Elucidation of $N^1$-methyladenosine as a Potential Surrogate Biomarker for Drug Interaction Studies Involving Renal Organic Cation Transporters

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ABSTRACT

Endogenous substrates are emerging biomarkers for drug transporters, which serve as surrogate probes in drug-drug interaction (DDI) studies. In this study, the results of metabolome analysis using wild-type and Oct1/2 double knockout mice suggested that $N^1$-methyladenosine ($m^1A$) was a novel organic cation transporter (OCT) 2 substrate. An in vitro transport study revealed that $m^1A$ is a substrate of mouse Oct1, Oct2, Mate1, human OCT1, OCT2, and multidrug and toxin exclusion protein (MATE) 2-K, but not human MATE1. Urinary excretion accounted for 77% of the systemic elimination of $m^1A$ in mice. The renal clearance (46.9 ± 4.9 ml/min per kilogram) of exogenously given $m^1A$ was decreased to near the glomerular filtration rates by Oct1/2 double knockout or Mate1 inhibition by pyrimethamine (16.6 ± 2.6 and 24.3 ± 0.6 ml/min per kilogram, respectively), accompanied by significantly higher plasma concentrations. In vivo inhibition of OCT2/MATE2-K by a single dose of 7-[(3R)-3-(1-aminocyclopropyl)pyrrolidin-1-yl]-1-[1R,2S]-2-fluorocyclopropyl]-8-methoxy-4-oxoquinoline-3-carboxylic acid in cynomolgus monkeys resulted in the elevation of the area under the curve of m1A (1.72-fold) as well as metformin (2.18-fold). The plasma m1A concentration profile showed low diurnal and interindividual variation in healthy volunteers. The renal clearance of m1A in younger (21–45 year old) and older (65–79 year old) volunteers (244 ± 58 and 169 ± 22 ml/min per kilogram, respectively) was about 2-fold higher than the creatinine clearance. The renal clearances of m1A and creatinine were 31% and 17% smaller in older than in younger volunteers. Thus, m1A could be a surrogate probe for the evaluation of DDIs involving OCT2/MATE2-K.

SIGNIFICANCE STATEMENT

Endogenous substrates can serve as surrogate probes for clinical drug-drug interaction studies involving drug transporters or enzymes. In this study, m1A was found to be a novel substrate of renal cationic drug transporters OCT2 and MATE2-K. N1-methyladenosine was revealed to have some advantages compared to other OCT2/MATE substrates (creatinine and N1-methyl nicotinamide). The genetic or chemical impairment of OCT2 or MATE2-K caused a significant increase in the plasma m1A concentration in mice and cynomolgus monkeys due to the high contribution of tubular secretion to the net elimination of m1A. The plasma m1A concentration profile showed low diurnal and interindividual variation in healthy volunteers. Thus, m1A could be a better biomarker of variations in OCT2/MATE2-K activity caused by inhibitory drugs.

Introduction

Drug transporters are one of the key determinants governing drug pharmacokinetics; thus, they provide sites for drug-drug interactions (DDIs) with concomitantly administered drugs. Inhibition of transporters, which facilitate the drug clearance from the systemic circulation, causes an increase in the systemic exposure of substrate drugs and thus could elevate the risk of adverse reactions. To avoid the risk, the regulatory authorities in the United States, European Union, and Japan have strongly recommended conducting separate clinical studies for investigational inhibitors using probe drugs to firmly evaluate the DDI risks in humans according to their guidelines. Recently, some endogenous substrates have emerged to serve as surrogate probes for clinical DDI studies. Using these endogenous substrates does not require exogenous administration of probe drugs, which enables the early assessment of DDI risks and contributes to overcoming the in vitro–in vivo gap in the DDI predictions (Mariappan et al., 2017; Chu et al., 2018; Rodrigues et al., 2018).

Organic cation transporter (OCT) 2 is predominantly expressed on the basolateral side of human renal proximal tubular epithelial cells and is responsible for the renal influx of water-soluble cationic compounds.
such as metformin, tetraethylammonium (TEA), and cisplatin (Morrissey et al., 2013). In turn, the efflux of those drugs into urine is mediated by multidrug and toxin extrusion proteins (MATEs) expressed on the apical side of the proximal tubules (Otsuka et al., 2005; Masuda et al., 2006). Human MATEs have two isoforms, MATE1 and MATE2-K, and they play a key role in the tubular secretion of cationic drugs in cooperation with OCT2. To date, dolutegravir, crizotinib, and vandetanib can inhibit OCT2 at the clinical dose to increase the systemic exposure of metformin (Reese et al., 2013; Johansson et al., 2014; Arakawa et al., 2017), whereas MATE inhibitors, such as pyrvinium (PYR), cimetidine, and trimethoprim also reduce renal clearance of metformin (Elsby et al., 2017), and can exacerbate the intracellular accumulation and nephrotoxicity of cisplatin.

We have reported serum creatinine levels and renal clearances of creatinine and N1-methylnicotinamide (NMN) to reflect the transport activity of OCT2/MATEs using 7-{(3R)-3-(1-aminocyclopropyl)pyrroldin-1-yl]-1-{(1R,2S)-2-fluorocyclopropyl]-8-methoxy-4-oxoquinoline-3-carboxylic acid (DX-619) and PYR as inhibitors (Imamura et al., 2011; Ito et al., 2012). Indeed, single or multiple doses of OCT2/MATE inhibitors were reported to be accompanied by an increased level of serum creatinine or reduction in renal creatinine clearances, although some inhibitors did not achieve significant unbound concentrations to inhibit OCT2 and/or MATEs considering in their vitro half-maximal inhibitory concentration. Nevertheless, creatinine and NMN have some disadvantages when used as potential biomarkers. Tubular secretion accounts for at most 20% of the renal creatinine clearance (Breyer and Qi, 2010). The plasma concentration of NMN, a metabolite of niacin (or nicotinamide), exhibited a diurnal change and was almost identical between PYR-treated healthy volunteers and the control group, despite extensive inhibition of the urinary excretion (Ito et al., 2012). Recently, it was also reported that trimethylamine-N-oxide, a carnitine-derived metabolite, is a substrate of OCT2 (Teft et al., 2017) and MATE1 (Gessner et al., 2018). However, the contribution of tubular secretion to the renal clearance of trimethylamine-N-oxide was not clearly observed in humans based on its renal clearance and glomerular filtration rate. Its plasma concentrations also showed extensive diurnal variation, which was presumably caused by food ingestion (Miyake et al., 2017).

To expand our knowledge on the endogenous probes of OCT2/MATEs, we first investigated the novel endogenous substrate of OCT2 by conducting a metabolome analysis using plasma and urine specimens from wild-type (WT) and Oct1/2 double knockout (dKO) mice. After validation of OCT2/MATE-mediated transport of the candidate compound, N1-methylenedenosine (m1A), its utility as a surrogate probe in clinical DDI studies was evaluated in both animals and humans.

Endogenous Probe of Organic Cation Transporters

Materials and Methods

Materials and Cell Lines. N1-methylenedenosine was purchased from Carbosynth (Berkshire, UK). [3H]1-methyl-4-phenylpyridinium (MPP+) (80 Ci/mmol) was obtained from PerkinElmer (Waltham, MA). DX-619 was synthesized at Daiichi-Sankyo Co., Ltd. (Tokyo, Japan). Metformin hydrochloride was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and reagents were commercially available and of analytical grade.

Human embryonic kidney 293 (HEK293) cells stably expressing mouse OCT (mOCT) 1, mOCT2, mouse MATE1 (mMate1), human OCT (hOCT) 1, hOCT2, human MATE (hMATE) 1, and hMATE2-K were described and cultured as previously (Ito et al., 2010). Animals. Oct1/2 dKO mice (Jonker et al., 2003) were purchased from Taconic Farms (Germantown, NY). PBV/Ncl mice (CLEA Japan, Tokyo, Japan) were employed as Oct1 and Oct2 gene WT controls. The mice were maintained in an air-conditioned room with lighting at 12 hours intervals, fed a standard animal diet, and received water ad libitum. The mice employed in the current study were from 8 to 12 weeks old. The studies using mice were conducted in accordance with the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, University of Tokyo).

Three male cynomolgus monkeys at 3 to 4 years of age were supplied by Shin Nippon Biomedical Laboratories, Ltd. (Tokyo, Japan). They were housed in a temperature- and humidity-controlled room set to a 12-hour light/dark cycle and given access ad libitum to water and a standard laboratory diet. The study using monkeys was carried out in accordance with the guidelines of the Animal Care and Use Committee of Daiichi-Sankyo Co., Ltd.

Metabolome Analysis. The serum and urine samples from WT and Oct1/2 dKO mice were measured with the Metabolon analytical system (Metabolon Inc., Durham, NC). To identify structurally named and unknown molecules, Metabolon applied a nontargeted semiquantitative liquid chromatography– tandem mass spectrometry (LC-MS/MS) and gas chromatography/mass spectrometry platform. Identification codes, which start with the letter X, were imparted to unnamed and structurally unknown metabolites.

In Vitro Transport Study Using cDNA Transfected Cells. Cells were washed and preincubated with Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM NaHCO3, 4.8 mM KCl, 1.0 mM KH2PO4, 1.2 mM MgSO4, 12.5 mM HEPES, 5 mM glucose, and 1.5 mM CaCl2) at 37°C for 15 minutes, then, substrates were added to the wells to initiate uptake. The pH of the buffer solution was adjusted to 7.4 for OCT-expressing cells and 8.0 for MATE-expressing cells. Incubation buffer was removed and ice-cold Krebs-Henseleit buffer was added to halt the uptake at the designated times. For quantification of m1A, cells were recovered in 2 mM ammonium acetate (pH 5.0) using a cell scraper and disrupted with a Biorupter (UCD-250HSA: Cosmo Bio, Tokyo, Japan). The specimens were then mixed with a 3-fold volume of methanol for protein precipitation and centrifuged for 5 minutes at 20,000g. The supernatant was diluted with a 4-fold volume of 2 mM ammonium acetate (pH 5.0) and subjected to LC-MS/MS analysis. For quantification of metformin, cells were recovered in 0.1% formic acid and disrupted as well. A 3-fold volume of acetoneitrile was added, and after centrifugation the supernatant was subjected to LC-MS/MS analysis. A Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) was employed to determine the protein concentration with bovine serum albumin as the protein standard.

Kinetic Analysis of In Vitro Data. The data gathered from the saturation study were fitted to the following equation assuming the Michaelis-Menten equation:

\[
CL_{\text{uptake}} = \frac{V_{\text{max}}}{K_m + S} 
\]

where \(CL_{\text{uptake}}\) denotes uptake clearance, calculated by subtracting the \(CL_{\text{uptake}}\) in the empty vector–overexpressing cells from that in the transporter-expressing cells; and \(V_{\text{max}}, S,\) and \(K_m\) represent the maximum transport velocity at the saturating substrate concentration, substrate concentration, and the Michaelis constant, respectively.

The inhibition constants of various inhibitors for OCT2 and MATE2-K were calculated using the following equation:

\[
CL_{\text{uptake}(+\text{inhibitor})} = \frac{CL_{\text{uptake}(\text{control})}}{1 + I/C} 
\]
where $C_{\text{uptake (inhibitor)}}$ and $C_{\text{uptake (control)}}$ indicate the uptake with or without inhibitors, respectively; and $\gamma$ and $\kappa_i$ denote the inhibitor concentration and inhibition constant, respectively.

To calculate the kinetic parameters, nonlinear least-squares fitting was performed using the MULTI program (Yamaoka et al., 1981) with the damped Gauss-Newton method algorithm.

**Transport Study Using Kidney Slices.** Slices (300 $\mu$m in thickness) of whole kidneys were prepared from WT and Oct1/2 dKO mice, and incubated at 37°C for 5 minutes on a 12-well plate with 1 ml of oxygenated buffer for preincubation. To initiate uptake, the slices were incubated at 37°C with 1 ml of oxygenated buffer containing 100 $\mu$M m1A for 10 minutes. Then, the slices were rapidly removed from the incubation buffer, washed twice with ice-cold buffer to halt the uptake, blotted on filter paper, and weighed. The specimens were homogenized and deproteinized, followed by the determination of m1A concentration by LC-MS/MS. The uptake activity of Oct1 and Oct2 in the mouse kidney slices was checked with $[^3]H$MPP$^+$ as the positive control. To quantify the radiolabeled compound, slices were dissolved in 1 ml of Soluene-350 (Packard Instruments, Meriden, CT) overnight at 55°C. The specimens were mixed with a scintillation cocktail (Hionic Fluor; Packard Instruments) and the radioactivity in the specimens was determined with a liquid scintillation counter (LS6000SE; Beckman Coulter, Brea, CA).

**In Vivo Study of m1A in WT and Oct1/2 dKO Mice.** Under anesthesia with isoflurane, m1A (100 nmol/min per kilogram) was infused through the jugular vein. Blood samples were obtained through the jugular vein at the indicated times after the initiation of infusion and immediately centrifuged at 12,000 g for 5 minutes to collect the plasma fraction. The urethral meatus was fixed with 1 ml of Soluene-350 (Packard Instruments, Meriden, CT) overnight at 55°C for 5 minutes on a 12-well plate with 1 ml of oxygenated buffer for preincubation. To initiate uptake, the slices were incubated at 37°C with 1 ml of oxygenated buffer containing 100 $\mu$M m1A for 10 minutes. Then, the slices were rapidly removed from the incubation buffer, washed twice with ice-cold buffer to halt the uptake, blotted on filter paper, and weighed. The specimens were homogenized and deproteinized, followed by the determination of m1A concentration by LC-MS/MS. The uptake activity of Oct1 and Oct2 in the mouse kidney slices was checked with $[^3]H$MPP$^+$ as the positive control. To quantify the radiolabeled compound, slices were dissolved in 1 ml of Soluene-350 (Packard Instruments, Meriden, CT) overnight at 55°C. The specimens were mixed with a scintillation cocktail (Hionic Fluor; Packard Instruments) and the radioactivity in the specimens was determined with a liquid scintillation counter (LS6000SE; Beckman Coulter, Brea, CA).

**Pharmacokinetic Analysis in the Steady-State Infusion Study.** The total body clearance of plasma ($CL_{\text{p,tissue}}$) and the renal clearance with respect to the plasma concentration ($CL_{\text{p}}$) were obtained as follows:

$$CL_{\text{p,tissue}} = \frac{\text{Dose}}{AUC_p}$$

(1)

$$CL_{\text{p}} = \frac{X_{\text{urine}}}{AUC_p}$$

(2)

where Dose and $X_{\text{urine}}$ indicate the amount of compounds administered in 2 hours and excreted into urine, respectively; and $AUC_p$ indicates the area under the curve (AUC) of the time-plasma concentration for compounds from 0 to 120 minutes and determined with the trapezoidal rule. The apparent tissue-to-plasma concentration ratio ($K_{p,tissue}$) was determined as follows:

$$K_{p,tissue} = \frac{C_{\text{tissue}}}{C_p}$$

(3)

where $C_{\text{tissue}}$ and $C_p$ indicate the tissue and plasma concentrations of compounds at 120 minutes after administration, respectively.

**In Vivo Inhibition Study of OCT2 and MATEs Using DX-619.** A crossover study design, where the same three monkeys were employed over a couple of studies, was used with a 2-week washout period between each study. Monkeys were kept under overnight fasting conditions and then provided food after blood sampling at 6 hours, in which they received water ad libitum during the periods. DX-619 (30 mg/kg) in a 0.5% (w/v) methylcellulose (in water) suspension was administered through oral gavage at a dose volume of 5 ml/kg. Additionally, 2 hours after administration of DX-619, metformin was administered orally at 5 mg/kg (dosing volume: 5 ml/kg) in a 0.5% (w/v) methylcellulose suspension. Blood samples were obtained through the femoral vein, collected in tubes coated with heparin in $-2$ (before metformin dosing), 0.25, 0.5, 1, 2, 4, 6, and 24 hours after metformin dosing. The specimens were kept on ice before being centrifuged to obtain plasma specimens (15,000 rpm, 2 minutes at 4°C). The AUC of the test compounds was determined with the trapezoidal rule.

**Clinical Samples.** The study protocol was approved by the Ethics Review Boards of The University of Tokyo, Kyushu University, and Sagioka Memorial...
Hospital. Written informed consent was confirmed from all participants prior to their inclusion in this study. The plasma and urine samples were those from our previously performed clinical DDI study (Miyake et al., 2017). Briefly, the study was a single-arm, nonrandomized study where eight younger and seven older male subjects were enrolled. All participants were in healthy condition and not on any medication. They received the following drugs in a cassette after overnight fasting for the investigation of the effect of aging on drug transporter activities and cytochrome pigment enzymes, which was the primary objective of the clinical study: alprazolam 0.2 mg, atorvastatin 3 mg, chlorzoxazone 10 mg, pitavastatin 0.4 mg, telmisartan 5 mg, and valsartan 10 mg. Aliquots of plasma specimens were filtered using the Centrifree Ultrafiltration device (Merck Millipore, Burlington, MA) to evaluate the plasma protein binding of m1A. The participants’ information is summarized in Supplemental Table 1.

Pharmacokinetic Analysis in the Human Clinical Study. The area under the plasma concentration-time curve from 0 to 24 hours was determined with the trapezoidal rule. The CLr,p values of m1A and creatinine was determined by dividing the amount excreted into the urine from 0 to 24 hours ($X_{urine, 0-24 h}$) by the area under the plasma concentration-time curve from 0 to 24 hours (AUC0–24 h) value as follows:

$$CLr_p = \frac{X_{urine, 0-24 h}}{AUC_{0-24 h}}$$

Fig. 1. In vitro transport study of m1A. The uptake of m1A (100 μM) by organic cation transporters was determined in the absence (solid lines) or presence (dotted lines) of inhibitor (5 mM TEA). The incubation buffer also contained 10 μM dipyridamole to inhibit the ENT-mediated m1A uptake. Each symbol and bar represents the mean ± S.E. (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001, the absence vs. presence of 5 mM TEA in transporter-overexpressing cells, Student’s two-tailed unpaired t test.

Fig. 2. Effect of various inhibitors on hOCT2- and hMATE2-K-mediated m1A uptake. The uptake of m1A (100 μM) for 60 minutes in HEK293 cells stably expressing hOCT2 or hMATE2-K was determined in the absence and presence of inhibitors (trimethoprim, pyrimethamine, and cimetidine) at the designated concentrations. The transporter-specific uptake of m1A was calculated by subtracting the uptake by empty vector-transfected cells from that by transporter-expressing cells and shown as a proportion to the uptake value in the absence of inhibitors. The solid line represents the fitted line obtained by nonlinear regression analysis as described in Materials and Methods. Each symbol and bar represents the mean ± S.E. (n = 3).
Quantification of Test Compounds in Biologic Specimens by LC-MS/MS. The plasma and urine samples were diluted with 10- and 100-fold volumes of water, respectively. The kidney specimens were homogenized in a 3-fold volume of saline. For quantification of m1A, samples were mixed with a 3-fold volume of methanol and centrifuged at 20,000 g for 10 minutes. The supernatants were mixed with a 4-fold volume of 2 mM ammonium acetate (pH 5.0) and subjected to LC-MS/MS analysis. To quantify rhodamine 123 and creatinine, a 3-fold volume of acetonitrile containing 10 nM creatinine-d3 (internal standard) was added, followed by centrifugation, and then the supernatant was subjected to LC-MS/MS analysis. Samples were analyzed on the QTRAP5500 system (AB SCIEIX, Toronto, Canada), equipped with Prominance UPLC (Shimadzu, Kyoto, Japan), in electrospray ionization mode. The measurement conditions are summarized in Supplemental Tables 2 and 3.

Quantification of Metformin and DX-619 in Monkey Plasma by LC-MS/MS. Sample analysis was conducted on the API4000 system (AB SCIEIX), equipped with ACQUITY UPLC (Waters, Milford, MA), in electrospray ionization mode. Each plasma sample was prepared as previously described, followed by mixing with water, acetonitrile, and internal standard solution [niflumic acid 15 ng/ml in acetonitrile:methanol (75:25)]. After vortexing, the mixtures were filtered by employing MultiScreen Solvinert phobic PTFE 0.45 g and centrifuged at 20,000 × g.

Sample analysis was conducted on the API 4000 system (AB SCIEX), and in electrospray LC-MS/MS. To quantify rhodamine 123 and creatinine, a 3-fold volume of acetonitrile containing 100 nM creatinine-d3 (internal standard) was added, followed by centrifugation, and then the supernatant was subjected to LC-MS/MS analysis. Samples were analyzed on the QTRAP5500 system (AB SCIEIX, Toronto, Canada), equipped with Prominance UPLC (Shimadzu, Kyoto, Japan), in electrospray ionization mode. The measurement conditions are summarized in Supplemental Tables 2 and 3.

Statistical Analysis. Data are presented as the mean ± S.E.M. for the in vitro and in vivo experiments, and the mean ± S.D. for the clinical study. The statistical analysis was performed with Student’s t test, Welch’s two-sample t test and Dunnett’s post hoc test to detect significant differences between two groups where appropriate (*P < 0.05; **P < 0.01; ***P < 0.001).

Results

Investigation of Candidate Compounds by Metabolomics Analysis of WT and Oct1/2 dKO Mice. Of the 819 compounds surveyed, the compounds that showed higher plasma concentrations or lower urine concentrations in the knockout mice are summarized in Table 1. Among them, m1A was selected for further analysis because its major elimination pathway in rats was urinary excretion (Dutta and Chheda, 1987) and its serum level was significantly associated with OCT2 single nucleotide polymorphisms (Shin et al., 2014).

Uptake of m1A by Organic Cation Transporters. To verify the m1A transport by OCT2 and other renal cation transporters, an in vitro transport study was performed using transporter-overexpressing HEK293 cells. In the m1A transport studies, 10 µM dipyridamole [an inhibitor of equilibrative nucleoside transporters (ENTs)] was added to the incubation buffer to inhibit ENT-mediated m1A uptake in HEK293 cells (Supplemental Fig. 1A). Using [3H]MPP+ and metformin, it was confirmed that dipyridamole had no effect on OCT- and MATE-mediated uptake (Supplemental Fig. 1B).

The result is summarized in Fig. 1. The m1A uptake in HEK293 cells individually expressing moct1, moct2, and mMate1 was significantly higher than that in empty-vector transfected cells. Among the human transporters, hOCT1, hOCT2, and hMATE2-K were responsible for the uptake of m1A, but hMATE1 was not. The hOCT2-mediated m1A transport was not affected by G808T mutation, a well-known single nucleotide polymorphism of the transporter (Zolk, 2012; Yoon et al., 2013) (Supplemental Fig. 2). The m1A uptake was not saturated at the concentration of 1 mM in all renal cation transporters other than mMate1, in which the K<sub>i</sub> and V<sub>max</sub> values of m1A were 246 ± 14 µM and 76.7 ± 3.0 pmol/min per milligram protein, respectively (Supplemental Fig. 3, A and B). Similarly, 1 mM m1A did not inhibit the metformin uptake by moct1, moct2, hOCT2, or hMATE2-K, although m1A concentration-dependent inhibition of metformin uptake by mMate1 was observed with the K<sub>i</sub> value of 89.5 ± 15.1 µM (Supplemental Fig. 3C).

The K<sub>i</sub> values of trimethoprim, PYR, and cimetidine for hOCT2 and hMATE2-K–mediated m1A uptake were determined (Fig. 2). Concerning substrate dependency, the K<sub>i</sub> values of these drugs for m1A uptake were compared with those for creatinine and metformin uptake (Mathialagan et al., 2017) (Table 2). Compared with metformin, the K<sub>i</sub> value of cinemidine for hOCT2 was 33 times smaller and that of trimethoprim for hMATE2-K was 2.7-fold greater when m1A was used as the test probe.

m1A Uptake by Mouse Kidney Slices. Renal cortical slices of WT and Oct1/2 dKO mice were used to elucidate the contribution of OCTs to m1A in the kidney. The effects of Oct1/2 dKO and TEA are shown in Fig. 3. The uptake of m1A into mouse kidney slices was depleted by Oct1/2 dKO or 5 mM TEA, in the same way as [3H]MPP+ (positive control of the transport activity of OCT). In contrast, it was not affected by 250 µM probenecid, a typical organic anion transporter inhibitor.

The Contribution of Oct1/2 to the Kinetics of m1A in Mice. The plasma concentrations of endogenous m1A were measured using LC-MS/MS in WT and Oct1/2 dKO mice, which were 69.0 ± 6.2 and 173 ± 8 nM, respectively (mean ± S.E., n = 3, P < 0.001). To confirm

![Graph](https://via.placeholder.com/150.png)

**Fig. 3.** m1A uptake by renal cortical slices of WT and Oct1/2 dKO mice. The uptake of m1A (100 µM) for 10 minutes in renal cortical slices of WT and Oct1/2 dKO mice was determined in the absence or presence of 250 µM probenecid or 5 mM TEA. Each bar represents the mean ± S.E. (n = 3). **P < 0.01, Dunnett’s post hoc test.
the key role of Oct1/2 in the clearance and tissue distribution of m1A in vivo, an infusion study was carried out with WT and Oct1/2 dKO mice. The dose of m1A (100 nmol/min per milligram) was selected to achieve concentrations higher than the endogenous level, but below its Kₘ value for mMate1. The kinetic data are shown in Fig. 4 and Table 3. The systemic clearance (CLᵦₑₑₑₚ) was mainly explained by the renal clearance (CLᵦₑᵦₑₑₚ) and decreased significantly by Oct1/2 deletion, causing about 2-fold increase of m1A concentration in the plasma. The CLᵦₑₑₑₑₚ in Oct1/2 dKO mice was comparable with the glomerular filtration rate (Jonker et al., 2003). Regarding the tissue distribution of m1A, the kidney-to-plasma ratio (Kᵦₚₚkidney) in Oct1/2 dKO mice exhibited a decreasing tendency compared with that in WT mice. On the other hand, the liver-to-plasma ratio was almost equal between these mice.

**The Contribution of Mate1 to the Kinetics of m1A in Mice.** We also performed a PYR treatment study using WT mice to check the contribution of Mate1 to the renal secretion of m1A. PYR was administered to mice by a bolus injection 30 minutes prior to the initiation of the m1A infusion, since PYR has a long half-life in the administered to mice by a bolus injection 30 minutes prior to the m1A infusion, since PYR has a long half-life in the systemic circulation (Ito et al., 2010). The dosage amount of PYR (20 μmol/kg) was chosen to inhibit mMate1 strongly, as reported previously (Kito et al., 2019). A reference Mate1 substrate, rhodamine 123, was simultaneously given to mice with m1A. The kinetic change in the elevation in the m1A by the PYR treatment is summarized in Fig. 5 and Table 4. The plasma concentration of m1A was about 1.5-fold higher in the WT mice. The dose of m1A (100 nmol/min per milligram) was selected to achieve concentrations higher than the endogenous level, but below its Kᵠₚ value for mMate1. The kinetic data are shown in Fig. 4 and Table 3. The systemic clearance (CLᵦₑₑₑₑₚ) was mainly explained by the renal clearance (CLᵦₑᵦₑₑₚ) and decreased significantly by Oct1/2 deletion, causing about 2-fold increase of m1A concentration in the plasma. The CLᵦₑₑₑₑₚ in Oct1/2 dKO mice was comparable with the glomerular filtration rate (Jonker et al., 2003). Regarding the tissue distribution of m1A, the kidney-to-plasma ratio (Kᵦₚₚkidney) in Oct1/2 dKO mice exhibited a decreasing tendency compared with that in WT mice. On the other hand, the liver-to-plasma ratio was almost equal between these mice.

**Effect of DX-619 on the Kinetics of m1A and Metformin in Monkeys.** To elucidate the significance of OCT2 and MATE2-K on the m1A kinetics in primates, a single-dose study was carried out with male cynomolgus monkeys administered metformin with or without DX-619, which inhibits those transporters at its therapeutic dose. Prior to this study, m1A transport by cynomolgus monkey OCT2 was confirmed using cDNA-transfected HEK293 cells (Supplemental Fig. 4).

The plasma-concentration profiles of m1A, metformin, creatinine, and DX-619 after oral administration of metformin (5 mg/kg) with and without DX-619 pretreatment (30 mg/kg) are summarized in Fig. 6. The DX-619 dose (30 mg/kg, by mouth) was designed to achieve sufficient plasma concentrations exceeding by far the reported Kᵠ value (0.94 μM) for hOCT2-mediated creatinine uptake (Imamura et al., 2011). The administration of DX-619 resulted in an increase in the AUCᵦₑₑₑₑₚ of m1A and metformin (Table 5), although not statistically significant (P = 0.06 and 0.07, respectively). The plasma concentration of creatinine exhibited an increasing tendency in the DX-619–treated period.

**Determination of the Renal Clearance of m1A and Creatinine in Younger and Older Volunteers.** To obtain insight into the dispersion of m1A in humans, m1A and creatinine concentrations in the plasma and urine samples from younger and older healthy volunteers were determined with LC-MS/MS. Binding of m1A to the plasma protein was not detected. As is the case with creatinine, the plasma concentration of creatinine showed low diurnal and interindividual variation, and no significant difference between the younger and older subjects. The amount of m1A excreted into urine was significantly lower in older subjects (Fig. 7A), leading to a reduction in renal clearance (Table 6). The AUCs of the plasma m1A concentration and creatinine clearance showed a moderately negative correlation (Fig. 7B, left panel). The renal clearance of m1A was about 2-fold higher than the creatinine clearance (Table 6), and the AUCs also showed a moderate correlation (Fig. 7B, right panel).

**TABLE 3**
Pharmacokinetic parameters of m1A in WT and Oct1/2 dKO mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>Oct1/2 dKO</th>
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<tbody>
<tr>
<td>Cᵠₚₚ₁₂₀ₐ₀ (μM)</td>
<td>1.63 ± 0.23*</td>
<td>2.45 ± 0.11*</td>
</tr>
<tr>
<td>AUCᵠₚₚ₁₂₀ₐ₀ (μmol per min/l)</td>
<td>138 ± 15*</td>
<td>203 ± 9*</td>
</tr>
<tr>
<td>Xᵠₚₒₚ (nmol)</td>
<td>220 ± 27*</td>
<td>103 ± 13*</td>
</tr>
<tr>
<td>Cᵠₑₑₑₑ (μM)</td>
<td>3.31 ± 0.49*</td>
<td>5.63 ± 0.28*</td>
</tr>
<tr>
<td>Xᵠₑₑₑₑₑₑ (ml/g of liver)</td>
<td>2.08 ± 0.36</td>
<td>2.30 ± 0.09</td>
</tr>
<tr>
<td>Kᵠₑₑₑₑₑₑ (μM)</td>
<td>15.6 ± 6.7</td>
<td>9.27 ± 0.91</td>
</tr>
<tr>
<td>Kᵠₑₑₑₑₑₑₑ (ml/g of kidney)</td>
<td>8.81 ± 2.70</td>
<td>3.76 ± 0.23</td>
</tr>
<tr>
<td>KLₑₑₑₑₑₑₑₑ (ml/min)</td>
<td>2.10 ± 0.13**</td>
<td>1.11 ± 0.04**</td>
</tr>
<tr>
<td>CLₑₑₑₑₑₑₑₑ (ml/min per kilogram)</td>
<td>61.1 ± 2.7**</td>
<td>36.4 ± 1.3**</td>
</tr>
<tr>
<td>CLₑₑₑₑₑₑₑₑ (ml/min)</td>
<td>1.61 ± 0.16**</td>
<td>0.511 ± 0.077**</td>
</tr>
<tr>
<td>CLₑₑₑₑₑₑₑₑₑₑₑ (ml/min per kilogram)</td>
<td>46.9 ± 4.9**</td>
<td>16.6 ± 2.6**</td>
</tr>
<tr>
<td>GFR (Jonker et al., 2003)</td>
<td>0.462 ± 0.045</td>
<td>0.462 ± 0.065</td>
</tr>
</tbody>
</table>

Cᵠₑₑₑₑₑₑₑₑ, kidney concentration; Cᵠₚₚ₁₂₀ₐ₀–plasma concentration of compound at 120 minutes after administration; GFR, glomerular filtration rate; Kᵠₑₑₑₑₑₑ, liver-to-plasma ratio; Xᵠₑₑₑₑₑₑ, amount of compound excreted in urine.

*P < 0.05; **P < 0.01; ***P < 0.001, Student’s two-tailed unpaired t test; value in Oct1/2 dKO mice vs. WT mice.
Condition in which analyte peaks of m1A and LC-MS/MS analysis. For that reason, we optimized the analytical method of methyladenosine has the same mass-to-charge transition as m1A in LC-MS/MS analysis. For that reason, we optimized the analytical condition in which analyte peaks of m1A and N6-methyladenosine are detected separately (Supplemental Table 1) and could reproduce the significant alteration of plasma m1A concentration in Oct1/2 dKO mice. N6-methyladenosine concentration was under the lower limit of quantification (3 nM) in any specimen. Plasma concentrations of various kinds of fatty acids were also higher in Oct1/2 dKO mice. However, it was assumed to be the effect of Oct1 knockout, which causes hepatic thiamine deficiency and thereby enhances fatty acid oxidation to compensate for the reduced glycolysis (Chen et al., 2014).

m1A is a modified nucleoside that originally presents at position 58 in transfer RNA (tRNA). For the stabilization of the tRNA tertiary structure, the N6 position of adenine 58 is methylated to introduce a positive charge into the TψC loop of tRNA. Under cell stress conditions, tRNA is damaged in order to unfold and then enzymatically cleaved into tRNA-derived stress-induced RNA, followed by degradation to mononucleosides including m1A (Mishima et al., 2014). We considered another possibility for the synthesis of m1A, such as direct methylation of adenosine by gut flora, which motivated us to compare the endogenous level of m1A in plasma and various tissues between germ-free mice.

Discussion

Endogenous substrates were recently regarded as surrogate probes for the clinical assessment of DDI risk in drug development. While OCT2 and MATEs are the major drug transporters involved in the proximal tubular secretion of organic cations, sensitive and convenient biomarkers for them have not yet been found. Therefore, the goal of this study was to identify novel endogenous metabolites in plasma that could be used in DDI studies of renal OCT2/MATEs in healthy subjects.

We employed Oct1/2 dKO mice for the metabolomic analysis to search for candidate metabolites because OCT1 is also expressed in the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice. m1A was suggested to be a potential probe, and the basolateral membrane of proximal tubular epithelial cells along with OCT2 in mice.
mice and specific-pathogen-free mice (Supplemental Fig. 5). The results showed no significant differences in the plasma, liver, and kidney concentrations, whereas significant differences in the intestine and decreasing tendency in the brain were observed. On the other hand, the abundance of trimethylamine-N-oxide, whose precursor trimethylamine is produced by gut flora–mediated metabolism, was diminished in germ-free mice, indicating that the contribution of gut flora to the m1A disposition is negligible. In addition, in all tissues tested, endogenous m1A was detected at about 10-fold higher concentration than in plasma.

The in vitro transport study revealed that m1A is a substrate of not only mOct2 and hOCT2, but also mOct1, mMate1, hOCT1, and hMATE2-K (Fig. 1). Except for mMate1 with a $K_m$ value of 246 ± 14 μM, renal cation transporter-mediated m1A uptake was not saturated even at the concentration of 1 mM (Supplemental Fig. 3), indicating the low-affinity and high-capacity nature of m1A transport by those transporters. Considering the substrate dependency of the $K_i$ values (Hacker et al., 2015; Lechner et al., 2016; Mathialagan et al., 2017), we compared the $K_i$ values of the three inhibitors for m1Au p t a k e with those for other typical substrates. Trimethoprim and PYR exhibited almost identical inhibition constants for all substrates, whereas the $K_i$ value of cimetidine varied according to each substrate. Further studies are necessary to determine whether this finding is in vivo relevant in future clinical studies.

Next, we investigated the physiologic significance of OCTs in m1A transport with Oct1/2 dKO mice. We have clarified that TEA-sensitive uptake of m1A was completely abolished in the kidney slices from Oct1/2 dKO mice (Fig. 3). The results of the infusion study showed that the systemic clearance of m1A was dominated mostly by urinary excretion. Approximately 80% of intravenously given m1A was recovered unchanged in the urine in WT mice. The renal clearance of m1A was decreased to the value comparable with the glomerular filtration rate in Oct1/2 dKO mice (Table 3), causing its accumulation in the plasma (Fig. 4). A decreasing tendency of the $K_{p,kidney}$ value of m1A was observed in Oct1/2 dKO mice, but there was too large a variation in this parameter in WT mice to obtain statistical significance for unknown reasons. In contrast, the liver-to-plasma ratio was much lower than the $K_{p,kidney}$ value and almost identical between WT and Oct1/2 dKO mice, indicating little contribution of mOct1 to the hepatic uptake of m1A. The importance of mMate1 in the urinary excretion of m1A was suggested by the in vivo study using the MATE1 inhibitor PYR. PYR significantly decreased the renal clearance of m1A, accompanied by the increased kidney-to-plasma ratio and plasma concentration of m1A (Fig. 5), attributable to a significant reduction in

<table>
<thead>
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<th>TABLE 5</th>
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<td>The AUCs of metformin and m1A in control and DX-619-treated monkeys</td>
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</table>

<table>
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<tr>
<th>Treatment</th>
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<th>2</th>
<th>3</th>
<th>Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1A (nM·h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>364</td>
<td>216</td>
<td>219</td>
<td>266 ± 49</td>
</tr>
<tr>
<td>+ DX-619</td>
<td>468</td>
<td>361</td>
<td>464</td>
<td>431 ± 35</td>
</tr>
<tr>
<td>Fold change</td>
<td>1.29</td>
<td>1.67</td>
<td>2.12</td>
<td>1.72 ± 0.24</td>
</tr>
<tr>
<td>Metformin (μM·h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>43.5</td>
<td>11.1</td>
<td>38.3</td>
<td>31.0 ± 10.0</td>
</tr>
<tr>
<td>+ DX-619</td>
<td>91.9</td>
<td>26.5</td>
<td>77.6</td>
<td>65.3 ± 19.9</td>
</tr>
<tr>
<td>Fold change</td>
<td>2.11</td>
<td>2.39</td>
<td>2.03</td>
<td>2.18 ± 0.11</td>
</tr>
</tbody>
</table>
the luminal efflux. On the other hand, the kinetics of exogenously given m1A was almost identical between WT and Mdr1a/1b/Abcg2 triple knockout mice (Supplemental Fig. 6; Supplemental Table 4), confirming the predominant role of mMate1 in the efflux of m1A across the brush border membrane in mice.

Recently, cynomolgus monkeys have been used as a surrogate model to evaluate pharmacokinetic changes by the inhibition of renal drug transporters (Tahara et al., 2006; Shen et al., 2016). Monkey cation transporters exhibit a high similarity to their human counterparts, represented by amino acid sequence, transport properties such as substrate selectivity and pH dependency, and inhibition constants of various inhibitors (Shen et al., 2016). In accordance with these similarities, intravenous infusion of PYR caused the reduction of metformin renal clearance in cynomolgus monkeys (Shen et al., 2016), as is the case for oral PYR pretreatment in humans (Kusuhara et al., 2011). In our study, administration of DX-619, a potent OCT2 and MATE1/2-K inhibitor, resulted in an increase in AUCp of both m1A and metformin (Fig. 6), indicating the potency of plasma m1A concentration.

![Fig. 7. Plasma concentrations and renal clearance of m1A in younger and older volunteers. Plasma concentration profiles and urinary excretion of m1A and creatinine (A) in younger and older volunteers (B) were determined. Each symbol and bar represents the mean ± S.D. of eight and seven subjects. *P < 0.05; **P < 0.01; ***P < 0.001, younger vs. older, Student’s two-tailed unpaired t test.]

### TABLE 6
Pharmacokinetic parameters of m1A in younger and older Japanese volunteers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>m1A</th>
<th>Creatinine</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Younger</td>
<td>Older</td>
</tr>
<tr>
<td>AUC0–24 h</td>
<td>1.22 ± 0.22</td>
<td>1.29 ± 0.14</td>
</tr>
<tr>
<td>Xurine, 0–24 h</td>
<td>17.2 ± 1.4</td>
<td>13.0 ± 0.9***</td>
</tr>
<tr>
<td>CLr,p</td>
<td>244 ± 58</td>
<td>169 ± 22**</td>
</tr>
</tbody>
</table>

AUC0–24 h: area under the plasma concentration-time curve from 0 to 24 hours; Xurine, 0–24 h: amount excreted into the urine from 0 to 24 hours. *P < 0.05; **P < 0.01; ***P < 0.001.
were 31% and 17% lower in older volunteers than in younger volunteers, urinary excretion of m1A and creatinine in healthy younger and older serum elimination half-life of creatinine (3.85 hours) (Chiou and Hsu, 1975) shorter than metformin (4.0–8.7 hours) (Dunn and Peters, 1995) there might be other mechanisms to alter the kinetics of creatinine—of course, further studies in cynomolgus monkeys are needed.

Finally, we investigated the time profiles of plasma concentration and urinary excretion of m1A and creatinine in healthy younger and older volunteers (Fig. 7; Table 6). Like creatinine, the plasma concentrations of m1A showed minimal diurnal variation for 24 hours. The renal clearance of m1A was revealed to be approximately 2-fold higher than that of creatinine, indicating a greater contribution of the tubular secretion to the net urinary excretion of m1A. Correlation analysis suggested that the m1A AUC and renal clearance reflected renal function, including OCT2/MATE2-K activity. The renal clearances of m1A and creatinine were 31% and 17% lower in older volunteers than in younger volunteers, respectively. This is probably why the plasma m1A concentrations were slightly higher in older volunteers, although this was not the case with creatinine.

The major challenge remaining is to examine the effect of OCT2/MATE2-K inhibitors or genetic variants on m1A kinetics in humans. It was previously reported that the serum m1A level was significantly associated only with genetic variants of OCT2 in a metabolomic and genome-wide association study (Shin et al., 2014), whereas nonsynonymous single nucleotide polymorphism in the coding region was not included in those variants (Supplemental Table 5). In accordance with genome-wide association study, no effect of G808T mutation on hOCT2–m1A interaction was observed in HEK293 parental cells (Supplemental Fig. 1A). To conclude, the performance of m1A as a surrogate probe of OCT2 and MATE2-K needs further clinical studies using their inhibitors.

In addition, the contribution of other transporters to the disposition of m1A remains to be revealed. In our experiment, ENT-meditated m1A uptake was observed in HEK293 parental cells (Supplemental Fig. 1A). Although ENTs did not appear to have major roles in the renal secretion of m1A in mice, the effect of ENT inhibition on the systemic exposure of m1A needs further investigation, considering the ubiquitous expression of ENTs (Young et al., 2013). The m1A transport by organic anion transporters expressing in basolateral membrane of proximal tubular cells must also be checked, which we could not precisely evaluate using transporter-overexpressing HEK293 cells because of the inhibition of organic anion transporters by dipyridamole (data not shown).

In conclusion, we found m1A was a novel endogenous substrate of OCT2 and MATE2-K. m1A is a renal excretion-type compound that undergoes tubular secretion via those transporters in animals and humans. This study has provided the potency of m1A as a biomarker for OCT2/MATE2-K in future clinical DDI studies.

Authorship Contributions

Participated in research design: Miyake, Mizuno, Ieiri, Maeda, Kusuhara.

Conducted experiments: Miyake, Takehara, Mochizuki, Kimura, Matsuki, Irie, Watanabe, Ando.

Contributed new reagents or analytic tools: Kato.

Performed data analysis: Miyake, Takehara, Watanabe, Ando, Kusuhara.

Wrote or contributed to the writing of the manuscript: Miyake, Mizuno, Takehara, Ando, Kusuhara.

References


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as a biomarker for the evaluation of OCT2/MATE2-K–mediated DDIs. This is a salient feature not found in other endogenous substrates of OCT2/MATEs, such as NMN and creatinine. In practice, the plasma concentration is a better biomarker than renal clearance or urinary recovery, considering the simplicity of sample collection. The plasma creatinine concentration also exhibited an increasing tendency in the DX-619–treated period. Although metformin was eliminated from the systemic circulation at 24 hours after DX-619 administration, the plasma creatinine concentration remained high. Considering that in humans the serum elimination half-life of creatinine (3.85 hours) (Chiou and Hsu, 1975) shorted than metformin (4.0–8.7 hours) (Dunn and Peters, 1995) there might be other mechanisms to alter the kinetics of creatinine—of course, further studies in cynomolgus monkeys are needed.

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