**; **Figure 5**). MATE1-mediated m<sup>1</sup>A uptake and MATE1/2-K-mediated creatinine

**Table 1 Adjusted geometric means and GMR (90% CI) for metformin and the endogenous biomarkers**

	Metformin			1-NMN <sup>a</sup>			m <sup>1</sup> A			Creatinine			PDA	
	AUC (μM × h)	CL <sub>r</sub> (ml/min)	CL <sub>r</sub> (ml/min)	AUC (nM × h)	CL <sub>r</sub> (ml/min)	CL <sub>r</sub> (ml/min)	AUC (nM × h)	CL <sub>r</sub> (ml/min)	CL <sub>r</sub> (ml/min)	AUC (μM × h)	CL <sub>r</sub> (ml/min)	AUC (nM × h)	CL <sub>r</sub> (ml/min)	
Baseline, placebo	N/A	N/A	413.2	386.8	413.2	174.5	2,673.1	174.5	131.1	2,370.8	131.1	N/D	N/D	
Control, metformin	72.5	468.3	388.8	652.6	388.8	161.6	2,489.9	161.6	126.3	2,119.6	126.3	291.2	545.1	
PYR 10 mg, + Met	74.2	411.6	360.0	402.6	360.0	145.0	2,576.0	145.0	103.8	2,494.9	103.8	214.2	584.6	
PYR 25 mg, + Met	89.8	308.9	247.2	361.4	247.2	128.3	2,694.6	128.3	95.3	2,545.3	95.3	177.2	603.1	
PYR 75 mg, + Met	122.9	210.2	161.3	451.2	161.3	84.9	2,654.5	84.9	95.2	2,303.1	95.2	164.7	423.7	
Control vs. baseline														
GMR	N/A	N/A	0.94	1.69	0.94	0.93	0.93	0.93	0.96	0.89	0.96	N/A	N/A	
90% CI	N/A	N/A	0.83, 1.07	1.39, 2.05	0.83, 1.07	0.84, 1.02	0.89, 0.98	0.84, 1.02	0.88, 1.05	0.86, 0.93	0.88, 1.05	N/A	N/A	
PYR 10 mg vs. control														
GMR	1.02	0.88	0.93	0.62	0.93	0.90	1.03	0.90	0.82	1.18	0.82	0.74	1.07	
90% CI	0.91, 1.15	0.80, 0.96	0.82, 1.05	0.51, 0.75	0.82, 1.05	0.82, 0.99	0.99, 1.08	0.82, 0.99	0.75, 0.90	1.13, 1.23	0.75, 0.90	0.52, 1.05	0.91, 1.26	
PYR 25 mg vs. control														
GMR	1.24	0.66	0.64	0.55	0.64	0.79	1.08	0.79	0.75	1.20	0.75	0.61	1.11	
90% CI	1.10, 1.39	0.60, 0.72	0.56, 0.72	0.49, 0.67	0.56, 0.72	0.72, 0.87	1.03, 1.13	0.72, 0.87	0.69, 0.83	1.15, 1.25	0.69, 0.83	0.43, 0.87	0.94, 1.30	
PYR 75 mg vs. control														
GMR	1.69	0.45	0.41	0.69	0.41	0.53	1.07	0.53	0.75	1.09	0.75	0.57	0.78	
90% CI	1.51, 1.90	0.41, 0.49	0.37, 0.47	0.57, 0.84	0.37, 0.47	0.48, 0.58	1.02, 1.12	0.48, 0.58	0.69, 0.82	1.04, 1.13	0.69, 0.82	0.40, 0.81	0.66, 0.92	

1-NMN, N1-methylnicotinamide; AUC, area under the plasma concentration–time curve; CI, confidence interval; CL<sub>r</sub>, renal clearance; GMR, geometric mean ratio; m<sup>1</sup>A, N<sup>1</sup>-methyladenosine; Met, metformin; N/A, not applicable; N/D, not determined; PDA, pyridoxic acid; PYR, pyrimethamine.

<sup>a</sup>Based on 12-hour data.



**Figure 1** Effect of pyrimethamine on the plasma concentrations, and amount excretion of metformin (a), and renal clearance (b). Plasma concentrations and urinary excretion of metformin were determined in the subjects treated with or without pyrimethamine (10, 25, and 75 mg). Each symbol represents the mean and SEM ( $n = 12$ ). Urinary excretion during the intervals designated was shown in box-and-whisker plot. (b) Renal clearance determined in healthy volunteers treated with or without an oral dose of pyrimethamine (10, 25, and 75 mg) were shown in box-and-whisker plots. +PYR, pyrimethamine administrated group.

uptake could not be observed in our expression system. The absolute values of the uptake are summarized in **Table S3**. After correction of the unbound fraction of pyrimethamine in the plasma ( $0.13^{20}$ ), the  $K_{i,app}$  was similar to the *in vitro*  $K_i$  values of metformin,  $m^1A$ , and 1-NMN for MATE1 and MATE2-K (**Table 2**).

#### Inhibition of OAT1 and OAT3 by pyrimethamine

As a negative control, we determined the  $CL_r$  of PDA, a pyridoxine metabolite. Renal OATs, OAT1 and OAT3, are considered to mediate tubular secretion of PDA.<sup>32,33</sup> Pyrimethamine is a weak OAT1 and OAT3 inhibitor at its therapeutic dose with half-maximal inhibitory concentration of  $241 \pm 55 \mu M$  and  $9.69 \pm 2.27 \mu M$ , respectively (**Figure S3**). Low inhibition potency to OAT3 was consistent with our previous report.<sup>34</sup> Pyrimethamine decreased the AUC of PDA in a dose-dependent manner, but was shown to weakly inhibit the PDA  $CL_r$  (22% decrease) at the highest dose (**Table 1** and **Figure S4**).

#### Effect of transporter genotype on the plasma concentrations of the probe drugs and endogenous substrates

Genetic polymorphisms have been reported to alter the kinetics of OCT2 and MATE substrate drugs. As such, genetic polymorphisms were assessed and pharmacokinetic profiles were segregated in terms of subject genotype. None of the tested polymorphisms, OCT2 rs316019, MATE1 rs2289669, rs2453579, rs2252281, and MATE2-K rs12943590, provided significant separation (**Figure S5**).

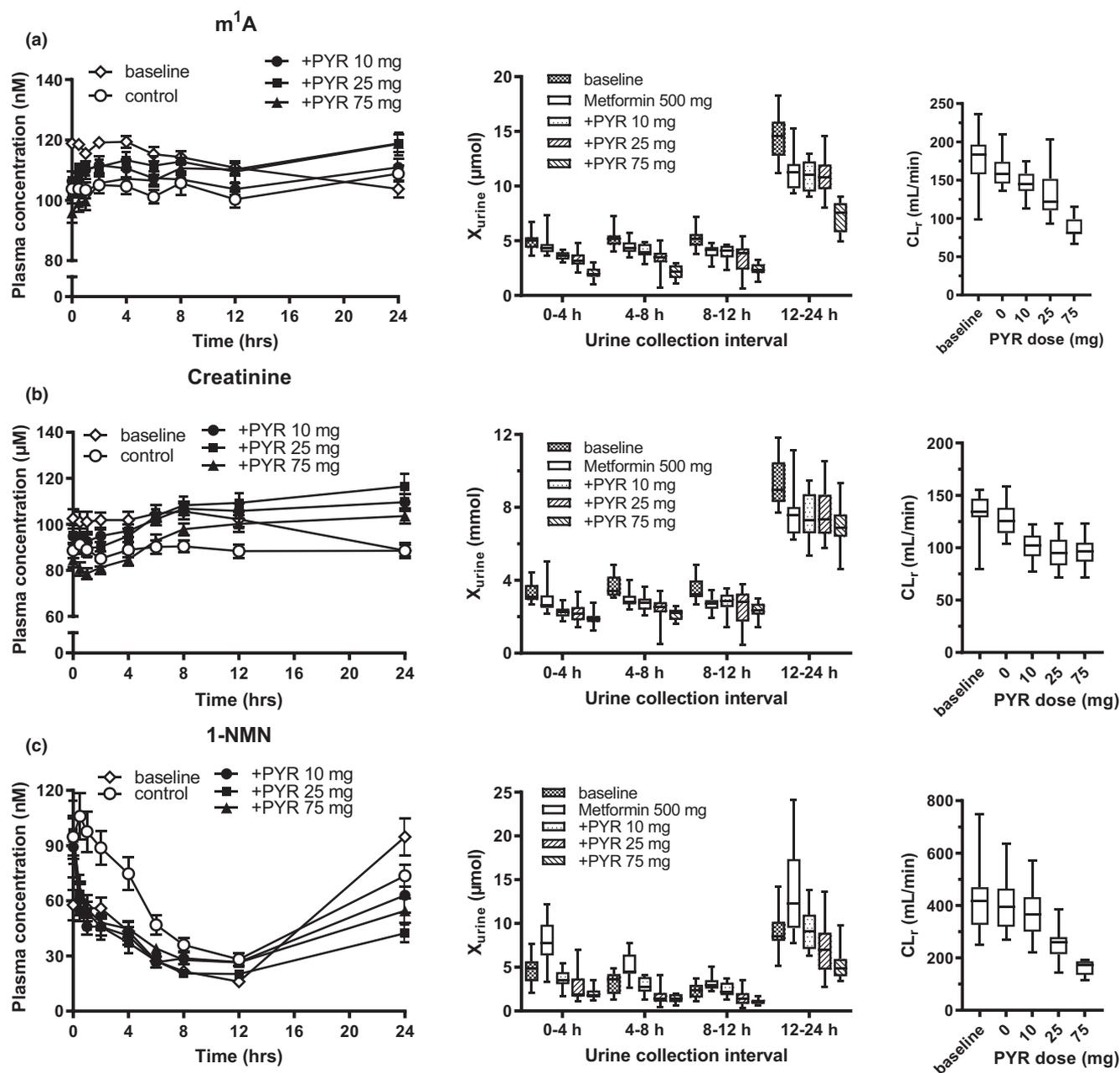
#### DISCUSSION

OCT2 and MATE1/2-K play a pivotal role in the renal elimination of water-soluble cationic compounds. This study aimed to identify endogenous biomarkers appropriate for assessing MATE1/2-K inhibition by examining the effect of increasing doses of pyrimethamine on their  $CL_r$  in comparison to metformin, a frequently used clinical probe to examine *in vivo* inhibition of either or both OCT2 and MATE1/2-K.

The decreases in metformin  $CL_r$  supported the dose-dependent inhibition of MATE1/2-K by pyrimethamine (**Table 1**;

**Figure 1**). In addition, pyrimethamine may weakly inhibit the oral absorption of metformin because the amount recovered in the urine over 24 hours divided by the dose ( $f_c$ ) and bioavailability ( $F$ ) were lower at the highest pyrimethamine dose compared in this phase (**Table S1**). The effect of pyrimethamine on the AUC and  $CL_r$  differed across the endogenous compounds (**Table 1**). A minimal increase in AUC was observed for creatinine and  $m^1A$  despite significant dose-dependent reductions in  $CL_r$  (**Figure 3**). Therefore, consideration should be given to these biomarkers for further analysis. Metformin administration negligibly impacted the  $CL_r$  of NMN,  $m^1A$ , and creatinine ( $< 7\%$ ; **Figure 3a**). It was evident that there was a notable difference in 1-NMN AUC, but not  $m^1A$  and creatinine AUC, between the baseline and control phases (**Figure 3a**). Without mass balance data, it is not practical to address the underlying mechanism. Because the plasma concentrations of 1-NMN in the baseline phases were almost similar to those in pyrimethamine-treated phases during the duration to calculate AUC (**Figure 2c**), metformin administration may accompany with increments of 1-NMN AUC by unknown mechanism, which was diminished by pyrimethamine administration. It is also possible that there happened to be interday variation in the supply of 1-NMN as well as its precursor nicotinamide. The plasma concentrations of 1-NMN did not show a continuous increase for hours in this study, which was reported by Mueller *et al.*,<sup>35</sup> although the subjects were fasted in both studies when they received drug administration. Of note, the same author's value for metformin  $CL_r$  was like the result in this study after normalization of the body weights of the participants (9%). In contrast, the  $CL_r$  of 1-NMN reported previously<sup>35</sup> was a 38% smaller value by unknown reason. Further studies are necessary to elucidate if ethnicity of participants can be critical in selection of appropriate MATE1/2-K biomarkers.

Unlike  $m^1A$  and 1-NMN, a reduction in  $CL_r$  of creatinine appeared to reach a plateau at 25 mg pyrimethamine dose (**Figure 2**; **Figure 3a**). Consequently, the  $CL_r$  ratio of  $m^1A$  and 1-NMN was reasonably correlated with that of metformin

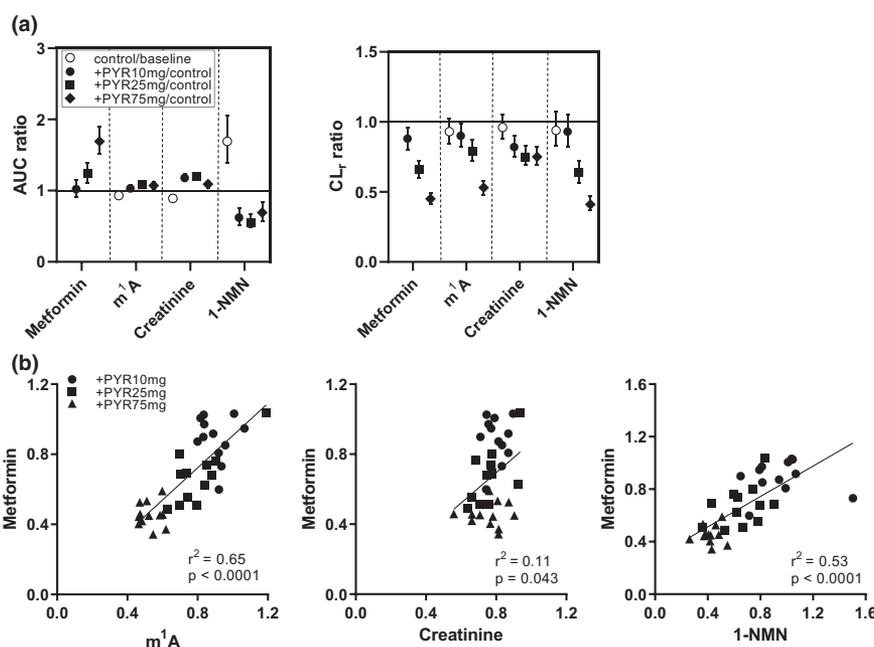


**Figure 2** Effect of pyrimethamine on the plasma concentration-time profiles and amount excreted into the urine and  $CL_r$  of endogenous OCT2/MATE substrates. Plasma concentrations of  $m^1A$  (a), creatinine (b), and 1-NMN (c) were determined at designated times in healthy volunteers in the baseline phase, and drug treatment phases (metformin with or without an oral dose of pyrimethamine 10, 25, and 75 mg). Each symbol represents the mean and SEM ( $n = 12$ ). Amounts excreted into the urine during the intervals designated and  $CL_r$  were shown in box-and-whisker plot.  $m^1A$ ,  $N^1$ -methyladenosine; 1-NMN,  $N^1$ -methylnicotinamide; +PYP, pyrimethamine administration;  $CL_r$ , renal clearance.

according to the degree of transporter inhibition, whereas correlation of creatinine  $CL_r$  was poor (Figure 3b). Furthermore, nonlinear regression analysis provided estimates of  $K_{i,app}$  values of pyrimethamine, which were similar between metformin and the endogenous compounds ( $m^1A$  and 1-NMN), and also to the *in vitro*  $K_i$  values for MATE1/2-K after correction of  $K_{i,app}$  for the plasma unbound fraction of pyrimethamine (0.13; Table 2). These results convincingly demonstrate that  $m^1A$  and 1-NMN are quantitative biomarkers for MATE1/2-K-mediated DDI. Considering the long half-life of pyrimethamine in the plasma

(Figure S1), mean plasma concentrations ( $C_{av}$ ) were used in this analysis. Because we have used  $C_{max}$  for the analysis of other transporters, such as OATP 1B1/3-mediated DDI caused by a single dose of rifampicin,<sup>36</sup> the analysis using the maximum pyrimethamine concentrations were also conducted. In this instance, it was determined that the fitted parameters were not greatly affected (Figure S6).

The reason for the substrate dependence in the dose-dependent effect of pyrimethamine on  $CL_r$  between creatinine and other substrates remains unknown. Previously, the  $K_i$  values of



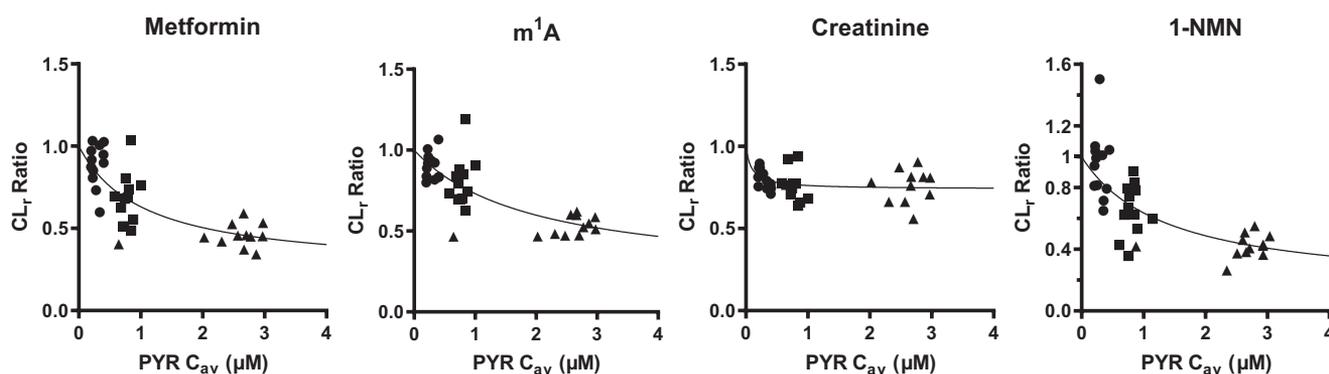
**Figure 3** Effect of pyrimethamine on AUC ratio and CL<sub>r</sub> ratio, and correlation between CL<sub>r</sub> ratio of endogenous OCT2/MATE substrates against that of metformin. (a) AUC ratio and CL<sub>r</sub> ratio with 90% confidence intervals were shown. Data were taken from **Table 1**. (b) The correlation of the CL<sub>r</sub> ratio among the test compounds is shown with correlation coefficient and *P* value. m<sup>1</sup>A, N<sup>1</sup>-methyladenosine; 1-NMN, N<sup>1</sup>-methylnicotinamide; AUC, area under the curve; CL<sub>r</sub>, renal clearance; PYR, pyrimethamine.

pyrimethamine for MATE1 and MATE2-K-mediated creatinine uptake were reported as 0.17 and 0.22 μM, respectively.<sup>37</sup> Such results are not too different from the corresponding values determined in this study using other test substrates (**Table 2**). OAT2 has also been reported as a creatinine transporter in the kidneys, however, the inhibition potency of pyrimethamine for OAT2 was very weak (half-maximal inhibitory concentration > 100 μM) to account for this substrate dependence.<sup>37,38</sup> Perhaps most importantly, the low contribution of the tubular secretion to the urinary excretion (*f*<sub>PYR</sub>, 26%) may limit application of creatinine to such quantitative analysis, when compared with other compounds (**Table 2**).

The absolute value of m<sup>1</sup>A CL<sub>r</sub> under control conditions was only 1.3-fold greater than the corresponding creatinine clearance,

and 1.7-fold greater than the creatinine clearance at 75 mg, which could approximate glomerular filtration rate. Assuming this represents contribution of the tubular secretion (41%), it could not account for *f*<sub>PYR</sub> (**Table 2**). Because CL<sub>r</sub> of m<sup>1</sup>A at the highest pyrimethamine dose was below the CL<sub>r</sub> of creatinine (**Table 1**; **Figure 2**), m<sup>1</sup>A may undergo reabsorption from the urine. Considering that m<sup>1</sup>A is an adenosine metabolite, a nucleoside transport system might account for such reabsorption. Indeed, it has been suggested that m<sup>1</sup>A is a substrate of SLC29A1/equilibrative nucleoside transporter 1 in HEK293 cells.<sup>27</sup> However, it has been confirmed that the inhibitory effect of pyrimethamine on this transporter is minimal (**Figure S3**).

Given that the AUC of m<sup>1</sup>A and 1-NMN were not increased along with CL<sub>r</sub> reduction (**Figure 2**), their major elimination



**Figure 4** Nonlinear regression analysis CL<sub>r</sub> ratio of the test compounds and pyrimethamine C<sub>av</sub>. *K*<sub>*i,app*</sub> of pyrimethamine with regard to C<sub>av</sub>, and *f*<sub>PYR</sub> were determined by iterative nonlinear regression analysis, as described in Methods. Rigid lines represent the fitted lines. The fitted parameters were summarized in **Table 2**. 1-NMN, N<sup>1</sup>-methylnicotinamide; C<sub>av</sub>, mean plasma concentration; CL<sub>r</sub>, renal clearance; *f*<sub>PYR</sub>, fraction sensitive to inhibition by pyrimethamine; *K*<sub>*i,app*</sub>, apparent inhibition constant of pyrimethamine as the total concentration; PYR, pyrimethamine.

**Table 2**  $K_{i,app}$  with regard to pyrimethamine  $C_{av}$ ,  $f_{PYR}$ , and *in vitro*  $K_i$  of test compounds

Compound	Clinical data		<i>In vitro</i> $K_i$ ( $\mu$ M)		
	$K_{i,app}$ , $\mu$ M	$f_{PYR}$	OCT2	MATE1	MATE2-K
Metformin	1.05 $\pm$ 0.39 (0.137)	0.766 $\pm$ 0.103	37.9 $\pm$ 5.6	0.154 $\pm$ 0.029	0.0941 $\pm$ 0.016
<i>N</i> <sup>1</sup> -methyladenosine	1.94 $\pm$ 0.96 (0.252)	0.792 $\pm$ 0.188	0.466 $\pm$ 0.043	ND	0.0680 $\pm$ 0.015
Creatinine	0.0914 $\pm$ 0.0661 (0.0119)	0.260 $\pm$ 0.026	1.57 $\pm$ 0.24	ND	ND
<i>N</i> <sup>1</sup> -methylnicotinamide <sup>a</sup>	1.37 $\pm$ 0.63 (0.178)	0.863 $\pm$ 0.157	41.2 $\pm$ 9.1	0.125 $\pm$ 0.026	0.0821 $\pm$ 0.018

$K_{i,app}$  and  $f_{PYR}$  were determined by iterative nonlinear regression analysis using renal clearance ratio, and average pyrimethamine concentrations, as described in the Methods. *In vitro*  $K_i$  were determined by iterative nonlinear regression analysis using the data shown in **Figure 5**. Number in the parenthesis represents the  $K_{i,app}$  corrected by the unbound fraction of pyrimethamine in the plasma (0.13). Each parameter represents the fitted parameter with computer calculated SD.  $K_i$  was calculated from  $IC_{50}$ , assuming  $IC_{50}$  approximates  $K_i$  when substrate concentration is lower than the  $K_m$ .  $C_{av}$ , mean plasma concentration;  $f_{PYR}$ , fraction sensitive to inhibition by pyrimethamine;  $K_i$ , inhibition constant;  $K_{i,app}$ , apparent inhibition constant of pyrimethamine as the total concentration; ND, not determined.

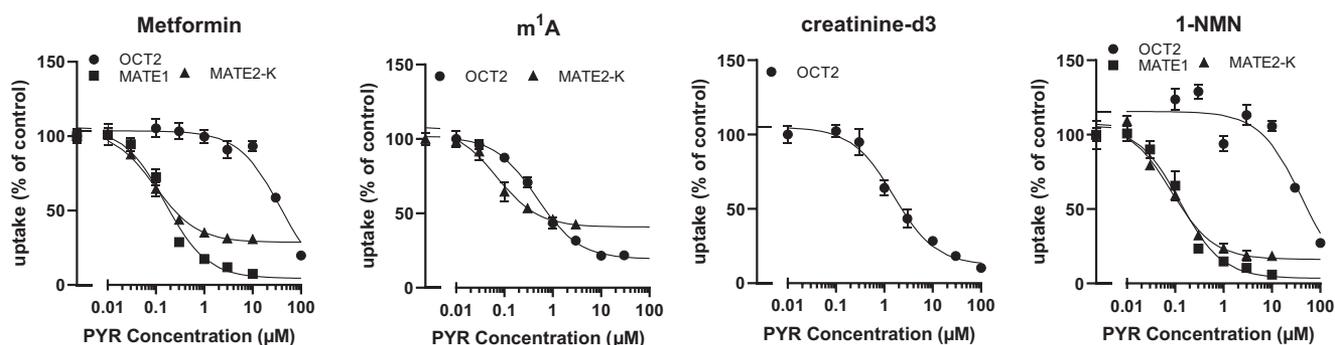
<sup>a</sup> $CL_r$  calculated from time zero to 12 hours was used in the analysis.

pathway is unlikely to be urinary excretion, as unchanged form through pyrimethamine may also affect the synthesis of 1-NMN. Even though urinary excretion is not major for 1-NMN and *m*<sup>1</sup>A, it is still possible to use changes in their  $CL_r$  as an index of MATE inhibition. High metabolic activity of 1-NMN in human hepatocytes was previously reported.<sup>39</sup> Urinary excretion of 1-NMN was decreased along with an increase in the replacement index of human hepatocytes in human hepatocytes transplanted chimeric mice, suggesting the hepatic metabolism is the major elimination pathway of 1-NMN. As observed for OCT2, pyrimethamine unlikely inhibits OCT1-mediated hepatic uptake of 1-NMN at the doses in this study, considering its  $K_i$  for OCT1.<sup>40</sup> The major elimination pathway of *m*<sup>1</sup>A remains undefined in humans although its concentration in serum was associated with OCT2 single-nucleotide polymorphism.<sup>27</sup>

We also determined  $CL_r$  for PDA, a recently identified OAT1 and OAT3 endogenous biomarker,<sup>32,33</sup> in the same subjects to exclude the possibility of nonspecific inhibition of the kidney function by pyrimethamine (**Table 1; Figure S4**). Based on *in vitro* inhibition data, pyrimethamine is a weak inhibitor of OAT1 and OAT3 (**Figure S3**). Consistently, pyrimethamine showed only a weak inhibitory effect on  $CL_r$  of PDA at the highest dose, supporting the specificity of pyrimethamine for MATE1/2-K in the

kidneys (**Table 1; Figure S4**). This approach expands the application of endogenous biomarkers in assessing DDIs. Dosing several probe drugs in a cocktail is a way to assess the DDI risk of multiple transporters simultaneously in the same subject.<sup>41,42</sup> However, equivalence of the pharmacokinetic parameters of probe drugs between single dose and simultaneous dosing as a cocktail would need to be validated prior to its use.<sup>42</sup> Leveraging of multiplexed endogenous biomarkers is one possible way to facilitate simultaneous DDI assessment across multiple drug transporters early in clinical development without the potential need for DDI studies with individual probes or probe drug cocktails.

As described, this study highlighted the difference in pyrimethamine dose response across different endogenous MATE1/2-K substrates to identify the appropriate endogenous biomarker for these transporters, and presented integrative analysis of the dose dependence by nonlinear regression analysis. The  $CL_r$  of 1-NMN and *m*<sup>1</sup>A showed similar performance as DDI biomarkers for MATE1/2-K, however, because neither 1-NMN nor *m*<sup>1</sup>A present as plasma biomarkers, it is essential to collect urine with appropriate intervals considering the elimination half-life of the investigational drug in question, which is a limiting factor for their application to drug development. In addition, because diurnal change in the plasma concentration of 1-NMN was more evident than that of *m*<sup>1</sup>A, plasma



**Figure 5** Effect of pyrimethamine on OCT2, MATE1, and MATE2-K. Uptake of metformin (10  $\mu$ M), *m*<sup>1</sup>A (100  $\mu$ M), creatinine-*d*3 (100  $\mu$ M), and 1-NMN (10  $\mu$ M) was determined in the presence of pyrimethamine at various concentration in the stable expression system of OCT2, MATE1, and MATE2-K using HEK293 cells. Incubation time was 2 minutes except for *m*<sup>1</sup>A (30 minutes) to determine the uptake. Absolute values of the uptake are summarized in **Table S3**. Each symbol represents the mean and SEM ( $n = 3$ ). Rigid lines represent the fitted line. Nonlinear regression analysis was conducted as described in **Supplementary Methods**. 1-NMN, *N*<sup>1</sup>-methylnicotinamide; *m*<sup>1</sup>A, *N*<sup>1</sup>-methyladenosine; PYR, pyrimethamine.

sampling at multiple timepoints between 12 and 24 hours postdose is warranted to calculate reliable  $CL_r$  values if a 24-hour assessment is needed. In this regard, as previously described for OATP1B, it is envisioned that physiologically-based pharmacokinetic (PBPK) model-based analysis will aid the design of clinical studies.<sup>41</sup> A PBPK model for metformin has already been constructed and it is able to describe the metformin-cimetidine DDI involving MATE1 inhibition.<sup>43</sup> Likewise, the construction of PBPK models for 1-NMN and  $m^1A$  will advance the prediction of OCT2 and MATE1/2-K mediated DDI in drug development.

In conclusion, the present study assessed the performance of creatinine, 1-NMN, and  $m^1A$  as candidate OCT2 and MATE1/2-K biomarkers, and strongly supports the rationale to leverage the  $CL_r$  of 1-NMN and  $m^1A$  as quantitative MATE1/2-K biomarkers for the DDI risk assessment in healthy volunteers. Further studies are required to assess the performance of both 1-NMN and  $m^1A$  clinical biomarkers for perpetrator drugs that present different OCT2, MATE1, MATE2-K inhibition signatures vs. pyrimethamine.

#### SUPPORTING INFORMATION

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

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#### CONFLICT OF INTEREST

E.K., L.L., S.M., L.M.H., M.V., V.L., and A.D.R. are employees of Pfizer Inc., USA. R.R. was employed by Pfizer Inc at the time of the study. C.M. is an employee of Pfizer Inc., Japan. All other authors declared no competing interests for this work.

#### AUTHOR CONTRIBUTIONS

T.M., E.K., A.D.R., Y.S., and H.K. wrote the manuscript. M.V., A.D.R., E.K., C.M., K.F., Y.S., and H.K. designed the research. T.M., L.L., L.M.H., S.M., R.R., L.S.W., and J.G.J. performed the research. T.M., A.D.R., Y.S., H.K., V.H.L., and M.V. analyzed the data.

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